Increased Episomal Replication Accounts for the High Rate of Adaptive Mutation in *recD* Mutants of *Escherichia coli*

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Manuscript received November 13, 1998
Accepted for publication February 8, 1999

ABSTRACT

Adaptive mutation has been studied extensively in FC40, a strain of *Escherichia coli* that cannot metabolize lactose (Lac$^-$) because of a frameshift mutation affecting the *lacZ* gene on its episome. *recD* mutants of FC40, in which the exonuclease activity of RecBCD (ExoV) is abolished but its helicase activity is retained, have an increased rate of adaptive mutation. The results presented here show that, in several respects, adaptive mutation to Lac$^+$ involves different mechanisms in *recD* mutant cells than in wild-type cells. About half of the apparent increase in the adaptive mutation rate of *recD* mutant cells is due to a RecA-dependent increase in episomal copy number and to growth of the Lac$^-$ cells on the lactose plates. The remaining increase appears to be due to continued replication of the episome, with the extra copies being degraded or passed to *recD*$^+$ recipients. In addition, the increase in adaptive mutation rate in *recD* mutant cells is (i) dependent on activities of the single-stranded exonucleases, RecJ and ExoI, which are not required for (in fact, slightly inhibit) adaptive mutation in wild-type cells, and (ii) enhanced by RecG, which opposes adaptive mutation in wild-type cells.

MOST spontaneous mutations are assumed to be the result of random errors made by DNA polymerases during genomic replication. However, spontaneous mutations also arise in static populations exposed to nonlethal selection (Cairns *et al.* 1988). Because these mutations can appear to be specific to the selective pressure applied, the phenomenon has come to be known as "adaptive" mutation (reviewed in Foster 1993). Adaptive mutation has been extensively studied in FC40, a strain of *Escherichia coli* that cannot utilize lactose (Lac$^-$) but that readily reverts to lactose utilization (Lac$^+$) when lactose is its sole energy and carbon source. Lac$^+$ revertants of FC40 do not arise during starvation in the absence of lactose (Cairns and Foster 1991), but it has recently been found that mutations giving phenotypes other than Lac$^+$ occur during lactose selection (Foster 1997; Torkelson *et al.* 1997). Thus, cells appear to have mechanisms uncoupled from genomic replication that can produce genetic variants during periods of nutritional deprivation; but, at least in the case of FC40, the process is not directed toward beneficial phenotypes. However, we continue here to call the selected Lac$^+$ revertants of FC40 "adaptive" simply to distinguish them from mutations occurring during nonselective growth and from nonselected mutations occurring during lactose selection. This meaning of "adaptive mutations" is the same as that used by evolutionists to distinguish beneficial from neutral or deleterious mutations.

FC40 is deleted for the *lac-pro* region on its chromosome but carries the *lac-pro* region on an F′ episome. The Lac$^-$ allele, Φ(*lacI33-lacZ*), has a +1 bp frameshift affecting the *lacZ* gene. Mutation to Lac$^+$ during lactose selection has the following characteristics:

i. While deletions, duplications, and frameshifts revert the Lac$^-$ allele during growth, adaptive Lac$^+$ mutations are mainly −1 bp frameshifts in runs of iterated bases (Foster and Trimarchi 1994; Rosenberg *et al.* 1994).
ii. Adaptive but not growth-dependent reversion to Lac$^+$ requires recombination functions, specifically the RecABCD pathway for double-strand break (DSB) repair (Cairns and Foster 1991; Foster 1993; Harris *et al.* 1994).
iii. *E. coli*'s two enzyme systems that catalyze the branch migration of recombination intermediates, RuvAB and RecG, both contribute to normal recombination (West 1997), but RuvAB promotes and RecG opposes adaptive Lac$^+$ mutation (Foster *et al.* 1996; Harris *et al.* 1996).
iv. The high level of adaptive reversion to Lac$^+$ in FC40 requires that the Lac$^-$ allele be on the episome; if the same allele is at its normal position on the chromosome, adaptive reversion to Lac$^+$ occurs at about 1/100 the rate and is no longer *recA*-dependent (Foster and Trimarchi 1995a; Radicella *et al.* 1995).
v. Defects in conjugal functions cause a 10-fold reduc-

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tion in adaptive mutation (Foster and Trimarchi 1995a; Galitski and Roth 1995), although in this case most of the remaining mutations are *recA*-dependent (Foster and Trimarchi 1995a). However, actual episome transfer is not required for adaptive mutation (Foster and Trimarchi 1995a,b).

In two respects adaptive mutation in FC40 is similar to normal growth-dependent mutation: (i) Adaptive Lac$^+$ mutations are produced by DNA polymerase III, *E. coli*'s replicative polymerase (Foster *et al.* 1995; Harris *et al.* 1997a); (ii) The methyl-directed mismatch repair (MMR) pathway corrects mismatches in hemi-methylated DNA in favor of the methylated strand (reviewed in Modrich and Lahue 1996). In FC40, both growth-dependent and adaptive mutation rates are increased about 100-fold by defects in MMR and reduced 2- to 5-fold by overproducing components of the MMR pathway (Foster and Cairns 1992; Foster *et al.* 1995, 1996), although there is some disagreement about this latter result (see Harris *et al.* 1997b; Foster 1999).

Because of the involvement of the RecABCD pathway, models of adaptive mutation in FC40 have centered on DSB repair. Conjugal DNA replication is initiated by a nick at *oriT*, the conjugal origin, but this nick occurs even in the absence of a conjugal signal and persists in stationary-phase cells (Firth *et al.* 1996; Frost and Manchak 1998). Kuzminov (1995a) proposed that a double-strand end is created when a replication fork initiated at one of the episome's vegetative origins collapses at this nick. Alternatively, DSBs can occur when a replication fork stalls (Kuzminov 1995b; Michel *et al.* 1997; Seigneur *et al.* 1998) or as a result of DNA damage at the nick or elsewhere (Bridges 1998). Rec ABCD-catalyzed recombinational repair of the DSB could initiate DNA synthesis either by restoring a normal replication fork (Kuzminov 1995a) or by allowing the 3′ single-strand end to prime DNA synthesis (Asai *et al.* 1994). Replication errors produced at this point could be the source of adaptive mutations (reviewed in Foster 1998).

Other models for adaptive mutation in FC40 have postulated that conjugal DNA synthesis, which occurs by unidirectional strand-displacement replication of the episome, gives rise to the mutations (Foster and Trimarchi 1995a; Galitski and Roth 1995; Radicella *et al.* 1995). However, these models have no clearly defined role for recombination in general, or for RecBCD in particular. In addition, genetic defects that completely inhibit conjugation reduce but do not eliminate adaptive mutation (Foster and Trimarchi 1995a; Foster *et al.* 1996). One explanation for this discrepancy is that these defects prevent episome transfer but do not completely inhibit nicking at *oriT*.

An alternative model is that during lactose selection the Lac$^-$ allele is amplified in tandem by RecA-dependent unequal recombination of an initial duplication (Cairns and Foster 1991; Andersson *et al.* 1998). Mutations could occur during the DNA synthesis that produces the amplification, and a Lac$^+$ mutation that allowed the cell to grow would necessarily be retained. RecBCD can be incorporated by proposing that amplified arrays are produced if during DSB repair the broken end recombines with a homologous region upstream of itself, setting up a loop of iterative replication (Roth *et al.* 1996). Although 2% of the late-appearing Lac$^+$ colonies of FC40 consist almost entirely of cells that have amplified their Lac$^-$ allele (Foster 1994; Foster *et al.* 1995), it remains to be seen if amplification can account for the majority of the Lac$^+$ revertants of FC40. If so, to be consistent with the constant rate of adaptive mutation in FC40 (Cairns and Foster 1991), amplification and deamplification (or degradation of the amplified region) must be in equilibrium (Foster 1994).

RecBCD, also known as exonuclease V (ExoV), is a multifunctional enzyme with helicase and nuclease activities (Kowalczykowski *et al.* 1994). *recD* mutants of FC40, in which exonuclease activity is abolished but helicase activity is retained, have an increased rate of adaptive mutation (Harris *et al.* 1994). In this report we investigate further the role of RecBCD in adaptive reversion of FC40. We show that, relative to wild-type cells, *recD* mutant cells have two- to fourfold more copies of the episome. This increased number of Lac$^-$ alleles results in higher residual β-galactosidase levels, allowing *recD* mutant cells to grow slowly on lactose. This growth, combined with the increased number of mutational targets per cell, accounts for much, but not all, of the higher rate of adaptive mutation of *recD* mutant cells. The remaining increase in mutation rate appears to be due to continued replication of the episome, with the extra copies being degraded or passed to *recD*$^+$ recipients.

MATERIALS AND METHODS

Bacterial strains: The strains used are derivatives of FC36, a rifampicin-resistant (Rif^R) isolate of P90C [=F$^-$ *ara* Δ(*lac-proB*)$_{X111}$ *thi*; Coulondre and Miller 1977; Cairns and Foster 1991]. The Rif^R Lac$^-$ revertible strain, FC40, and the Rif^S Lac$^-$ nonrevertible scavenger, FC29, have been previously described (Cairns and Foster 1991). Additional strains used in this study (and references to alleles) are listed in Table 1; all are isogenic to FC40. Except where noted, derivatives were constructed by first moving chromosomal alleles into the F$^-$ background by P1 bacteriophage-mediated transduction. The Φ(*lacI33-lacZ*) *proAB*$^+$ episome was then mated into the various backgrounds by selecting for proline prototrophy. Finally, if required, the episome-carrying strains were transformed with plasmid DNA. Standard techniques were used (Miller 1972; Ausubel *et al.* 1988).

recD mutant strains were confirmed by their ability to give large plaques when used as an indicator for *red gam* Chi^o λ bacteriophage (Chaudhury and Smith 1984). *recA*, *ruv*, and *recG* mutant strains were confirmed by their sensitivity to UV light. *recD1009* was moved by P1 transduction of *recD1009* *argA*::Tn*10* (Chaudhury and Smith 1984) followed by selec-

TABLE 1

The strains used in this study

Strain	*recD* allele[a]	Other alleles[a]	Other drug markers	Donor strain	Obtained from	Reference to strain or allele
FC40						Cairns and Foster (1991)
FC465		*recG258*::*d*Tn*10*Kan		N2731	S. T. Lovett	Foster *et al.* (1996)
FC836		Δ(*ruvA-ruvC*)*65*	*eda57*::Cam	AM887	F. W. Stahl	Mandal *et al.* (1993); Foster *et al.* (1996)
FC877		*recA938*::Tn*9-200*		DB1319	CGSC	Foster (1997)
FC897		Δ(*ruvA-ruvC*)*65* *recG258*	*eda57*::Cam			
FC973	*recD1903*::*d*Tn*10*			FS3206	F. W. Stahl	Biek and Cohen (1986)
FC975	*recD1903*	*recG258*				
FC977	*recD1903*	Δ(*ruvA-ruvC*)*65*	*eda57*::Cam			
FC1012	*recD1009*	Tet^S on episome		AC203	F. W. Stahl	Chaudhury and Smith (1984); Foster (1997)
FC1051	*recD1903*	*recA938*				
FC1053	*recD1903*	*recG258 sulA11*		DM1187	E. Eisenstadt	Mount (1977)
FC1112		*recJ284*::Tn*10*		JC12123	S. T. Lovett	Lovett and Clark (1984)
FC1114		*recJ2051*::*d*Tn*10*Kan		RDK2129	S. T. Lovett	Lovett and Sutera (1995)
FC1168	*recD1903*	*recJ2051*				
FC1185		pJC765 = precJ$^+$		JC14281	S. T. Lovett	Lovett and Clark (1985)
FC1186	*recD1903*	pJC765				
FC1187		*recJ205*/pJC765				
FC1188	*recD1903*	*recJ2051*/pJC765				
FC1271		*xonA300*::Cat		STL2664	S. T. Lovett	Razavy *et al.* (1996)
FC1272	*recD1903*	*xonA300*				
FC1274	*recD1009*	*ruvA60*::Tn*10*		N2057	A. Kuzminov	Shurvinton *et al.* (1984)
FCJ65		*traD411*::Kan on episome		pKI256	K. Ippen-Ihler	Foster and Trimarchi (1995a)
FCJ207	*recD1903*	*traD411* on episome				

[a] For clarity, drug-resistance insertions in genes are identified only once.

tion for spontaneous arginine prototrophy and screening for sensitivity to tetracycline (Tet^S). *sulA11* strains were made by transducing a *pyrD34 zcc-282*::Tn*10* intermediate (alleles obtained from S. T. Lovett, Brandeis University) to uracil prototrophy and then screening for Tet^S and suppression of filamentation after exposure to nalidixic acid. Other alleles were moved by selecting for their drug resistances.

Adaptive mutation experiments: The media and protocols used for the large-scale adaptive mutation experiments were as previously described (Cairns and Foster 1991; Foster 1994). Cells were grown to saturation in M9-0.1% glycerol medium; the number of revertible cells plated on lactose plates was controlled by the volume plated. The semiquantitative assay used in many of the experiments reported here was described in Foster *et al.* (1996). Briefly, 10-μl aliquots (about 10^7 cells) of cultures grown to saturation in M9-0.1% glycerol were spread on each quadrant of a M9-lactose plate. Each assay consisted of three or more independent cultures of the strain to be tested plus appropriate controls. Plates were incubated at 37°, and Lac$^+$ colonies were counted daily starting on day 2. Adaptive mutation rates to Lac$^+$ were calculated as the mean number of Lac$^+$ colonies appearing each day from days 3 to 5. Throughout, the error given is the standard error of the mean (SEM); statistical calculations were as given in Bulmer (1979) and Zar (1984).

Incubation in lactose: Aliquots (1 ml) containing about 4×10^8 cells from three independent cultures of FC40 and FC973 grown in M9-0.1% glycerol medium were each embedded in low-melt agarose microbeads (Koob and Szybalski 1992). The beads were washed three times in M9 salts, added to 1 ml M9-0.1% lactose medium plus Xgal (5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside, a chromogenic substrate of β-galactosidase) and distributed into 8 wells of a 24-well microtiter dish (for a total of 24 wells for each strain). After 3 days incubation, some wells contained only clear beads, some had a mixture of clear and blue beads, and some wells were completely blue (because a Lac$^+$ revertant arose from the residual cells on the outside of the beads or because proliferating Lac$^+$ cells occasionally escape from the beads). Blue and clear wells were harvested separately, and any remaining blue beads among the clear beads were removed with a pipette tip.

Amplification and episome transfer: To determine if Lac$^+$ phenotypes were the result of amplification of the Lac$^-$ allele, Lac$^+$ colonies were gridded onto M9-lactose plates, replicated onto Luria-Bertani (LB) plates, grown overnight, replicated again onto LB plates, grown 6 hr, and then replicated onto lactose-MacConkey plates (on which medium Lac$^+$ colonies are red). This procedure allows amplified Lac$^-$ alleles to deamplify, giving white patches on lactose-MacConkey plates (Tlsty *et al.* 1984). Episome transfer into scavenger cells was detected by single-colony purifying Lac$^+$ colonies on M9-lactose plates and checking three independent colonies from each original Lac$^+$ colony for Rif^S on LB plus Rif plates. Media were as in Miller (1972).

DNA manipulations: Pulsed-field gel electrophoresis (PFGE) was performed with a CHEF-DR II PFGE system (Bio-Rad, Hercules, CA). Cells were embedded in agarose microbeads and the DNA was purified as described (Koob and Szybalski 1992). Beads were either used immediately or stored at 4° in 0.5 EX (0.5 m EDTA, pH 8, plus 0.01% Triton X-100). For restriction enzyme digests, the agarose beads were washed three times in TEX (10 mm Tris-HCl, 1 mm EDTA, 0.01% Triton X-100), three times in the restriction enzyme buffer, and digested 4–6 hr with the restriction enzyme according to the manufacturer's instructions. After digestion, the beads were washed and resuspended in TEX. Because large circular DNA molecules do not resolve by PFGE, the size of the episome was determined after linearization with S1 nuclease (Barton *et al.* 1995). Agarose beads were washed twice with TE (10 mm Tris-HCl, 1 mm EDTA), twice with S1 buffer (50 mm NaCl, 30 mm NaAcetate, pH 4.5, 5 mm $ZnSO_4$), 0.2, 0.5, and 0.7 units of S1 were added, and digests were incubated for 45 min at 37°. The reaction was stopped with ice-cold 0.5 EX.

For other procedures, genomic DNA was extracted with Isoquick kits (Orca Research Inc.) and plasmid DNA was extracted with Wizard kits (Promega, Madison, WI). Probes for Southern blots were generated from plasmids carrying Φ(*lacI33-lacZ*) or *dnaQ*$^+$. The *lac* probe was either a 1320-bp *Eco*RV fragment (which hybridizes to one *Eco*RV band) or a 1482-bp fragment amplified using the primers 5′ TGAATTA CATTCCCAACCGCGT 3′ and 5′ CGCTCATCCGCCACA TATCC 3′ (which hybridizes to two *Eco*RV bands); the *dnaQ* probe was a 667-bp fragment amplified with the primers 5′ AGTCTGACATAAATGACCGCT 3′ and 5′ ACGCGTAACT TACTTGCCTGA 3′ (which hybridizes to one *Eco*RV band). The probes were purified by agarose gel electrophoresis, extracted with Qiaex beads (QIAGEN, Valencia, CA), and labeled with [α-^{32}P]dCTP using the NEBlot kit (New England Biolabs, Beverly, MA). Southern transfers were by capillary action onto Zeta-Probe membranes (Bio-Rad), following the manufacturer's instructions for transfer and DNA hybridization. Autoradiographs of Southern hybridizations and photographs of ethidium bromine-stained agarose gels were digitalized and the bands were quantified using the Scion image program (Scion Corp., Frederick, MD).

β-Galactosidase assays: Assays were performed as described (Miller 1972). To detect low levels of β-galactosidase, incubation times of up to 24 hr were used, during which the assay tubes were capped to prevent evaporation.

RESULTS

The rate of adaptive mutation to Lac$^+$ of *recD* mutant cells increases with time: During incubation in lactose medium, Lac$^+$ revertants of the wild-type strain, FC40, accumulate at a fairly constant rate. This is true on agar plates [if contaminants in the agar are consumed by nonreverting Lac$^-$ scavenger cells (Cairns and Foster 1991)] and in liquid medium (Foster 1994). The constant rate implies that neither the number of Lac$^-$ cells nor the average number of their Lac$^-$ alleles increases during the time that adaptive Lac$^+$ mutations are occurring. In contrast, with a *recD* mutant derivative of FC40, FC973, the rate at which Lac$^+$ mutants accumulate increased with time during lactose selection (Figure 1A).

To determine if this increase was due to cell growth, plugs were taken from the lactose plates and the number of viable Lac$^-$ cells was determined. Figure 1B shows that, as previously reported (Cairns and Foster 1991; Foster 1994), the number of wild-type FC40 cells was static for 3 days. As is typical in these experiments, the Lac$^-$ population then began to increase as early arising Lac$^+$ colonies started crossfeeding nutrients to the Lac$^-$ cells. In contrast, the number of Lac$^-$ *recD* mutant cells on the lactose plates started to increase after day 2, achieving a fourfold increase by day 4 (Figure 1B). Because fewer of the *recD* mutant cells had been plated, there were fewer Lac$^+$ colonies on these plates through day 3 than on the plates with wild-type cells. Thus it is unlikely that the increase in Lac$^-$ *recD* mutant cells was due to crossfeeding.

Most Lac$^+$ revertants take about 2 days to make colonies (Cairns and Foster 1991). This was confirmed for the *recD* mutant by mating a Lac$^+$ episome (a true revertant) into the F$^-$ parents of FC40 and FC973, growing these cells in M9-glycerol medium, and plating dilutions on M9-lactose plates in the presence of 10^9 Lac$^-$ scavenger cells. After 22 hr no Lac$^+$ colonies of either strain were visible, whereas by 42 hr, 95 and 82% of the Lac$^+$ colonies of the wild-type and *recD* mutant strains, respectively, had appeared. Because the mutational events that give rise to adaptive Lac$^+$ revertants are the same −1 bp frameshifts in *recD* mutants as in wild-type cells (Rosenberg *et al.* 1994), and in wild-type cells all of these revertants produce the same amount of β-galactosidase (Foster and Trimarchi 1994), most Lac$^+$ revertants of the *recD* mutant will take 2 days to produce visible colonies on lactose plates.

For Figure 1C, the number of Lac$^+$ colonies appearing each day was divided by the number of Lac$^-$ cells on the plates two days earlier to give the mutation rate per 10^8 cells per day. As discussed above, this rate is fairly constant in wild-type cells. In contrast, after day 3, the mutation rate per cell per day increased linearly in the *recD* mutant. Although the error bars are wide (reflecting variation in both the numbers of Lac$^+$ colonies and the numbers of Lac$^-$ cells), by day 6 the mutation rate to Lac$^+$ of the *recD* mutant was nearly 30-fold greater than that of the wild-type strain.

The copy number of the episome is increased in *recD* mutant cells: Many plasmids, including a derivative of F (miniF), amplify in *recD* mutants (Biek and Cohen 1986; Cohen and Clark 1986; Seelke *et al.* 1987). The results described above would be explained if the episome, or just the *lac* allele, was continuously amplifying in *recD* mutant cells during incubation on lactose. Thus, we used several techniques to determine the copy number of the episome and the *lac* allele in wild-type and *recD* mutant cells.

The F episome is normally maintained at one to two copies per cell (Frame and Bishop 1971; Stetson and Somerville 1971; Movva *et al.* 1978; Gordon *et al.* 1997; Cooper and Keasling 1998). Table 2 gives the

levels of β-galactosidase in Lac$^-$ and Lac$^+$ cells of FC40 and various derivatives. When the Φ(*lacI33-lacZ*) allele is on the chromosome, Lac$^-$ cells have about one Miller unit of β-galactosidase (W. A. Rosche and P. L. Foster, unpublished results). Assuming the level of β-galactosidase reflects the number of Lac$^-$ alleles, the results in Table 2 indicate that FC40 cells have two copies, which corresponds well to the estimated number of episomes per cell. In contrast, *recD* mutant cells have about eight copies of the Lac$^-$ allele. *recG* mutant cells have a 100-fold higher adaptive mutation rate than wild-type cells, but unlike *recD* mutants, Lac$^-$ *recG* mutant cells do not grow or show an increasing mutation rate to Lac$^+$ during lactose selection (Foster *et al.* 1996; Harris *et al.* 1996). Table 2 indicates that *recG* mutant cells have three to four copies of the Lac$^-$ allele. In a *recD recG* double mutant the copy number of the Lac$^-$ allele appears to be nearly additive relative to each of the single mutants (the *recD recG* mutant strain will be discussed further below).

Most Lac$^+$ revertants of FC40 produce about 200 Miller units of β-galactosidase (Foster and Trimarchi 1994). The data in Table 2 demonstrate that this does not change when Lac$^+$ cells are grown for 24 hr in lactose, *i.e.*, there is no indication that growth in lactose selects for amplification of the Lac$^+$ allele. The Lac$^+$ derivatives of the other strains behaved similarly, maintaining a higher copy number than wild-type cells but without further increase when grown in lactose.

We also quantified with Southern blots the number of copies of the *lac* allele in wild-type and *recD* mutant cells. A blot of several dilutions of total cellular DNA cut with the restriction endonuclease *Eco*RV (which cuts within the *lac* allele) was hybridized with probes to the episomal *lac* allele and to the chromosomal *dnaQ* gene. *dnaQ* was used to normalize for the amount of DNA because, after growth in minimal medium, most of these

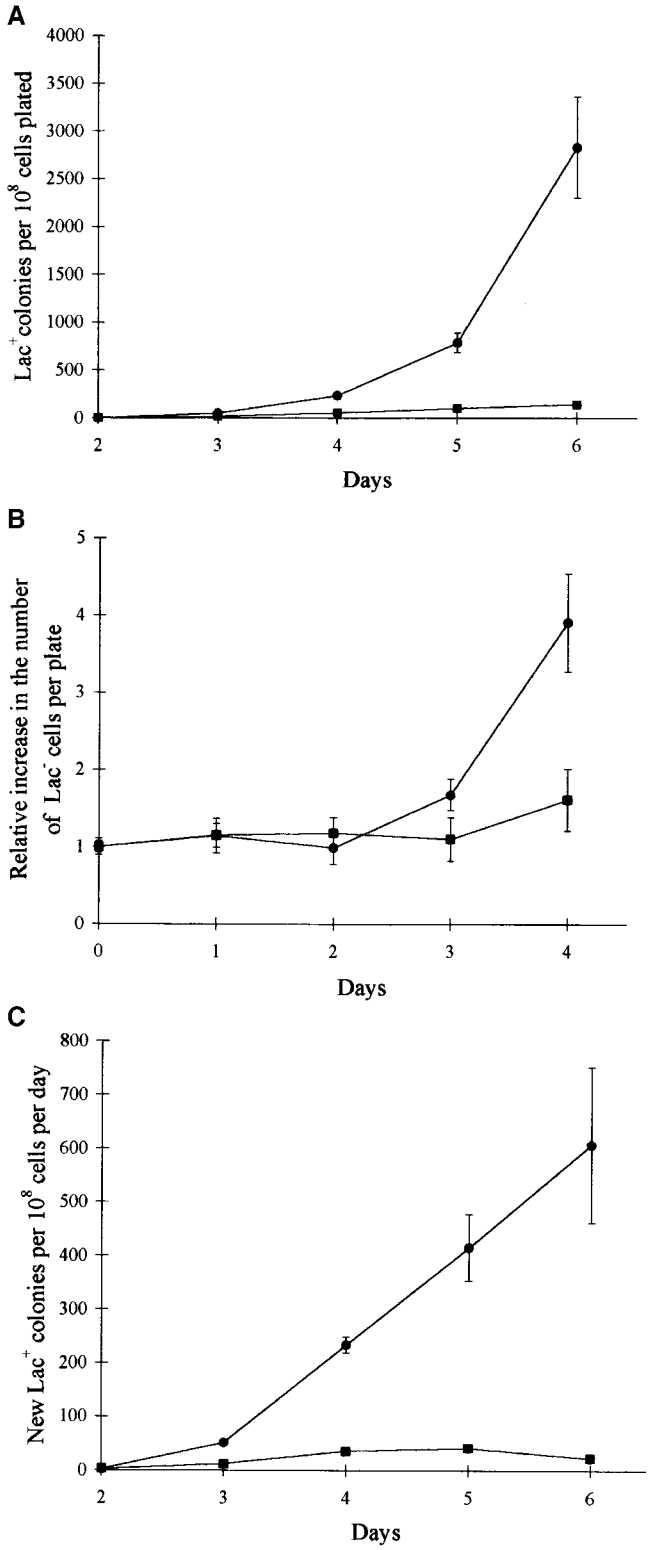

Figure 1.—Adaptive mutation to Lac$^+$ is increased in *recD* mutant cells. Three aliquots from five independent overnights each of the wild-type strain, FC40 (squares), and the *recD* mutant strain, FC973 (circles), were plated on M9-lactose minimal plates with 1.5×10^9 cells of the scavenger strain, FC29. Plugs were taken from three plates of each strain each day from days 0–4 and dilutions plated on LB + Rif plates to determine the viable cell number. These plates were then replicated onto MacConkey-lactose plates and the number of Lac$^+$ cells was subtracted from the total. The initial numbers of cells plated were 1.3×10^8 of FC40 and 1.0×10^7 of FC973. Lac$^+$ colonies were counted every day (plates from which plugs were taken were not further counted). (A) The cumulative number of Lac$^+$ revertants per day per 10^8 cells initially plated. Points are means and error bars are ±SEM (some of which are smaller than the symbols). (B) The relative increase in Lac$^-$ cells per plate each day. For each strain the mean number of cells on the plate each day has been divided by the mean number present on day 0. Error bars are SEM calculated from the combined variances of the counts on each day and the counts on day 0. (C) The number of new Lac$^+$ revertants per day per 10^8 cells. The mean number of new Lac$^+$ colonies appearing each day was divided by the mean number of Lac$^-$ cells on the plates two days earlier. Error bars are SEM calculated from the combined variances of the Lac$^+$ colony counts and the Lac$^-$ cell counts (some of which are smaller than the symbols).

TABLE 2

Levels of β-galactosidase in various genetic backgrounds

	Genotype	β-Galactosidase in Miller units[a] (fold increase over wild type)	
		Grown in glycerol	Grown in lactose
Lac$^-$ strains			
FC40	Wild type	2.3	ND[b]
FC973	*recD1903*	8.9 (3.8)	ND
FC465	*recG258*	4.0 (1.7)	ND
FC975	*recD1903 recG258*	11.9 (5.1)	ND
Lac$^+$ derivatives[c]			
FC40	Wild type	206	196
FC973	*recD1903*	690 (3.4)	461 (2.3)
FC465	*recG258*	383 (1.9)	305 (1.6)
FC975	*recD1903 recG258*	842 (4.1)	723 (3.7)

[a] See Miller (1972).
[b] Not done.
[c] All strains carried the same episome, which is a true revertant of *lacI33*.

cells will contain only one chromosome (Akerlund *et al.* 1995), and *dnaQ* is an essential gene located well away from the origin of replication. The ratios of the densities of these hybidization spots indicated that the copy number of the *lac* allele relative to the *dnaQ* gene was 2.6- to 4.2-fold higher in *recD* mutant cells than in wild-type cells (data not shown). DNA isolated from *recD* mutant cells was highly degraded, which increased the background hybidization (see discussion), so these numbers may be underestimates. But, they are in reasonable agreement with the β-galactosidase results.

PFGE revealed that the copy number of the entire episome is higher in *recD* mutant cells than in wild-type cells (Figure 2). The episome present in these cells, F′128, carries two recognition sites for the *Sfi*I restriction endonuclease, one in the Tra region (GenBank accession no. U01159) and one near the *codBA* operon (GenBank accession no. AE000140 U00096). From PFGE of a limited S1 nuclease digest (to linearize the episome) we determined that F′128 is about 200 kb in size (data not shown). A *Sfi*I digest of total cellular DNA gave episomal fragments of approximately 70 and 130 kb (Figure 2), the larger of which carries the *lac* allele. Figure 2 shows that both these bands were more intense than monomer chromosomal fragments in wild-type cells, and even more intense in *recD* mutant cells. *recG* cells had an intermediate level of intensity, and *recG recD* double mutant cells were indistinguishable from *recD* mutant cells by this technique. In all backgrounds the intensities of the two episomal bands are the same, indicating that the increases in the copy number of the *lac* allele that we detected by the methods described above are due to increases in the numbers of episomes in the mutant backgrounds.

We quantified the intensity of ethidium bromide staining with a PFGE gel that better separated the middle-sized bands. The intensity of the 130-kb episomal band relative to the nearest larger chromosomal band was 2.0 for wild-type cells and 3.9 for *recD* mutant cells, again in reasonable agreement with the β-galactosidase results.

The copy number of the episome increases further during lactose selection: It is difficult to do biochemical or molecular analyses of Lac$^-$ cells during incubation

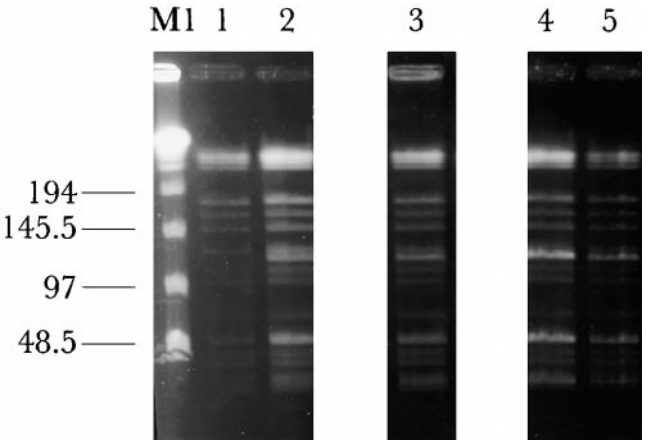

Figure 2.—The entire episome is amplified in *recD* mutant cells. The various strains were grown to saturation in M9-0.1% glycerol medium, embedded in agarose microbeads, and their DNA was extracted and digested with *Sfi*I restriction endonuclease. The parameters for pulsed-field gel electrophoresis were as follows: 1% PFGE-grade agarose gel at 5V/cm and 14°; the gel was run for 22.5 hr with an initial pulse time of 5 sec increasing linearly to 20 sec by the end of the run. The gel was stained with ethidium bromide. Lane M1, λ-ladder molecular weight markers (New England Biolabs); lane 1, the F$^-$ Δ (*lac-proB*) parental strain FC36; lane 2, the F′ *recG* mutant strain FC465; lane 3, the F′ wild-type strain FC40; lane 4, the F′ *recD* mutant strain FC973; lane 5, the F′ *recG recD* mutant strain FC975. Numbers on the left are kilobases. The episomal bands of 70 and 130 kb are visible in lanes 2–5. Only one gel is shown with irrelevant lanes masked.

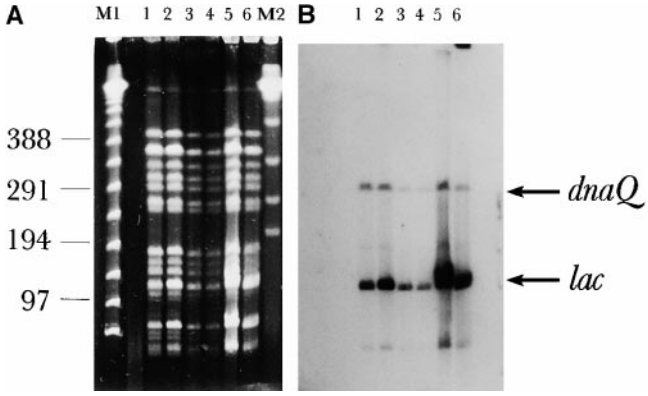

Figure 3.—The episome continues to amplify in *recD* mutant cells during incubation in lactose. FC40 (wild-type) and FC973 (*recD* mutant) cells were embedded in agarose microbeads, incubated for 3 days in M9-lactose liquid medium plus Xgal, and Lac$^+$ and Lac$^-$ cells were harvested (see materials and methods). The DNA was purified and digested with *Sfi*I restriction endonuclease. The parameters for PFGE were as follows: 1% PFGE-grade agarose gel at 5V/cm and 14°; the gel was run for 35 hr with an initial pulse time of 15 sec increasing linearly to 50 sec by the end of the run. After electrophoresis, a Southern blot was performed using radioactive probes to the chromosomal *dnaQ* gene and the episomal *lac* allele. (A) Ethidium-stained pulsed-field gel: lane M1, λ- ladder molecular weight markers (New England Biolabs); lane 1, Lac$^-$ FC40 from day 0; lane 2, Lac$^-$ FC973 from day 0; lane 3, Lac$^+$ FC40 from day 3; lane 4, Lac$^-$ FC40 from day 3; lane 5, Lac$^+$ FC973 from day 3; lane 6, Lac$^-$ FC973 from day 3; M2, *Saccharomyces cerevisiae* chromosomes (New England Biolabs). Numbers on the left are kilobases. (B) Southern blot of the gel shown in A with the *dnaQ* and *lac* bands indicated.

in lactose because Lac$^+$ revertants arise and take over the culture. However, we enriched for Lac$^-$ and Lac$^+$ cells by first embedding Lac$^-$ cells in low-melt agarose microbeads, incubating them for 3 days in minimal lactose medium plus Xgal, and separating white and blue microbeads enriched for Lac$^-$ and Lac$^+$ cells, respectively (see materials and methods). The DNA was prepared for PFGE as usual, and after PFGE (Figure 3A), a Southern blot of the gel was hybridized to the *lac* and *dnaQ* probes (Figure 3B). The degree of hybridization was quantified (using a darker exposure than shown for lanes 3 and 4) and the ranges of four separate determinations of the comparative intensities of the *lac* and *dnaQ* bands are given in Table 3. These results indicate that during incubation in lactose the copy number of the *lac* allele relative to the chromosomal *dnaQ* gene does not detectably change in wild-type cells, but increases two- to threefold in *recD* mutant cells. As mentioned above, because of the intense background in the *recD* lanes this may be an underestimate. The two episomal bands did not differ in their intensity by ethidium bromide staining (Figure 3A); thus in *recD* mutant cells the copy number of the entire episome, not just the *lac* allele, increases during lactose selection.

During lactose selection, DNA segments containing the *lac* allele can be amplified in tandem in some cells,

TABLE 3

The copy number of the *lac* allele during incubation in lactose

Strain	Genotype	Days in lactose	Ratio of the *lac* allele to the *dnaQ* gene[a]
FC40 Lac$^-$	Wild type	0	2.8–3.4
FC40 Lac$^-$		3	2.5–3.5
FC40 Lac$^+$		3	2.3–3.3
FC973 Lac$^-$	*recD1903*	0	4.4–4.7
FC973 Lac$^-$		3	9.5–11.4
FC973 Lac$^+$		3	5.5–9.7

[a] Four separate measurements of the densities of the *lac* and *dnaQ* hybridizing bands were made; the ratio of *lac* to *dnaQ* was computed for each, and the ranges of the ratios are given.

producing heterogeneously sized arrays (Tlsty *et al.* 1984; Andersson *et al.* 1998). In Figure 3 such arrays would appear as a series of bands either above or below the *lac* fragment, depending on whether the amplified region included the closest *Sfi*I site. To improve sensitivity, DNA from agarose-embedded cells was digested with *Eco*RV, which cuts within the *lac* allele, and used for Southern blots, as described above. For both strains, the amount of *lac* DNA on day 3 relative to day 0 was the same as obtained with PFGE (data not shown). Therefore, if the *lac* allele is amplified independently of the rest of the episome, this amplification makes too small a contribution to the total amount of *lac* DNA to be detected by these methods, at least during the first 3 days of incubation in lactose.

The copy number of the episome or of the Lac$^-$ allele does not increase to the extent that *recD* mutant cells become phenotypically Lac$^+$. Amplified Lac$^-$ alleles giving a Lac$^+$ phenotype are unstable when lactose selection is relieved (Tlsty *et al.* 1984). We previously found that 2% of the late-arising Lac$^+$ colonies of FC40 were composed mainly of cells with an unstable Lac$^+$ phenotype (Foster 1994) and confirmed that these represented cases of amplification by oligonucleotide probing (Foster *et al.* 1995). In the study presented here, 1 out of 50 (2%) and 0 out of 67 (<1.5%) late-arising Lac$^+$ colonies of FC40 and FC973, respectively, were composed mainly of unstably Lac$^+$ cells.

The results so far can be summarized as follows. *recD* mutant cells normally have two- to fourfold more copies of the episome than wild-type cells (Table 2 and Figure 2). When incubated in lactose, the copy number of the episome further increases, at least doubling by day 3 (Table 3 and Figure 3). The increase in β-galactosidase from each or both of these factors allows Lac$^-$ *recD* mutant cells to grow slowly on lactose minimal medium, achieving a twofold increase in cell number by day 3 (Figure 1B). Thus, 3 days after plating the same number of cells on a lactose plate, the *recD* mutant population will have 8–16 more copies of the Lac$^-$ allele than will

TABLE 4

The effect of recombination functions on adaptive mutation to Lac$^+$

Strain	Other genotype	n^a	Day 2 Lac$^+$/sector^b	Days 3–5 Lac$^+$/sector/day^c	Lawn^d
recD$^+$ strains					
FC40		4	0.3 ± 0.3	19 ± 2	Normal
FC877	*recA938*	4	1 ± 1	1 ± 0.3	Normal
FC836	(*ruvAC*)*65*	4	0	4 ± 1	Normal
FC465	*recG*258	3	8 ± 2	257 ± 31	Normal
FC897	*recG*258 (*ruvAC*)*65*	4	0	5 ± 1	Normal
recD$^-$ strains					
FC973		8	3 ± 1	70 ± 7	Heavy
FC1051	*recA938*	8	0.1 ± 0.1	2 ± 0.5	Normal
FC977	(*ruvAC*)*65*	4	0	0.3 ± 0.2	Normal
FC1274	*ruvA60*	8	0	2 ± 0.6	Late^e
FC975	*recG258*	4	0	23 ± 6	> Normal
FC1053	*recG258 sulA11*	8	0	11 ± 2	> Normal

[a] Number of independent cultures tested. The results of four experiments have been combined.
[b] Mean number of Lac$^+$ colonies appearing on day 2, ± SEM where appropriate.
[c] Mean number of Lac$^+$ colonies appearing each day after day 2 for as long as the colonies could be counted, ±SEM.
[d] The appearance of the lawn of Lac$^-$ cells during days 3–5. Normal, the light lawn normally made by FC40 cells.
[e] The lawn did not appear until day 5.

the wild-type population, accounting for much, but not all, of the apparent increase in the accumulation of Lac$^+$ revertants (Figure 1A). And, these factors appear insufficient to account for the steady increase in mutation rate per cell with time (Figure 1C).

Genetic requirements for the increased rate of adaptive mutation of *recD* mutant cells: To screen a large number of different gene functions for their effects on adaptive mutation, we routinely use a semiquantitative assay (see materials and methods). In these experiments, no nonreverting scavenger cells are used, and wild-type Lac$^-$ cells produce a light lawn of background growth during the 5 days of the experiment. In contrast, *recD* mutants produce a lawn that is so heavy by day 5 that new Lac$^+$ colonies can barely be seen (in which case the counts from day 5 are not included in the results). However, the semiquantitative test has two advantages over a large-scale test. First, the appearance of the lawn allows visible assessment of how different genetic backgrounds affect the ability of Lac$^-$ cells to grow on lactose, which, barring growth defects, may indicate their numbers of episomes. Second, unlike *recD*$^+$ strains, if certain *recD* mutant derivatives are plated with scavengers, the genotype of the scavenger strain influences the apparent mutation rate to Lac$^+$ (further discussed below). Because no scavengers are used in the semiquantitative test, this complication is avoided. The results presented below were obtained with this semiquantitative test.

Recombination functions: Adaptive mutation to Lac$^+$ in FC40 and in *recD* mutant cells is dependent on RecA and RecB (Cairns and Foster 1991; Harris *et al.* 1994). Table 4 shows that, as in FC40 (Foster *et al.* 1996; Harris *et al.* 1996), adaptive mutation in *recD* mutant cells also depends on RuvABC, enzymes for translocation and resolution of recombination intermediates. As mentioned above, in otherwise wild-type cells, adaptive mutation is greatly enhanced by loss of RecG (Foster *et al.* 1996; Harris *et al.* 1996), which participates in an alternative pathway to resolve recombination intermediates (reviewed in West 1997). In contrast, the adaptive mutation rate of *recD recG* double mutants was essentially the same as wild-type cells (Table 4). (We were unable to construct a *recD recG ruv* triple mutant.) Because populations of the *recD recG* double mutant contained many filamentous cells, we also tested a *recD recG sulA* triple mutant and found that the *sulA* defect, which suppresses DNA damage-induced filamentation (Mount 1977), did not relieve the low adaptive mutation rate of the *recD recG* double mutant (Table 4).

Single-stranded DNA exonucleases: Mutations in *recD* result in hyperrecombination of λ-phage (in the absence of chi sites) and plasmids, but have only a modest effect on transductional and conjugational recombination of the chromosome (Chaudhury and Smith 1984; Biek and Cohen 1986; Lovett *et al.* 1988). However, in *recD* mutants, chromosomal recombination becomes more dependent on RecJ than in wild-type cells (Lloyd *et al.* 1988; Lovett *et al.* 1988). RecJ is a single-stranded exonuclease with 5′ to 3′ polarity (Lovett and Kolodner 1989) and *recJ* derivatives of FC40 appear to have a somewhat higher than normal rate of adaptive mutation to Lac$^+$ (Harris *et al.* 1994; Table 5), although we believe that at least some *recJ* derivatives readily ac-

TABLE 5

The effect of single-strand exonucleases on adaptive mutation to Lac$^+$

Strain	Other genotype	n^a	Day 2 Lac$^+$/sector[b]	Days 3–5 Lac$^+$/sector/day[c]	Lawn[d]
recD$^+$ strains					
FC40		4	1 ± 0.5	41 ± 3	Normal
FC1112	*recJ284*	4	2 ± 1	56 ± 4	Normal
FC1114[e]	*recJ2051*	4	4 ± 0.3	61 ± 4	Normal
FC1185	p*recJ*$^+$	4	1 ± 0.3	26 ± 4	Normal
FC1187	*recJ2051*/p*recJ*$^+$	4	3 ± 1	27 ± 3	Normal
FC1271[f]	*xonA300::Cat*	5	12 ± 3	62 ± 8	Normal
recD$^-$ strains					
FC973		4	1 ± 0.4	87 ± 10	Heavy
FC1168	*recJ2051*	4	0	5 ± 1	Normal
FC1186	p*recJ*$^+$	4	2 ± 1	116 ± 8	≫ Normal[g]
FC1188	*recJ2051*/p*recJ*	4	0.3 ± 0.3	84 ± 21	Heavy, but late
FC1272	*xonA300::Cat*	6	0.3 ± 0.3	14 ± 4	≫ Normal[g]

[a] Number of independent cultures tested. The results of three experiments have been combined.

[b] Mean number of Lac$^+$ colonies appearing on day 2, ± SEM where appropriate.

[c] Mean number of Lac$^+$ colonies appearing each day after day 2 for as long as the colonies could be counted, ±SEM.

[d] The appearance of the lawn of Lac$^-$ cells during days 3–5. Normal, the light lawn normally made by FC40 cells.

[e] In other experiments with this strain and with additional transductions of the *recJ2051* allele, a variable number of cultures gave high mutation rates. We believe that the cells in these cultures acquired additional mutations that gave an increased adaptive mutation rate. This variability was not observed in constructions with the *recD* mutant or with *recJ284*.

[f] The results given are for five fresh transductants into FC40; a sixth transductant had an adaptive mutation rate lower than wild-type cells. Similar results were obtained in other constructions with this allele, suggesting that additional mutations occur that lower the adaptive mutation rate. However, this variability was not observed in constructions with the *recD* mutant. The relatively high number of Lac$^+$ revertants appearing on day 2 in this experiment was probably a chance occurrence as it was not observed in other experiments.

[g] The lawn was lighter than with *recD* mutant cells, but heavier than with wild-type cells.

quire other mutations that increase the adaptive mutation rate. But, as shown in Table 5, loss of RecJ activity nearly eliminated adaptive mutation in *recD* mutant cells. This loss was complemented by supplying *recJ*$^+$ on a multicopy plasmid.

Exonuclease I, encoded by the *xonA* gene (also known as *sbcB*), is a single-stranded exonuclease with 3′ to 5′ polarity. Loss of ExoI activity reduces conjugal recombination two- to threefold (Viswanathan and Lovett 1998). A Δ*xonA* derivative of FC40 had a slightly enhanced adaptive mutation rate (Table 5) although, in contrast to *recJ* mutant cells, *xonA* mutant cells appear to acquire additional mutations that decrease their adaptive mutation rate. In *recD* mutant cells, however, loss of ExoI activity was similar to loss of RecJ activity, reducing adaptive mutation to below wild-type levels (Table 5).

Conjugal functions: Mutations in genes required for F conjugation reduce the rate of adaptive mutation of FC40 about 10-fold (Foster and Trimarchi 1995a). The high mutation rate of *recD* mutant cells was likewise reduced by a *traD* mutation, although not to as great a degree. In a semiquantitative test, a *traD* derivative of FC973 produced 20 ± 4 Lac$^+$ mutants per day from days 3 to 5, and the lawn was delayed, not becoming heavy until day 5.

Genetic requirements for episome amplification in *recD* mutant cells: Unless a genetic defect also confers a growth defect, the lawn produced on lactose plates by *recD* mutant derivatives probably reflects their episome copy number. By this visual assay, episome amplification in *recD* mutant cells is independent of ExoI, but dependent on RecA, RuvABC, RecJ (Tables 4 and 5), and TraD (see above). Southern blots of *Eco*RV-digested DNA confirmed that the increased number of *lac* alleles in *recD* mutant cells was eliminated in *recA* and *recJ* derivatives and reduced by the *traD* mutant (*ruv* mutants were not tested). Figure 4 shows one such Southern blot of DNA from *recJ* and *recD recJ* strains. (The *lac* probe used hybridizes to two *Eco*RV fragments, each carrying a part of the *lac* allele.) The densities of the *lac* bands compared to the *dnaQ* bands confirms that RecJ does not affect the episomal copy number in wild-type cells, but episome amplification in *recD* mutant cells is dependent on RecJ.

***recD* mutant cells have an increased rate of mutation at another episomal gene during lactose selection:** When wild-type cells carry a revertible Tet^S allele on their epi-

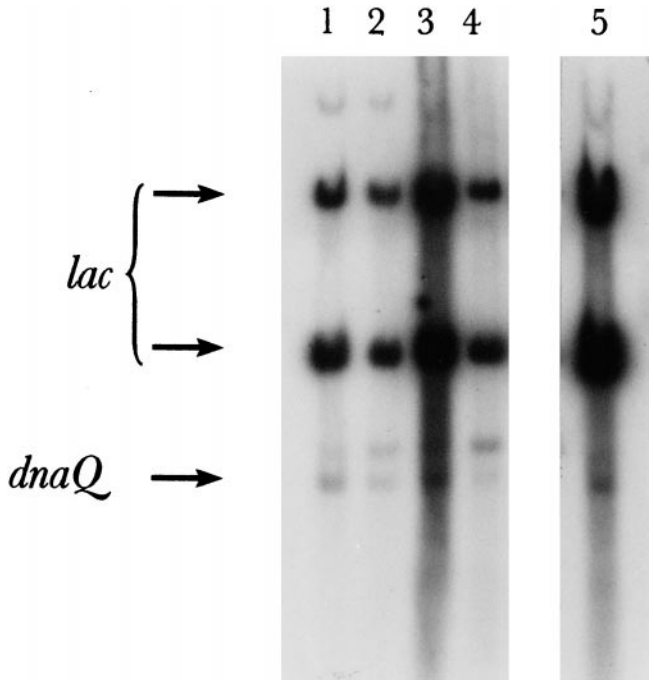

Figure 4.—RecJ is required for maximal amplification of the episome in *recD* mutant cells. DNA extracted from the various strains grown to saturation in M9-glycerol medium was digested with *Eco*RV restriction endonuclease. After electrophoresis, a Southern blot was performed using radioactive probes to the episomal *lac* allele (which, in this case, hybridizes to two *Eco*RV fragments) and to the chromosomal *dnaQ* gene. Lane 1, FC40 (wild type); lane 2, FC1114 (*recJ* mutant); lane 3, FC973 (*recD* mutant); lane 4, FC1168 (*recD recJ* mutant); lane 5, FC1188 (*recD recJ* mutant complemented by pJC765, which carries *recJ*$^+$). Lane 5 is from the same blot, but intervening lanes have been excised.

some, nonselected Tet^R mutants appear during lactose selection at about 70% of the rate at which Lac$^+$ revertants appear (Foster 1997). To determine the effect of *recD* on nonselected reversion, we used a different allele of *recD*, *recD1009* (formerly designated *recB1009*; Chaudhury and Smith 1984). Five independent cultures of the *recD1009* mutant carrying the Tet^S Φ(*lacI33-lacZ*) episome were plated on minimal lactose plates (with scavengers) in triplicate. One set of five was overlaid with tetracycline plus glycerol and Xgal on day 0, one set of five was likewise overlaid on day 3, and one set of five was not overlaid. The mean number of Lac$^+$ mutants per 10^8 cells plated (±SEM) was 0 on day 2 and 3037 ± 87 by day 5, in close agreement with the results obtained with *recD1903* (Figure 1A). The mean number of Tet^R mutants per 10^8 cells plated (±SEM) was 6 ± 3 on day 0 and 2743 ± 232 on day 3. Thus, while *recD* mutant cells are accumulating Lac$^+$ revertants at a high rate, they are also accumulating revertants of a nonselected gene on the episome at a nearly equivalent rate.

Transfer of episome DNA increases the apparent rate of adaptive mutation to Lac$^+$ in *recD* mutant derivatives: As mentioned above, the results presented in Tables 4 and 5 were obtained in semiquantitative experiments without scavenger cells, in contrast to our typical large quantitative experiments. With most derivatives of FC40, the two assays give equivalent results (*e.g.* see Foster *et al.* 1996). This was also the case with *recD* mutant cells, but not when *recD* was combined with other mutant alleles. Typically, the apparent mutation rate to Lac$^+$ was higher and more variable in large experiments with scavengers than in the semiquantitative tests. Particularly dramatic were results with the *recD recG* mutant strain, which had an adaptive mutation rate equivalent to the wild-type strain in semiquantitative tests (Table 4) but a much higher and variable adaptive mutation rate to Lac$^+$ in large-scale tests (data not shown).

Although all the cells are male, a low level of episome transfer takes place during lactose selection, which can be detected by the appearance of Lac$^+$ episomes in the scavenger population (Foster and Trimarchi 1995a; Radicella *et al.* 1995; Peters *et al.* 1996). This could be transfer of a complete episome or of fragments that recombine with the recipient's resident episome (Foster and Trimarchi 1995b). After a large-scale test in which the *recD recG* mutant strain had an apparent adaptive reversion rate higher than the *recD* mutant strain, Lac$^+$ colonies were purified on lactose minimal medium and tested for resistance to rifampicin. If the Lac$^+$ episome were in the scavenger cells, the Lac$^+$ cells would be Rif^S. With FC40, 10% (5/48) of the Lac$^+$ colonies that arose after day 2 were Rif^S, somewhat higher in this experiment than the 4% we previously found (Foster and Trimarchi 1995b). With the *recD* mutant, 43% (23/54) of the Lac$^+$ colonies that arose after day 2 were Rif^S; and an additional 2 Lac$^+$ colonies were mixed Rif^S and Rif^R. With the *recD recG* double mutant, 100% (46/46) of the Lac$^+$ colonies that arose after day 2 were Rif^S.

These results could mean that *recD* and *recD recG* mutant cells are more likely to transfer their episomes, or that the recovery of Lac$^+$ mutants is poor unless the Lac$^+$ episome (or some form of it) is transferred into the scavenger. To help distinguish between these possibilities, we repeated the experiment but with preexisting Lac$^+$ cells (described above) plated with scavengers on lactose plates. In this case, <3% (0/36) of the Lac$^+$ colonies of the wild-type strain were Rif^S; 8% (3/36) of the Lac$^+$ colonies of the *recD* mutant strain were Rif^S; and 97% (35/36) of the Lac$^+$ colonies of the *recD recG* double mutant strain were Rif^S and one colony was mixed. Thus, it appears that a high rate of episome transfer is intrinsic to *recD* mutants, particularly when they are also defective in *recG*, which may indicate that the episome (or some form of it) is unstable in these mutant cells (see discussion).

Table 6 gives the result for a semiquantitative test in which about 10^7 cells of each revertible strain were spread on a quadrant of a lactose plate that had been prespread with 10^9 cells of nonrevertible strains with

TABLE 6

The effect of the scavenger genotype on adaptive mutation to Lac$^+$

Revertible strain	Revertible strain's genotype	Scavenger strain's genotype	n^a	Day 2 Lac$^+$/sector^b	Days 3–5 Lac$^+$/sector/day^c	Lawn^d
FC40	Wild type	Wild type	4	1 ± 1	4 ± 1	None
		recD1903	4	2 ± 1	5 ± 1	None
		recG258	4	1 ± 0.4	6 ± 1	None
		recD1903 recG258	4	1 ± 0.3	8 ± 1	None
FC465	*recG258*	Wild type	4	1 ± 1	212 ± 14	None
		recD1903	4	2 ± 1	173 ± 6	None
		recG258	4	4 ± 1	182 ± 6	None
		recD1903 recG258	4	2 ± 1	130 ± 10	None
FC973	*recD1903*	Wild type	4	0.5 ± 0.3	139 ± 27	Heavy
		recD1903	4	0	120 ± 26	Heavy
		recG258	4	1 ± 1	69 ± 15	Light
		recD1903 recG258	4	1 ± 1	58 ± 11	Light
FC975	*recD1903 recG258*	Wild type	4	1 ± 1	51 ± 14	None
		recD1903	4	2 ± 2	47 ± 13	None
		recG258	4	1 ± 1	30 ± 9	None
		recD1903 recG258	4	2 ± 2	6 ± 2	None

[a] Number of independent cultures tested.
[b] Mean number of Lac$^+$ colonies appearing on day 2, ±SEM where appropriate.
[c] Mean number of Lac$^+$ colonies appearing each day after 2, ±SEM.
[d] The appearance of the lawn of Lac$^-$ cells during days 3–5.

various genotypes. For both *recD* mutant and *recD recG* mutant revertible strains, the apparent adaptive mutation rate to Lac$^+$ was reduced slightly if the scavengers were *recG* mutants. If the scavengers were *recD recG* double mutants, the adaptive mutation rate of the *recD recG* double mutant dropped to the level observed when no scavengers were used.

DISCUSSION

The results presented here indicate that, in several respects, adaptive mutation to Lac$^+$ involves different mechanisms in *recD* mutant cells than in wild-type cells. About half of the apparent increase in the adaptive mutation rate of *recD* mutant cells can be attributed to a RecA-dependent increase in episomal copy number and growth of the Lac$^-$ cells on the lactose plates. However, these factors appear insufficient to account for the continuing increase in mutation rate with time during lactose selection (Figure 1C). In addition, the increase in adaptive mutation rate in *recD* mutant cells is (i) dependent on activities of the single-stranded exonucleases, RecJ and ExoI, which are not required for (in fact, slightly inhibit) adaptive mutation in wild-type cells and (ii) enhanced by RecG activity, which opposes adaptive mutation in wild-type cells.

Loss of ExoV activity affects the replication and stability of certain nonconjugal plasmids (Bassett and Kushner 1984; Biek and Cohen 1986; Cohen and Clark 1986; Seelke *et al.* 1987; Niki *et al.* 1990). The following model emerged from these studies. Nicks occurring at the replication fork during plasmid replication produce double- and single-stranded DNA ends that are normally destroyed by exonucleases, particularly ExoV. In the absence of ExoV, a long-lived 3′ end can be a substrate for RecA-catalyzed duplex invasion. If another plasmid molecule is invaded, rolling-circle (σ-form) replication is initiated, resulting in the accumulation of linear multimers of the plasmid. If the plasmid has a site-specific recombination activity, linears are resolved into covalently closed circular (CCC) forms. Plasmid amplification is inhibited by exonucleases I and III in *recBC* mutants, but occurs in the presence of these exonucleases in *recD* mutants.

With a few modifications, a similar model can explain many of the results presented here. Normal θ-form DNA replication of F is initiated at the vegetative origins (*oriS* and *oriV*), whereas σ-form DNA synthesis is initiated by a TraI-catalyzed nick at the conjugal origin, *oriT* (Willetts and Skurray 1987). Our current model for adaptive mutation in wild-type FC40 is that a replication fork initiated at one of the vegetative origins collapses when it encounters the persistent nick at *oriT*. The collapsed arm is a substrate for RecBCD, whose exonuclease and helicase activities create an invasive 3′ end that is homologous to a region distal to the *lac* region. RecA catalyzes strand invasion and, after both strands have invaded homologous duplex DNA, the replication fork is restored and replication resumes. Mutations are produced as this new fork traverses the *lac* region (Kuzminov 1995a; Foster *et al.* 1996, Figure 7).

In *recD* mutant cells, the nick at *oriT* would frequently

initiate the same sequence of events postulated for nonconjugal plasmids. The helicase activity of RecBC(D^-) on the collapsed arm of the replication fork would produce a long 3′ single-stranded end that could invade a different episome molecule and initiate rolling-circle replication. However, the consequences of this replication appear to be different from that observed with nonconjugal plasmids. We did not detect linears by PFGE of undigested DNA. After the episome was linearized with limited S1 nuclease digestion, there was no detectable difference between wild-type and *recD* mutants in the amount of episome multimers (although we would not have resolved multimers greater than 3×). And, in contrast to other plasmids, which seem to amplify out of control in *recD* mutants, the steady-state copy number of the episome did not appear to increase beyond about eight in *recD* mutant cells (Tables 2 and 3). These differences can be explained by characteristics specific to the F episome.

Two mechanisms would tend to produce monomers from rolling-circle replication of the episome. First, F has a site-specific recombination system at *oriV* (Willetts and Skurray 1987), which (after second strand synthesis) should convert linears and multimers to monomer CCC forms (Seelke *et al.* 1987). Second, if TraI acts as it does during conjugation (Lanka and Wilkins 1995), it may terminate rolling-circle replication after one round by nicking and sealing the new strand at *oriT*.

Like other low-copy plasmids, the copy number of F is controlled by interactions between a required replication protein (Rep) and structural components of the vegetative origin (iterons; Helinski *et al.* 1996). This mechanism would not control rolling-circle replication. Thus, the fact that the steady-state copy number of F is limited in *recD* mutant cells means either that there is some other control on copy number or that initiation of rolling-circle replication depends on θ-form replication initiated from a copy-controlled vegetative origin. However, the increase with time in the adaptive mutation rate in *recD* mutants (Figure 1C) implies that, despite the limits on the steady-state copy number, episomal replication continues at an ever increasing rate during lactose selection. If that is so, the additional copies must be removed from the cells.

Extra episome copies could be removed from *recD* mutant cells by degradation by nucleases other than ExoV. As can be seen in Figures 3 and 4, DNA extracted from *recD* mutant cells contains a large amount of degraded DNA (to which both the *lac* and *dnaQ* probes hybridize, either specifically or nonspecifically). Similar degradation has been seen by others (Biek and Cohen 1986; Niki *et al.* 1990). Linear DNA produced by σ-form replication would provide a ready substrate for single-strand exonucleases. In addition, after second-strand DNA synthesis, the helicase activity of $RecBCD^-$ or other helicases on linear duplex DNA would produce substrates for single-strand exonucleases. RecJ and ExoI have been shown to be active in such circumstances (Rinken *et al.* 1992), which may explain, in part, why in their absence, Lac^+ mutation in *recD* mutant cells is inhibited (Table 5; see below). While this explanation would allow for the maintenance of a constant number of episomes, it fails to explain why the rate of replication continues to increase.

Extra episomal copies could also be removed by conjugation, which would not only allow the maintenance of a steady state number per cell, but would also allow for their ever-increasing rate of replication. Invasion of a second episome by a 3′ end close to *oriT* is similar to the initiation of conjugal replication. Our results, and those of others (Peters *et al.* 1996), indicate that *recD* cells readily pass episomes to $recD^+$ male cells. This tendency is exacerbated by additional mutations affecting DNA metabolism, such as *recG* and *xonA*. Thus, the activities of TraI and its associated conjugal proteins may result in the transfer to neighboring cells of all or part of the DNA strand displaced by σ-form replication. Because scavenger cells are in excess, this process could continue and accelerate during the course of a typical experiment. If the displaced strand carries a Lac^+ mutation (produced during a previous round of replication), the recipient would become Lac^+. The genotype of the scavenger cells would then determine the rate of appearance of Lac^+ revertants, *e.g.*, when the scavenger cells are *recD recG* mutants they have more copies of their own episome and would be less likely to serve as recipients, or less likely to retain the transferred episome, and fewer Lac^+ revertants would appear (Table 6).

The *recD* mutant cells may be heterogeneous in their number of episomes, so in the absence of scavengers episome transfer could still occur to neighbors with fewer episomes. Note, we typically observed in semiquantitative tests that the mutation rate to Lac^+ appeared to decline in the last two days of the experiments, when fewer recipients may be available.

Although it is appealing to assume that rolling-circle replication accounts for the high rate of Lac^+ mutation in *recD* mutant cells, the genetic requirements indicate that the situation is more complex. In particular, as in wild-type cells, adaptive mutation to Lac^+ in *recD* mutant cells is dependent on RuvABC, enzymes that catalyze the branch migration and resolution of four-stranded recombination intermediates (Holliday junctions; Table 4). If Lac^+ mutations were being produced by rolling-circle replication, site-specific recombination and the activity of TraI should insure the stable retention of at least some of the Lac^+ episomes without the need for homologous recombination. In addition, in contrast to wild-type cells, adaptive mutation to Lac^+ in *recD* mutant cells requires RecG, but only in the absence of recipient scavengers. These requirements can be accommodated by the following model, illustrated in Figure 5, which is based on McGlynn *et al.* (1997).

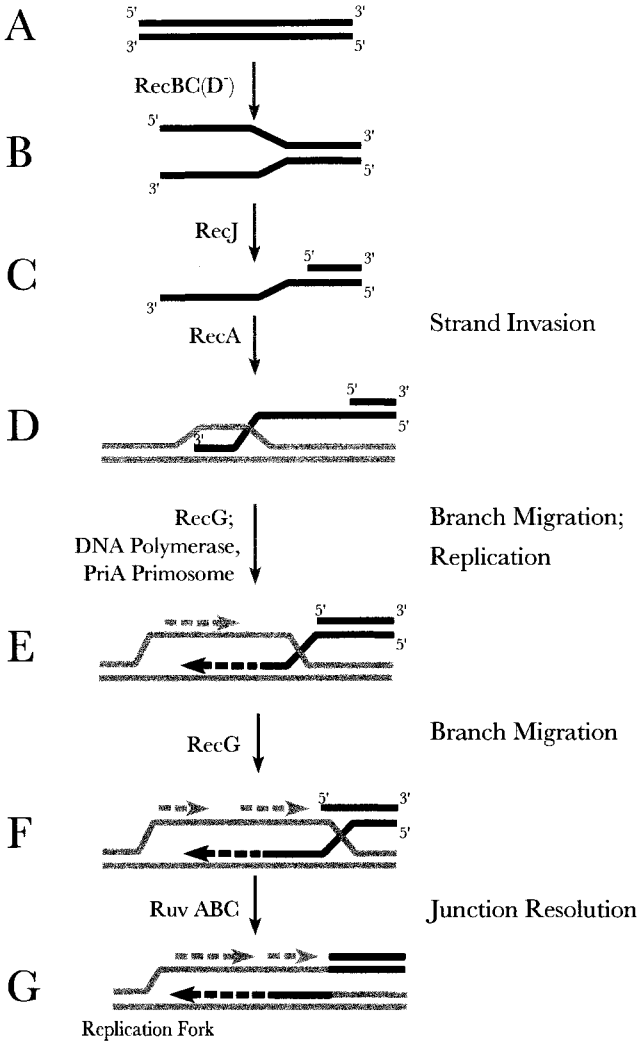

Figure 5.—A model for adaptive mutation in *recD* mutant cells. (Not drawn to scale.)

As discussed above, after replication-fork collapse in wild-type cells, RecBCD's nuclease and helicase activities produce an unpaired 3′ end that can invade the homologous duplex region of the same episome distal to the *lac* region. But, in *recD* mutant cells, RecBCD$^-$ helicase activity (Figure 5B) plus RecJ's 5′ to 3′ exonuclease activity (Figure 5C) produce a long unpaired 3′ end that is homologous to the proximal region between *oriT* and *lac* (Figure 5D). This 3′ end can invade another episome, forming a D-loop (Figure 5D). D-loops are recognized by RecG and by PriA, an essential primosome component. Both of these proteins have helicase activities, but they have antagonistic roles in recombination (Al-Dieb *et al.* 1996; McGlynn *et al.* 1997). By translocating the D-loop away from the invasion point toward duplex DNA, RecG prevents PriA's helicase activity from driving the D-loop in the direction of leading strand synthesis, while allowing the primosome to assemble and initiate lagging-strand synthesis (Figure 5E). Thus, RecG tends to oppose the initiation of rolling-circle replication and favor the establishment of a normal replication fork. The region of new synthesis expands until the 5′ partner of the invading strand (that has been resected by RecJ) also invades, producing a Holliday junction (Figure 5F). Now a substrate for RuvABC, the Holliday junction is translocated and resolved (McGlynn *et al.* 1997). The two linked episomes would eventually be resolved by site-specific or homologous recombination.

In this model, Lac$^+$ mutations would be produced as the new lagging-strand synthesis traverses *lac*. Indeed, DNA synthesis initiated by PriA *in vitro* can proceed in the absence of the proofreading subunit of DNA polymerase III (Marians *et al.* 1998); if the replication complex were assembled without the proofreader *in vivo*, this synthesis would be highly error prone. As we previously hypothesized (Foster *et al.* 1996), new mutations could also be protected from MMR correction if RuvAB translocated the Holliday junction toward the reassembled replication fork, producing doubly unmethylated DNA.

In *recD recG* mutants, PriA's helicase activity is unimpeded by RecG, so the 3′ invasion would initiate rolling-circle replication. This replication would also produce mutations, but, for the reasons given above, the amount of new synthesis is limited by the episome's copy-number control system. However, in the presence of wild-type scavengers, the *recD recG* cells could overcome mating inhibition and pass extra episomal copies to the cells that surround them. Given repeated rounds of rolling-circle replication, these passed episomes eventually would have Lac$^+$ mutations. That 40% of the Lac$^+$ mutants of *recD*$^-$ *recG*$^+$ cells were also in the scavengers suggests that this type of replication may frequently occur even in the presence of RecG.

As mentioned above, the roles of ExoI and RecJ in this process may be to degrade extra episomal copies, allowing more replication to proceed. However, unlike ExoI, RecJ is required both for amplification and for mutation (Table 5; Figure 4). This implies that RecJ is required at an early stage, *e.g.*, to resect the 5′ end after RecBCD$^-$ helicase activity (Figure 5C). ExoI could also have a more direct role in mutation by occasionally resecting the 3′ end past the *lac* region, which would essentially recreate the situation pertaining in wild-type cells (see Foster *et al.* 1996, Figure 7).[1]

The model in Figure 5 predicts that PriA is essential

[1]Recent results indicate that during recombination RecJ and ExoI act postsynaptically, probably by degrading the strands displaced during branch migration (Friedman-Ohana and Cohen 1998). Because such activities would tend to prevent the establishment of a replication fork, they may promote recombination but inhibit mutation (and, indeed, they do inhibit mutation in wild-type cells; Table 5). Although more complicated models are possible, the simplest conclusion is that in *recD* mutant cells, the antimutagenic activities of RecJ and ExoI are overshadowed by additional mutagenic activities, such as proposed here.

for adaptive mutation in *recD* mutants. Although not included in previous models, PriA is presumably also required in wild-type cells to initiate lagging-strand synthesis during recombination. A *priA* null derivative of FC40 (obtained from T. Kogoma) was, as predicted, defective for adaptive mutation (P. L. Foster, unpublished results). However, *priA* mutants are defective for all forms of recombination, are SOS induced, and are enfeebled for growth (Nurse *et al.* 1991; Kogoma *et al.* 1996; Sandler *et al.* 1996), so it is not clear how meaningful this result is on its own. Other alleles of *priA* may be more informative.

As mentioned in the Introduction, tandem amplification of the *lac* allele is an alternative to recombination-initiated replication as the source of the DNA synthesis that gives rise to adaptive Lac$^+$ mutations (Cairns and Foster 1991; Foster 1994; Roth *et al.* 1996). Andersson *et al.* (1998) proposed that if 0.03% of the cells amplified the *lac* allele 100-fold, and (because they have more β-galactosidase) these amplifying cells increased in number 1000-fold, the requisite amount of *lac* replication would be achieved. If so, to maintain the constant rate of adaptive mutation in wild-type cells (which requires a constant rate of replication), the total number of amplifying alleles in the population must attain steady-state almost immediately (Foster 1994). Our results could be explained if, in a *recD* mutant, the life of an amplified array were prolonged, allowing a net increase in the number of *lac* copies and an accelerating rate of mutation. We do not favor this hypothesis for two reasons. First, we did not detect an increase in *lac* DNA that could not be accounted for by the increase in the number of episomes in *recD* cells. Second, if amplified arrays were stabilized, some of the amplifying cells should be able to produce visible colonies; but, we did not detect in *recD* mutant cells an increase in the number of Lac$^+$ colonies that were unstably Lac$^+$. In addition, it would still be necessary to account for the opposing effects that RecG, RecJ, and ExoI have in wild-type and *recD* mutant cells.

In summary, the results presented here indicate that, as proposed for other plasmids, RecD plays an important role in controlling replication of the F episome and maintaining its proper copy number. RecD most likely does this by insuring that DSBs are repaired by intramolecular, not intermolecular, recombination. RecD mutant cells differ from wild-type cells not only in their high rate of adaptive mutation, but also in that their adaptive mutation rate continuously increases with time. Our results indicate that this increase is due to growth of the cells, episome amplification, and continued episome replication.

We thank J. H. Miller and the people listed in Table 1 for bacteriophage and bacterial strains and J. Cairns, J. W. Drake, A. Kuzminov, R. G. Lloyd, and F. W. Stahl for useful discussions. We are also grateful to the anonymous reviewers for improvements to this article. This work was supported by National Science Foundation grant no. MCB97838315.

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Communicating editor: R. Maurer