Mutant Bias in Nonlethal Selections Results From Selective Recovery of Mutants

Spencer A. Benson, Amy M. DeCloux and Joelle Munro

Department of Microbiology, University of Maryland, College Park, College Park, Maryland 20742–4451

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ABSTRACT

We have characterized a nonlethal selection for mutations that allow *Escherichia coli* to grow on large maltodextrins (Dex$^+$) in the absence of the *lamB* encoded maltoporin LamB. These Dex$^+$ mutations occur before and after imposition of the selection and the selection does not result in a general increase in mutagenesis. The recovered Dex$^+$ mutations are almost exclusively mutations that alter the *ompF* gene that encodes a major *E. coli* porin, OmpF even though analogous mutations in the homologous *ompC* gene, which encodes the OmpC porin, can confer a Dex$^+$ phenotype. We show that the bias for *ompF* mutations results from a biased recovery and that the genetic background of the starting strain and the selection itself influences the type of mutants that are recovered. When we use a strain carrying an amber mutation in the *lamB* gene we observe the same preference for *ompF* mutations as when we start with a *lamB* deletion strain. In addition, we show that there is no preferential mutagenesis of the *lamB* gene during the selection which induces transcription of the *lamB* gene. We present evidence that the biased recovery of mutants observed in this selection does not result from adaptive or directed mutagenesis and that the phenotypic fitness which allows recovery of Dex$^+$ mutants involves more than the increased ability to take up maltodextrins.

HOW bacteria genetically respond to environmental conditions is an important and fundamental question in evolution and bacterial physiology. Several reports have suggested that under conditions of nutrient limitation mutations that are advantageous are recovered at a disproportionately high rate. These findings have been reported for selections involving utilization of lactose (CAIRNS and FOSTER 1991; CAIRNS, OVERBRAUGH and MILLER 1988), β-glucosides (HALL 1988) Mu-excisions (SHAPIRO 1984), and restoration of tryptophan prototroph (HALL 1990, 1991). It has been suggested that under such conditions bacteria can direct or influence the type and/or rate at which mutations occur (SHAPIRO 1984; CAIRNS, OVERBRAUGH and MILLER 1988). This is referred to as directed (CAIRNS, OVERBRAUGH and MILLER 1988), "Cairnsian" (HALL 1990) or adaptive mutagenesis (HALL 1991) and has precipitated a discussion of the results and possible mechanisms in the scientific literature (BENSON, PARTRIDGE and MORGAN 1988; CHARLESWORTH *et al.* 1988; STAHL 1988; HALL 1990 1991). Alternative explanations for these experimental observations that do not invoke the idea of adaptive mutagenesis have been proposed (LENSKI 1989).

We have previously reported that the selection for growth on large maltodextrins (Dex$^+$) results in a strong bias in the type of mutants we recover (BENSON, OCCI and SAMPSON 1988). Specifically we observed that Dex$^+$ mutants with alterations in the *ompF* gene, which encodes the OmpF porin, are recovered almost exclusively even though mutations in the homologous *ompC* gene, which encodes the OmpC porin, can confer a Dex$^+$ phenotype. We have previously suggested that this might also be a case where adaptive mutagenesis influences or determines the type of mutants that are recovered (BENSON, PARTRIDGE and MORGAN 1988). The idea that starving cells could specifically or preferentially mutate a gene (*ompF*) whose product is directly involved in getting many necessary nutrients into the cells is attractive. Moreover, the Dex$^+$ selection system is similar to other selection systems where adaptive mutagenesis has been reported in that the majority of the mutant colonies appear many days after imposition of the selection. It differs in that the proposed adaptive Dex$^+$ mutations occur in a gene whose regulation is not directly linked to the selection itself and mutations in other loci can yield a Dex$^+$ in an *ompF*$^-$ host. Thus it seemed appropriate to determine if adaptive mutagenic was directing the mutagenic events and to test if adaptive mutagenesis operated in systems where the medium does not directly control transcription of the gene where the necessary mutation must occur. In this paper we explore several possible explanations for the observed mutant bias and show that phenotypic fitness, genetic background and selection conditions play important roles in determining the types of mutants recovered and that adaptive mutagenesis does not appear to occur.

MATERIALS AND METHODS

Media and chemicals: Microbiological media, chemicals, maltodextrins and antibiotics were purchased, prepared and

used as described previously (BENSON, OCCI and SAMPSON 1988; SAMPSON, MISRA and BENSON 1989). L broth, L agar and M63 minimal medium are described in SILHAVY, BERMAN and ENQUIST (1984). 4XM63 medium was prepared by adding four times the normal amount of 10 × salts solution to the medium. The 2 × and 0.5 × maltodextrin media were prepared by doubling or reducing by one-half the amount of maltodextrin added to the medium.

Bacterial strains, phages and strain constructions: Bacterial strains are described in Table 1. Bacterial strains were constructed by P1 transductions as described in SILHAVY, BERMAN and ENQUIST (1984). The Tn*10* and Tn*5* determinants were selected using resistance to tetracycline (25 μg/ml) or kanamycin (25 μg/ml), respectively. DME557 was constructed using the *zcb*01::Tn*10* determinant to introduce the *ompF-lacZ* and *pyrD*$^-$ genes into strain MCR106. SBM18 was constructed by introducing the *ompF*-1::Tn*5* mutation into RAM191 from MH450. RAM102 was constructed by introducing the *zei*06::Tn*10* into MCR106. The *ompF*(Dex) and *ompC*(Dex) alleles shown in Table 1 have been described (BENSON, OCCI and SAMPSON 1988; MISRA and BENSON 1988a,b). The numbers refer to the amino acid positions in the mature peptides and the letter to the single letter amino acid code. JMM strains were constructed by introducing the various *ompC*(Dex) alleles into MCR106 using the linked (50%) *zei*06::Tn*10* marker. SBM140 was constructed by introducing the *ompC*(Dex)Δ103-110(Am)102 mutation from SBM102 into SBM110 using the linked *zei*06::Tn*10* marker. SBM9532 and SBM158 were constructed by introducing the *malB* region carrying the *lamB*(Am)5 mutation into MCR106 or SBM110 in a two step construction where the recipient was made Mal$^-$ with a *malK*::Tn*5* and then transduced to Mal$^+$ Dex$^-$ with a P1 lysate grown on a strain carrying the *lamB*(Am)5 mutation.

Bacteriophages P1^{vir}, λ^{vir}, Φ80pSuIII (Φ80p*supF*$^+$) (SILHAVY, BERMAN and ENQUIST 1984), the OmpF specific phages K20 (BASSFORD *et al.* 1977) and Tu1a (DATTA, ARDEN and HENNING 1977), the OmpC specific phages SS4 (SCHNAITMAN and MCDONALD 1984) and Tu1b (DATTA, ARDEN and HENNING 1977) are from laboratory stocks. The T4(Am)39 phage as kindly provided by DIANE KIINO of the University of Chicago. Suppression of the T4(Am)39 mutation restores plaque forming ability.

Mutant selection: Spontaneous Dex$^+$ mutants were selected as described previously (BENSON, OCCI and SAMPSON 1988). Basically cells were grown overnight in L broth, collect by centrifugation, washed in an equal volume of 1XM63 medium and resuspended in 5 ml of 1XM63 medium. Samples (0.1–0.3 ml) were plated on M63 maltodextrin plates and incubated at 37°. The colony forming units (cfu) were determined by plating suitable dilutions on L medium. The position and number of the Dex$^+$ colonies was recorded at approximately 24-hr intervals by marking the back of the plate.

Amber mutations in the *ompF*(Dex)Δ111-123 allele were identified by selecting spontaneous mutants resistant to bacteriophage K20 and Tu1a. Generally, 0.1 ml of an overnight culture was mixed with approximately 1 × 10^9 plaque forming units (pfu) of each phage and the mixture plated in 3 ml of soft agar on an L plate. Amber mutations were identified by cross streaking the phage resistant mutants against the suppressor phage Φ80pSuIII that carries the *supF*$^+$ suppressor (SILHAVY, BERMAN and ENQUIST 1984) on a maltodextrin plate to identify isolates where the Dex$^+$ phenotype was restored by the Φ80pSuIII phage. The *ompF*(Dex)Δ111-123(Am)110 allele was then transduced into DME557 using the linked *pyrD*$^-$ marker, *pyrD*$^+$ transductants were screened for loss of the *ompF-lacZ* fusion (white on plates containing the chromogenic indicator 5-bromo-4-chloro-3-indolyl-β-D-galactoside, X-Gal) and acquisition of the suppressible Dex$^-$ phenotype to yield SBM110. Amber mutations in the *ompC*(Dex)Δ103-110 allele were isolated in a similar manner using the *ompC* specific phages Tulb or SS4 and the linked *zei*06::Tn*10* marker was used to move the mutation into the MCR106 genetic background to yield strain SBM102. To test if the Dex$^+$ mutants obtained from the amber containing strains; SBM102, SBM110, SBM140, SBM159 and SBM9532 resulted from reversal of the amber mutation, acquisition of a suppressor mutation, or a Dex$^+$ mutation at an undetermined locus the Dex$^+$ colonies were cross streaked over the porin specific phages [Tu1a (OmpF), Tu1b (OmpC) and λ (LamB)] and the T4(Am)39 tester phage. If they were sensitive to the porin-specific phage but were not sensitivity to the T4 tester phage they were scored as revertants. If they were sensitive to the T4 tester phage they were scored as suppressor mutants and if they remained resistant to both the T4 tester phage and porin specific phages, the Dex$^+$ phenotype was assumed to be the result of Dex$^+$ forward mutation. To confirm that we could identify revertants using these tests we randomly tested (>24) Dex$^+$ clones we had scored as revertants by transducing the appropriate gene (*ompF*, *ompC* or *lamB*) into a *lamB*$^-$ derivative. In all cases the Dex$^+$ transductants regained both phage sensitivity and a Dex$^+$ phenotype, confirming our original scoring.

The frequency of resistance to phages Tu1a, Tu1b, and K20, the antibiotic kasugamycin (Ksg) and the amino acid valine (Val) was determined by spreading an aliquot of washed cells on L plates that contained phages, (approximately 1 × 10^9 pfu), Ksg (40 μg/ml) or glucose plates containing 100 μg/ml valine. The frequency is expressed as the number of mutants per number of cfu plated. Cells were collected from maltodextrin selection plates by washing the lawn off the plate using 5 ml of 1XM63 medium.

Mapping of the Dex$^+$ mutations: To determine the genetic location of the Dex$^+$ mutations we introduced in parallel experiments the Δ*ompC*198::Tn*10* and the *ompF*-1::Tn*5* mutations from SBM18 and determined if either null mutation resulted in loss of the Dex$^+$ phenotype. To confirm that this was a valid test we randomly chose Dex$^+$ mutants and transduced the presumptive *ompF*(Dex) or *ompC*(Dex) allele nonselectively into a Dex$^-$ host using a linked marker. In 24/24 cases tested the assignment of the Dex$^+$ mutation was correct.

Growth test and competition studies: Plate growth tests and competition (reconstruction) tests were done as described previously (BENSON, OCCI and SAMPSON 1988). Basically a small number (50–100) of washed Dex$^+$ cells were spotted on to a freshly prepared lawn of Dex$^-$ MCR106 cells in 50–100 μl samples and the position of the spot marked. The plates were incubated at 37° and the number of Dex$^+$ colonies was recorded each day by marking the position the colonies. After 4 days representative samples (48–72) of the colonies from within the boundary of the spot were tested for the Tc^R marker present in the seeded cells. The percentage Tc^R colonies was used as a correction factor in calculating the number of colonies in the spot that resulted from the seeded cells.

Biochemical studies: The porin proteins were prepared and analyzed on linear sodium dodecyl sulfate (SDS)-polyacrylamide gels (11%) as previously described (LUGTENBERG *et al.* 1975). Cells were collected from maltodextrin plates by washing the plates with 5 ml of 1XM63 medium. The relative amount of the two porins was determined by densitometer measurements of Coomassie blue-stained gels

TABLE 1

Bacterial strains

Strain	Genotype	Source
MC4100	F⁻ *araD*139 Δ(*argF-lac*)4169 *rpsL*150 *relA flbB*5301 *ptsF*25 *deoC*1 *thi*-1 *rbsR*	CASADABAN (1976)
MCR106	MC4100 Δ*lamB*106	EMR and SILHAVY (1980)
MH450	MC4100 *ompF*-1::Tn*5*	HALL and SILHAVY (1981)
OC208	MCR106 *ompF*(Dex)Δ111–123	BENSON, OCCI and SAMPSON (1988)
OC211	MCR106 *ompF*(Dex)R132 → C	BENSON, OCCI and SAMPSON (1988)
OC214	MCR106 *ompF*(Dex)R42 → S	BENSON, OCCI and SAMPSON (1988)
DME553	MCR106 *ompF*Δ80	MISRA and BENSON (1989)
DME557	MCR106 Φ(*ompF'-lacZ*⁺)Hyb16-13 *pyrD zcb*-01::Tn*10*	S. BENSON
RAM102	MCR106 *zei-06*::Tn*10*	R. MISRA
RAM105	MCR106 *ompF*Δ80*zei-06*::Tn*10*	MISRA and BENSON (1988a)
RAM191	MCR106 Δ*ompC*198::Tn*10*	MISRA and BENSON (1988b)
SBM18	MCR106 *ompF*-1::Tn*5* Δ*ompC*198::Tn*10*	This study
SBM102	MCR106 *ompC*(Dex)Δ103–110(Am)102 *zei*-06::Tn*10*	This study
SBM110	MCR106 *ompF*(Dex)Δ111–123(Am)110	This study
SBM140	MCR106 *ompC*(Dex)Δ103–110(Am)102 *ompF*(Dex)Δ111–123(Am)110 *zei*-06::Tn*10*	This study
SBM158	MC4100 *lamB*(Am)5 *ompF*(Dex)Δ111–123(Am)110	This study
SBM9532	MC4100 *lamB*(Am)5	This study
JMM120	MCR106 *ompC*(Dex)R74 → S *zei*-06::Tn*10*	This study
JMM121	MCR106 *ompC*(Dex)VA Insert *zei*-06::Tn*10*	This study
JMM124	MCR106 *ompC*(Dex)R74 → G *zei*-06::Tn*10*	This study
JMM172	MCR106 *ompC*(Dex)R37 → H *zei*-06::Tn*10*	This study
JMM176	MCR106 *ompC*(Dex)R37 → C *zei*-06::Tn*10*	This study
JMM180	MCR106 *ompC*(Dex)D105 → G *zei*-06::Tn*10*	This study
JMM356	MCR106 *ompC*(Dex)R74 → S; D105 → G *zei*-06::Tn*10*	This study
JMM3256	MCR106 *ompC*(Dex)Δ103–108 *zei*-06::Tn*10*	This study

using a Hoefer densitometer and 365W software package (Hoefer Scientific, San Francisco, California).

RESULTS

Dex⁻ cells grow after plating on maltodextrin plates: *Escherichia coli* requires a functional LamB protein (the product of the *lamB* gene) to grow on maltodextrins larger than maltotriose (WANDERSMAN and SCHWARTZ 1982). The LamB protein facilitates the uptake of large maltodextrins across the outer membrane. Large maltodextrin molecules can not readily pass through the normal porins OmpF and OmpC porins due to their size. Strain MCR106 contains a 501-bp deletion within of the *lamB* gene and as such is unable to grow on maltodextrins (EMR and SILHAVY 1980). We have routinely used this strain and derivatives of it to obtain Dex⁺ mutants. We observed that the number of Dex⁺ mutants present on the selection plates did not correlate with the number of cells initially plated (Table 2). The reason for this is shown in Figure 1. Overnight inocula of 10^6 to 10^{10} cells grow to a density of approximately 10^{10} cells per plate by 24–36 hr post plating and then remain at this density. Presumably the growth of the Dex⁻ cells occurs due to the presence of contaminating sugars in the maltodextrin and the limited growth ability on maltodextrins of Dex⁻ strains.

Dex⁻ cells are present in the overnight culture: At about the time the lawn reaches confluent density (24–48 hr), Dex⁺ colonies begin to appear in the background. To estimate the frequency of Dex⁺ mutants present in the overnight culture we plated cells from six independent cultures at densities greater than 6×10^9 cells per plate to reduce the post plating growth and counted the number of Dex⁺ colonies present at 48 hr. For most cultures we could show that a threefold increase in the number of cells plated

TABLE 2

Appearance of Dex⁺ colonies

Strain [porin type]	Dilution	No. of Dex⁺ colonies 24 hr	48 hr	72 hr	96 hr
MCR106	10^0	0	32	11	7
[OmpF⁺ OmpC⁺]	10^{-2}	0	7	6	5
	10^{-4}	0	7	0	17
SBM102	10^0	0	17	30	24
[OmpC(Dex^{am}) OmpF⁺]	10^{-1}	0	6	15	30
	10^{-2}	0	4	24	18
SBM10	10^0	39	27	4	5
[OmpC⁺ OmpF(Dex^{am})]	10^{-1}	1	6	5	3
	10^{-2}	1	3	2	3
SBM140	10^0	>1000	ND	ND	ND
[OmpC(Dex^{am}) OmpF(Dex^{am})]	10^{-1}	100	205	215	41
	10^{-2}	5	45	40	19

Cell were grown and mutants selected as described in MATERIALS AND METHODS. The numbers at each time interval designate newly appearing colonies. The porin phenotype of each strain is given in brackets [ ]. ND = not determined.

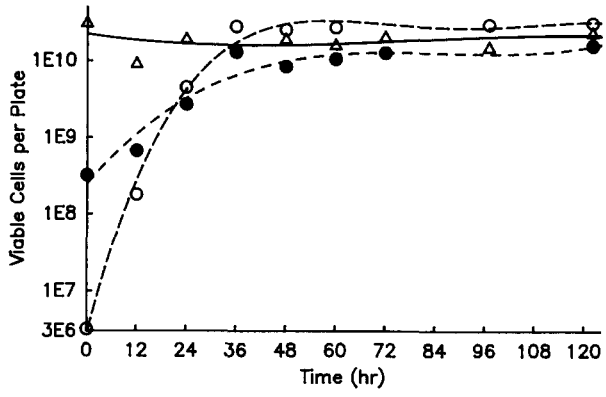

FIGURE 1.—Growth of MCR106 cells on maltodextrin selection plates. Cells were grown overnight in L broth and plated on 1XM63 maltodextrin agar media as described in MATERIALS AND METHODS at initial densities of 2.9×10^6 (open circles), 2.9×10^8 (closed circles) and 2.9×10^{10} (open triangles) cfu per plate. At various times samples of the bacterial lawn were removed and the number of cfu determined as described previously (BENSON, OCCI and SAMPSON 1988). The vertical axis is expressed in log 10.

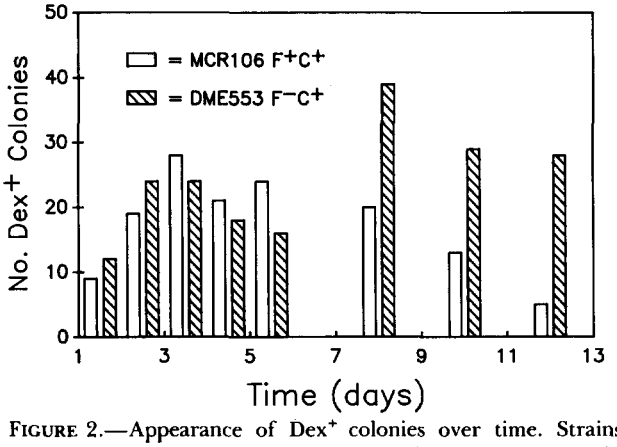

FIGURE 2.—Appearance of Dex$^+$ colonies over time. Strains MCR106 and DME553 were prepared and plated on maltodextrin medium as described in MATERIALS AND METHODS. The vertical bars represent the number of newly appearing Dex$^+$ colonies at each interval.

resulted in a threefold increase in the number of Dex$^+$ colonies present at 48 hr. The frequency of Dex$^+$ mutants ranged from 1.26×10^{-8} to 4.06×10^{-10} the average was 3.81×10^{-9}. These Dex$^+$ colonies presumably represent mutants that were present in the overnight culture or occurred immediately after plating. This frequency is consistent with what we have previously reported (BENSON, OCCI and SAMPSON 1988).

Dex$^-$ mutations continue to occur after the selection is imposed: Dex$^+$ colonies continue to appear on the selection plates after the density of cells in the lawn has reached a plateau (compare Figures 1 and 2 and Table 2). The number of newly appearing colonies at later times (*i.e.*, 96 hr or later) is independent of the number of initial cells plated (Table 2), thus Dex$^+$ colonies appearing at later times (after 96 hr) on plates seeded with a low (10^6 to 10^8) number of cells

TABLE 3

Frequency of phage-, valine- and kasugamycin-resistant mutants

Time (hr)	Tula resistance	Tulb resistance	Valine resistance	Kasugamycin resistance
0	4.8×10^{-5}	1.7×10^{-7}	7.6×10^{-5}	9.6×10^{-5}
48	1.8×10^{-6}	3.2×10^{-5}	1.6×10^{-6}	1.8×10^{-5}
96	4.0×10^{-6}	1.6×10^{-6}	1.6×10^{-6}	1.6×10^{-6}

Strain MCR106 was grown overnight in L broth (time = 0) plated on 1XM63 maltodextrin medium, and mutants identified as described in MATERIALS AND METHODS.

must represent mutants that occur after imposition of the selection. To determine if other types of mutations accumulated in the population the frequency of OmpF and OmpC phage resistant mutants and two nonporin phenotypes, kasugamycin resistance (Ksg^R) and resistance to valine (Val^R) were determined. Data from a representative experiment is shown in Table 3. The frequency of these mutants in the population did not change significantly during the selection and was similar to that of cultures grown overnight in L broth (Table 3).

Dex$^-$ mutants recovered from OmpF$^+$ OmpC$^+$ strains show a strong bias: We have characterized many Dex$^+$ mutants obtained from strain MCR106. Of more that 350 independent mutants analyzed from numerous selections done during the last 7 years we have obtained only two Dex$^+$ mutations that are not at *ompF*. One is the *ompC*(Dex)Δ103-110 allele (BENSON and DECLOUX 1985) and the other is the *imp4213* allele that defines a gene of unknown biochemical function (SAMPSON, MISRA and BENSON 1989). If we start with an isogeneic strain that lacks OmpF we have obtained Dex$^+$ mutations at the *ompC* locus (MISRA and BENSON 1988a,b) and identified a new gene *cog* (MISRA and BENSON 1989). There is no significant difference in the number of Dex$^+$ colonies that appeared during the selection for OmpF$^+$ OmpC$^+$ and OmpF$^-$ OmpC$^+$ strains (Figure 2). The final lawn density is approximately the same for both genetic backgrounds. DNA sequence analysis of over forty *ompF*(Dex) and *ompC*(Dex) mutations (BENSON, OCCI and SAMPSON 1988; MISRA and BENSON 1988a,b) shows that the type and frequency of the Dex$^+$ alterations obtained from each strain are analogous (Table 4). For both *ompF* and *ompC* we observed that a full spectrum of DNA alterations (transversions, transpositions, deletions and insertions) can confer a Dex$^+$ phenotype (Table 4). This suggest that no specific mutator gene or system is specifically involved in the formation of these alterations. In addition we find no evidence for a mutagenic hotspot in either gene.

Since we can isolate *ompC*(Dex) mutations using an OmpF$^-$ strain that are analogous to the *ompF*(Dex) mutations (Table 4) and occur at the same frequency it seemed paradoxical that the OmpF$^+$ strain did not

TABLE 4

Comparison of *ompF*(Dex) and *ompC*(Dex) mutations

Codon change	Mutation type	*ompC*(Dex)	*ompF*(Dex)
CGT to AGT (C to A)	Transversion	R74 → S (7)	R42 → S (1)
			R82 → S (4)
			R132 → S (3)
CGT to CAT (G to A)	Transition	R37 → H (1)	R82 → H (1)
CGT to GGT (C to G)	Transversion	R74 → G (1)	None
CGT to TGT (C to T)	Transition	R37 → C (4)	R82 → C (2)
		R74 → C (11)	R132 → C (1)
CGT to CCT (G to C)	Transversion	None	R132 → P (1)
GAC to GGC (A to G)	Transition	D105 → G (1)	D113 → G (1)
GTGGCT; GTGGCA	Insertions	VA after R74 (3)	None
	Deletions	Δ103–110 (2)	Δ108–114 (1)
			Δ111–123 (1)
			Δ114–129 (1)
			Δ118–130 (1)
			Δ118–133 (1)

Relevant homologous amino acid positions in OmpF and OmpC are; R42 and R37, R82 and R74, D113 and D105, R132 and R124, and the regions (112–122) and (104–114) respectively. The designation VA refers to a six base insertion resulting in the insertion of val and ala immediately following codon 74. The number in ( ) refers to the number of times the alteration has been independently isolated.

yield *ompC*(Dex) mutants. Two nonexclusive explanations for this finding are: The Dex$^+$ mutations at *ompF* confer a phenotypic selective advantage that is not conferred by Dex$^+$ mutations at *ompC* or the *ompF* gene exhibits adaptive mutagenesis in response to some aspect of the selection.

OmpF(Dex) mutations compete better: To test the first explanation we introduced the *ompC*(Dex) alleles into an *ompF*$^+$ background to yield OmpF$^+$ OmpC(Dex) strains. In every case the resulting JMM strain was Dex$^+$, eliminating the unlikely possibility that the *ompC*(Dex) alleles might be phenotypically silent in an *ompF*$^+$ host. To test if these OmpF$^+$ OmpC(Dex) strains were phenotypically equivalent to OmpF(Dex) OmpC$^+$ strains in their ability to form colonies in a lawn of Dex$^-$ cells we did competition tests as described in MATERIALS METHODS. To determine if the competition test was valid we first examined eight *ompF*(Dex) alleles for their ability to form colonies in a lawn of Dex$^-$ OmpF$^+$ OmpC$^+$ cells. All the *ompF*(Dex) alleles gave strong positive results, *e.g.*, formed colonies. The results for two of the *ompF*(Dex) alleles are shown in Table 5. We also tested if the OmpF$^-$ OmpC(Dex) parental strains could compete in a lawn of Dex$^-$ OmpF$^-$ OmpC$^+$ cells, all were competition positive under these conditions.

Under our routine selection conditions (1 × maltodextrin and 10^8 cells in the lawn) four of the *ompC*(Dex) mutants do not compete and two have a weak competition phenotype (Table 5). Only the R37→C substitution and the Δ103–110 deletion gave a strong competition phenotype (Table 5). These two alleles are phenotypically the strongest *ompC*(Dex) alleles we have characterized with respect to increased outer membrane permeability (MISRA and BENSON 1988b). The mutants with a weak competition phenotype (the Val-Ala [VA]) insertion and the D105→G substitution are intermediate in their permeability (Dex$^+$) phenotype (MISRA and BENSON 1988b). Strain JMM356 was included as a positive control, it contains two alterations in the *ompC* gene that in combination result in a Dex$^+$ phenotype that is comparable to the Δ103-110 mutation (MISRA and BENSON 1988b).

The results presented in Table 5 represent data from an average of at least three experiments. We routinely found that the absolute number of seeded *ompC*(Dex)$^+$ cells that formed colonies varied from experiment to experiment but that the relative competition phenotype of each strain with respect to each other and the controls was consistent. To better characterize the competition test we analyzed the effects of altering, two parameters that naturally change during the selection, the density of the lawn and the concentration of the maltodextrin. Changing either parameter does not alter the competition phenotype for strains that do not compete (Table 5). Altering one or both parameters had minor affects on competition for all strains that do compete (Table 5). How these parameters affected competition is different for each strain and no clear pattern of response was evident suggesting that both cell density and sugar concentration interplay to effect the ability of the seeded cells to form colonies. These findings suggest one reason that we did not recover OmpC(Dex) mutants from MCR106 is that most *ompC*(Dex) alterations do not confer a sufficient Dex$^+$ phenotype to allow their recovery. However these experiments do not eliminate the possibility that there is adaptive mutagenesis operating at *ompF* during the selection and suggest that both the density of Dex$^-$ cells in the

TABLE 5

Competition of the OmpC(Dex) mutants

Strain	Alteration	Against lawn of MCR106 10^8 concn. dextrin [1X]	[0.5X]	[2X]	MCR106 10^{10} concn. dextrin [1X]	[0.5X]	[2X]
JMM172	R37 → H	−	−	−	−	−	−
JMM176	R37 → C	+	++	++	+	++	+
JMM120	R74 → S	−	−	−	−	−	−
JMM124	R74 → G	−	−	−	−	−	−
JMM170	R74 → C	−	−	−	−	−	−
JMM180	D105 → G	wk	−	−	wk	−	−
JMM121	VA insert	wk	wk	+	−	−	−
JMM3256	Δ103–108	+	++	++	+	++	++
Controls							
JMM356	R74 → S; D105 → G OmpC(Dex)	+	++	++	+	++	++
OC211	R42 → S OmpF(Dex)	++	++	++	++	+	++
OC214	R132 → C OmpF(Dex)	+	++	++	++	++	++

Competition tests were done as described in MATERIALS AND METHODS. (−) means that <10% of the seeded cells formed colonies by 72 hr, (wk) means that 10–50% of the seeded cells formed colonies by 72 hr, (+) means that 50–80% of the seeded cells formed colonies by 72 hr, and (++) means that >80% of the seeded cells formed colonies by 72 hr. Each scoring represents the average of at least three independent competition experiments. Concn. = concentration. Strains are described in Table 1.

background lawn and the concentration of maltodextrins affect the ability of Dex^+ mutants to form colonies.

The porin level influences the type of mutants recovered: MCR106 cells grown overnight in L broth have approximately twice as much OmpC as OmpF protein in their outer membrane (Figure 3, lower panel). Within 8 hr after plating on 1XM63 maltodextrin medium the level of OmpC decreases and the level of OmpF correspondingly increases (data not shown), by 48 hr the level of OmpC is approximately 40% of the total porin (OmpF plus OmpC) and remains at this level (Figure 3, lower panel). These findings suggested that the increased level of OmpF in the outer membrane might account for the bias we observed for *ompF*(Dex) mutants. If this is true then under conditions where the OmpC porin is preferentially expressed *ompC*(Dex) mutants should be recovered. We made use of the fact that the expression of OmpF and OmpC is influenced by osmolarity (HALL and SILHAVY 1981) and determined a salt concentration (4XM63) where the level of the OmpC porin remains high (Figure 3, upper panel).

When MCR106 was plated on 4XM63 maltodextrin medium we observed a delay in the appearance of Dex^+ colonies, a decrease in the total number of Dex^+ colonies (Figure 4, upper panel) and a 40–50% decrease in the final bacterial lawn density as compared to the same culture plated on 1XM63 plates (data not shown). The delay in appearance of colonies results from slower growth on 4XM63 medium and the decrease in the density of the lawn presumably accounts for the decrease in the total number of Dex^+ colonies obtained (Figure 4). After 72 hr the number of newly appearing colonies on 4XM63 was about one-third that observed for the same culture plated on 1XM63 maltodextrin media (Figure 4, upper panel). The actual number of Dex^+ colonies fluctuated from plate to plate (Figure 4, lower panel) and for each culture (data not shown). We have mapped the location of the Dex^+ mutations for 59 independent Dex^+ isolates obtained from 4XM63 plates. Sixty-nine percent of the mutants (41 out of 59) carried *ompC*(Dex) mutations and 31% (18 out of 59) carried *ompF*(Dex) mutations. This included colonies that appeared at day 3 and day 5. We observed no correlation between when the colony appeared and the type of mutation it contained. All 59 of the Dex^+ mutations tested mapped to either *ompF* or *ompC*.

Does the level of gene expression influence the type of mutants that we recover: The above observations suggest that the amount of a specific porin biases the type of mutants we recover. However an alternate explanation is that it is not the level of the protein *per se* that drives the recovery bias but an increased level of transcription and this may represent a form of adaptive mutagenesis. The systems where adaptive mutagenesis has been reported involve selections that induce transcription of the gene where the proposed adaptive mutagenic events occur and the idea that transcription can lead to increased mutagenesis has been proposed as a possible mechanism to explain directed mutagenesis (DAVIS 1989). We reasoned that if there is transcription biased mutagenesis then the frequency of other types of mutations at *ompF* and *ompC* mutations might be different under 1XM63 and 4XM63 selections. To test this we determined the frequency of *ompF* and *ompC* phage resist-

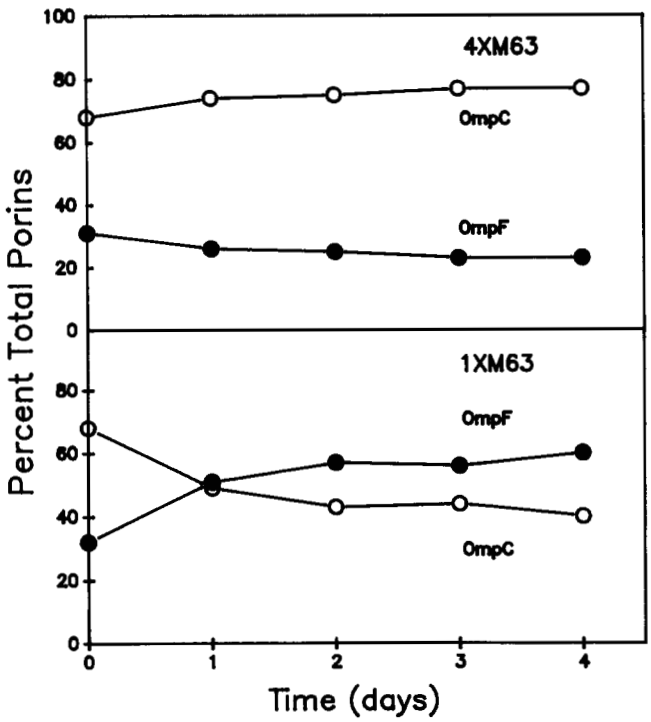

FIGURE 3.—Relative porin composition during the selection. MCR106 was grown and plated on maltodextrin plates as described in MATERIALS AND METHODS. At the indicated times cells were collected, and outer membrane proteins prepared and relative porin levels determined as described in MATERIALS AND METHODS. The lower panel shows the result for cells grown on normal (1XM63) maltodextrin medium and the upper panel for cells grown on (4XM63) medium. The zero time point reflects the composition of the starting cells grown overnight in L broth. The percentage was determined from the area of the each porin peak divided by the total area of the two peaks.

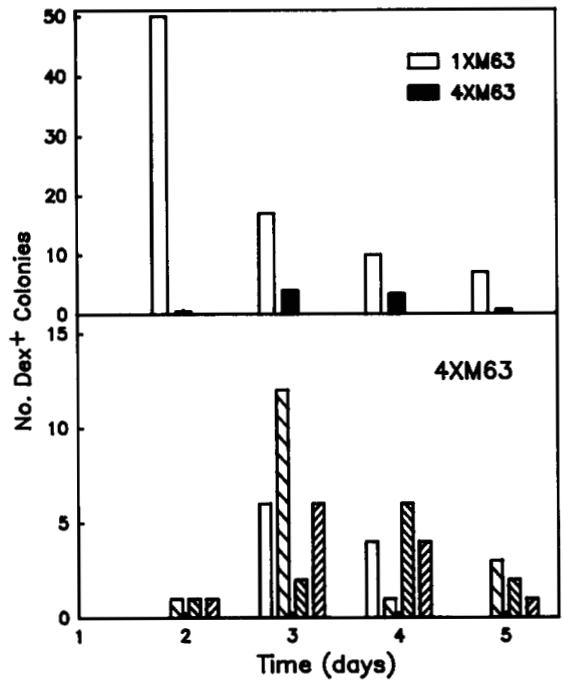

FIGURE 4.—Appearance of Dex$^+$ colonies on 4XM63 maltodextrin medium. Mutants were selected using strain MCR106 as described in MATERIALS AND METHODS. The upper panel shows the results for both normal (1XM63) and 4XM63 medium for an equal aliquot of the same culture. The lower panel shows the results for four different platings of the same culture on 4XM63 medium. The scale on the lower panel is approximately one-third that of the upper panel. Each time point shows the number of newly appearing mutants.

ant mutants in cells taken from 1XM63 and 4XM63 maltodextrin plates. There is no significant difference in the frequency of phage resistant mutants under the two conditions (Table 6), suggesting no preferential mutagenesis at either loci. However, since phage resistant mutants may require a different set of conditions for recovery than Dex$^+$ mutations we devised a second test involving selection for a Dex$^+$ phenotype. To test if adaptive mutagenesis is involved in biasing the type of mutants recovered we constructed strains carrying amber alleles in the *ompF*(Dex)Δ111-123 and *ompC*(Dex)Δ103-110 Dex$^+$ mutations which that have a strong competition phenotype (Table 5) and selected Dex$^+$ mutants using these strains. We reasoned that if adaptive mutagenesis was responsible for biasing the type of Dex$^+$ mutants recovered then reversal of an amber mutation in a competition positive *ompF*(Dex$^+$) allele should mimic that seen for forward mutations to a Dex$^+$ phenotype and be the predominant type of mutation recovered. Conversely if adaptive mutagenesis is not involved then reversal of the amber mutation in the Dex$^+$ allele should be observed at an intermediate level. Also, if adaptive mutagenesis is operating at *ompF* then reversal of an amber mutation in an *ompC*(Dex$^+$) allele should be a very rare event, *i.e.*, the forward mutation of *ompF*$^+$ to *ompF*(Dex) should be the predominate type of mutation recovered analogous to when we start with an OmpF$^+$ OmpC$^+$ strain.

The frequency and kinetics of appearance of Dex$^+$ colonies for strains carrying amber mutations in the *ompF*(Dex) and *ompC*(Dex) alleles is similar to that of MCR106 (OmpF$^+$ OmpC$^+$) (Table 2). Strain SBM140 which carries amber mutations in both the *ompF*(Dex) and *ompC*(Dex) genes has a greatly increased frequency of Dex$^+$ mutants in the overnight culture but only a slight increased frequency at later time points (Table 2). The increased frequency of Dex$^+$ cells in the overnight inoculum results from a disadvantage in growth ability that OmpF$^-$ OmpC$^-$ cells exhibit and the fact that porin plus strains rapidly out grow the porin minus strains (PUGSLEY and SCHNAITMAN 1978). Since both revertants and suppressor mutations yield a porin plus Dex$^+$ phenotype it is not surprising that the initial frequency of Dex$^+$ mutations is several orders of magnitude higher then that seen for the porin plus strains (Table 2)

For the SBM110 [*ompF*(Dex)Δ111-123(Am)110 OmpC$^+$] we found that only 32% of the mutants resulted from reversal of the amber mutation (Table 7). For SBM102 [OmpF$^+$ *ompC*(Dex)Δ103-

TABLE 6

Frequency of phage and valine resistant mutants on 1XM63 and 4XM63

Time (hr)	Tu1a resistance		Tu1b resistance		Valine resistance	
	1XM63	4X6M3	1XM63	4XM63	1XM63	4XM63
0	8.4×10^{-6}	8.4×10^{-6}	2.1×10^{-6}	2.1×10^{-6}	ND	ND
48	4.6×10^{-7}	5.3×10^{-7}	2.3×10^{-6}	4.0×10^{-7}	8.5×10^{-7}	5.3×10^{-7}
120	9.6×10^{-6}	1.9×10^{-6}	5.2×10^{-7}	2.2×10^{-7}	1.8×10^{-7}	2.3×10^{-7}

Mutant frequencies were determined as described in MATERIALS AND METHODS. ND = Not determined.

TABLE 7

Types of Dex$^+$ revertant colonies

Strain	Type of revertant	24 hr	96 hr	168 hr	Totals (%)
SBM102	OmpC^{rev}	1	7	8	16 (15)
[LamB$^-$ OmpF$^+$ OmpC(Dex^{am})]	Sup.	3	2	2	7 (6)
	Others	32	27	26	85 (79)
Total tested		36	36	36	108
SBM110	OmpF^{rev}	10	14	11	35 (32)
[LamB$^-$ OmpC$^+$ OmpF(Dex^{am})]	Sup.	6	7	11	24 (22)
	Others	20	15	14	49 (45)
Total tested		36	36	36	108
SBM140	OmpF^{rev}	20	3	13	36 (17)
[LamB$^-$ OmpF(Dex^{am}) OmpCDex^{am})]	OmpC^{rev}	0	0	5	5 (3)
	Sup.	51	6	28	85 (39)
	Others	1	63	26	90 (41)
Total tested		72	72	72	216

Dex$^+$ mutants were selected on 1XM63 maltodextrin medium and classified as described in MATERIALS AND METHODS. The porin phenotype of each strain is presented in brackets [ ]. ^{rev} indicates loss of the amber mutation, Sup. = acquisition of a suppressor phenotype.

110(Am)102] we found that 15% of the Dex$^+$ mutants recovered resulted from reversal of the amber mutation in *ompC* (Table 7). For strain SBM140 [*ompF*(Dex)Δ111-123(Am)110*ompC*(Dex)Δ103-110(Am)102] reversal of the *ompF* and *ompC* amber mutations make up 17% and 3% of the Dex$^+$ mutants obtained. These finding coupled with the phage resistance data (Table 6) argue that the bias recovery of *ompF*(Dex) mutants does not result from adaptive mutagenesis at the *ompF* locus and demonstrate that *ompC* mutations that confer a Dex$^+$ phenotype can occur and allow colonies to form in an *ompF*$^+$ genetic background on 1XM63 medium.

Increased expression of the *lamB* gene does not lead to increase mutagenesis: We wished to determine if the maltose system would exhibit adaptive mutagenesis in the Dex$^+$ selection. The LamB maltoporin is the normal means by which maltodextrins are taken into the cell and transcription of the *malB* operon that includes the *lamB* gene is induced by maltose (RANDALL-Hazelbauer and SCHWARTZ 1973; RAIBAUD *et al.* 1979) and maltodextrins. If adaptive mutagenesis is a general property of *E. coli* then we should see preferential recovery of mutations at *lamB* if they can result in a Dex$^+$ phenotype. To test this we used an amber mutation at codon five of the *lamB* signal sequence [*lamB*(Am)5]. This strain in Dex$^-$ and reversal of the UAG codon can result in a Dex$^+$ phenotype (D. Jackson, personal communication). When strain SBM9532 [*lamB*(Am)5 OmpF$^+$ OmpC$^+$] is plated on maltodextrin plates there is a greater than 50-fold induction of the *lamB* gene (data not shown). The frequency and kinetics of appearance of Dex$^+$ colonies is similar to that for strain MCR106 (B$^-$ F$^+$ C$^+$) and RAM105 (B$^-$ F$^-$ C$^+$)(data not shown). To identify which mutations yield a Dex$^+$ phenotype in this background mutants were characterized as described in MATERIALS AND METHODS. At early time points (days 1 and 2) alterations that reverse the *lamB* amber mutation were preferentially obtained. Their early appearance suggest they were present at the time of plating. At later times SBM9532 behaves in a manner analogous to strain MCR106 (*lamB*Δ106) *e.g.*, there is a biased recovery of *ompF* Dex$^+$ mutants and no bias for the reversal of the *lamB* lesion. Consequently we find no indication of adaptive mutagenesis or evidence that increased transcription results in increased recovery of mutations at the induced locus. We consistently observed that the Dex$^+$ cells present in the overnight culture were mutants where the *lamB*(Am)5 mutation had been reversed or suppressed. All of the *lamB*(Am)5 revertants tested (>12) have a competition positive phenotype (data not shown). Why we did not obtain *ompF*(Dex) mutants at

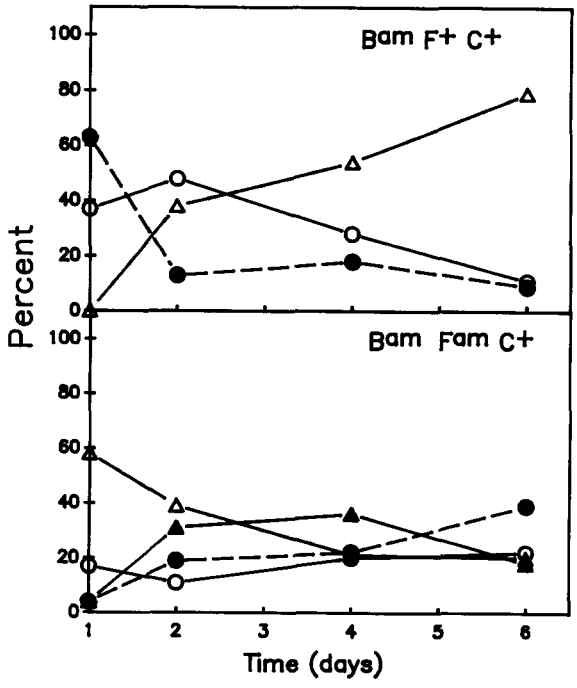

FIGURE 5.—Type of Dex$^+$ mutants obtained from a *lamB*^{am} strain. Dex$^+$ mutants were selected and classified as described in MATERIALS AND METHODS. Upper panel shows data obtained from SBM9532; the open circles show the percentage of mutants where the *lamB* amber mutation has been reversed; the closed circles, suppressor mutations; and the open triangles, *ompF*(Dex) mutants. Each time point represents the average of three independent experiments. At least 54 Dex$^+$ colonies were characterized from each time point except day one where only 19 colonies were tested. The lower panel shows data obtained from SBM158, the open circles show the percentage of mutants where the *lamB* amber mutation has been reversed; the closed circles, suppressor mutations; the open triangles, mutants where the *ompF* amber has been reversed; and the closed triangles, mutants that are not one of three described classes. Each time point represents the average of three independent experiments. At least 80 Dex$^+$ colonies were characterized from each time point except day one where only 24 colonies were characterized.

the early time points is not known. The predominant type of Dex$^+$ mutations obtained after 72 hr are *ompF*(Dex) mutations (Figure 5) suggesting that at later times the ability to take up maltodextrins is not the sole criteria determining the type of mutant recovered since cells with a wild-type LamB present in the outer membrane would have an increased ability to take up maltodextrins (MISRA and BENSON 1988a). Strain SBM110 (LamB$^-$ OmpF(Dex)^{am} OmpC$^+$) does not exhibit a bias recovery for *ompF* alterations (Table 7). To test if this also would be true for strain SBM158 (LamB^{am} OmpF(Dex)^{am} OmpC$^+$) Dex$^+$ mutants were selected and characterized (Figure 5). We find that as with strain SBM110 the bias recovery of mutants with alterations at *ompF* is not seen. Thus strains carry the *lamB*(Am)5 mutation behave like strains carrying the *lamB*Δ106 mutation, if the strain is OmpF$^+$ there is a strong bias for the recovery of *ompF*(Dex) mutants but if the strain is *ompF*(Dex)^{am}, [OmpF$^-$] this bias is not observed (Table 7 and Figure 5). A second *ompF*(Dex)^{am} mutation behaves in an analogous manner.

DISCUSSION

Conclusions: We draw the following conclusions. (1) Dex$^-$ cells continue to grow and divide after plating on maltodextrin until the lawn reaches saturation. This saturation limit is dependent upon the genotype of the cells plated and on the selection conditions themselves. (2) Dex$^+$ cells can be present in the culture prior to plating on selective media. (3) Dex$^+$ mutations occur after the selection is imposed, and after the bacterial lawn has reached saturation. (4) Dex$^+$ colonies carrying *ompF*(Dex) mutations are preferentially recovered from OmpF$^+$ cells although analogous alterations in the *ompC* gene confer a Dex$^+$ phenotype. (5) Increasing the level of OmpC porin in the outer membrane allows the preferential recovery of *ompC*(Dex) mutations in an OmpF$^+$ OmpC$^+$ background. (6) The level of transcription does not play a role in determining the type of mutants we recover. (7) The bias we observed results from selective advantages which are determined by a combination of factors or parameters including: genetic background, lawn density and medium composition.

The occurrence of Dex$^+$ mutants: Work of LURIA and DELBRUCK (1943) and LEDERBERG and LEDERBERG (1952) showed that mutations that confer resistance to lethal agents occur prior to exposure to the selective agent. The ability to demonstrate the generally accepted premise that mutations occur in the absence of selection for the Dex$^+$ selection system was more difficult since cells that are phenotypically Dex$^-$ have some capacity to grow under the initial conditions (Figure 1). This growth precluded the use of the LURIA and DULBRUCK fluctuation test for determining the rate of mutation during growth in nonselective media. It was not possible to obtain the necessary Poisson distribution of cultures with zero mutants since plating a low number of cells results in growth and mutation occurrence and conversely, if we plate sufficient cells (10^{10}) to prevent growth the population contains preexisting mutants. However, our analysis of the selection allowed us to design experimental conditions where we could estimate the frequency of Dex$^+$ mutants present in the overnight culture to be approximately 3×10^{-9} consistent with the fact that only a limited number of specific changes can confer a Dex$^+$ phenotype (Table 4) and with previously published estimates (BENSON, OCCI and SAMPSON 1988). Based on the facts that the number Dex$^+$ colonies fluctuates from culture to culture, the number of Dex$^+$ colonies in a given culture is proportional to the number of cells plated over a narrow range, and reconstruction experiments with *ompF*(Dex$^+$) cells that show these mutants form colonies in the bacterial lawn

within 48 hr after plating makes us confident that the mutants present at 48 hr post plating represent preexisting mutants that arose under nonselective conditions.

We have shown that Dex$^+$ mutants can occur after imposition of the selection since the number of newly appearing Dex$^+$ colonies after several days is independent of the number of initial cells plated (Table 2). The mutants that appear many days after the lawn has reached saturation might represent mutagenic events that occurred during the initial growth phase on the dextrin plate and are cells that require many days to form visible colonies. We think this unlikely because the rate of colony growth for late arising colonies is similar to that of the earlier colonies and competition studies with these mutants show that they form visible colonies in 24–48 hr when seeded into a lawn on Dex$^-$ cells. Thus we conclude that Dex$^+$ mutations occur after the number of cells in the lawn stops increasing.

How Dex$^+$ mutations occur after the number of cells ceases to increase is not known. The observation that seemingly stationary populations of cells yield events that result in mutants that form colonies or papilla is not unique to the maltodextrin selection system and is often observed by bacterial geneticist for a variety of selections. One explanation for the late arising mutants is that the population of cells is actively dividing and that the apparent stationary nature of the cell density results from equivalent cell death and division. We think that this explanation is unlikely since acridine straining of the population after it reaches saturation showed that all the cells appeared viable, we were unable to detect any dead cells, and the number of viable cells as determined by acridine staining correlates with the cfu (J. MUNRO, unpublished data). A similar conclusion was reach by Hall in a study of reversal of tryptophan auxotrophy using a different test to decide if cells were dividing (HALL 1990). The possibility that the selection induces a mutagenic state in all the cells in the population resulting in a high rate of general mutagenesis seems unlikely since we were unable to detect any increase in the frequency of phage resistance, kasugamycin resistance or valine resistance in the general population. However the mutant phenotypes tested for (such as the phage and kasugamycin resistances) may require significant growth to be adequately expressed or as in the case of Val^R may reflect as bias for the type of mutagenic event assayed (CHARLESWORTH *et al.* 1988). Thus, the conclusion that there is no increased in mutagenesis should be viewed as tentative. A similar conclusion has been arrived at by both HALL (1990) and CAIRNS, OVERBRAUGH and MILLER (1988) using different nonlethal selection systems. The mechanism of how the Dex$^+$ mutations occur in nondividing cells without a corresponding increase in mutations at other loci remains to be determined.

The bias recovery of *ompF*(Dex) mutants: The recovery of a Dex$^+$ mutation requires two events, first the mutation must occur and second it must provide a sufficient selective advantage to allow growth of a visible colony. The most striking finding for the maltodextrin selection system is that although the same alterations in both *ompF* and *ompC* can confer a Dex$^+$ phenotype we only recover *ompF*(Dex) mutations when we start with an OmpF$^+$ OmpC$^+$ strain. The competition studies (Table 5) suggest that many of the *ompC*(Dex) alleles do not have a strong enough selective advantage to allow colony formation in the presence of Dex$^-$ cells. The competition phenotype correlates with the strength of the permeability increase conferred by the porin mutations, *i.e.*, mutations with the greatest increase in outer membrane permeability have the strongest competition phenotypes. The explanation we favor for the biased recovery of *ompF*(Dex) mutations is that it results from a phenotypic advantage under these selection conditions due to the increased amount of OmpF relative to OmpC and OmpF having a slightly larger pore diameter (BENZ and BAUER 1988) that is functionally better at the uptake of maltose and maltotriose (MISRA and BENSON 1988a). This explanation is supported by the observation that when OmpC is the major porin species we see a bias for recovery of *ompC*(Dex) mutants. However, uptake of maltodextrin can not be the sole factor determining which cells from colonies since reversal of the *lamB*(Am) mutation is not favored at later time points over forward mutations to an OmpF(Dex) phenotype (Figure 5).

This contention is also supported by the observation that two of the *ompC*(Dex) alleles have a strong competition phenotype (Table 5) and are readily obtained from OmpF$^-$ OmpC$^+$ strains (Table 4) but not OmpF$^+$ OmpC$^+$ strains. Based on the competition phenotype we would expect to have routinely recovered mutants of this type from strain MCR106, why we fail to do so is not understood. One possibility is that the competition phenotype is misleading. The competition tests are biased, in that the seeded cells already have a full complement of the mutant porin. Porins are extremely abundant outer membrane proteins and it may take several generations before the mutant porins to replace the existing wild type proteins in the outer membrane. As such the competition experiments do not accurately reflect the early physiology of the mutant strain when only a small percentage of the porins will be in the mutant form. Dex$^+$ mutants at this early stage may have an insufficient or a negative selective advantage and as such may be unable to form a colony or may be lost from an exponentially growing population. The latter explanation could explain why we

fail to recover mutants of these types as preexisting mutants present in the overnight culture.

It should be noted that the *ompC*(Δ103–110)3256 allele was obtained from an OmpF$^+$ OmpC$^+$ strain (BENSON and DECLOUX 1985). However this strain carried a plasmid encoding a defective *lamB* gene and the presence of the plasmid may have altered the selection in some unknown fashion. We characterized this allele because we have obtained the same alteration in a selection using an OmpF$^-$ OmpC$^+$ strain that yielded the other *ompC*(Dex) mutations (MISRA and BENSON 1988b).

The other types of Dex$^+$ mutations that we have recovered are *imp4213* from MCR106 (OmpF$^+$ OmpC$^+$) and *imp208* and *cog174* mutations from strain RAM105 (OmpF$^-$ OmpC$^+$) (MISRA and BENSON 1989; SAMPSON, MISRA and BENSON 1989). The *imp* mutations are rare alleles and we have obtained only two of them (SAMPSON, MISRA and BENSON 1989). We have characterized the *imp4213* and the *cog174* alleles in the competition test and neither mutation yields a strong competition phenotype (data not shown). The finding that the *imp4213* mutation was isolated from MCR106 cells but does not have a strong competition phenotype underscores the limits of the competition assay and our ability to reconstruct the conditions that might be present during the selection process. In summary our findings strongly suggest that many factors interplay to determine which mutations actually yield colonies. It is likely that the importance of individual factors may change during the selection due to alterations in lawn density, sugar availability and other undefined parameters.

The alternative explanation for the bias recovery of certain mutant type is that the Dex$^+$ selection induces an adaptive mutagenic state where mutations with a strong phenotypic advantage preferentially occur and result in a bias in the type of mutants recovered. This explanation seems unlikely since altering parameters that directly affect phenotypic fitness of individual cells, *i.e.*, medium and porin composition alters the type of mutants we recover. In addition studies with amber mutations suggests there is no strong bias for specific mutagenic events at the DNA level at either *ompF* or *lamB*. The experiments involving the amber mutations suggest that altering the porin composition of the cell affects the spectrum of mutations that are recovered. The simplest explanation is that these changes affect the fitness level necessary for a Dex$^+$ cell to be able to form a visible colony in a dense lawn of Dex$^-$ cells.

The idea that increased transcription can result in preferential mutations during long term nonlethal selections does not appear to apply in the dextrin selection system. Although we see a slight preference in reversion of the *ompF*(Dex)^{am} as compared to the *ompC*(Dex)^{am} on 1XM63 medium this preference is not sufficient to account for the extreme bias we observe for the type of Dex$^+$ mutants we recovered from strain MCR106. More importantly, reversion of the *lamB* amber is not stimulated by plating on maltodextrins and in fact is less frequent than Dex$^+$ forward mutations at the *ompF* gene (Figure 5, upper panel). The latter finding suggest that unlike the *lac*, *trp* and *bgl* systems the dextrin selection system does not have the ability for directed or adaptive mutagenesis.

The role that the selection environment plays in the type of mutants recovered is obviously important and seldom defined or characterized. In this study we observe that the type of mutants recovered is influenced by environmental conditions as well as the genetic background of the starting strain. This suggest that porin composition, functionality, and expression level as well as medium composition and genetic background and possibly yet undefined factors interplay to determine the type of mutants that will be recovered. Minor changes in any of these parameters may alter the minimal selective advantage level a mutation must confer to allow out growth into a recoverable colony and affect the type and frequency at which a specific mutation is recovered.

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