Manx antisense RNA probe or a ^{35}S-ATP–labeled antisense RNA probe synthesized from the insert of the *MocuMA1* muscle actin cDNA clone (*20*).
15. The following 18-nucleotide oligomer phosphorothiolate-substituted ODNs, custom synthesized and purified by Oligos Etc. (Wilsonville, OR), were used in this study. Antisense ODN-1 (5′-ATCTTCATGTTTTGACGA-3′) and sense ODN-1 (5′-TCGTCAAAACATGAAGAT-3′) correspond to nucleotides +4 to +21 of the *Manx* cDNA sequence (*11*). In some experiments, a rhodamine moiety was covalently linked to the 3′ termini of antisense and sense ODN-1 to follow uptake and distribution in embryos. The control ODN was 5′-ATTTTGACTTGTGTTTCC-3′, which has a base composition similar to that of antisense ODN-1. In some experiments, we used antisense ODN-2 (5′-ATTATGATTGTTTAAATA-3′) or ODN-3 (5′-CCGAAGCAGCTCTTTCCA-3′), which correspond to nucleotides −15 to +2 and +167 to +184, respectively, in the *Manx* cDNA sequence (*11*), or a combination of the three antisense ODNs. The concentration of ODNs was determined empirically for different clutches of hybrid embryos. The standard procedure was to treat hybrids with 20 to 50 μM ODN at first cleavage (about 50 min after insemination) and incubate the embryos with the ODNs until hatching (10 to 12 hours after insemination). The morphology of the ODN-treated and control larvae was determined by light microscopy. Hybrid larvae were scored as containing restored urodele features if they contained either a brain sensory organ, a tail, or both structures. The effect of ODNs on restoration of urodele structures was confirmed by fixing larvae in 4% paraformaldehyde and examining hematoxylin and eosin–stained sections.
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25. This paper is dedicated to the memory of Alain Maron, whose expertise in collecting *M. occulta* and *M. oculata* was essential for these experiments. We thank J. Machula, D. Martasian, and J. Reardon for technical assistance; A. Toulmond, N. Sanséau, and L. Meijer for use of laboratory facilities at the Station Biologique; and R. Maxson for discussions on the use of antisense ODNs. Supported by NSF grants IBN 9304958 (B.J.S.) and DCB-915543 (W.R.J.) and NIH grant HD-13970 (W.R.J.).

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High Mutation Frequencies Among *Escherichia coli* and *Salmonella* Pathogens

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Here it is reported that the incidence of mutators among isolates of pathogenic *Escherichia coli* and *Salmonella enterica* is high (over 1 percent). These findings counter the theory, founded on studies with laboratory-attenuated strains, that suggests mutators are rare among bacterial populations. Defects in methyl-directed mismatch repair underlie all mutator phenotypes described here. Of nine independently derived hypermutable strains, seven contained a defective *mutS* allele. Because these mutant alleles increase the mutation rate and enhance recombination among diverse species, these studies may help explain both the rapid emergence of antibiotic resistance and the penetrance of virulence genes within the prokaryotic community.

Escherichia coli O157:H7, which is responsible for about 20,000 cases of food-related illnesses in the United States annually, was first recognized as a human pathogen in 1982 (*1*). Other foodborne pathogens have long been known but are creating new health concerns; for example, *Salmonella enteritidis* in eggs (*2*). Whether pathogens such as *E. coli* O157:H7 are truly new or are old but previously unrecognized, and whether *S. enteritidis* has changed sufficiently to fill new niches, are parts of the conundrum of emerging pathogens. The true nature of emerging pathogens can be comprehended only if the molecular mechanisms underlying the evolution of potential pathogens within natural populations are understood.

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Two forces known to govern genetic change are the mutation of nucleotide sequences within a genome and the horizontal transfer of existing sequences among genomes. In certain mutator phenotypes, namely those deficient in methyl-directed mismatch repair (MMR), these forces converge to increase the rate of genetic variation. MMR is a postreplicative repair system that corrects errors on newly synthesized DNA strands to ensure the precision of chromosome replication (*3*). MMR is also a major barrier to interspecies gene exchange (*4*). Consequently, bacteria defective in MMR show both an enhanced rate of mutation (a hypermutable phenotype) and an increase in recombination of diverged sequences; that is, they are more promiscuous (*4*). It therefore becomes relevant to the problem of emerging pathogens to determine the frequency of such mutator phenotypes among human pathogens.

Our aim was to survey isolates of *E. coli* and *S. enterica*, implicated in food-related outbreaks of disease, for hypermutable strains. We screened a portion of the Food and Drug Administration's bacterial pathogen collection and natural *E. coli* (*5*) and *S. enterica* (*6*) reference collections for the frequency of mutations conferring resistance to the antibiotic rifampicin. Under plating conditions that yielded 0 to 5 mutants per 10^8 cells for most isolates, 26 putative mutators exhibiting up to 1000-fold greater frequencies of rifampicin-resistant (Rif^R) mutants were observed in both *E. coli* and *S. enterica* (Table 1). Putative mutators were isolated from single colonies and tested for a general mutator phenotype by determination of the frequency of mutations that conferred resistance to each of

Table 1. Rifampicin-resistant mutants in isolates of *E. coli* and *S. enterica*. Cultures were grown in BHI broth overnight at 37°C; LB medium was used for plating, including selection for rifampicin resistance (100 μg/ml). Putative mutators displayed at least a 50-fold increase in mutation frequency as compared with control levels. Mutator percent is the number of mutator phenotypes per number of isolates examined times 100.

Strain	Number of isolates		
	Total analyzed	Putative mutator	Mutator (%)
	E. coli		
O157:H7	120	5	2 (1.7)
Other serotypes	20	3	1 (5.0)
ECOR collection	72	1	1 (1.4)
	S. enterica		
S. enteritidis	15	1	1 (6.7)
Other serovars	106	14	3 (2.8)
SARC collection	16	2	1 (6.2)

Table 2. Mutation frequency of hypermutable clones. Frequencies ± SD represent three to seven determinations from independent cultures. EC536 and SL12 served as controls for *E. coli* and *S. enterica*, respectively (27).

Isolate	Mutants per 10^8 cells		
	Rif^R	Spc^R	Nal^R
EC536	1.2 ± 0.4	0.2 ± 0.1	0.5 ± 0.2
EC503	114 ± 22	31 ± 8	403 ± 41
EC535	119 ± 23	35 ± 7	300 ± 57
DEC5A	425 ± 150	26 ± 2	195 ± 110
ECOR48	441 ± 45	Spc^R	177 ± 57
S. enteritidis (SL12)	2 ± 0.8	3 ± 0.2	0.8 ± 0.7
S. enteritidis (C396)	782 ± 101	70 ± 25	745 ± 163
S. berta (SL78)	738 ± 289	30 ± 3	148 ± 13
S. halmstad (SL58)	704 ± 265	338 ± 73	481 ± 269
S. arizonae (S2978)	798 ± 320	407 ± 83	199 ± 95
S. infantis (SL101)	981 ± 449	172 ± 40	113 ± 18

the antibiotics spectinomycin, nalidixic acid, and rifampicin. This screen identified nine hypermutable strains (Table 2). Antibiotic-resistant colonies in the remaining 17 strains were attributable to subpopulations of mutants (up to 10^{-5}) that resided in the original cultures. Each subpopulation was resistant to only one of the antibiotics. Clonality of preexisting mutants was confirmed by sib selection (7), in which mutants were isolated without exposure of bacteria to antibiotic selection.

The *E. coli* mutator collection comprises two O157:H7 strains, an O55:H7 isolate that is one of the diarrheagenic *E. coli* (DEC) clones (8), and ECOR48 (5), whereas the *Salmonella* series includes four different serovars and the SARC strain S2978 (6). The occurrence of nine independent hypermutable strains among 349 *E. coli* and *S. enterica* isolates surveyed indicates an incidence of 2.6%, distributed within a range of 1.4 to 6.7% (Table 1). Among natural populations at large, the incidence of hypermutable strains may be greater (9).

The magnitude of the increases in mutagenesis over control levels (10^2 to 10^3) is characteristic of *E. coli* and *S. typhimurium* strains defective in MMR. Four proteins essential for mismatch correction—MutH, MutL, MutS, and UvrD (or MutU)—are candidates for being defective in these mutators. Thus, hypermutable strains were transformed with plasmids containing each of the wild-type genes for these products. Complementation analysis showed that each of the nine mutators contained a defective MMR allele. Seven, including the four *E. coli* isolates, carried a *mutS* defect (Table 3).

Molecular studies of the *E. coli mutS* mutators were conducted to verify genetic results. Concordance with the sequence of *E. coli* K12 was established by cloning and sequencing of *mutS* from an *E. coli* O157:H7 nonmutator strain, EC536. Relative to *E. coli* K12, *mutS* of *E. coli* O157:H7 is unremarkable. That is, its sequence of 2735 base pairs (bp), encompassing the 2562-bp *mutS* gene, is 97.84% similar to that of its K12 counterpart, yielding 99.88% identical MutS products (10). However, long polymerase chain reaction (PCR) analysis (11) of the 12-kb *fhlA-mutS-rpoS* region revealed that the sequence 3′ to *mutS* is markedly different from that of *E. coli* K12 (Fig. 1). Nucleotide sequencing of a limited portion of the region between *mutS* and *rpoS* showed that EC536 contains novel DNA sequence (about 2750 bp) in place of 6098 bp of K12 sequence (12). Oligonucleotide probe analyses (13) confirmed that the unusual sequence identified in EC536 is present in all nonmutator O157:H7 strains studied and also in isolates of *E. coli* O55:H7, which is evolutionarily the closest sibling to O157:H7 (8). Contrary to nonmutator strains, analysis of *E. coli* mutators indicated the presence of larger deletions that directly affect *mutS*. Long PCR results showed that O157:H7 mutator EC503 contains a deletion of 7489 ± 48 bp relative to the K12 strain W3110, or approximately 4100 bp as compared with its O157:H7 progenitor (Fig. 1). Sequence analysis confirmed that a deletion of 7449 bp extends 212 bp into the 3′ end of *mutS*, with identical sequence (GAGGTAAT) residing at the deletion endpoints (14).

The incidence of hypermutable mutants among pathogenic strains (>1%) is alarmingly high considering that, at equilibrium, the frequency of deleterious alleles within a population is expected to be 10^{-5} to 10^{-3} (15). Our results, however, were partially presaged by chemostat competition analyses (16). Moreover, very recent studies show that when a series of *E. coli* B cultures are propagated for thousands of generations under glucose-limiting conditions, some convert to a mutator phenotype (17). Two previous studies noted mutators among natural *E. coli* populations (18), though neither suggested the possibility that a mutator phenotype may be found more frequently among human pathogens (19).

The mutators isolated here (*mutS*, *mutH*, and *uvrD* defects) portend an inti-

Table 3. Suppression of mutator activity by plasmid clones containing wild-type *mut* genes (28). Positive complementation is signified by >97% suppression of the mutator phenotype as assessed by spontaneous mutagenesis to rifampicin resistance. Percentage of suppression ± SD represents three or more determinations from independent cultures, except for *S. typhimurium* TW541, a known *mutS*-defective strain, which is the average of two experiments.

Isolate	pGW1899 (*mutH*$^+$)	pGW1842 (*mutL*$^+$)	pGW1811 (*mutS*$^+$)	pGT26 (*uvrD*$^+$)	pBR322*
EC503	56.4 ± 11.2	39.0 ± 7.6	97.3 ± 1.0	19.1 ± 11.9	40.2 ± 8.1
EC535	55.5 ± 10.0	38.2 ± 31.7	98.6 ± 0.6	44.2 ± 6.6	43.4 ± 17.9
DEC5A	44.2 ± 22.7	55.0 ± 4.0	99.3 ± 0.3	60.5 ± 12.8	62.1 ± 6.9
ECOR48	30.6 ± 18.6	26.6 ± 13.6	97.9 ± 1.9	46.0 ± 14.7	ND‡
S. enteritidis C396	46.7 ± 10.8	32.2 ± 9.1	98.9 ± 1.0	43.8 ± 14.7	ND
S. berta SL78	99.3 ± 0.6	46.4 ± 17.6	43.3 ± 8.8	61.6 ± 12.5	ND
S. halmstad SL58	50.4 ± 16.7	32.6 ± 8.4	99.2 ± 0.2	55.6 ± 8.0	ND
S. arizonae S2978	37.3 ± 9.4	45.5 ± 5.7	38.3 ± 17.9	98.5 ± 2.1	ND
S. infantis SL101	39.9 ± 19.0	50.9 ± 4.5	99.4 ± 0.8	18.6 ± 14.4	ND
S. typhimurium TW541†	60.0	69.1	99.4	33.7	37.3

*pBR322-transformed isolates were used to determine the extent of nonspecific suppression by vector DNA alone. †*Salmonella typhimurium* TW541 contains a Tn*10* insertion in the *mutS* gene and was used to distinguish complementation and nonspecific suppression. ‡ND, no determination.

mate role for methyl-directed mismatch repair in the emerging pathogen problem. Because MMR limits recombination between diverged sequences (*4*), inactivation of this system relaxes normal recombination barriers among species, offering a potential pathogen the opportunity to inherit, by horizontal transmission, useful genes from the reservoir of commensal and pathogenic bacteria at large. Promiscuity, then, might drive selection of these kinds of mutators among successful pathogens. Our findings offer an explanation of how virulence genes such as the *spa-inv* loci (*6*, *20*) and the *eae* and *slt* genes (*8*) have come to reside in *S. enterica* and *E. coli* O157:H7, respectively. It is of special interest that the left and right junctions of the 40-kb pathogenicity island found in *Salmonella*, but not in *E. coli*, originate in the 3′ portion of *fhlA* and the 5′ portion of *mutS*, respectively (*20*).

A mutator phenotype should be to the pathogen's advantage, affording strategies (mutation and recombination) that permit rapid variation in an unstable environment in order, for example, to escape immune surveillance or elude therapeutic intervention (antibiotic resistance). The ability to generate a large number of mutations for adaptation in a changing environment; the opportunity to establish and propagate in the new niche of its host (*21*); the increased potential for gene acquisition; and the requisite linkage of the mutator phenotype, in the absence of recombination, to any beneficial phenotype (*22*) spur selection of a mutator. In contrast, placed in a static environment, the same bacterium might be destined for extinction (that is, mutations in vital genes are lethal), although induction of suppressor mutations can quiet the mutator activity (*23*) and attenuate this outcome.

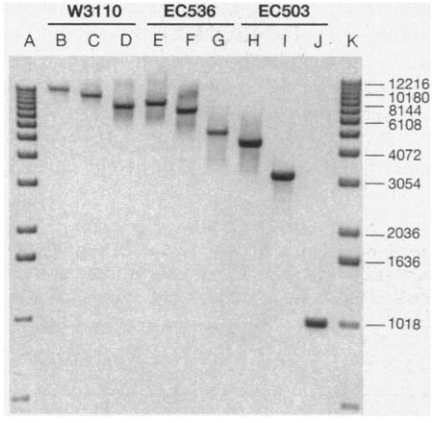

Fig. 1. Agarose gel analysis of long PCR products from the *mutS* region of *E. coli* strains W3110, EC536, and EC503 (*29*). 3′ primers at a fixed site in *rpoS* (R3) were paired with 5′ primers at a site in *fhlA* (F2), at the beginning of mutS (S8), or at its end (L119). Primer sets were designed to amplify products of 12,045 bp (F2-R3; lanes B, E, and H); 10,668 bp (S8-R3; lanes C, F, and I); and 8477 bp (L119-R3; lanes D, G, and J), based on *E. coli* K12 sequence (*10*). Lanes A and K, 1-kb ladder (Gibco BRL).

It would seem that the ultimate pathogen would possess an elevated mutation rate that is transient (or conditional), providing genetic variation during the first few hours when the pathogen must survive, invade, and colonize its host. Two aspects of our findings are relevant to this point. First, altered sequence found in *E. coli* O157:H7 and O55:H7 positions *rpoS*, which encodes the major stationary phase–specific sigma factor for RNA polymerase (*24*), closer to *mutS*, furnishing opportunities for control of MutS activity under the very conditions in which mutator activity may be advantageous (such as the stationary phase, nutrient starvation, and physicochemical stress). It is known that MutS is regulated downward in stationary-phase *E. coli* K12 cells, although the molecular means of its regulation are not understood (*25*). As *mutS* and *rpoS* are in an antipolar configuration, we are currently investigating whether an antisense strategy, the result of transcription read-through from the oppositely oriented *rpoS* gene, controls MutS activity under certain conditions of environmental stress in *E. coli* O157:H7. Second, given that most putative mutators among *E. coli* and *Salmonella* pathogens (Table 1) contained subpopulations with multiple but separate antibiotic resistances, stochastically an unlikely result, one wonders if these multiply mutated strains have passed through such a transient hypermutable state.

Finally, our findings appertain to current clinical situations in which antibiotic resistance is increasing dramatically among human pathogens (*26*). Although they are counter to the current paradigm that antibiotic resistance is due to the acquisition of plasmids harboring multiple drug-resistant determinants, our data show that chromosomal mutations might explain at least some of the multiply drug-resistant organisms found clinically. These same mutator phenotypes could help explain how the plasmid-borne resistance determinants first became linked (relaxed recombination between disparate species) and are now so readily inherited.

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12. Sizing of long PCR products (Fig. 1) yielded an apparent deletion of 3362 ± 190 bp for EC536, as compared with K12 strain W3110, and placed the deletion in the intergenic region between *mutS* and *rpoS*. Nucleotide sequencing of EC536 showed the endpoints of the K12 sequence at positions 32719 and 38818 (6098 bp) in the intergenic region (*10*).
13. Colony hybridization experiments with the use of oligonucleotides that annealed at a K12-O157:H7 border sequence (5′-CCCAAACAAAAGAAAAAGGCC) or within the novel sequence (5′-CTGCGTCATGCGGAAGCTTC and 5′-CACCGCATACTGTGGTTTGG) showed the presence of modified sequence in 15 independent O157:H7 strains and 4 O55:H7 strains. Negative probe results were obtained with K12 strains. Filter preparation and colony hybridization for oligonucleotide probe analysis were done according to the procedures of E. Kupchella, W. H. Koch, and T. A. Cebula [*Environ. Mol. Mutagen.* **23**, 81 (1994)].
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surrounding genes.
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28. Strains were transformed with multicopy plasmid clones harboring the wild-type gene for *mutH* (pGW1899), *mutL* (pGW1842), *mutS* (pGW1811), and *uvrD* (pGT26). pGW plasmids are described by P. P. Pang, A. S. Lundberg, and G. C. Walker [*J. Bacteriol.* **163**, 1007 (1985)], and pGT26 plasmids are described by G. Taucher-Scholz and H. Hoffmann-Berling [*Eur. J. Biochem.* **137**, 573 (1983)]. Mutators C396 and SL78 were transformed by phage P22 HT *int* transducing particles carrying plasmid clones [M. J. Orbach and E. N. Jackson, *J. Bacteriol.* **149**, 985 (1982)]. Other mutators were transformed by electroporation with the use of a Bio-Rad Gene Pulser apparatus and protocol supplied by the manufacturer.
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30. The authors thank M. J. Bessman, H. Ochman, R. M. Schaaper, P. I. Tarr, and T. S. Whittam for supplying bacterial strains and E. F. Boyd, E. C. Cox, P. E. Hartman, R. E. Lenski, P. Modrich, and P. D. Sniegowski for helpful discussions and comments.

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Neuronal Gene Expression in the Waking State: A Role for the Locus Coeruleus

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Several transcription factors are expressed at higher levels in the waking than in the sleeping brain. In experiments with rats, the locus coeruleus, a noradrenergic nucleus with diffuse projections, was found to regulate such expression. In brain regions depleted of noradrenergic innervation, amounts of c-Fos and nerve growth factor–induced A after waking were as low as after sleep. Phosphorylation of cyclic adenosine monophosphate response element–binding protein was also reduced. In contrast, electroencephalographic activity was unchanged. The reduced activity of locus coeruleus neurons may explain why the induction of certain transcription factors, with potential effects on plasticity and learning, does not occur during sleep.

In most brain regions, the expression of some transcription factors that could mediate long-term changes in neural function, such as the immediate-early genes (IEGs) encoding c-Fos and nerve growth factor–induced A (NGFI-A) (*1*), is much greater during the waking state than during sleep (*2–4*). The reasons why the expression of these genes is high during waking and low during sleep are not known. A key factor might be the activity level of neuromodulatory systems with diffuse projections to many regions of the brain, such as the noradrenergic locus coeruleus (LC) (*5*). During sleep, LC neurons fire regularly at very low levels, whereas during waking, LC neurons fire regularly at higher levels and emit phasic, short bursts of action potentials when triggered by salient events (*6*). These neurons release norepinephrine (NE), which can modify neural activity and excitability (*7*) as well as the expression of certain genes, including IEGs (*8*).

Does the LC play a role in the increased expression of transcription factors that occurs during the waking state? To analyze this possibility, we implanted electrodes in rats for electroencephalographic (EEG) and electromyographic recordings; we then administered a unilateral injection into the LC of 6-hydroxydopamine (6-OHDA), a neurotoxin that destroys catecholaminergic neurons (*9*). In rats in which the LC was not lesioned, we observed little or no Fos protein expression after 3 hours of sleep ($N = 7$; Fig. 1A), but there was a marked bilateral expression of Fos in cerebral cortex, hippocampus, and other brain areas after 3 hours of waking induced by sleep deprivation ($N = 8$; Fig. 1A) (*10*). In contrast, in sleep-deprived rats in which the LC of one side had been lesioned ($N = 9$), Fos expression was almost abolished in cortical areas and hippocampus on the lesioned side, whereas on the intact side, Fos levels were high and comparable to those observed in normal animals after periods of waking (Fig. 1B) (*11*). The extent and effectiveness of LC lesions were determined by examining quantitatively the disappearance of NE cell bodies in the LC and of NE fibers in target cortical regions (Fig. 1B) (*12*). On average, in cortical areas in which NE fibers were reduced by more than 80%, Fos expression after waking was reduced by 76 ± 8% (mean ± SEM, $P < 0.001$, Wilcoxon signed-rank test for matched pairs) with respect to the intact side (*13*). In all animals examined, Fos immunoreactivity decreased in close spatial correspondence with the disappearance of NE innervation, even at the level of individual NE fibers. The reduction of Fos protein on the lesioned side was accompanied by a comparable decrease of c-Fos mRNA as revealed by in situ hybridization ($N = 3$) (*14, 15*).

Because there is evidence that the release of NE may be responsible for the increased levels of IEG expression that are observed after stressful manipulations (*16*), we also examined Fos expression in rats with unilateral LC lesions that had been spontaneously awake for 3 hours in the dark without any external intervention. The rats were killed at least 2 to 3 weeks after surgery, when percentages of recording time spent in different behavioral states had returned to control values. As in animals killed after sleep deprivation in the light, Fos levels were high on the intact side but very low or absent in cortical areas depleted of NE fibers (77 ± 1% reduction, $N = 6$, $P < 0.001$) (Fig. 2, A and B). Thus, the expression of Fos in cortex and hippocampus was due to the waking state per se rather

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