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The effect of genomic position on reversion of a *lac* frameshift mutation (*lacIZ33*) during non-lethal selection (adaptive mutation)

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Summary

In a system described by Cairns and Foster, starvation of a particular leaky *lac* mutant (*lacIZ33*) in the presence of lactose appears to direct mutation in non-growing cells to sites that allow growth (adaptive mutation). This behaviour requires that the *lac* operon be located on an F′ plasmid. This position effect was investigated by placing the mutant *lac* operon at many sites in the genome of *Salmonella enterica* (*Typhimurium*; LT2) and testing reversion behaviour. Genomic position did not affect reversion during non-selective growth. When *lac* was at any of 550 chromosomal sites, starvation caused little or no enhancement of reversion. In the 28 strains with the *lac* on *Salmonella*'s conjugative plasmid (pSLT), selection enhanced reversion strongly, just as seen for strains with *lac* on an F′ plasmid. In 46 strains, the *lac* operon was inserted within a small chromosomal duplication, and selection stimulated RecA-dependent partial reversion by simple amplification (about 8×) of the mutant *lac* region. The position of *lac* on a conjugative plasmid is important to reversion because it allows more frequent gene duplication and amplification. These events are central to growth and reversion under selection because they increase the number of replicating *lac* alleles within each developing revertant clone.

Introduction

A particular *Escherichia coli lac* frameshift mutant (*lacIZ33*) appears to direct mutation to growth-promoting sites during starvation in the presence of lactose (Cairns *et al*., 1988; Cairns and Foster, 1991). This behaviour conflicts with the general view that all mutations arise at random with respect to phenotype (Mayr, 1982). It is widely believed that selection operates primarily to remove deleterious mutations and occasionally to favour a rare beneficial change, but never to stimulate or choose targets for mutation. Because of its potential to change our view of mutation, the Cairns–Foster system has been investigated in great detail. Work on this system (outlined below) has been reviewed extensively (Rosenberg, 1997; Foster, 1999; 2000; Hendrickson *et al*., 2002).

Several models have been proposed to explain the reversion behaviour of this system. According to the hypermutable state model, selection triggers an evolved mechanism that generally mutagenizes a subset of non-growing cells in the hope of producing a valuable mutation (Hall, 1992). This mechanism for selection-induced mutagenesis is suggested to enhance long-term survival.

In contrast, the amplification mutagenesis model proposes that selection has no direct effect on mutation rates, but acts only to favour the growth of cells with improved ability to use lactose. Improved growth results from amplification of the leaky mutant *lac* allele. This growth adds many replicating copies of the mutant *lac* allele to each developing clone. Once reversion occurs, selection holds that *lac*$^+$ allele and favours growth of cells that lose the array of mutant copies. These mutant alleles are deleterioucs because they contribute to loss of the revertant allele by segregation. If one is not aware of the intermediate growth, amplification, reversion and segregation, it appears that exposure to selective stress converts a non-growing haploid *lac* mutant to a *lac*$^+$ revertant. In its basic form, this model explains the apparent selection-directed mutagenesis without any change in the rate or target specificity of mutation. However, temporary mutagenesis does occur during this process (Torkelson *et al*., 1997; Rosche and Foster, 1999; Slechta *et al*., 2002).

The general mutagenesis associated with reversion in the Cairns system (and in no other bacterial selection system) is attributed to a deleterious side-effect of growth with a large amplification – SOS induction by DNA fragments released from amplified arrays by segregation. Because of *lac* amplification and mutagenesis, reversion under selection requires very little growth; this growth was missed in previous tests. This model has been presented in detail elsewhere with evidence that amplification is an

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essential intermediate in the process of reversion in the Cairns system (Andersson *et al.*, 1998; Hendrickson *et al.*, 2002)

The essential role of the F′ plasmid in the Cairns experiment is not explained by either the amplification model or the hypermutable state model. For selection to enhance reversion appreciably, the mutant *lac* allele must be on the F′ plasmid rather than at its normal chromosomal locus (Radicella *et al.*, 1995). Maximum yield of revertants requires expression of the plasmid *tra* operon, which encodes functions for conjugative replication and transfer (Foster, 1995; Galitski and Roth, 1995). There is conflicting evidence regarding the need for actual cell–cell transfer (Foster, 1995; Radicella *et al.*, 1995; Peters *et al.*, 1996; Godoy and Fox, 2000). It has been reported that genes on the F′ *lac* plasmid accumulate unselected mutations during starvation for lactose even in parental cells that do not themselves become Lac$^+$ (Foster, 1997). The *tetA* gene used for this experiment is within a Tn*10* element inserted 4 kb away from the *lac* operon in the *mhpC* gene (unpublished results). It seems likely that the target of unselected mutations was amplified and mutagenized with *lac* during selective growth. Insertions of Tn*10* on the F′ *lac* plasmid are prone to precise excision during conjugation and during reversion under selection of the nearby *lac* mutation. This suggests that transfer replication contributes to the course of reversion (Godoy and Fox, 2000). Particular F′ plasmids stimulate the low-frequency reversion of chromosomal mutations during selection (Godoy *et al.*, 2000). These observations suggest that some function encoded by the *tra* operon, internal replication from the transfer origin or perhaps conjugation itself contributes to reversion in the Cairns system.

To test the effect of genomic position on *lac* reversion and to search for chromosomal sites that might support selection-enhanced reversion, a transposon was constructed that includes the same *lac* operon used by Cairns and Foster (1991). This transposon was placed at many sites in the *Salmonella* genome, and the reversion behaviour of *lac* was tested for each site. The results suggest that the conjugative plasmid promotes duplication and amplification, essential intermediates in reversion that allow growth, add *lac* copies and stimulate induction of the mutagenic SOS system. We propose that DNA ends formed by the plasmid transfer replication system cause genes on the plasmid to duplicate and amplify frequently. Because *lac* is on an F′ plasmid in the Cairns experiment,

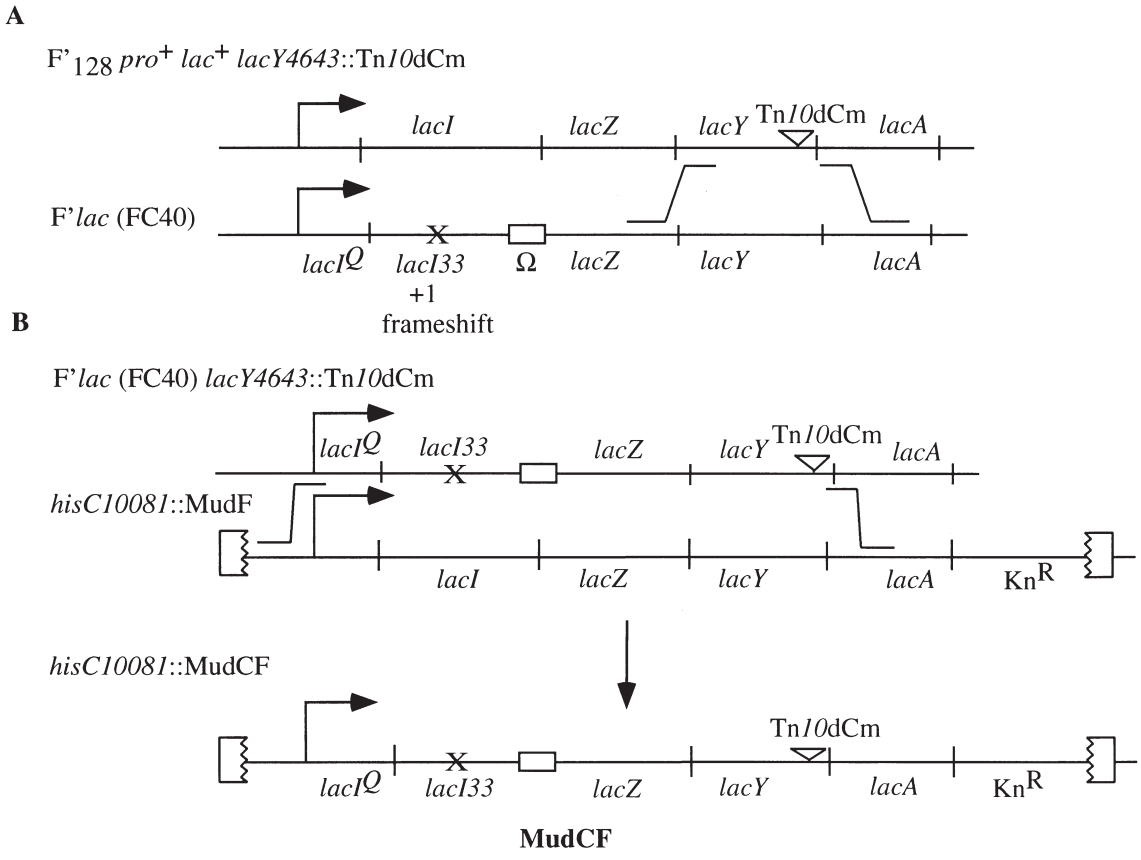

Fig. 1. Construction of MudCF. See *Experimental procedures* for details of the construction.

plasmid-enhanced amplification and associated SOS induction can drive the process of amplification, reversion and segregation with very little growth.

Results

Construction of the MudCF element and plan of the experiment

The *lac* operon used in the Cairns–Foster test system (Cairns and Foster, 1991) has three mutations: (i) a deletion (Ω) that fuses the *lacI* and *lacZ* coding sequences; (ii) a +1 frameshift mutation (*lacI33*) within the *lacI* sequence of the hybrid (*lacI-Z*) gene; and (iii) a $lacI^Q$ promoter mutation that provides for constitutive transcription of the chimeric *lacI-Z* gene. This triply mutant *lac* operon was placed within a Mu-derived transposon and allowed to transpose to a variety of sites in the *Salmonella* genome (see *Experimental procedures*). The structure and construction of the (MudCF) transposon is shown in Fig. 1. Each of 625 strains with *lac* at an independently selected position was tested for reversion to Lac^+ under both selective and non-selective conditions.

Reversion behaviour of the lac *operon within MudCF*

Figure 2A shows the reversion behaviour of a *Salmonella* strain (TT18302) with *lac* on the F′ plasmid used by Cairns and Foster (1991) and another strain (TT19199) with the same *lac* region within a chromosomal MudCF element. The chromosomal MudCF shows very little reversion under selection. A *Salmonella* strain with MudCF on an F′*nadA* plasmid (TT20850) shows reversion behaviour very similar to that of the original F′*lac* strain (Fig. 2B). Figure 2A and B shows that significant numbers of revertants arise under selection when the *lac* operon is on an F′ plasmid, but not when the same mutant allele is located in the chromosome. This confirms, for a second chromosomal site, for a second F′ plasmid and for *Salmonella*, the results obtained by Radicella *et al*. (1995) in *E. coli*. In both strains with *lac* on the plasmid, reversion under selection is dependent on RecA function (Fig. 2B). It should be noted that all these strains are derived from *Salmonella* and all carry the pSLT plasmid, which represses the *tra* operon of the F plasmid (Galitski and Roth, 1995). Therefore, the rates reported here are lower than those seen in *Salmonella* strains carrying the F′*lac* plasmid without pSLT (Galitski and Roth, 1995).

Reversion of the MudCF lac *mutation at various sites in the* Salmonella enterica *genome*

The MudCF element was allowed to transpose to 625 independent sites (*Experimental procedures*). Independent Kn^R insertion mutants were tested in a preliminary reversion assay (see *Experimental procedures*), which scored the number of revertants on day 7. Based on this screen, the 625 strains were sorted into three classes (I, II and III). Selected members of class I and all members of classes II and III were tested more extensively to quantify their reversion phenotypes (see *Experimental procedures*). Mutants from each class are described below.

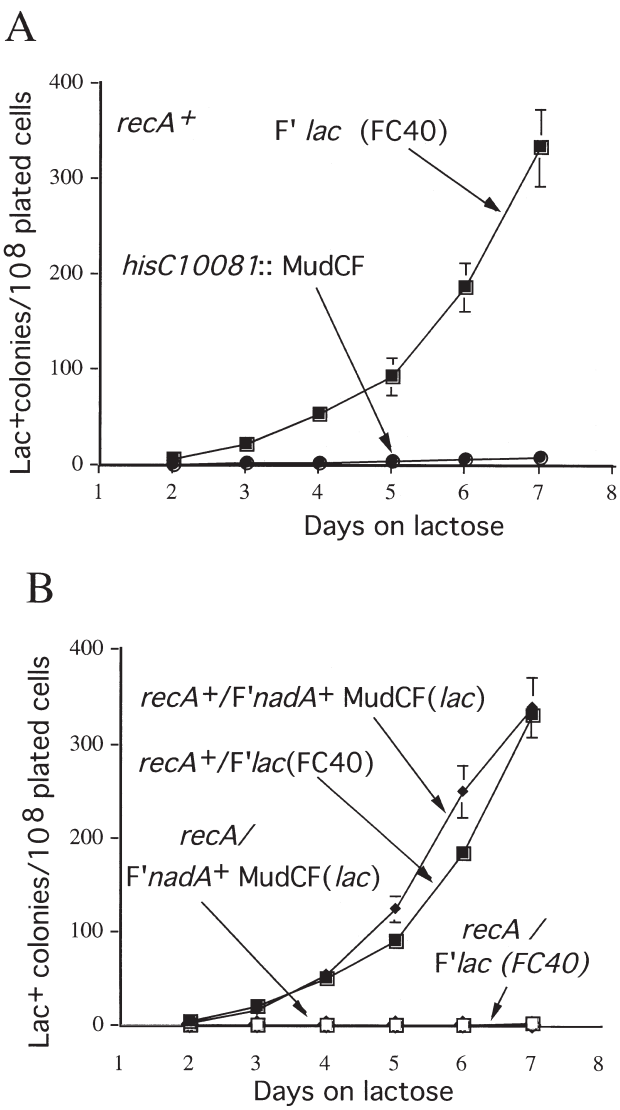

Fig. 2. Reversion of the *lac* mutation of MudCF when in the chromosome or on an F′ plasmid.
A. Chromosomal *lac* region. Closed squares, TT18302 (rec^+/F′*lac*); closed circles, TT19199 (*hisC*:: MudCF).
B. Plasmid-borne *lac* region. Closed squares, TT18302 (rec^+/F′*lac*); open squares, TT18306 (rec^-,F′*lac*); closed diamonds, TT20850 (rec^+/F′*nadA* MudCF); and open diamonds, TT23006 (rec^-/F′*nadA* MudCF).

Class I – most chromosomal insertion strains show little or no late reversion

Class I strains (550/625) carry a simple MudCF insertion

at some point in the *Salmonella enterica* chromosome. These strains show a few Lac$^+$ revertants on day 2, but do not accumulate a significant number of additional revertants with time. This reversion behaviour is like that seen by Radicella *et al.* (1995) for *E. coli* strains carrying the same mutant *lac* operon at its normal chromosomal location and is like that of the chromosomal *hisC*::MudCF insertion in *Salmonella* (Fig. 2A).

Reversion behaviour of a typical class I strain (TT20850) is shown in Fig. 3A. No class I site showed more than 10% of the final number of revertants seen on day 7 for a *Salmonella* strain carrying the original F′ plasmid (TT18302). The small accumulation of Lac$^+$ revertants seen in a few strains was not dependent on RecA function (data not shown). Twelve class I insertions have been sequenced, as described in *Experimental procedures*; their positions in the chromosome are listed in Table 1 and shown in Fig. 5.

One chromosomal insertion showed an atypical reversion pattern (Fig. 3B). This strain (TT20864) had a 10-fold higher number of revertants on day 2 than the strain with the F′*lac*, but accumulated only fivefold more revertants over the following days. Reversion in this strain was not recombination dependent. The MudCF element in this strain is inserted within the *mutS* gene (identified by its 100% linkage to *mutS421*::Tn*10* and by its frequent generation of Rif^R mutants). The MutS defect of this strain enhances reversion of a chromosomal *lac* gene during pregrowth (seen on day 2) but allows very little late accumulation of revertants under selective conditions. This

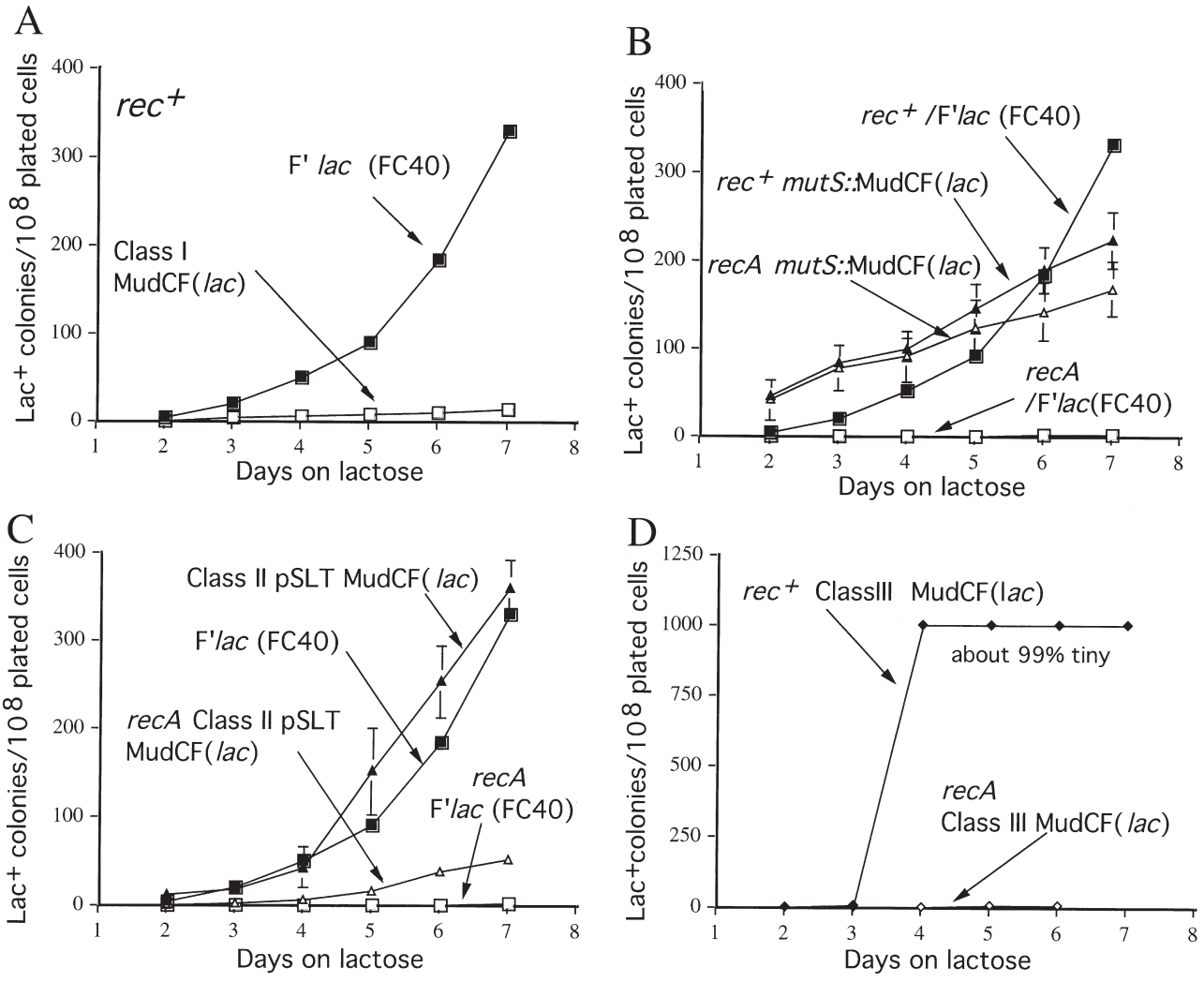

Fig. 3. Reversion of the *lac* operon of MudCF inserted at several different positions.
A. A typical class I MudCF insertion. Closed squares, TT18302 (rec$^+$/F′*lac*); open squares, TT20853 (rec$^+$, *oadB10*::MudCF).
B. A *mutS177*::MudCF insertion. Closed squares, TT18302 (rec$^+$/F′*lac*); open squares, TT18306(rec$^-$/F′*lac*); closed triangles, TT20864 (rec$^+$, *mutS*::MudCF); open triangles, TT23007 rec$^-$, *mutS*::MudCF).
C. A typical class II insertion. Closed squares, TT18302 (rec$^+$/F′*lac*); open squares, TT18306 (rec$^-$/F′*lac*); closed triangles, TT23009 (rec$^+$/pSLT MudCF); open triangles, TT23011 (rec$^-$/pSLT MudCF).
D. A typical class III insertion. Open triangles, TT22996 (rec$^+$/*dbpA*::MudCF); closed triangles, TT23014 (rec$^+$, *dbpA*::MudCF).

Table 1. Sequenced positions of class I insertions.

Strain	Target gene	Basepair position within ORF	Chromosomal position (min)
TT20853	*oadB*	1138	17′
TT20854	*orf-6528*	209	32′
TT20855	*nhaC*	1166	37.5′
TT20856	*dsdC*	450	52.7′
TT20857	*fdnG*	1751	87.7′
TT20858	*orf-2472*	434	38.7′
TT20859	*yfeR*	689	52.8′
TT20860	*kdpD*	1507	15.5′
TT20861	*orf-3310*	1533	55.5′
TT20862	*mrcB*	1354	3.6′
TT10863	*eutJ*	655	53.3′

effect of a *mutS* defect on reversion of a chromosomal *lac* gene is different from that seen by Foster and Cairns (1992), who tested a *lac* allele on the F′ plasmid. They noted the increase in the number of early (day 2) revertants and, in addition, saw that Lac$^+$ revertants accumulated faster under selection than seen for *mutS*$^+$ strains (Foster and Cairns, 1992). We believe both results are consistent with the amplification mutagenesis model and that the effect of a *mutS* defect on selected reversion depends on whether *lac* amplification and growth is permitted. We suggest that *lac* on the plasmid allows amplification and growth, whereas *lac* in the chromosome allows little or no amplification and growth (see *Discussion*).

Class II – sites within plasmid pSLT allow RecA-dependent reversion under selection

The class II insertions (28/625) were identified by their high frequency of reversion under selection and were mapped (see *Experimental procedures*) to *Salmonella*'s virulence plasmid, pSLT, a conjugative plasmid related to the F plasmid and carried by *Salmonella* strain LT2 (Sanderson *et al*., 1983; 1996; Sanderson and Stocker, 1987). Plasmid pSLT is a Fin$^+$ plasmid which represses its transfer functions with the FinDP complex, which can also repress the conjugative functions of the F plasmid (Gasson and Willetts, 1975; Sanderson *et al*., 1983). The F′ plasmid was isolated from nature as a regulatory mutant lacking the finD component of the complex (Cheah and Skurray, 1986). Even though pSLT represses its own fertility (is Fin$^+$), it can transfer to other strains with low efficiency on minimal medium (Ahmer *et al*., 1999).

Strains containing class II MudCF insertions showed a reversion pattern similar to that seen for strains with the mutant F′*lac* plasmid and a fertility-repressing pSLT (TT18302). Late revertants accumulate with time on selective medium, and the accumulation depends on RecA function (Fig. 3C). The position of MudCF within pSLT does not appear to be critical to enhanced reversion under selection. Two class II insertions were sequenced and found to be in *orf-691* (36 kb from OriT) and *trbB* (20 kb from OriT) on the pSLT plasmid (total size 94 kb). The *trbB* gene product is a putative conjugation protein, but null mutations in the homologue of this gene on the F′ plasmid cause no conjugation deficiency (Kathir and Ippen-Ihler, 1991). In the first mutant hunt, all MudCF insertions that showed a class II reversion pattern proved to be in pSLT. In a later hunt (see below), eight insertions of MudCF in pSLT were isolated without regard to reversion behaviour, and all proved to revert efficiently under selection (the class II phenotype).

Unstable Lac$^+$ cells are found in class II late revertant colonies but rarely in class I revertants

The amplification mutagenesis model suggests that selection enhances reversion by allowing cells with a *lac* amplification to grow within a clone initiated by a cell with a *lac* duplication; reversion events occur within each such growing clone. After reversion and segregation, faster growing stable haploid Lac$^+$ segregants overgrow the colony and reduce the relative frequency of amplification cells in the colony. Despite this overgrowth, some of the original amplification cells can be found as a minority type in every final revertant colony (Andersson *et al*., 1998; Hendrickson *et al*., 2002).

Such cells with multiple copies of the (leaky) *lac* allele can be recognized because they have an unstable Lac$^+$ phenotype resulting from frequent loss of the amplification by recombination. This is scored by suspending colonies from the selection plate in liquid, diluting and plating on rich medium containing Xgal; unstable Lac$^+$ cells form blue colonies with multiple white sectors (see *Experimental procedures*). The instability scored in this way does not result from loss of the F′ plasmid, which is extremely stable. In the original strains used by Cairns and Foster (Cairns and Foster, 1991; Hendrickson *et al*., 2002) and in analogous strains made in *Salmonella* (Andersson *et al*., 1998), the *lac* operon is on an F′*lac* plasmid. In these strains, revertant colonies accumulate over several days, and unstable Lac$^+$ cells are found at some frequency in every Lac$^+$ revertant colony arising under selection. These unstable cell types are not seen in day 2 revertant clones formed by mutants that arose during non-selective pregrowth (Andersson *et al*., 1998; Hendrickson *et al*., 2002). Contradictory reports that these amplification cells are found in only a few revertant colonies (Hastings *et al*., 2000; McKenzie *et al*., 2001) are in error because too few cells were tested from each colony to score the frequency of colonies with amplification-bearing cells reliably (Hendrickson *et al*., 2002).

For class II strains with *lac* on the pSLT plasmid, unstable Lac$^+$ cells were also seen in every late (day 5) revertant colony. Most late class II revertant colonies were

Table 2. Percentage unstable Lac$^+$ cells in early (day 2) and late (day 5) Lac$^+$ revertant colonies from class I (TT19199) or class II (TT23009) strains.

	Class I				Class II		
Colony	No. of cells tested	No. of unstable Lac$^+$ cells	% of cells with an unstable Lac$^+$ phenotype	Colony	No. of cells tested	No. of unstable Lac$^+$ cells	% of cells with an unstable Lac$^+$ phenotype
Day 2 revertants							
1	1017	0	0	1	3016	0	0
2	1029	0	0	2	3134	5	0.2
3	1316	0	0	3	2176	0	0
4	927	1	0.1	4	3137	1	0.03
5	1114	0	0	5	3336	0	0
6	1916	2	0.1	6	2917	0	0
7	875	0	0	7	3600	1	0.03
8	1112	0	0	8	1928	0	0
9	1129	1	0.1	9	2006	0	0
10	1715	0	0	10	2127	1	0.05
Day 5 revertants							
1	1197	2	0.2	1	3214	310	10.3
2	1212	0	0	2	2916	42	1.4
3	1475	1	0.1	3	3012	110	3.7
4	1010	2	0	4	3347	215	6.4
5	798	0	0.2	5	2476	12	0.5
6	876	0	0	6	1918	17	0.8
7	927	1	0.1	7	2216	100	4.5
8	1119	0	0	8	2817	28	1.0
9	1476	0	0	9	3006	39	1.3
10	1628	0	0	10	796	796	100

found to contain 0.4–10% unstable Lac$^+$ cells (Table 2), but the variation in the fraction of cells with an unstable phenotype is large. This is expected because the process of reversion – amplification, reversion, segregation – involves a series of stochastic events. In contrast, class I strains gave very few late (day 5) revertants, and these revertants showed very little evidence of amplification (Table 2). Most of these revertants showed no amplification cells, and the rest showed amplifications only at an extremely low frequency. Class I revertants (like day 2 revertants in all strains) appeared to arise in cells that did not carry a *lac* amplification. We presume that most late-appearing revertants of class I strains actually arose during pregrowth and were slow to form a colony under selection. As predicted by the amplification mutagenesis model, essentially no unstable Lac$^+$ cells were found in early (day 2) revertants of either class I or class II strains. These revertants are thought to reflect reversion events that occurred under non-selective pregrowth conditions that do not favour prior amplification.

The above behaviour suggests that *lac* mutations on the conjugative plasmid pSLT revert under selection, as do those on the F′ plasmid; in both cases, amplification precedes reversion (Hendrickson *et al*., 2002). Thus, significant reversion under selection is invariably associated with a *lac* operon located on a conjugative plasmid and with unstable Lac$^+$ cells in the revertant clone. The correlation between amplification under selection and plasmid location will be pursued below.

Class III insertion strains yield frequent, partially Lac$^+$ revertants under selection

Class III insertion strains (46/625) are like those of class I, in that both carry the *lac* operon at sites in the chromosome and both fail to accumulate full-sized revertants with time under selection. Class III strains, however, give rise to over 1000 small, partially Lac$^+$ revertant colonies that appear on day 4 and do not increase in number thereafter (Fig. 3D). These tiny colonies do not develop into full-sized revertants over the course of a 14-day experiment. If one ignored these tiny colonies and scored only full-sized revertant clones, class III mutations would be indistinguishable from class I.

When tiny class III revertant colonies are picked from selection plates, resuspended, diluted and plated for single colonies on selection plates seeded with scavenger cells (LT2), they require 4 days to reform a colony. This suggests that the events that initiated class III revertants occurred during pregrowth, and the 4 day delay was required for colony formation. When cells from a tiny revertant colony are diluted and plated onto non-selective media with Xgal, 100% of the cells in the tiny colonies show an unstable Lac$^+$ phenotype, based on forming blue colonies with multiple white sectors. In *recA* derivatives of class III strains, these tiny colonies do not appear even after 21 days of incubation on selective medium (Fig. 3D). Thus, the partial revertants of class III strains require RecA function to form the initial mutant during pregrowth

and/or to support their slow development under selection. We propose that the role of RecA is to allow duplication and amplification of *lac*.

Revertants of class III strains carry an amplification of the lac *region*

The unstable Lac$^+$ phenotype and the RecA dependence of the class III partial revertants suggested that they might carry an amplification of the *lac* operon. Consistent with this, their unstable Lac$^+$ phenotype became stable after the introduction of a *recA* allele (*recA643*::T-POP). The inferred amplifications were first verified by quantitative Southern hybridization and later by pulsed field gel electrophoresis (PFGE; below). Apparently, these amplifications did not occur in class I strains, which accumulated neither partial nor complete revertants while under selection. The particular chromosomal position of class III insertions appeared to permit or promote amplification of the inserted *lac* region, but did not allow full reversion.

The nature of the class III insertions was inferred from a combination of PFGE, genetic mapping and sequencing of MudCF insertions. We conclude that each of the class III insertions of MudCF is within a duplicated chromosomal region. The insertions fall into two subclasses. Each class IIIA insertion is at the join point of its own particular duplication, formed in the course of MudCF transposition, and each class IIIB insertion is at a different site within one copy of one particular duplication that happened to pre-exist in the strain used for the transposition experiment.

Sequencing revealed that each class IIIA insertion is at the join point of a different duplication. That is, the sequences at the left and right flank of the insertion were normally at separated sites in the bacterial chromosome in the same orientation but reversed order (see Fig. 4). The end-points of these duplications show a strong tendency to be in or near the *his* operon (Fig. 5). In the figure, each bracket indicates the region duplicated in the parent strain with MudCF lying between the two copies. There is no single chromosomal site common to all class IIIA duplications. As expected for an insertion at the join point, each class IIIA insertion is 100% linked to the class III phenotype; that is, when the insertion is transduced into a new recipient, all transductants inherit the duplication and the reversion behaviour of the parent. We believe the preference for the *his* region reflects a bias in target selection by MudCF when it transposes into the chromosome from the homologous linear transduced fragment carrying the *hisC*::MudCF insertion. None of the duplication join points corresponds to the insertion site of the donor MudCF (see *Discussion*)

Class IIIB insertions are standard MudCF insertions in one copy of a region (15 kb) near the *dbpA* gene between minutes 36.2 and 36.9. This region happened (unbeknown to us) to be duplicated in the particular strain (TR6611) used for the initial mutant hunt (see Fig. 5). All class IIIB insertions are within this region, and insertions of this type are not found when one starts with a different parental strain lacking the pre-existing duplication. Four class IIIB insertions have been sequenced, and their positions are listed in Table 3 and illustrated in Fig. 5. Sequence data show that class IIIB insertions are not at a duplication join point, but all these insertions show transductional linkage to a duplication join point. Transduction of a class IIIB insertion from the original background into an LT2 recipient lacking the parental duplication yields some transductants with a class I phenotype and others with a class III phenotype. We conclude that the class IIIB phenotype can be transduced when the fragment carrying the insertion also carries the join point of the duplication (Roth *et al*., 1996). If the MudCF insertion is inherited without the duplication join point, the resulting recombinant strains show a class I reversion phenotype.

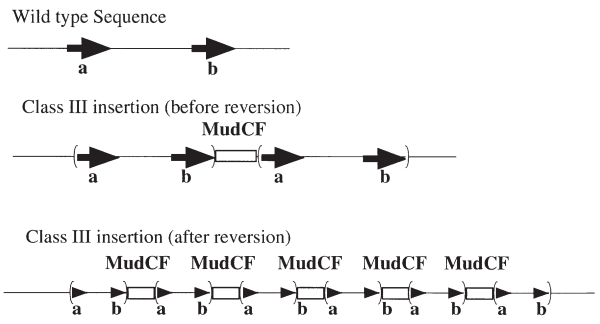

Fig. 4. Structure of amplified class IIIA MudCF insertions. The insertions are located at the junction of a duplication join point, making them prone to further amplification (via homologous recombination).

The pre-existing amplification is present in variable copy number in every cell of the parent strain. DNA from 16 different *recA* derivatives of the parent strain TR6611 were digested with *Xba*I and subjected to PFGE. Each isolate lacks the wild-type 457 kb band and has a new band with a size between ≈ 500 kb and 650 kb (data not shown), suggesting that the 15 kb amplification is present in about two to seven copies. Digestion of a class IIIB insertion strain with *Spe*I, which cuts within the MudCF, yields a very intense 30 kb band not present in digestions of the LT2 control. As MudCF is 15 kb long, the chromosomal duplicated region is inferred to be 15 kb. How this duplication arose in the parent strain and why it is maintained is unknown, but the amplification appears to expand and contract in the parent strain without any consciously imposed selection. No other strain tested showed a tendency to duplicate this region.

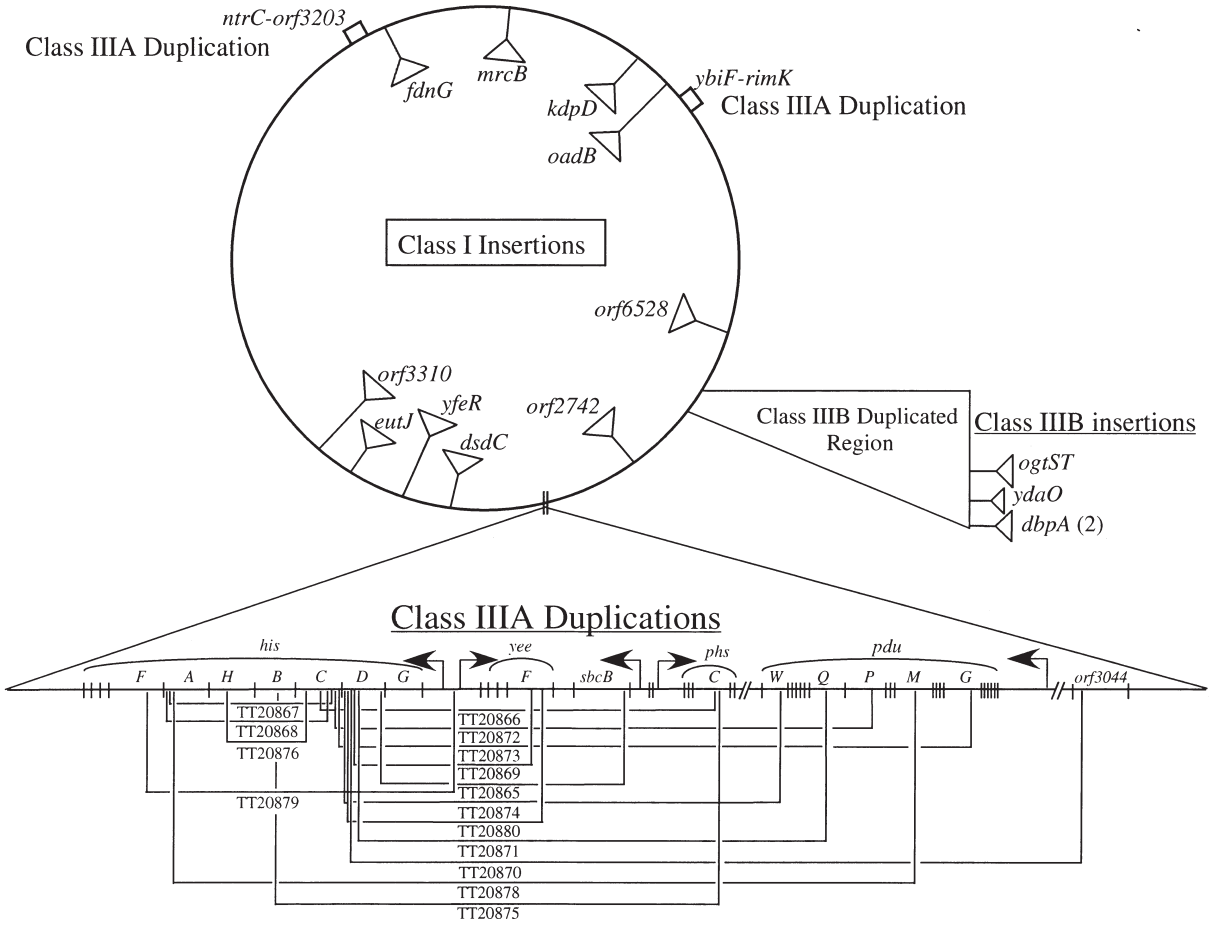

Fig. 5. Map positions of all sequenced insertions. Brackets indicate duplication join points. Triangles indicate simple insertions. The region of DNA instability in TR6611 is enlarged to indicate the positions of class IIIB insertions.

Southern hybridization of several of the parental (Lac⁻) class IIIA and IIIB strains and their revertants showed two to five copies of the *lac* operon in the parental (Lac⁻) strains and 8–10 copies in their Lac⁺ revertants (data not shown). To visualize the inferred amplifications, PFGE was performed on *recA* derivatives of revertants from 15 different class III strains (including both class IIIA and B types). The RecA function was eliminated to prevent recombination events that could introduce heterogeneity in the size of the amplified array. In RecA⁺ strains, variability in the size of the amplified array within one culture distributes the affected fragment among many positions and prevents its visualization. All 15 class III (A and B) revertants (*recA*) showed a 100–1000 kb increase in the size of a 457 kb *XbaI* fragment characteristic of wild-type strains. This wild-type fragment carries the region between map minutes 35 and 44 of the *Salmonella* chromosome, including the *his* operon. The same 457 kb fragment was affected in all of the initially isolated class III insertions tested (both IIIA and IIIB types) because both the favoured regions are contained in this 9 min region of the chromosome. Eighteen additional class III insertions are near *his* based on sequence data from one end of the MudCF element. The remaining 12 class III insertions have not been assigned to IIIA or IIIB.

Significance of the class III duplication insertions

The class IIIA and IIIB insertions place *lac* at chromosomal positions that allow some amplification under selection. We suggest that the significant feature of these insertions lies in their provision of direct order repeats flanking *lac* that can support unequal recombination events and allow progressive amplification under selection. If this is correct, then the clustering of class IIIA insertions near the *his* operon and clustering of class IIIB insertions near the *dbpA* gene do not reflect any importance of these particular regions in amplification. Rather, it reflects the fact that these regions provide (for different reasons) the duplication status required for amplification under selection. That is, the bias of class IIIA insertions to *his* reflects a bias of MudCF to transpose and create

Table 3. Sequenced positions of class III insertions.

Strain	Gene nearest Mud *attR*[a]	Position in gene[b]	Gene nearest Mud *attL*	Position in gene	Orientation[c]
Class IIIA					
TT20865	*sbcB*	549	*hisD*	527	B
TT20866	*phsC*	764	*hisC*	81	A
TT20867	*hisC*	51	*hisA*	56	A
TT20868	*hisC*	40	*hisA*	98	B
TT20869	*yeeF*	1033	*hisD*	1201	B
TT20870	*orf304*	379	*hisD*	1236	B
TT20871	*pduQ*	337	*hisD*	1115	B
TT20872	*pduP*	1362	*hisC*	24	B
TT20873	*pduG*	162	*hisC*	4	B
TT20874	*pduW*	293	*hisD*	1297	B
TT20875	*phsA*	420	*hisB*	375	B
TT20876	*hisC*	240	*hisH*	555	A
TT20877	*ntrC*	904	*orf-3204*	95	B
TT20878	*pduM*	435	*hisA*	38	B
TT20879	*hisL*	2	*hisF*	125	B
TT20880	*yeeF*	110	*hisD*	1273	B
TT20881	*rimK*	60	*ybiF*	455	A
Class IIIB					
TT20882	*dbpA*	538	*dbpA*	555	A
TT20883	*ydaO*	328	*ydaO*	310	B
TT22995	*ogtST*	40	*ogtST*	34	A
TT22996	*dbpA*	1013	*dbpA*	993	B

a. Open reading frames were identified on the Integrated Genomics WIT ERGO site (www.integratedgenomics.com).
b. Number given is the last normal base in the coding sequence before the Mud sequence. Some of these are estimated when the actual junction sequence was not obtained.
c. For MudCF in orientation A, *lac* transcription proceeds clockwise in the chromosome map as usually drawn.

duplications in this region (see *Discussion*), and the bias of class IIIB insertions to the *dbpA* region reflects the position of an unusual pre-existing parental duplication.

To pursue this question in more detail, a second set of MudCF insertions was isolated in a strain (LT2) that has no tendency to duplicate the chromosomal region near *dbpA* at 36′. This hunt yielded 288 independent transpositions of MudCF, none of which are of the class IIIB type. Before reversion testing, transduction crosses were made to determine whether MudCF was located on the pSLT plasmid or the chromosome. All eight insertions in pSLT showed a class II reversion phenotype. Of the chromosomal insertions, 266 were in class I and 14 in class III. Five of the class III insertions were sequenced from both ends, and all proved to carry the MudCF at duplication join points (class IIIA). Three insertions were adjacent to *his* sequences as was seen in the first mutant hunt, but two were at the join point of duplications far from the *his* locus (Table 2; Fig. 9). Seven additional insertions are also in the *his* region (class IIIA) based on sequence from one end of the MudCF element. Thus, duplicated regions other than *his* can stimulate amplification and give a class III reversion phenotype. No class IIIB insertions (near *dbpA*) were found among 12 characterized insertions in this strain lacking the pre-existing duplication near *dbpA*. Over half the class III insertions from the first hunt were in class IIIB.

Construction of a class III insertion far from his *and* dbpA

The interpretation of the class III insertions described above predicts that a class III reversion phenotype (partial reversion by amplification) would be shown by any strain in which the MudCF (*lac*) element is located within a duplicated region. We tested this prediction using a duplication of the *nadB* gene that extends from *nadB* to *gua* and carries a MudA insertion at the join point (T. Tsang, unpublished results). The MudCF was transduced into this strain replacing the join point MudA element. This generated a 3.4 min duplication with MudCF at the join point. Large revertant clones appeared on day 2, but their number did not increase over the next 6 days. This strain showed a class III reversion phenotype with over 1000 tiny colonies appearing on day 4.

Discussion

In the Cairns system, large effects of selection on reversion have been observed only in strains carrying the *lac* operon on an F′ plasmid (Cairns and Foster, 1991; Radicella *et al*., 1995). Effects seen previously for a chromosomally located *lac* operon are minor, comparable with the residual level of reversion seen in some of the class I MudCF insertions described here (Radicella *et al*., 1995; Rosche and Foster, 1999; Godoy *et al*., 2000). The

experiments described here were done to characterize this position effect on *lac* reversion. None of the 700 chromosomal sites tested allowed selection-enhanced *lac* reversion comparable with that seen when *lac* is located on an F′ plasmid. In contrast, all 60 sites on the pSLT plasmid allowed enhanced reversion indistinguishable from that seen with the F′*lac* plasmid. These data support the conclusion of Radicella *et al*. (1995) that reversion in the Cairns system requires that the *lac* operon under selection be located on a conjugative plasmid. Their conclusion is extended to several hundred more chromosomal sites (which also fail to show enhanced reversion) and to 60 sites on another conjugative plasmid pSLT (which do show selection-enhanced reversion). We conclude that the location of *lac* on a conjugative plasmid is central to the behaviour of this system.

Class III insertions (with sequence repeats flanking *lac*) can achieve sufficient amplification under selection to allow the appearance of tiny colonies, whereas class I insertions (which lack major repeats) cannot. This suggests that most chromosomal genes are limited in their ability to form the sort of duplication required for reversion under selection. Although class III strains achieve some *lac* amplification under selection, they do not achieve the high amplification and induced reversion seen when *lac* is on a conjugative plasmid (Tlsty *et al*., 1984; Whoriskey *et al*., 1987; Andersson *et al*., 1998; Hastings *et al*., 2000). This suggests that chromosomal sites are limited in their ability to expand an amplified array even when the initiating duplication is provided. However, when *lac* is on a conjugative plasmid, both duplication and high-copy-number amplification occur even when no repeated sequences are provided. The propensity of sequences on a conjugative plasmid to amplify has been reported previously (Chandler *et al*., 1979; 1982; Silver *et al*., 1980; Tlsty *et al*., 1984; Peterson and Rownd, 1985a,b; Whoriskey *et al*., 1987). Conversely, the difficulty of achieving high amplification of chromosomal genes has also been demonstrated (Normark *et al*., 1977; Edlund and Normark, 1981). We conclude that the role of the conjugative plasmid in the Cairns experiment is (at least in part) to promote the events that are rare for chromosomal sites – duplication formation and high-level amplification.

According to the amplification mutagenesis model, high-level amplification during growth under selection is an essential intermediate in the process of reversion in the Cairns system. Segregation from the amplified array releases fragments that induce SOS mutagenesis and further contributes to the number of Lac^+ revertants. Amplification precedes every reversion event under selection in both *E. coli* and *Salmonella* (Hendrickson *et al*., 2002). We interpret the results presented here as evidence that chromosomal sites are less prone to duplication and amplification than sites on a conjugative plasmid and, therefore, are less prone to reversion under selection. The difficulty in amplifying chromosomal genes may be because spontaneous duplications in the chromosome are usually extremely large (Anderson and Roth, 1987). The major involvement of RecA in reversion can be understood, as it is required both for formation and segregation of amplifications and for SOS induction (as co-protease for cleaving LexA). In view of these roles and the growth of the reverting cells, it seems unnecessary to postulate that mutations are made during recombination events (Foster, 1999; Bull *et al*., 2000). The role of RecA seems rather to be in allowing duplication, amplification and segregation events and in contributing to induction of the SOS regulon. Reversion during non-selective pregrowth does not involve prior amplification or SOS mutagenesis and, as seen here, is unaffected by the genomic position of the *lac* operon; as expected, it is also unaffected by lack of Rec functions or inability to induce SOS (Slechta *et al*., 2002).

We suggest that the plasmid *tra* functions stimulate the formation of duplications and amplifications by producing DNA ends that recombine illegitimately to generate a duplication and legitimately to stimulate further amplification and segregation of an existing duplication. The proposed role of plasmid transfer replication was suggested earlier (Galitski and Roth, 1995; Radicella *et al*., 1995) and was subsequently confirmed and extended (Foster and Trimarchi, 1995; Peters *et al*., 1996; Godoy and Fox, 2000). A proposal shown in Fig. 6 suggests that transfer replication occurs either within one cell or in the course of a homosexual cell–cell transfer as suggested by Peters *et al*. (1996). In initiating transfer replication, the plasmid is nicked, and a 5′ ended single strand is displaced. This single strand can be copied to generate a double-stranded ended linear molecule. This double-strand end (suggested by the RecBC requirement for reversion) can invade the parental plasmid (either the parent from which it was made or an identical copy in another mutant cell). These F-produced ends may stimulate illegitimate exchanges (which form the initial duplication) as seen in Fig. 6. Once a duplication is present in the plasmid, ends generated in the same way can stimulate unequal homologous recombination events that generate further amplification or segregation. They might even lead to rolling circle replication that would produce a high number of *lac* copies in a short time, the ‘do-loop’ model suggested previously (Roth *et al*., 1996). Thus, according to the amplification model, normal plasmid transfer functions increase the frequency of copy number variants on which selection operates to promote growth and stimulate reversion.

Origin of class IIIA insertions

Phage Mu transposition is prone to produce deletions

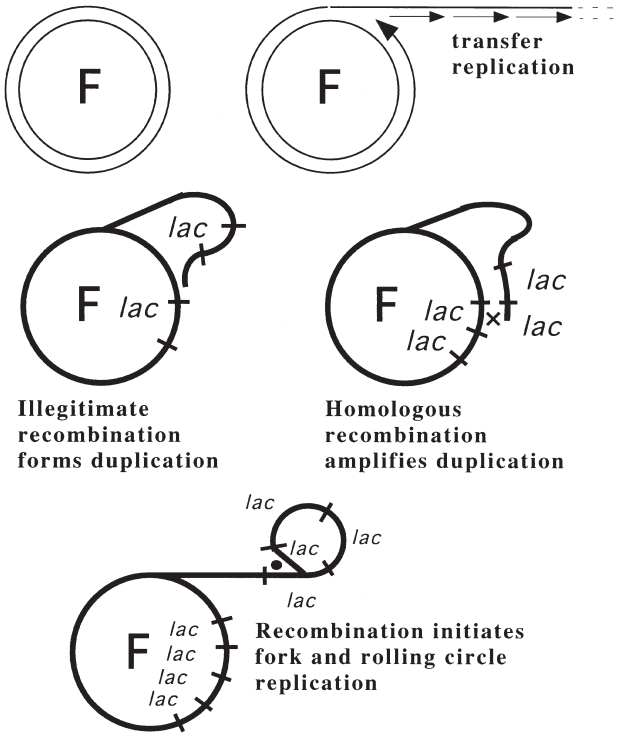

Fig. 6. Transfer replication provides DNA ends. See text for description.

(and presumably also duplications) adjacent to the insertion site. These rearrangements are thought to occur when a conservative transposition from the injected linear molecule is followed by a replicative transposition to a nearby site before phage repression is established (Danielle *et al*., 1972; Cabezon *et al*., 1978; Howe and Schumm, 1981). When a Mu element transposes from a P22-transduced fragment, as did MudCF in our experiments, conservative transposition fails, and replicative transposition from the linear donor molecule breaks the target chromosome, leaving a copy of the Mu element at each of the broken ends (Sonti *et al*., 1993). This break is usually repaired by recombination between the Mu copies but can also be healed by secondary transposition events that generate a deletion or duplication (Sonti *et al*., 1993). We suggest that the preferential targeting of MudCF transposition from a transduced *his* region to distinct sites in the homologous chromosomal *his* region may reflect recombination events between the transduced donor fragment and the chromosome that are initiated before the production of transposase. MudCF may actually transpose from a partially integrated donor fragment and seek targets that are close to the donor site in the chromosome. No transductants with the original donor insertion are recovered because they would be histidine auxotrophs, and all *his* auxotrophs were discarded (to avoid a common recombinant class). The observed duplications in and near *his* cause no histidine requirement.

This explanation leads to the prediction that many simple (class I) insertions should have been found in and near the *his* operon. Presumably, most such strains were discarded because of their auxotrophy or because they carried both the original insertion and a nearby new insertion. We have not tried to identify rare *his* auxotrophs with a secondary simple insertion nearby.

The reversion behaviour of a chromosomal lac *operon in a* mutS *strain*

The unusual *mutS*::MudCF insertion showed enhanced *lac* reversion during pregrowth but very little additional reversion over the course of the selection experiment. This behaviour is expected because the MutS function is known to correct mismatches made during replication and growth, and MutS-deficient strains are mutators. We suggest that cells with a chromosomal *lac* region are impaired for amplification and thus grow very little under selection, explaining why the MutS defect has a small effect on a chromosomal *lac* region under selective conditions. In contrast, the *mutS* mutation studied by Foster and Cairns (1992) was tested in an F′*lac* strain and found to stimulate reversion both during pregrowth and on selective medium. We suggest that the *lac* operon on the F′ plasmid amplified and allowed growth under selection, allowing the *muts* defect to stimulate reversion. As expected for growing cells, the *mutS* lesion enhanced reversion during growth under non-selective (preplating) conditions, regardless of the position of the *lac* operon, because this growth did not require amplification.

Experimental procedures

Strains

All strains are derivatives of *Salmonella enterica* (*Typhimurium*; LT2) and are listed in Table 4. The element Tn*10*dT-POP is a transposition-defective derivative of Tn*10* that directs tetracycline-inducible transcription out of both ends of the element (Rappleye and Roth, 1997). Other transposons are described in the relevant sections below.

Media and chemicals

The minimal medium was NCE salts (Berkowitz *et al*., 1968), containing 0.2% of the appropriate carbon source (glycerol or lactose) plus nutrient supplements at the concentrations recommended by Davis *et al*. (1980). The rich medium was NB (Difco Laboratories) supplemented with 5 g l^{-1} NaCl. Media were solidified with 1.5% BBL agar. Final concentrations of antibiotics in rich media were: 50 μg ml^{-1} kanamycin sulphate (Kn); 20 μg ml^{-1} tetracycline (Tc); 10 μg ml^{-1} chloramphenicol

Table 4. Strain list.

Strain	Genotype	Source
LT2	Wild type	Laboratory collection
TR6611	*ara-9*	= SGSC180
TT2243	*his-712*(Del:DCBHAFIE) *arg-501 ser-821/zzc-65*::Tn*10* (in cryptic plasmid)	Laboratory collection
TT7216	*hisA9944*::MudI	Laboratory collection
TT9540	*nadA56*/F′152 *nadA*$^+$	Laboratory collection
TT10378	LT2/pLP103-22 (Mu A$^+$)	
TT10604	*pro-47*/F′128 *pro*$^+$ *lac*$^+$ *lacY4643*::Tn*10*dCm (LacY$^+$)	Laboratory collection
TT17208	l*euD21*/pSLT$^+$	Laboratory collection
TT17209	*leuD21*/pSLT$^-$	Laboratory collection
TT17551	*hisI10095*::Tn*10*dTc	Laboratory collection
TT18302	*leuD21 proB1657*::Tn*10*/F′ *pro*$^+$ *lacI*^{Q} *lacI33 lacI*Ω*lacZ*	Cairns and Foster (1991)
TT18306	*leuD21 proB1657*::Tn*10 recA1*/F′ *pro*$^+$ *lacI*^{Q} *lacI33* l*acI*Ω*lacZ*	
TT18519	*ara-9 hisC10081*::MudF	Laboratory collection
TT18783	*hisG10175*::Tn*10*dTc	Laboratory collection
TT19199	*ara-9 hisC10081*::MudCF	
TT20360	DUP1731[(*leuA1179*)*MudJ*(*nadC220*)] *recA642*::T-POP1	Laboratory collection
TT20848	*leuD21 proB1657*::Tn*10*/F′ *pro*$^+$ *lacI33*Ω *lacZ lacY4643*::Tn*10*d-Cm	
TT20849	*hisA9944*::MudI *hisC10081*::MudCF	
TT20850	*nadA56*/F′152 *nadA*$^+$ *zzc-9172*::MudCF	
TT20851	*mutS421*::Tn*10*	
TT20853	*ara-9 oadB10*::MudCF Class I	
TT20854	*ara-9 orf-6528*::MudCF Class I	
TT20855	*ara-9 nhaC*::MudCF Class I	
TT20856	*ara-9 dsdC51*::MudCF Class I	
TT20857	*ara-9 fdnG51*::MudCF Class I	
TT20858	*ara-9 orf-2472*::MudCF Class I	
TT20859	*ara-9 yybE*::MudCF Class I	
TT20860	*ara-9 kdpB111*::MudCF Class I	
TT20861	*ara-9 orf-3310*::MudCF Class I	
TT20862	*ara-9 mrcB21*::MudCF Class I	
TT20863	*ara-9 eutJ356*::MudCF Class I	
TT20864	*ara-9 mutS177*::MudCF	
TT20865	*ara-9* DUP1996[(*sbcB*)*MudCF*(*hisD*)] Class IIIA	
TT20866	*ara-9* DUP1997[(*phsC*)*MudCF*(*hisC*)] Class IIIA	
TT20867	*ara-9* DUP1998[(*hisC*)*MudCF*(*hisA*)] Class IIIA	
TT20868	*ara-9* DUP1999[(*hisC*)*MudCF*(*hisA*)] Class IIIA	
TT20869	*ara-9* DUP2000[(*yeeF*)*MudCF*(*hisD*)] Class IIIA	
TT20870	*ara-9* DUP2001[(*orf-3044*)*MudCF*(*hisD*)] Class IIIA	
TT20871	*ara-9* DUP2002[(*pduQ*)*MudCF*(*hisD*)] Class IIIA	
TT20872	*ara-9* DUP2003[(*pduP*)*MudCF*(*hisC*)] Class IIIA	
TT20873	*ara-9* DUP2004[(*pduG*)*MudCF*(*hisC*)] Class IIIA	
TT20874	*ara-9* DUP2005[(*pduW*)*MudCF*(*hisD*)] Class IIIA	
TT20875	*ara-9* DUP2006[(*phsC*)*MudCF*(*hisB*)] Class IIIA	
TT20876	*ara-9* DUP2007[(*hisC*)*MudCF*(*hisH*)] Class IIIA	
TT20877	DUP2008[(*ntrC*)*MudCF*(*orf-3204*)] Class IIIA	
TT20878	DUP2009[(*pduM*)*MudCF*(*hisA*)] Class IIIA	
TT20879	DUP2010[(*hisG*)*MudCF*(*hisF*)] Class IIIA	
TT20880	DUP2011[(*rimK*)*MudCF*(*ybiF*)] Class IIIA	
TT20881	DUP2012[(*yeeF*)*MudCF*(*hisD*)] Class IIIA	
TT20882	*ara-9 dbpA1*::MudCF Class IIIB	
TT20883	*ara-9 ydaO*::MudCF Class IIIB	
TT22995	*ara-9 ogtST11*::MudCF Class IIIB	
TT22996	*ara-9 dbpA2*::MudCF Class IIIB	
TT23006	*nadA56 recA642*::T-POP1/F′152 *nadA*+ *zzc-9172*::MudCF	
TT23007	*ara-9 mutS177*::MudCF *recA642*::T-POP1	
TT23009	*ara-9 zzc-9174*::MudCF Class II	
TT23011	*ara-9 recA642*::T-POP *zzc-9174*::MudCF Class II	
TT23014	*ara-9 recA642*::T-POP *dbpA2*::MudCF Class IIIB	

(Cm). The chromogenic β-galactosidase substrate Xgal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Diagnostic Chemicals) was used at either 25 μg ml^{-1} in minimal media or 40 μg ml^{-1} in NB for the sectoring assay. For the frameshift reversion test, 20 μl of a solution of frameshift mutagen ICR-191 (1 mg ml^{-1}) was spotted in the centre of a plate spread with cells of the strain to be tested. Restriction enzymes were from New England Biolabs. Unless otherwise specified, chemicals were obtained from Sigma Chemical.

Transductional methods

Transductional crosses were mediated by the high-frequency generalized transducing phage P22105-1 (Schmieger, 1971). Transductant clones were purified and freed of remaining phage by single colony isolation on green indicator plates (Chan *et al*., 1972). Phage sensitivity was tested by cross-streaking cells with the clear plaque P22 mutant H5.

Construction of MudCF

The *lac* operon of the strain (FC40) used by Cairns and Foster (1991) was placed within a Mu-derived MudF transposon by a transductional cross. To provide a selective marker for this cross, a *lacY*::Tn*10*dCm insertion (phenotypically LacY$^+$, at the distal end of the *lacY* gene) was introduced into the F′*lac* plasmid carried in *Salmonella* strain TT18302; this cross is illustrated in Fig. 1A. Chloramphenicol-resistant transductants were screened to identify recombinants that retained all the mutations of the recipient *lac* operon. The desired type was Lac$^-$, could be induced to revert by the frameshift mutagen (ICR-191) and gave Lac$^+$ revertants whose β-galactosidase level was not increased by IPTG. The structure of this strain (TT20848) is shown in Fig. 1. Except for chloramphenicol resistance, the growth phenotype and reversion behaviour of the strain carrying the *lacY*::Tn*10*d-Cm insertion (TT20848) was indistinguishable from that of strains with original F′*lac* plasmid (data not shown).

Using the above strain as donor, the mutant *lac* operon was transduced from the F′*lac* plasmid into the chromosomal MudF element (*hisC10081*::MudF) in strain TT18519. The MudF element is a transposition-defective derivative of phage Mu that includes a complete wild-type *lac* operon with a functional *lacI* gene and a normal *lac* control region. Selection was made for Cm resistance, and transductants were screened for relevant mutations described above (see Fig. 1B). A recombinant was saved (TT19199) that carried the three mutations of the original *lac* operon within the Mud transposon. The new transposon is designated MudCF, as its *lac* region is that used by Cairns and Foster (1991).

Transposition of MudCF

Transposition of MudCF was achieved by the method of Hughes and Roth (1988). To apply this method, a transposition-competent Mud element (MudI) in the *hisA* gene (*hisA9944*::MudI; TT7216) was introduced near the *hisC10081*::*MudCF* insertion. MudI is a Mu-derived Amp^R transposon with heat-inducible transposition functions (Hughes and Roth, 1988). A P22 lysate was grown at 30°C on the strain with both insertions (TT20849). Some fragments transduced by this lysate include the MudCF element and the transposase genes of the nearby MudI element. These fragments introduce the MudCF element with a transitory source of transposase that allows (at 37°C) the element to transpose from the linear transduced fragment into random sites on the recipient chromosome. The donated fragment can also recombine with the chromosome to regenerate an auxotrophic insertion in the histidine operon; these auxotrophic recombinants are about half the total Kn^R transductants. To obtain insertions at new sites, a P22 lysate grown on strain TT20849 was used to transduce Kn^R into a recipient strain (TR6611); this cross was performed at 37°C to induce transposase and allow MudCF to transpose from the transduced fragment into the chromosome. All His$^-$ transductants (caused by recombination) were discarded, and 625 independent prototrophic transposition transductants were saved from the initial mutant hunt. Later mutant hunts are described in *Results*.

Testing lac *reversion behaviour of MudCF insertion strains*

Initially, the reversion behaviour of each MudCF strain was tested qualitatively to identify those that showed substantial reversion to Lac$^+$ under selection. Each strain was pregrown overnight in 1.5 ml of NCE with 0.2% glycerol. Cells were pelleted and resuspended in the same volume of NCE medium. Approximately 3×10^8 cells from the strain being tested were plated with a 10-fold excess of Lac$^-$ scavenger cells (LT2 = TR10000) on NCE lactose Xgal medium and incubated for 7 days at 37°C. The scavenger cells are added to consume any residual carbon sources in the agar or excreted by Lac$^+$ revertant colonies. Lac$^+$ revertants on each plate were counted on day 7, and this number was compared with the number of day 7 revertants seen for control strains TT18302 (F′*lac*) and TT19199 (*hisC10081*::MudCF). All MudCF strains that accumulated more revertant colonies than the chromosomal *lac* mutation (and some that did not) were subjected to a more extensive reversion experiment.

In these more extensive tests, 10 independent cultures of each strain were grown in NCE 0.2% glycerol, pelleted and resuspended as described above. The strains being tested (3×10^8 cells) were plated with a 10-fold excess of scavenger cells on NCE lactose Xgal plates. Revertants were counted every day between days 2 and 7. The reversion patterns seen in these experiments confirmed the majority insertion type (class I), which showed little or no reversion under selection. These more extensive tests made it easier to distinguish the two additional insertion types (classes II and III) that showed appreciable enhancement of *lac* reversion under selection.

Scoring unstably Lac$^+$ mutant cells

To identify unstably Lac$^+$ cells in revertant colonies, each revertant colony was removed from the assay plate using the small end of a Pasteur pipette to remove an agar plug containing the entire colony (plugging). These agar plugs were placed in NCE medium with no carbon source, and the cells were removed from the agar plug by 15 s of vortex mixing followed by 15 min standing at room temperature, and then a second 15 s of vortex mixing. The cell suspensions were diluted and plated for single colonies on solid NB medium containing 40 μg ml^{-1} Xgal. The plates were incubated for 2 days at 37°C and for one additional day at 25°C to allow optimum colour development. Colonies were inspected with a dissecting microscope to identify those with blue and white sectors, which indicated instability of the *lac* phenotype. To test recombination dependence of reversion, a *recA* insertion

(*recA642*::T-POP, TT20360) was transduced into each strain, selecting tetracycline (Tc) resistance. These *recA* derivatives were tested for reversion to Lac^+ as described above to evaluate the importance of RecA function for reversion.

Pulsed field gel electrophoresis (PFGE)

Agarose plugs containing whole cells were prepared by standard methods (Bergthorsson and Ochman, 1995). To prepare for restriction digestion, plugs were incubated for 30 min in the digestion buffer appropriate for the enzyme being used. After this equilibration step, the restriction enzyme was added (*Xba*I or *Bln*I, 20 units per reaction), and the plugs were incubated overnight at 37°C. To stop the digestion, plugs were treated with 5 µl of 0.5 M EDTA. Slices of these digested plugs were used for PFGE. In general, the gels were run with pulse times from 30 to 90 s for 24 h at 150 V in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA). Standard gel concentration was 0.9% SeaKem GTG agarose.

Mapping of MudCF insertions

Transductional crosses were used to identify insertions on the pSLT plasmid (class II insertions). Phage P22 lysates grown on MudCF insertion strains were used to transduce kanamycin resistance (Kn^R) into two strains, TT17208 ($pSLT^+$) and TT17209 ($pSLT^-$). A donor insertion on pSLT would form transductants with TT17208, but not with TT17209. A chromosomal insertion could be transduced into both recipients. A lysate grown on strain TT2243 (*zzc-65*::Tn*10*, on pSLT) was used as a control in each cross (data not shown).

Positions of MudCF insertions was determined by sequencing single primer polymerase chain reaction (PCR) products from one or both ends of the MudCF element (Hermann *et al*., 2000). DNA was isolated from insertion strains grown overnight in 1 ml of NB broth. Cells were pelleted and resuspended in 200 µl of Quick DNA buffer (10 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.2% SDS) and incubated for 5 min at 100°C. Debris was removed by centrifugation (10 min at 13 000 r.p.m.), and the supernatants were stored at –20°C. These DNA samples were diluted 100-fold and used in single primer PCR reactions with the following conditions; 20 cycles of <1 s at 94°C, <1 s at 55°C, 1 min at 72°C followed by 30 cycles of <1 s at 94°C, <1 s at 40°C, 1 min at 72°C followed by 30 cycles of <1 s at 94°C, <1 s at 55°C, 1 min at 72°C. PCR primers were TP251 (5′-GCAAGCCCCA CCAAATCTAATCCCA-3′) for priming replication from the *attL* end of MudCF into adjacent chromosomal sequence, and primer TP81 (5′-GAAACGCTTTCGCGTTTTTCGTGCG-3′) was used to initiate outward replication from the *attR* end of MudCF. PCRs were carried out in an Idaho Technologies air cycler. PCR products were treated with single-strand exonuclease (1 µl of a 1 unit μl^{-1} solution of ExoI) for 1 h at 37°C to remove excess primers, purified with a Qiaquick PCR purification kit and sequenced using a nested primer. Nested sequencing primers were TP240 (5′-CCGAATAATCCAAT GTCC-3′) for PCR products made with TP251, and TP79 (5′-GTTTTTCGTGCGCCGCTTC-3′) for PCR products made with TP81.

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