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Selected Lac$^+$ revertants in the Cairns system are initiated by pre-existing cells with multiple copies of the F'*lac* plasmid

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Abstract

The origin of mutations under selection has been intensively studied using the Cairns system, in which cells of an E. coli *lac* mutant are plated on lactose and give rise to 100 Lac^+ revertants over several days. These revertants have been attributed variously to stress-induced mutagenesis of non-growing cells or to selective improvement of pre-existing weakly Lac^+ cells with no mutagenesis. Most revertant colonies (90%) contain stably Lac^+ cells, while others (10%) contain cells with an unstable amplification of the leaky mutant *lac* allele. Evidence is presented that both stable and unstable Lac^+ revertant colonies are initiated by pre-existing cells with multiple copies of the F'*lac* plasmid, which carries the mutant *lac* allele. The tetracycline analogue AnTc inhibits growth of cells with multiple copies of the *tetA* gene. Populations with *tetA* on their F'*lac* plasmid include rare cells with an elevated plasmid copy number and multiple copies of both the *tetA* and *lac* genes. Pre-growth of such populations with AnTc reduces the number of cells with multiple F'*lac* copies and consequently the number of Lac^+ colonies appearing under selection. Revertant yield is restored rapidly by a few generations of growth without AnTc. We suggest that pre-existing cells with multiple F'*lac* copies divide very little under selection but have enough energy to replicate their F'*lac* plasmids repeatedly until reversion initiates a stable Lac^+ colony. Pre-existing cells whose high-copy plasmid includes an internal *lac* duplication grow under selection and produce an unstable Lac^+ colony. In this model, all revertant colonies are initiated by pre-existing cells and cannot be stress-induced.

Introduction

Knowing the source of mutants that arise under selective conditions is critical to understanding evolution and therefore the origins of cancer and the progress of infectious disease. A genetic system to address this problem was designed by Cairns and Foster (CAIRNS and FOSTER 1991; CAIRNS *et al.* 1988). In this system, a population of bacterial tester cells carries a leaky *lac* frameshift mutation on a low-copy F'*lac* plasmid. Following pre-growth under permissive conditions in liquid medium, 10^8 of these cells are plated on selective medium containing lactose as sole carbon source. Over a week, one hundred Lac$^+$ revertant colonies accumulate above the non-growing plated population. These colonies are of two types. One includes stable Lac$^+$ mutant cells whose mutant *lac* allele has been corrected by a compensating frameshift mutation (FOSTER and TRIMARCHI 1994; ROSENBERG *et al.* 1994). The other includes cells with multiple tandem copies of the original leaky mutant allele (ANDERSSON *et al.* 1998). Two general models have been proposed to explain the origin of these colonies. The evidence presented below suggests some problems with both models.

Stress-induced mutagenesis in non-growing cells. The first explanations of the Cairns system proposed that Lac$^+$ revertants are generated by an evolved mechanism that senses growth cessation (stress) and creates mutations in hopes of genetically resolving the physiological limitation (CAIRNS and FOSTER 1991; CAIRNS *et al.* 1988; TORKELSON *et al.* 1997). The reversion rate of the *lac* mutation during non-selective growth is 10^{-8}/cell/division. The population of 10^8 cells plated on lactose does not grow but gives rise to roughly 100 Lac$^+$ revertant colonies over 6 days. The number of revertants could be explained if the 10^8 plated cells experienced a 100-fold increase in genome-wide frameshift mutation rate (assuming one division per week). This simple explanation was ruled out because non-revertant starved cells in the lawn show very little evidence of chromosomal mutagenesis (FOSTER 1994; TORKELSON *et al.* 1997). The absence of new mutations in the lawn has been explained in two ways – "directed mutation" and "hypermutable states."

"Directed mutation" suggests that induced mutagenesis is focused preferentially on the *lac* region (FOSTER and CAIRNS 1992). This could explain why so few mutations are seen in the genome at large. Direction of mutations to functionally relevant targets is mechanistically difficult to imagine (STAHL 1988), but it seems clear that the number of Lac$^+$ revertants under

selection increases much more than the number of associated mutations (TORKELSON *et al.* 1997). Furthermore most of the recovered Lac$^+$ revertants show little or no general mutagenesis (ROSCHE and FOSTER 1999). It seems likely that if any mutagenesis occurs in this system, it is somehow focused preferentially on the F'*lac* plasmid, which carries the *lac* mutation that is under selection (FOSTER 1997).

"Hypermutable states" suggests that starvation causes an increase in the genome-wide mutation rate but only affects about 1 in a 1000 of the starved population (10^5 of the plated 10^8 cells) (HALL 1990; TORKELSON *et al.* 1997). Focusing general mutagenesis on a few cells (instead of a small genomic region), can also explain why unselected mutants were hard to detect in the starved population as a whole. If all *lac* revertants were caused by genome-wide mutagenesis of a small subset of the population, the general rate of frameshift mutations (which correct *lac*) in the chosen cells would need to increase 10^5-fold over that seen in growing cells. That is, about 100 *lac* reversion events would have to occur in the mutagenized sub-population (10^5 cells) in one division – a rate of 10^{-3}/cell/division. This rate would be a 10^5-fold increase over that measured during non-selective growth (10^{-8}/cell/division). Increasing the genome-wide mutation rate 10^5-fold seems impossibly costly in associated lethal mutations (ROTH 2006). Not surprisingly, demonstrable genome-wide mutagenesis (a 200-fold rate increase) is only experienced by about 10% of Lac$^+$ revertants, while 90% of revertants appear to form without such general mutagenesis (ROSCHE and FOSTER 1999).

Thus the Cairns system shows very little evidence of an increase in general mutation rate. The mutagenesis seen in a few revertants has been attributed to the error-prone polymerase DinB, whose removal reduces over-all revertant number only about 4-fold (MCKENZIE *et al.* 2001). When DinB is eliminated, the number of stable revertants drops about 10-fold, while unstable revertant number is unaffected. Without DinB, the residual number of *lac*$^+$ revertant colonies is equally distributed between stable and unstable types. The contribution of DinB to the number of stable revertants and to associated chromosomal mutations requires that the *dinB* gene be located on the same F' plasmid that carries the mutant *lac* allele (ROUSH *et al.* unpublished; SLECHTA *et al.* 2003). This requirement is consistent with the possibility suggested below that general mutagenesis requires co-amplification of *dinB* with *lac* as described below.

Selective amplification in growing cells. If growth limitation acts purely as a selective agent, he behavior of the Cairns system can be explained with no increase in mutation rate. That is, partially revertant cells can arise at standard rates during growth prior to selection and grow slowly after plating on lactose medium. Cells within these clones can improve their growth rate by acquiring common mutations at unenhanced rates. That is, the number of mutation targets within a clone may increase by growth and amplification while the mutation rate per act of replication remains unchanged. Initially, the critical pre-existing cells were thought to carry a common duplication of the leaky *lac* allele (ANDERSSON *et al.* 1998). Under selection, these cells were thought to grow and improve by further amplification steps, which occur at a rate of 0.01/cell/division (REAMS *et al.* 2010). Each colony growing with an amplified *lac* allele has an exponentially increasing opportunity to acquire a spontaneous *lac* frameshift mutation -- more *lac* copies/cell and more cells/colony (HENDRICKSON *et al.* 2002). The yield of revertant cells under selection is thus stimulated by increases in target copy number within colonies rather than an increase in mutation rate/target. When a frameshift mutation generates a *lac*$^+$ allele, growth improves, allowing loss of amplified non-revertant *lac* alleles and overgrowth of the revertant colony by haploid *lac*$^+$ cells.

There is considerable evidence that this amplification-under-selection model operates in many situations (ELDE *et al.* 2012; NÄSVALL *et al.* 2012; PRANTING and ANDERSSON 2011; QUINONES-SOTO *et al.* 2012; ROTH 2011), including the Salmonella version of the Cairns system. Amplification-under-selection can explain the selective evolution of new genes (BERGTHORSSON *et al.* 2007), a process that has been experimentally demonstrated (NÄSVALL *et al.* 2012). However, the Cairns system of *E. coli* shows so little growth that it has been hard to directly demonstrate selective improvement and conclusively eliminate stress-induced mutagenesis.

To help decide this issue, a fundamental difference between induced mutagenesis and selection-only models is tested here. Stress-induced mutagenesis models propose that growth limitation induces mutagenesis which produces new mutants that initiate colonies appearing under selection. In contrast, selective improvement models propose that revertant colonies are initiated by cells that arise during non-selective pre-growth. These cells arise before exposure to selective conditions. Under selection, these pre-existing cells develop into visible colonies with no change in mutation rate. It is already clear that the unstable Lac$^+$ revertants are initiated by cells formed before selection. Short duplications that underlie the selected *lac* amplifications are

found in the unselected pre-growth culture at sufficient frequency to explain unstable revertants (REAMS *et al.* 2012). In a few cases, particular amplification junction sequences found in unstable revertants have been directly associated with duplication junctions present in the pre-growth population (KUGELBERG *et al.* 2006). It is not known whether the stable Lac$^+$ revertant clones are also initiated prior to exposure to selective conditions.

Are all revertants initiated prior to exposure to selection? So far, the strongest support for initiation of *lac* revertants during selection (regardless of mechanism) is the demonstration that revertant number is not subject to Luria-Delbrück fluctuation (CAIRNS and FOSTER 1991). These experiments are very clear and have been repeated in our lab with the same results (Emiko Sano, unpublished results). Luria and Delbrück used this test to demonstrate that mutants detected by stringent bacterial laboratory selections arise prior to plating. In their classic experiments, the frequency of selectively detected mutants varied from one parallel culture to the next due to chance timing in the occurrence of the first mutation. This frequency fluctuation showed that selection detects pre-existing mutants and does not stimulate their formation (LURIA and DELBRUCK 1943). In the Cairns system, the failure to see fluctuation was reasonably interpreted as evidence that in this system, unlike standard lab selections, new mutants do not pre-exist but are initiated only after cells are immobilized on the selection plate. Such mutants could, at least in principle, be caused by "stress-induced mutagenesis." Alternatively, the absence of fluctuation could reflect a problem detecting the responsible pre-existing mutants. For reasons described below, duplications and amplifications cannot be detected by a fluctuation test. This calls into question the strongest previous evidence for stress-induced mutagenesis.

Gene duplications are carried at a steady state frequency during non-selective growth (REAMS *et al.* 2010). This steady state results from a balance between a high formation rate, on one hand, and an even higher loss rate and fitness costs (on the other). The forces that dictate this steady state obscure frequency fluctuations due to timing of mutation events. That is, these forces bring back to steady state any high or low duplication frequency caused by early or late duplication events. Fluctuation tests cannot reveal whether or not revertants are initiated by duplications or amplifications that arose prior to plating. If copy number variants initiate *lac* revertants in the Cairns system, some other test is required to determine whether or not these cells arise prior to plating on selective medium.

Evidence reported here suggests that Lac+ revertant colonies are initiated by pre-existing cells with multiple copies of the whole F'*lac* plasmid. Reducing the number of such cells in the pre-growth population reduces the number of both stable and unstable Lac+ revertant colonies appearing under selection. It was initially proposed that the pre-existing *lac* gene copy number increases were tandem duplications of the local *lac* region of the plasmid (ANDERSSON *et al.* 1998; HENDRICKSON *et al.* 2002; KUGELBERG *et al.* 2006). Contrary to this expectation, the critical cells seem to have more copies of the entire F'*lac* plasmid. This finding directs attention to the biology of the F' plasmid as a way to explain behavior of the Cairns system. Early experiments on the Cairns system showed that appearance of revertants under selection depends on the DNA replication origin specific to conjugative transfer (GALITSKI and ROTH 1995; GODOY and FOX 2000; PETERS *et al.* 1996; RADICELLA *et al.* 1995). This suggested that mutants might arise during conjugative mating, but direct tests showed that the frequency of whole plasmid transfer is low (FOSTER and TRIMARCHI 1995a; FOSTER and TRIMARCHI 1995b; MAISNIER-PATIN unpublished results). The importance of the transfer replication origin has not been explained. We propose that transfer replication produces cells with multiple plasmid copies (a form of amplification). Under selective conditions, these cells have insufficient energy to initiate chromosome replication, but they can replicate their plasmid copies repeatedly until a mutation arises. In this model, F'*lac* plasmid over-replication, rather than an increased mutation rate may be responsible for the mutations that restore ability to use lactose.

Materials and Methods

Bacterial strains and plasmids. All experiments were done with *E. coli* K12 strains whose genotypes is Table 1. *Salmonella typhimurium* LT2 strains and plasmids used in this study are also described in Table 1.

Table 1 - Bacterial strains and plasmids

Strain Numbers	Genotypes/Description	Reference/Source
<u>*E. coli* K12</u>		
TR7177	*ara thiA* Δ(*lac-proB*)$_{XIII}$ /F'$_{128}$ *proAB*+ Δ(*lacI-Z*), same as FC29	(CAIRNS and FOSTER 1991)
TR7178	*ara thiA* rif^R Δ(*lac-proB*) $_{XIII}$ / F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω), same as FC40	(CAIRNS and FOSTER 1991)

TT26180	F- *ara thiA* rif^R Δ(*lac-proB*) $_{XIII}$, same as FC36	(CAIRNS and FOSTER 1991)
TT26323	*ara thiA* rif^R Δ(*lac-proB*) $_{XIII}$ / F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω) *yebB11*::Tn*10*dTc	this study
TT26324	*ara thiA* rif^R Δ(*lac-proB*) $_{XIII}$ / F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω) *yagU211*::Tn*10*dTc	this study
TT26325	*ara thiA* rif^R Δ(*lac-proB*) $_{XIII}$ / F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω) *ychA11*::Tn*10*dTc	this study
TT26326	*ara thiA* rif^R Δ(*lac-proB*) $_{XIII}$ / F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω) *tnpR11*::Tn*10*dTc	this study
TT26327	ara thiA rif^R Δ(lac-proB) $_{XIII}$ / F'$_{128}$ proAB+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω) *lacA4659*::Tn*10*dTc	this study
TT26328	*ara thiA* rif^R Δ(*lac-proB*) $_{XIII}$ *btuR482*::Tn*10*dTc/ F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω)	this study
TT26900	FC40/ pSMP71 (pOU71::*tetRA-gfp-cat*)	this study
TT26905	ara *thiA* rif^R Δ(*lac-proB*) $_{XIII}$ / F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω) *zzf-1831*(*mhpC*)::Tn*10*dTc-*gfp-cat yebB*11::Tn*10*dTc	this study
TT26911	FC40/ pSMP73 (pGB2:: *tetRA-gfp-cat*)	this study
TT26916	*ara thiA* rif^R Δ(*lac-proB*) $_{XIII}$ / F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω) *zzf-1831*(*mhpC*)::Tn*10*dTc-*gfp-cat*	Lab. collection
TT26932*	*ara thiA* rif^R Δ(*lac-proB*) $_{XIII}$ / F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω) *yebB11*::Tn*10*dTc	this study
TT26933*	*ara thiA* rif^R Δ(lac-proB) $_{XIII}$ / F'$_{128}$ proAB+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω) *lacA4659*::Tn*10*dTc	this study
TT26935	ara *thiA* rif^R Δ(*lac-proB*) $_{XIII}$ / F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω) *lacA*4659::Tn*10*dTc *yebB*11::Tn*10*dTc	this study
<u>*S. typhimurium* LT2</u>		
TT18784	*hisG10175*::Tn*10*dTc *hisC9955*::MudJ	Lab. Collection
TT25387	*leuD21 proAB670*::Spec	Lab. Collection
TT26310	*leuD21 proAB670*::cam *recA651*::rif/ F'$_{128}$ *proAB*+ l*acI*^{q} *lacI33*(fs) *lacIZ*(Ω)	Lab. Collection
TT26311	*leuD21 proAB670*::cam *recA651*::rif/ F'$_{128}$ *proAB*+ *lacI*^{q} *lacI33*(fs) *lacIZ*(Ω)/ pNK2881	this study
<u>Plasmid</u>		
pNK2881	Tn*10* transposase under *tac* promoter, Ap^R	(WAY *et al.* 1984)
pGB2	pSC101 origin, Sp^R	(CHURCHWARD *et al.* 1984)
pOU71	Temperature-dependent R1 origin, Ap^R	(LARSEN *et al.* 1984)
pSMP71	pOU71::*tetRA-gfp-cat*, Ap^R, Tc^R, Cm^R	this study
pSMP73	pGB2::*tetRA-gfp-cat* , Sp^R, Tc^R, Cm^R	this Study

* reconstructed duplicates

Growth media and chemicals. Minimum medium was no-citrate E (NCE) medium described previously (BERKOWITZ *et al.* 1968). Rich medium was Luria-Bertani (LB) or nutrient broth (NB). Antibiotics were added at the following concentrations: tetracycline, 20 μg/ml; and

chloramphenicol, 20 μg/ml. Anhydrotetracycline (AnTc) was used at concentrations described in the text. Plates were solidified with 1.5% agar. Non-selective NCE medium was supplemented with 0.2% glycerol, thiamine and other nutrients at concentrations described previously (DAVIS 1980). For lactose selection plates, NCE agar was supplemented with 0.1% lactose and thiamine. The chromogenic β-galactosidase substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was used at a concentration of 25 μg/ml (minimal medium) or 40 μg/ml (rich medium).

Growth rates. Growth of the strains with and without AnTc was measured in a 96-well plate using a Synergy HT Multi-Detection Microplate Reader (BioTek Instruments). Each strain was grown to saturation in 2.0-mL NCE with 0.2% glycerol and thiamine. To determine growth rates, an aliquot of 0.002-mL from each overnight culture was dispensed into 0.2-mL NCE glycerol (thiamine) contained in a well of the 96-well microplate. Cells were placed in the microplate reader for incubation at 37°C for 20 hours. The microplate reader measured the optical density of the cell cultures at a 650 nm wavelength every 15 minutes. Doubling times (Dt) were calculated using equation [1]; where (N_1) is the number of cells at the initial time point (t_1) and (N_2) is the number of cells at final time point (t_2).

Equation [1] $$Dt = \frac{t_2 - t_1}{3.3 (\log N_2 - \log N_1)}$$

Competitions. Each strain was grown to saturation in 2.0-mL NCE with 0.2% glycerol and thiamine. After overnight growth, cells were washed in NCE salts. The two competing strains were inoculated at an approximate 1:1 ratio in 10mL NCE glycerol (thiamine) medium with or without AnTc. After 24h of growth, cultures were diluted 1:1000 into fresh medium and allowed to compete for an additional 24h. Samples were taken at 0, 24, and 48h. Three independent replicates were performed for each competition. Viable cell titer was determined on NB agar plates and NB agar plates containing tetracycline.

Reversion tests. All reversion experiments were performed as previously described (ANDERSSON *et al.* 1998). One day prior to plating the tester cells, lactose minimum selection plates were seeded with approximately $2x10^9$ Lac^- cells (scavenger strain TR7177). All tester cultures were

inoculated with cells from an independent single colony. All pre-selection cultures were grown to saturation at 37C in glycerol minimal medium with aeration by shaking. Each reversion experiment was done with at least five replicates for each strain tested. When used, AnTc (216nM) was included in the pre-growth medium. Cells were sedimented and resuspended twice in NCE minimal medium prior to plating. This protocol was used for all reversion assays performed.

Construction of random Tn*10*dTc insertional mutants on F'$_{128}$. Generalized transduction with P22 was used for random mutagenesis. The P22 lysate used for delivery of Tn*10*dTc was obtained by growing P22 (mutant HT105/1 *int-201*) on a *Salmonella* strain carrying a defective Tn*10* transposon (lacking transposase function). The F'$_{128}$ strain TT26311 used as recipient was first transformed with the plasmid pNK2881, which carries the Tn*10* transposase gene under control of the *tac* promoter (KLECKNER *et al.* 1991; WAY *et al.* 1984). The resulting tetracycline resistant transductants were pooled and conjugated to a F$^-$ strain of Salmonella (TT25387) to screen for transposition events that specifically occurred on F'$_{128}$. Subsequent crosses consisted of a generalized transduction by P22 to move each Tn*10*dTc element to a new F'$_{128}$ that had not been subjected to transposition events. Finally the whole plasmid was conjugated to a F- strain of *E. coli* (TT26180). The insertion points of Tn*10*dTc were determined by direct sequencing of a single-primer PCR product from one end of the Tn*10*dTc element using TP93 (5'-ACCTTTGGTCACCAACGCTTTTCC-3'). Sequencing primer was TP133 (5'-CAAGATGTGTATCCACCTTAACTTAATG-3').

Cloning of the "*tetRA-gfp-cat*" cassette and determination of copy number. Plasmid pOU71 (Larsen et al., 1994) and pGB2 (Churchward et al., 1984) were cleaved at a unique restriction site with the restriction enzymes *EcoR*I and *Sma*I respectively. The linearized plasmids were made blunt-ended with Klenow enzyme (NEB Biolabs) and dephosphorylated by calf intestinal alkaline phosphatase (NEB Biolabs), prior to purification with the Qiaquick PCR Purification Kit. The cleaved plasmids were then ligated with a "*tetRA-gfp-cat*" DNA fragment, which was amplified by PCR from template DNA prepared from strain TT26916. This strain carries an engineered mini-Tn*10*dTc transposon where *gfp* and *cat* genes have been inserted at the 3' end Tn*10*dTc (Sun et al., 2009). The primers used were TP2889 (5'-TTAAGACCCACTTTCACATTT-3', binds end of *tetR*)

and TP2891 (5'- GTCATTTCTGCCATTCATCC-3', binds downstream of the *cat* gene). Prior to ligation the PCR fragment were purified using the Qiaquick PCR Purification Kit (Qiagen) and phosphorylated using T4 kinase (NEB Biolabs). All Cm^R transformants were also tet^R and the new plasmids (pSMP71 and pSMP73) purified using a Qiagen plasmid prep kit were introduced by transformation into TR7178. The relative copy number of the "*tetRA-gfp-cat*" cassette was determined by quantitative PCR using an Applied Biosystems 7900HT real-time PCR platform. The reactions were performed using SYBR Green PCR Master Mix (Applied Biosystems) and primers TP2874 (5'- CTTTCCGTCCGTTTCATCAC -3') and TP2875 (5'- TTCTTTCCACTGCGGGTTAG-3') to amplify 150bp of *pck* used as the reference chromosomal gene and primers TP2829 (5'- GACGGTGAGCTGGTGATATG -3') and TP2830 (5'- CGGAAATCGTCGTGGTATTC-3') to amplify 107bp of the *cat* gene located on the plasmid.

Results

Inhibiting growth of cells that carry a gene amplification. The *tetA* gene of transposon Tn*10* encodes an efflux pump whose expression provides resistance to the antibiotic tetracycline (Tet). Over-expression of this pump inhibits cell growth (ECKERT and BECK 1989; MOYED *et al.* 1983), perhaps by compromising proton motive force. Because of this inhibition, cells with multiple (5-10) copies of *tetA* are less resistant to tetracycline than cells with one copy (COLEMAN *et al.* 1983). Even cells with a single induced copy of *tetA* can be counter-selected, but this requires the presence of lipophilic metal chelators, which allow even low levels of TetA to compromise membrane integrity perhaps by extracting metal ions (BOCHNER *et al.* 1980). In the absence of these chelators, the non-toxic analogues that induce *tetA* expression do not impair growth of cells with a single Tn*10* copy, but can inhibit cells that over-express TetA from many gene copies.

Here *lac* tester strains with *tetA* near *lac* on the F'*lac* plasmid are pre-grown with anhydrotetracycline (AnTc), which induces expression of *tetA,* but is not directly toxic to cells (ECKERT and BECK 1989; MOYED *et al.* 1983). Induction of *tetA* preferentially inhibits growth of cells with multiple copies of the Tn*10* element. When the amplified region includes both *tetA* and the *lac* genes, growth with the inducer reduces the frequency of cells with many *lac* copies. These inducers were use to test the importance of *lac* gene amplification for reversion in the Cairns system (HENDRICKSON *et al.* 2002; STUMPF *et al.* 2007).

In the previous tests, Tn*10* (including *tetA*) was inserted at various positions on the F'*lac* plasmid and an inducer of *tetA* (chlortetracycline, ChTc) was included in the selective plate. Presence of ChTc reduced revertant yield if the *tetA* gene was located near *lac*, but (in our hands) caused a progressively smaller reduction when *tetA* was inserted farther from *lac* on the same plasmid (HENDRICKSON *et al.* 2002). These results were interpreted as evidence that the process of *lac* reversion on the selective plate included amplification of a small region of the F'*lac* plasmid that included both *tetA* and *lac.* Such amplifications of *lac* were thought to improve growth on lactose, as suggested by the amplification-selection model described above. Cells in which *tetA* was co-amplified with *lac* became sensitive to inhibition by the analogue, which reduced the number of revertant colonies. The reduction was greatest when *tet* was placed near *lac* on the plasmid, favoring co-amplification. This result supported the initial selection model in which selective amplification of *lac* under selection permitted point mutations and stable Lac^+ revertants. Stumpf and Foster later confirmed that ChTc in the selection medium caused a drop

in revertant yield, but did not confirm the effect of *tetA* position *vis a vis lac*. They concluded that local *lac* amplifications within the F' plasmid did not contribute to origin of revertants on the plate (STUMPF *et al.* 2007).

In revisiting these experiments, we found that results are sensitive to slight variations in protocol that we have not been able to control. Especially important is how cells are handled during the pre-growth period and whether pre-growth cultures enter stationary phase. Some experiments duplicated our previous results and some duplicated those of Stumpf and Foster and some differed from both. It seems possible that both published results are correct under some conditions that we cannot yet duplicate reliably. We suspect that part of the problem is that when the inducer ChTc is present in the selective lactose medium, cells face two opposite selections -- for and against amplification. That is, amplification of the *lac tetA* region favors growth on lactose by giving more copies of the limiting *lac* allele, but the same amplification increases cell sensitivity to ChTc by producing more of the toxic TetA protein. The balance of these opposite selections may make the results sensitive to slight variations in conditions. In the experiments reported here, cells are exposed to the *tetA* inducer (AnTc) during non-selective growth prior to plating on lactose. Results are more robust when cells are exposed to a single selection. The presence of AnTc in the pre-growth culture removes cells with multiple *lac tet* copies prior to plating.

We used this induce to test whether pre-existing cells with multiple copies of the *lac* region are critical for initiating revertant colonies. Before exposure to the lactose selection, cultures were grown with AnTc to reduce the number of cells with many copies of *tetA* (and co-amplified *lac*). Cultures grown in this way showed fewer Lac^+ revertants after plating on selective medium. We expected decreases in revertant number only in strains with Tn*10*dTc near *lac* as seen previously (HENDRICKSON *et al.* 2002), but instead we observed a drop in revertant number regardless of the relative positions of *lac* and Tn*10*dTc in the plasmid. This agrees with observations of Stumpf and Foster (STUMPF *et al.* 2007) and suggests that the pre-existing *lac* amplification critical to revertant initiation is provided by increased copy number of the entire F'*lac* plasmid .

Copy number variation in bacterial populations. The frequency of cells with a duplication comes to a high steady state (10^{-4}) in an unselected population (REAMS *et al.* 2010). The process of tandem duplication and amplification is diagrammed in Figure 1.

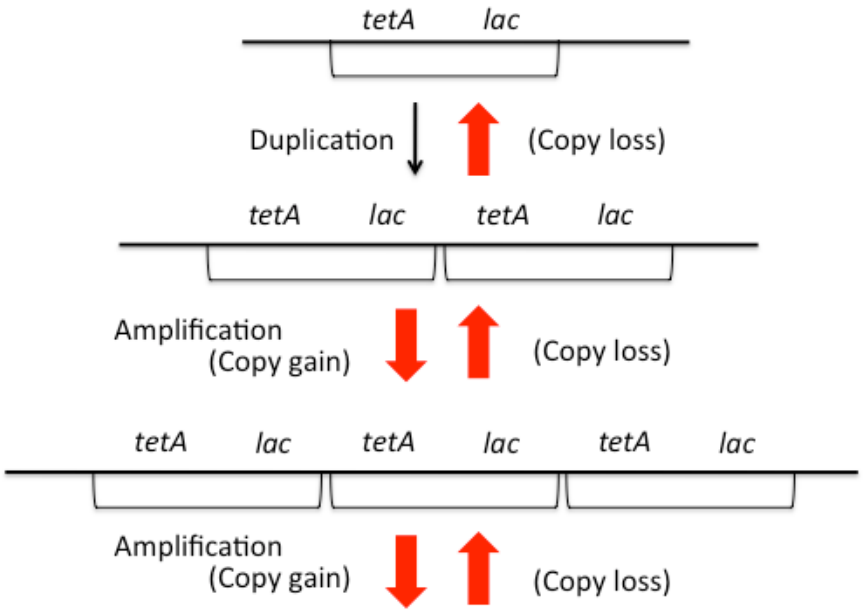

Figure 1 The process of gene amplification by tandem duplication. The above sequence of events is essentially a random walk with two boundary conditions – amplification initiation is limited by a low initial duplication rate and expansion is limited by increasing fitness cost and copy loss rate in higher amplifications. Only single copy changes are depicted, but exchanges between more distant copies can cause larger copy number shifts. Each amplification and loss step (red arrows) depends on recombination between a pair of long repeats. These recombination-dependent events are expected to occur at the same rate, which is higher than that of RecA-independent initial duplication formation (black arrow). The whole system (frequency of all copy number variants) comes to a steady state as a unit due to the high loss rate and increasing fitness cost (MAISNIER-PATIN *et al.* unpublished results; REAMS *et al.* 2010). Fitness cost can be offset (and steady state frequencies can rise) when growth is limited by the copy number of gene within the amplified region.

The frequency of duplications comes to a steady state due to a balance between the initial duplication formation rate and the higher loss rate combined with the fitness cost of the duplication. This has been demonstrated experimentally and modeled mathematically (REAMS *et al.* 2010). Each further increase in copy number is expected to occur at a fixed rate similar to that of duplication loss. Higher amplification is expected to cause higher fitness cost. As a result, the

entire system of copy number variants comes to a steady state with higher copy number variants present at lower steady-state frequencies. The steady state frequency of higher copy number variants has been modeled mathematically and demonstrated experimentally (S Maisnier-Patin, M. Savageau and J.R. Roth unpublished). Thus, growth of rare high copy-number variants is expected to be inhibited by AnTc even in a population whose overall growth rate is not strongly affected. In effect, the analogue gives a higher fitness cost to those cells with a sufficiently high copy number of any region that includes *tetA*, causing a decrease in their steady state frequency, but having very little effect on the frequency of cells with lower copy number.

The same steady state considerations are expected to apply to any system of highly reversible variants in *lac* copy number. This includes not only the tandem amplifications described above, but also cells with differences in plasmid copy number, plasmid multimer size (PINKEL *et al.* 1998; PROJAN *et al.* 1983) or rolling-circle replication (COHEN and CLARK 1986; MANN and SLAUCH 1997). That is, steady state frequencies of copy number variants are expected whenever the array of copy number variants expands and contracts at characteristic fixed rates and causes characteristic fitness costs.

The effect of AnTc on growth of cells with Tn*10*dTc (*tetA*). A normally-regulated single *tetA* gene has very little effect on fitness in the absence of induction (NGUYEN *et al.* 1989). Over-expression of multiple *tetA* copies from a foreign promoter can be lethal (ECKERT and BECK 1989), but induced expression of multiple *tetA* copies by tetracycline or its analogues seems to inhibit growth without killing (MOYED *et al.* 1983). A demonstration of this sensitivity is in Figure 2, which shows sensitivity to AnTc of an *E. coli* strain carrying an R1 plasmid with a temperature-sensitive copy number control. The strain described was grown at 39 ^{o}C where it showed a *tetA* gene copy number of 15, measured by quantitative PCR. The same strain is resistant to AnTc at 37^{o}C when its copy number is about 3. We estimate that 10 copies of *tetA* are needed for the analogue anhydrotetracycline (AnTc) to completely inhibit cell growth in the minimal glycerol medium used here.

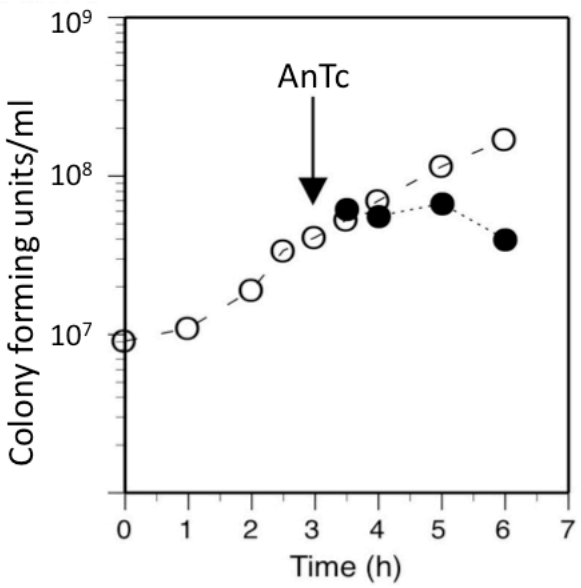

Figure 2 Inhibition by AnTc of growth of a strain with high *tetA* copy number.
The strain used (TT26900) is a derivative of the standard reversion tester FC40 (TR7178) carrying a non-conjugative R1 plasmid having a tetracycline resistance cassette cloned from Tn*10*. The cassette includes the *tetR tetA* genes and their regulatory sequences. Cells were grown in NCE glycerol (0.1%), open circles, with anhydrotetracycline (AnTc) added at 216nM when indicated (filled circles). Samples were withdrawn diluted and plated on LB medium.

Thus the bulk of cells in a population with Tn*10*dTc on the F' *lac* plasmid may not be sensitive to AnTc, but rare cells with more copies of the plasmid or its *tetA* region will be inhibited. When Tn*10*dTc (with *tetA)* is inserted near *lac,* the steady state frequency of cells with a duplication of the *tetA – lac* region is about 10^{-4}, based on a trapping assay that detects any cell with two or more heritable copies of *lac* (REAMS *et al.* 2010). Since the F'_{128} *lac* plasmid is normally carried at 1- 2 copies per cell, duplication-bearing cells are likely to have about 4 copies of *lac* and *tetA.* Cells with a plasmid dimer may also have about 4 copies of this region if their copy number is similarly controlled. Alternatively, if plasmid copy number control breaks down or conjugative plasmid replication switches to rolling circle replication, occasional cells might

have multiple copies of the entire plasmid. Any of these situations are likely to be unstable and subject to the shifts in copy number shown in Figure 1. Since so few cells are expected to be sensitive to growth inhibition, one does not expect a major effect of AnTc on overall strain growth rate. However the steady state plasmid copy number will be affected since impaired copy number variants are continuously replaced at a high rate. Growth rates are presented in Figure 3 below.

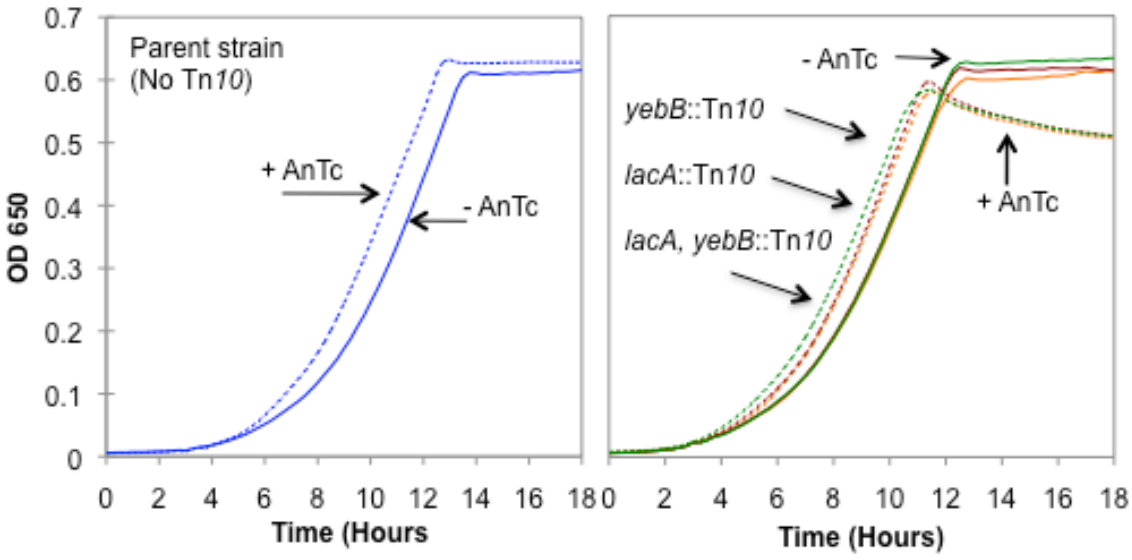

Figure 3 Effect of AnTc on growth of strains with Tn*10*dTc on the F' plasmid.

The strain without Tn*10* (at left) is the standard tester strain (TR7178). Strains with Tn*10*dTc (right) have an insertion either in *lac* (TT26933), in the *yebB* gene, far from *lac* (TT26932) or at both positions (TT26935). Growth was measured in minimal NCE medium with glycerol using an automated plate reader.

As expected, AnTc in the medium had very little effect on the overall doubling time whether or not Tn*10* is present in the plasmid. While growth rates of most cells are insensitive to inhibition by AnTc, one expects that growth of rare cells with more *tetA* copies would be inhibited. This inhibition is expected to remove cells with high *tetA* copy number from the equilibrium system diagrammed in Figure 1. Growth results are presented in Figure 3, where culture OD_{650} is plotted linearly to make differences in final density more obvious. The growth rates are indistinguishable during exponential phase, but strains with Tn*10*dTc on the F'*lac* showed slightly decreased number of cells (OD_{650}) at stationary phase following growth with AnTc. This drop was also seen in viable cell assays. It should be noted the Tn*10*dTc-containing strains grown with AnTc reached a maximum cell density earlier and their cell number (and viable counts) dropped slightly with time in stationary phase. Results similar to these were seen for several different single and double Tn*10*dTc insertions at various positions in the F' plasmid. Strains with two Tn*10*dTc insertions in F'*lac* did not show a significant increase in their sensitivity to AnTc when tested directly.

Growth competitions allowed better visualization of small effects of AnTc. Strains with and without Tn*10*dTc in their F'*lac* plasmid were grown together for two passages. (See Figure 4.) Cultures were inoculated with a 1:1 mixture of the two strains, grown in minimal glycerol medium with or without AnTc and then diluted 1000-fold in the same medium at each passage. Strains with Tn*10*dTc on the F' plasmid showed a competitive disadvantage when grown in the presence of AnTc over two cycles. The competitive disadvantage could be due either to slightly slower overall growth rate, to occasional cell death or to slower emergence from stationary phase between cycles. The strain with two Tn*10*dTc insertions in F seemed to show a slightly clearer competitive disadvantage, an effect noted for other strains.

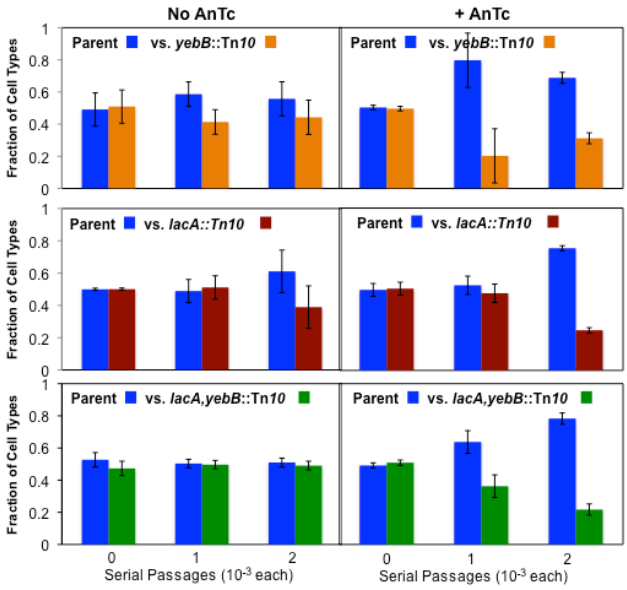

Figure 4. Competition between strains with and without Tn*10*dTc. To better observe effects of AnTc on growth, pairs of strains with and without a Tn*10*dTc insertion in F'*lac* were mixed and grown together in minimal glycerol medium with or without AnTc. The cultures were grown for two passages and the ratio of cells with and without Tn*10*dTc was scored by plating diluted cultures on rich medium with and without tetracycline. Strains: parent with no *Tn10* (TR7178); one insertion *yebB*::Tn*10*dTc (TT26932); one insertion of Tn*10*dTc in *lacA* (TT26933); and two Tn*10*dTc insertions, one in *lacA* and the other in *yebB*::Tn*10*dTc (TT26935).

One of the two Tn*10*dTc insertions used here (*lacA*) is very close to *lacZ* about 1kb away from *lac*Z, while the other (*yebB*) is at the opposite side of the plasmid, near the origin of replication. The two insertions had essentially the same effect on the sensitivity of growth with AnTc. Effects of AnTc on general growth in these strains are small as expected if inhibition bears only on the few cells with multiple copies of *tetA*. The similar sensitivity of strains with Tn*10*dTc at different positions suggests that different points on the plasmid have the same probability of amplification either individually or as part of a general increase in plasmid copy number. Whenever such an amplification happens to include *lac* as well as Tn*10*dTc(*tetA*), one expects growth with AnTc to also reduce the number of cells in the population with multiple *lac* copies.

We had expected that *tet* – *lac* amplification would primarily occur by tandem arrays of small regions of the plasmid and the effect of AnTc on *lac* copy number (reduction in revertant yield) would be seen only in strains with Tn*10*dTc near *lac.* Contrary to this expectation, tests below show that position of *tetA* on the plasmid made little difference in revertant yield, suggesting that sensitivity to AnTc may be due to occasional amplification of the entire plasmid.

Effect of AnTc on revertant yield in the Cairns system. The tester strain used in the Cairns system carries an F'*lac* plasmid with a *lac* frameshift mutation (CAIRNS and FOSTER 1991). When 10^8 cells of this strain were pre-grown on glycerol and plated on lactose, roughly 100 revertant colonies appear over the course of 6 days. The selective amplification model proposes that these revertants are initiated by pre-existing cells with multiple copies of *lac.* These duplications and amplifications arise during the pre-growth period and their frequency approaches a steady state (REAMS *et al.* 2010).

To test the effect of pre-growth with AnTc on revertant yield, cells with Tn*10*dTc on the F'*lac* plasmid were pre-grown in glycerol medium containing various concentrations of AnTc and were then plated on lactose plates to detect revertants. Following growth to stationary phase, cells were washed to remove AnTc and plated on lactose medium without AnTc. Figure 5 shows that pre-growth with AnTc concentrations above 10nM reduced the number of reverant colonies that accumulated over the following 6 days on selective lactose plates. Revertant yields typically dropped between 5 and 10-fold following growth with sufficient concentrations of AnTc.

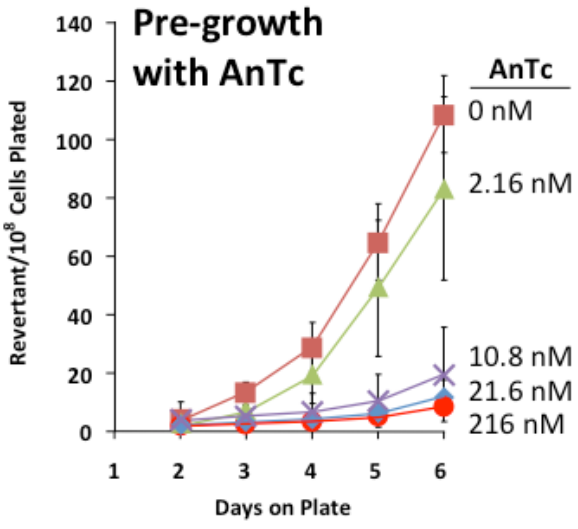

Figure 5 Concentrations of AnTc in pre-growth medium that reduce revertant yield Reversion was tested as described in Materials and Methods using strain TT26327 with a Tn*10*dTc insertion in *lacA* gene of plasmid F'$_{128}$*lac.* The revertant colony number is presented per viable cell of the tester strain plated.

In Figure 5 and most of the reversion tests described below, the number of viable cells plated is used to normalize results of the reversion test. That is, plotted revertant numbers are expressed per viable cell plated. This normalization has been done in much of the analysis of the Cairns system because many recombination mutants reduce viability and normalizing to viable cells plated assures that the number of revertants scored is derived from the same number of cells. We have done this normalization here to make our results comparable to those early experiments. However it should be noted that in these experiments, the population grown with AnTc has been enriched for cells that lack the amplification, thus normalization understates the effect of AnTc on revertant number. Viability decreases following growth with AnTc did not exceed two-fold. With this normalization, revertant numbers were typically reduced 7-fold by AnTc, without normalization 10-fold decreases were typical. Similarly, lawn viability on the selection plate was tested for cells pre-grown with and without AnTc and found to be essentially constant over 5 days.

The major effect of AnTc is seen during non-selective pre-growth. The reduction in revertant yield in strains with Tn*10*dTc was greatest when AnTc was included in otherwise non-selective pre-growth medium. This is shown in Figure 6, which describes the effects of exposure to AnTc before selection, during selection or both. In strains lacking Tn*10*dTc (top panel), revertant yield was not affected by AnTc added either before or during selection (in lactose plates). In strains with Tn*10*dTc on the F'*lac* plasmid, addition of AnTc to the selective lactose plates caused only a slight decrease in revertant number, as reported previously (HENDRICKSON *et al.* 2002; STUMPF *et al.* 2007). However, pre-growth of these strains with AnTc prior to plating caused strong reduction in the number of revertants produced later on selective medium. This effect of AnTc added during non-selective pre-growth in glycerol routinely caused a 5-10 fold reduction in revertant yield (bottom panel). This is recorded following normalization for viability, which understates the AnTc effect.

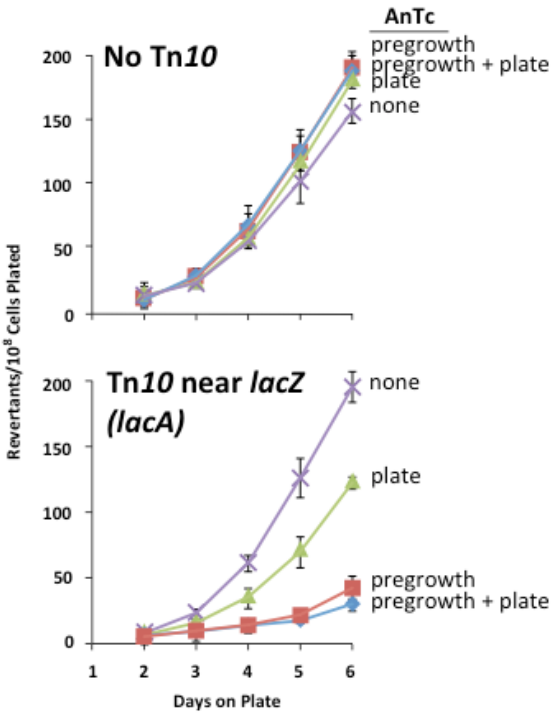

Figure 6. Effect of AnTc on revertant yield requires Tn*10*dTc and exposure during pre-growth. The strain without Tn*10*dTc (TR7178) is the original tester strain of Cairns and Foster (CAIRNS and FOSTER 1991) and the strain with Tn*10*dTc in *lac* (TT26327) carried an insertion at the distal end of the *lac* operon of the F'$_{128}$ plasmid. Cells were pre-grown for 20 generations in minimal glycerol with and without AnTc (216nM) and revertant number is expressed per viable cell plated.

The effect of AnTc on *lac* reversion requires having Tn*10*dTc on the F'*lac* plasmid. The effect of AnTc on revertant yield seen above was attributed to reducing the number of cells in the pre-growth culture that have co-amplified of *lac* and with *tetA* genes. Initially it was expected that amplification was due to multiple tandem copies of the mutant *lac* region. This expectation predicts that Tn*10*dTc (*tetA*) would have to be located on the F'*lac* plasmid close enough to *lac* to be frequently co-amplified. Figure 7 (top panel) shows that AnTc has little effect on revertant yield when Tn*10*dTc is inserted in the chromosome (*btuR*) rather than on F.

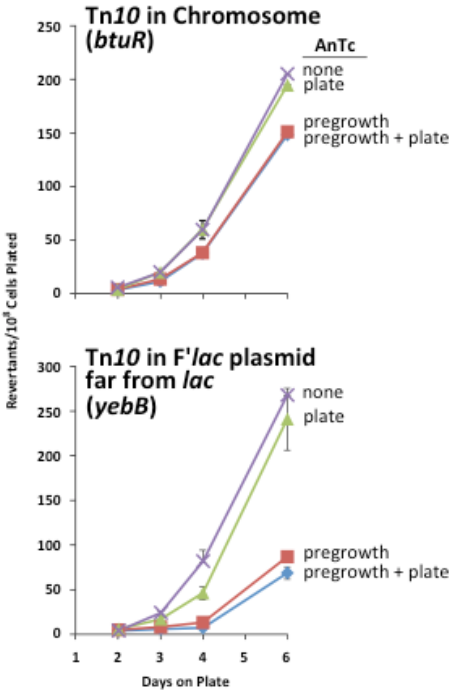

Figure 7 Effect of Tn*10*dTc position on sensitivity of reversion to AnTc. Cells were pre-grown to stationary phase in glycerol with or without AnTc (216 nM) and then washed and plated on selective lactose plates with or without AnTc (216nM). The strain at top carries Tn*10*dTc in the chromosomal *btuR* gene (TT26328). The strain at the bottom carries Tn*10*dTc in the *yebB* gene (TT26323). Revertant number is expressed relative to viable cells plated. This normalization understates the effect of pre-growth with AnTc, by including only cells that have survived the effects of AnTc and eliminating those multi-copy cells lost during pre-growth.

Unexpectedly, as seen in the bottom panel of Figure 7, reversion was strongly reduced when the *F'lac* Tn*10*dTc was inserted at a position far from *lac* (*yebB::*Tn*10*dTc). The *yebB*::Tn*10*dTc insertion is near the plasmid vegetative replication origin at the greatest possible distance from *lac* on the F'*lac* plasmid (Figure 8). In the belief that Lac$^+$ revertants were initiated by cells with a tandem duplication of a small region of the plasmid that included *lac,* we expected that AnTc would only reduce revertant yield in strains whose Tn*10*dTc(tetA) was inserted close enough to *lac* to be co-amplified. The observed effect of AnTc on strains with a Tn*10* far from *lac*, suggested that the cells responsible for initiating revertant colonies must carry multiple copies of the entire F'*lac* plasmid, not just the immediate *lac* region. This position-independent effect of Tn*10* on revertant number was noted previously by Stumpf and Foster (STUMPF *et al.* 2007).

Effect of Tn*10*dTc at various positions on the F' plasmid. To pursue the effect of Tn*10*dTc position on revertant inhibition, a series of insertions at varying distances from *lac* were tested for their ability to make reversion sensitive to pre-growth with AnTc. Figure 8 shows a map of F'$_{128}$*lac* plasmid with the positions of various insertions. Each strain was pre-grown either with or without AnTc and then plated without AnTc on selective lactose medium. Regardless of the position of Tn*10*dTc on the plasmid, pre-growth with AnTc reduced the yield of Lac$^+$ revertants.

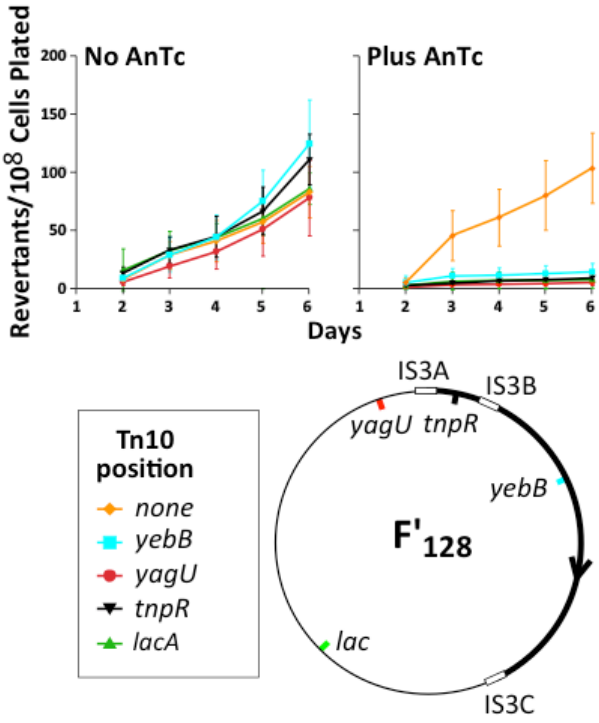

Figure 8 Insertions of Tn*10*dTc at points far from *lac* on the F' plasmid allowed AnTc to inhibit revertant yield. A series of tester strains with Tn*10*dTc inserted at various points in the F'*lac* plasmid were compared for their yield of Lac$^+$ revertants following pre-growth with and without AnTc. These strains are described in Table 1. Revertant yields following pre-growth without and with AnTc are described in top left and right parts of the figure. The map position of the Tn*10*dTc insertions are described at the lower left.

No AnTc effect is seen when Tn*10*dTc and *lac* are on different plasmids. This was tested by inserting a Tn*10*dTc(*tetA*) on an R1 plasmid in a strain with the *lac* mutation on the standard F'*lac* plasmid. The R1 plasmid used here (and in Figure 2) carries a temperature-sensitive replication origin (LARSEN *et al.* 1984) and is present at copy number of 3 during growth at 37°C, approximately the same number (1-2) as F'*lac*. As seen in Figure 9 (top right), AnTc has little effect on *lac* reversion when Tn*10*dTc and *lac* are on separate plasmids.

Effect of two Tn*10*dTc elements on the plasmid with *lac.* The bottom panel of Figure 9 describes the effect of two Tn*10*dTc insertions on the same plasmid. The strain at the left has one Tn*10*dTc element inserted in *lacA.* The strain on the bottom right has two Tn*10*dTcs, one inserted far from *lac* (*yebB*::Tn*10*dTc) and the other inserted 5kb from *lacA* (*zzf-1831*::Tn*10*dTc-GFP-Cm) (SUN *et al.* 2009). These two strains were tested together and the strain carrying both *mhpC31* and *yebB* insertions was more sensitive to pre-growth with AnTc, as one might expect if cells with more *tetA* copies are more sensitive to inhibition. Both Tn*10*dTc insertions in the double mutant are defective for transposase and are placed in opposite orientation, so they are not expected to contribute to duplication by either transposition or recombination. The higher revertant yield in the strain with inverse order Tn*10*dTc copies may reflect a previous observation that strains with inverse order repeats are prone to dimer formation (LYU *et al.* 1999).

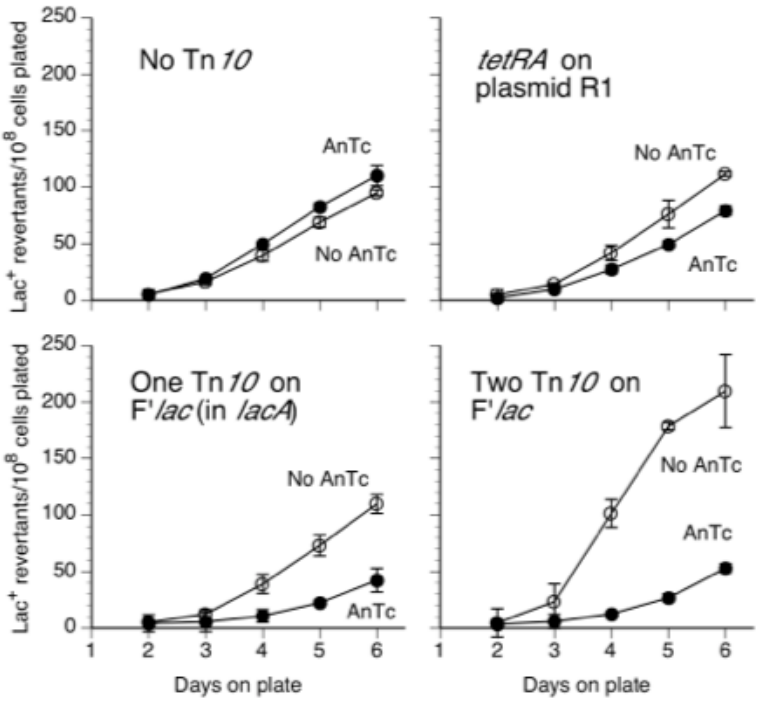

Figure 9 Effect of Tn*10*dTc on *lac* reversion under selection

The figure compares strains with *tetA* in plasmid R1 to strains with one and two copies of Tn*10*dTc *in* F'*lac.* The standard tester strain (TR7178) with F'*lac* but no Tn*10* is at top left. The strain at top right (TT26900) carries plasmid R1 with a cloned *tetR tetA* cassette derived from Tn*10*. The strain at the lower left has Tn*10*dTc inserted in *lacA* and is the same strain described in Figures 6 and 8. The strain at the lower right (TT26905) has two *tetA* genes, one within insertion *yebB*::Tn*10*dTc far from *lac* and the other within a derivative of *zzf-1881*::Tn*10*d*Tc* inserted in inverse order about 5kb away from *lac* – this derivative has Cm resistance marker and a GFP gene (SUN *et al.* 2009).

Populations inhibited by AnTc regained revertant yield when passaged without AnTc. The effects of AnTc described above were interpreted as evidence that reversible *lac* copy number variants such as tandem amplifications or plasmid multimers arise during pre-growth and initiate the revertant colonies that develop and appear during a week under selection. This reversibility

causes copy number variants to come to a steady state frequency in non-selective conditions when a balance is attained between high rates of formation and loss (REAMS *et al.* 2010). This instability of copy number was suggested to explain why revertant number, like duplication frequency, is not subject to Luria-Delbruck fluctuation (CAIRNS and FOSTER 1991; REAMS *et al.* 2010). That is, the forces that drive frequency to steady state obscure fluctuations due to the time of initial mutant formation. An alternative possibility is that pre-growth in the presence of AnTc favors some conventional stable mutants that are impaired for reversion under selection (e. g. plasmid loss or *lac* deletion). Several lines of evidence argue strongly against this possibility.

The reversibility of the AnTc-induced reduction in revertant number was shown by growing tester cells first with AnTc and then diluting 10-fold to inoculate a second passage without AnTc. The revertant yield of the two cultures is shown in the top two panels of Figure 10. The initial tester strain was pre-grown 7 generations either without (top left) or with AnTc (top right) and the revertant yield was reduced by AnTc as expected. The AnTc-grown population was then diluted 10-fold and grown 3-4 generations without AnTc. During this brief period, the strain regained full ability to revert under selection (lower left). We interpret this as evidence that copy number variants form and are lost at high rates. The analogue AnTc inhibits growth of the variants with highest copy number and thereby reduces the steady state frequency of these variants in the population. However the high steady state frequency is restored quickly by interconversion of copy number variants once the inhibitor is removed. The loss rate of large duplications in the chromosome and on the F'*lac* plasmid is about 10^{-2}/cell/generation (REAMS *et al.* 2010). We do not yet know the formation or loss rates for the plasmid copy number variants inferred here.

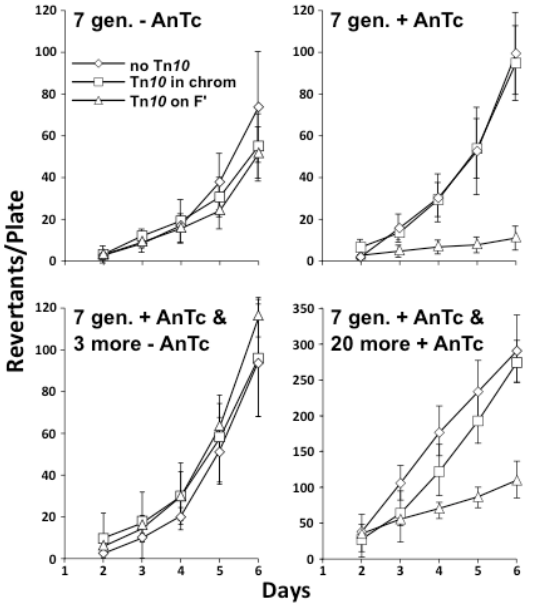

Figure 10 The effect of AnTc on revertant yield is reversed by growth without AnTc.

Pre-existing cells responsible for initiating revertants appearing under selection are thought to have multiple copies of the F'*lac* plasmid (including the *tetA* gene). The stability of such cells was tested using a strain with Tn*10*dTc in *lacA* (TT26327), a strain with Tn*10*dTc inserted into the chromosomal *btuR* gene (TT26328) and a strain with no Tn*10* (TR7178). All strains were pre-grown for 7 generations without (top left) or with AnTc (top right) and then are the used in a standard reversion experiment. Immediately following growth with AnTc all three mutants were diluted 10-fold into fresh medium lacking AnTc, grown 3 generations and retested for reversion (lower left). The same strain was passaged 6 times by 10-fold dilution into glycerol medium containing AnTc (20 additional generations with AnTc).

Irreversible changes in behavior are seen after longer growth in the presence of AnTc.

When cells with Tn*10*dTc near *lac* (in *lacA*) on the F'*lac* plasmid were passaged 6 additional times (each a 10-fold dilution) in the presence of AnTc, they begin to regain ability to generate Lac^+ revertants. See Figure 10 (lower right). This restoration of revertant yield is accompanied by loss of Tn*10*dTc (Tet^R) from the selectively-held F'*lac* plasmid by about 10% of cells. Cells that lost Tet^R retained the leaky Lac^+ phenotype characteristic of the parent mutation. The loss of Tn*10*dTc from F'*lac* renders cells insensitive to growth inhibition by AnTc and allows the *lac* copy

number to increase in the population. Thus, induction of the *tetA* gene by AnTc appears to impose a small growth inhibition on the entire population. We infer that AnTc acts primarily on the high copy sub-population, increasing their fitness cost and reducing their frequency in the population. Since these disadvantaged cells are continually replaced by new amplifications, the effect of AnTc is ultimately felt by the whole population. That is, any cell sub-lineage that loses Tn*10*dTc from the plasmid can produce cells with multiple F'*lac* copies that escape inhibition by AnTc.

Pre-existing cells with a *lac* amplification initiate both stable and unstable Lac$^+$ revertants

The Lac$^+$ revertant colonies that accumulate under selection in the Cairns system are of two types. About 90% of colonies are comprised primarily of stable *lac*$^+$ revertant cells that have acquired a point mutation that corrects the parental +1 frameshift. About 10% of revertant colonies contain cells with an unstable amplification of the original leaky *lac* allele. The initial growth-under-selection model proposed that cells with a tandem duplication of the parent *lac* region grow under selection and develop in one of two ways. They either acquire a point mutation and form a stable revertant, or they acquire a deletion that reduces amplification repeat size and fitness cost, allowing higher amplification and formation of an unstable revertant (KUGELBERG *et al.* 2010). The results presented here suggest that revertant colonies are initiated by cells with more copies of the whole plasmid rather than a tandem amplification of the *lac* region. This raises the question of whether whole plasmid amplification is required for both stable and unstable revertant types.

The yield of Lac$^+$ revertants drops (typically about 7-fold) following pre-growth with AnTc to reduce the frequency of cells carrying F'*lac* at high copy number. In standard reversion experiments, about 2- 10% of total revertants appearing on Days 5-6 are unstably Lac$^+$ (ANDERSSON *et al.* 1998; HASTINGS *et al.* 2000). To determine whether pre-growth with AnTc reduces the number of unstable revertants, we tested 156 total revertant colonies following pre-growth in the presence of AnTc of strains with *yebB*::Tn*10*dTc or *lacA*::Tn*10*dTc insertions on their F'*lac* plasmids. Of 156 revertants tested (66 from *yebB* and 99 from *lacA*) unstably Lac$^+$ cells were found in 17 (6 from yebB and 11 from *lacA*). This is just over 10% of the total revertants. Thus while AnTc reduced total revertant number about 7-fold, it had no effect on the fraction that contains unstably Lac$^+$ cells. This suggests that presence of AnTc in the pre-growth

culture reduces the yield of both stable and unstable revertants to the same extent and both types of revertants have a common starting point prior to imposition of selection.

DISCUSSION

These results suggest that both stable and unstable Lac$^+$ revertants appearing under selection in the Cairns system are initiated by cells formed during non-selective pre-growth that carry a high copy number of the F'*lac* plasmid. This suggests shortfalls in both stress-induced mutation and selective amplification models for the origin of mutants. First, revertants are not initiated under selection as proposed by stress-induced mutagenesis. Second, the initiating cells appear to carry multiple copies of the entire F'*lac* plasmid not a tandem amplification of just the *lac* region, as proposed by the amplification-under-selection model.

The results shed light on two aspects of the Cairns system that have been used to explain the paucity of evidence for general mutagenesis. The amplification of the whole F'*lac* plasmid suggests that "directed mutation" may mean increased likelihood mutations on this plasmid due to repeated replication and copy number increase and not to mutagenesis. The subset of plated cells suggested to enter the "hypermutable state" may mean those pre-existing cells with extra copies of the F'*lac* plasmid. The results presented are consistent with early evidence that the F' plasmid plays a central role in the reversion process. We will discuss these results in the context a new model for the process of reversion under selection.

There is a caveat in our interpretation of the effects of AnTc. The results are interpreted as evidence that growth with AnTc reduces the number of cells able to initiate a revertant. This interpretation is based on evidence that AnTc inhibits growth of cells with high TetA levels (ECKERT and BECK 1989; MOYED *et al.* 1983) and this inhibition increases with added *tetA* gene copy number (S. Maisnier-Patin, M. Savageau, J. R. Roth, unpublished results). An alternative interpretation, which we cannot eliminate, is that all cells grown with AnTc, regardless of their TetA level, are placed in a shared physiological state that reduces the probability of their surviving or reverting when placed under selective conditions. We know that pre-growth with AnTc does not impair survival of starved cells, but we cannot eliminate the possibility that all

AnTc-grown cells have a stochastically reduced probability of reversion. We think this alternative is unlikely because it requires that AnTc have two independent effects – one in inhibiting growth of cells with high TetA protein and a second in reducing the probability of reversion under lactose selection. These alternative particulate and stochastic interpretations of population behavior are common to many biological situations (e.g. assays of phage titer, bacterial colony-forming ability and cancer stem cell transplantation).

The interpretation of these experiments depends on AnTc only impairing growth of cells with multiple copies of *tetA* or *F'lac tetA.* That is, the bulk of the tester population retains the F'*pro+ lac tetA+* plasmid during growth with AnTc. A simple alternative would be to suggest that AnTc causes plasmid loss and leads to more cells with no *lac* allele capable of reversion. Several lines of evidence presented here support the idea that plated cells still carry the F'pro$^+$ *lac* tetA+ plasmid. a) The population is grown on minimal glycerol medium, which selects for retention of the plasmid *pro*+ allele. b) The plated population demonstrably retains the plasmid-encoded Pro+ Tet^R phenotypes and the leaky Lac$^+$ phenotype of the mutant *lac* allele. c) Following revertant loss due to pre-growth with AnTc, the tester population regains full ability to produce revertants within only 3 generations of additional growth without AnTc. This rapid recovery is inconsistent with complete loss of the *lac* allele.

The nature of the pre-existing cells that initiate revertant colonies remains uncertain. It is inferred that these cells have multiple copies of the entire F'*lac* because reversion was sensitive to AnTc, regardless of the position of Tn*10*dTc vis a vis the plasmid *lac* operon. Cells might acquire multiple plasmid copies in several ways. Stochastic loss of plasmid copy number control could lead to occasional cells with multiple independent plasmid copies. Stochastic failure of dimer resolution mechanisms could lead to accumulation of plasmid multimers formed by plasmid-plasmid recombination or sister strand exchange during replication. Multiple plasmid copies could be generated by temporary rolling-circle replication. Many plasmids are known to occasionally switch into rolling circle mode in which their replication fork proceeds repeatedly around the basic circular plasmid producing a linear double-stranded concatamer of repeated plasmid genome copies (VIRET *et al.* 1991). Such replication underlies growth of many viruses and is known to occur stochastically in cells carrying circular plasmids. On the F' plasmid, rolling circle replication forks could be derived from forks initiated at F' plasmid transfer origin, whose forks normally produce a 5'-ended single strand for conjugation. Conversion to a full stable

replication fork could allow this origin to produce non-transferable double-stranded linear products. Cells with a stable rolling circle fork could form reversibly and incur a fitness cost, leading to a steady state frequency of such cells in the pre-selection population. A final possibility is copy addition by repeated acts of plasmid transfer.

Early work on the Cairns/Foster system revealed that revertant yield depends heavily on presence of a functional F'*lac* conjugation replication origin (*oriT*)(GALITSKI and ROTH 1995; GODOY and FOX 2000; PETERS *et al.* 1996; RADICELLA *et al.* 1995). Essentially all Lac$^+$ reversion is eliminated in cells that lack the plasmid TraI protein, an endonuclease that nicks plasmid DNA at the plasmid *oriT* site, displacing a 5' ended single-stranded plasmid copy for transfer into the recipient cell. Reversion also depends heavily on other plasmid-encoded proteins needed to signal readiness to mate and to assemble the transfer replication complex – e.g. the TraD proteins, pilus structure (FOSTER and TRIMARCHI 1995a; FOSTER and TRIMARCHI 1995b). These dependencies demonstrate the importance of conjugation abilities of the F' plasmid. However, direct tests of mating suggested that actual DNA transfer is not required on the selection plate for formation of the majority of revertants (FOSTER and TRIMARCHI 1995a; FOSTER and TRIMARCHI 1995b). An increase in plasmid copy number due to successive mating between F′ cells mating was suggested previously (PETERS *et al.* 1996), but not pursued in the light of evidence against transfer. This evidence did not eliminate mating events prior to plating, transfer between immediate siblings or exceptional establishment of a transfer replication fork within a single cell or cell filament.

A new model for the origin of mutations under selection in the Cairns system. This model accounts for the results described here in terms of generally accepted properties of the Cairns system.

1. The bulk of the plated population with a standard number of *lac* copies is unable to grow under lactose selection, due to insufficient energy to trigger initiation of standard chromosome replication.
2. Rare variants with multiple copies of the whole *F'lac* plasmid have enough energy to divide once, but produce daughter cells without sufficient extra *lac* copies to grow exponentially. However, non-dividing cells with multiple F'*lac* plasmid copies may have enough energy to continuously replicate their F' plasmid from unregulated origins. The cells that do this may be

any or all of the forms discussed above. These cells replicate their F'*lac* repeatedly with little or no cell division until a reversion event occurs at any time within several days. As soon as reversion occurs, energy is resupplied by the *lac*$^+$ allele, cell division starts and the clone expands exponentially, maintaining its revertant allele under selection.

3. Occasional pre-existing cells have multiple copies of a plasmid that already carries an internal tandem duplication of *lac*. Cells with a short duplication on F'*lac* are present at about 10^{-4} in an unselected population (REAMS *et al.* 2012). The join points of these duplications are like those of the amplifications found in unstable revertants (KUGELBERG *et al.* 2010; KUGELBERG *et al.* 2006; SLACK *et al.* 2006). The extra *lac* copies provided by multiple copies of a plasmid with an internal amplification is sufficient to allow immediate exponential growth under selection and development of an unstable revertant colony with a expanded tandem *lac* amplification. The frequency of short *lac* duplications prior to selection is sufficient to account for the observed number of unstable Lac$^+$ revertants.
4. Cells with multiple copies of the F'*lac* plasmid also have multiple copies of the *dinB* gene, which encodes an error-prone polymerase and happens to lie on that plasmid. Thus DNA replication in both the stable and unstable lineages occurs in the presence of elevated levels of the mutagenic DinB polymerase. In lineages leading to stable revertants, the chromosome does not replicate extensively and mutagenesis is focused on the replicating F' plasmid. In unstable lineages (10% of total), many of the *lac* duplications co-amplify the nearby *dinB* gene. These cells grow and replicate their entire chromosome in the presence of elevated DinB. Thus mutagenesis affects the entire chromosome. While DinB mutagenesis contributes to the yield of stable Lac$^+$ revertants, its effect is essentially an artifact due to the chance location of *dinB* on F'*lac* where it can be co-amplified with *lac* during replication under selection.

Summary The history of work on the Cairns system is remarkable in that a large body of excellent data has accumulated and been interpreted in two diametrically opposed ways – mutagenesis or selection. This body of information is largely agreed to by all participants in the discussion and contains very few internal contradictions. However, most experiments have been directed at verifying one model or the other rather discriminating between alternatives. The results and model described above may help refocus the discussion.

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