Requirement of the *Manx* Gene for Expression of Chordate Features in a Tailless Ascidian Larva

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An evolutionary change in development was studied in two closely related ascidian species, one exhibiting a conventional tadpole larva and the other a modified tailless larva. Interspecific hybridization restores chordate features to the tailless larva. The zinc finger gene *Manx* is expressed in cells that generate chordate features in the tailed species but is down-regulated in the tailless species. *Manx* expression is restored in hybrid embryos. Antisense oligodeoxynucleotide treatment inhibited *Manx* expression and chordate features in hybrid embryos, which suggests that *Manx* is required for development of the chordate larval phenotype in ascidians.

Many animal phyla exhibit closely related species with different modes of development (*1*). These species are attractive systems in which to study the evolution of developmental mechanisms, because secondary changes arising by genetic drift are minimized. Comparative studies indicate that changes in oogenesis, cleavage, axis and cell fate determination, gastrulation, and inductive cell interactions are responsible for evolutionary modifications in development (*2*). Although the molecular mechanisms are poorly understood, it has been proposed that genes with conserved DNA recognition motifs, such as homeodomains and zinc fingers, play critical roles in these processes (*3*). Here, we show that the zinc finger gene *Manx* is involved in an evolutionary change in ascidian development.

Most ascidian species develop by means of a tailed (or urodele) larva. The tadpole larva has a head containing a dorsal brain and a pigmented sensory organ (otolith) and a tail containing a notochord, spinal cord, and striated muscle cells (Fig. 1A) (*4*). These chordate features are lacking in a small number of ascidian species that develop by means of a tailless (or anural) larva (Fig. 1C) (*5*). The anural species have been derived at least five different times from ancestors with urodele larvae, and closely related species can exhibit the urodele or anural mode of development (*6*). *Molgula oculata*, a urodele developer (Fig. 1A), and its sister species, *M. occulta*, an anural developer (Fig. 1C), can be cross-hybridized in the laboratory (Fig. 1B) (*7*, *8*). The pattern and timing of cleavage, gastrulation, and neurulation is the same in these species, yet the anural embryo normally fails to differentiate an otolith, notochord, and tail muscle cells. Eggs and embryos of *M. occulta* have evolutionarily modified egg cytoplasmic determinants, inductive cell interactions, and morphogenetic movements (*9*). However, chordate features—namely the brain sensory organ, notochord, muscle, and tail—are restored in interspecific hybrids produced by fertilizing *M. occulta* eggs with *M. oculata* sperm (Fig. 1B) (*7*, *8*). This is an example of an experimentally induced atavism: the reappearance of an ancestral character that was lost during evolution (*10*). The *M. oculata* genome is required for this restoration (*11*), which suggests that the anural larva evolved by loss-of-function mutations in *M. occulta* genes.

The *Manx* gene was isolated in a subtractive screen of cDNA libraries designed to identify maternal genes expressed differentially in *M. oculata* and *M. occulta* (*12*). This screen yielded the (urodele) *uro-1*, *uro-2*, and *uro-11* (*Manx*) cDNA clones, which encode genes that are expressed in *M. oculata* but are silent or down-regulated in *M. occulta*. *Manx* is a single-copy gene encoding 2.0- and 2.3-kb mRNAs that are expressed both maternally and zygotically. The two *Manx* transcripts encode the same protein; the larger transcript is derived by splicing two additional exons to the 5′ leader of the smaller *Manx* mRNA (*13*). The predicted Manx protein contains a nuclear localization signal and a zinc finger motif (*12*), which suggests that it may function as a transcription factor. Zygotic *Manx* mRNA is present only during a short interval between late cleavage stages and neurulation. During this period, *Manx* transcripts accumulate in prospective notochord, neural tube, tail muscle, and posterior ectoderm cells, which undergo concerted differentiation and morphogenetic movements to generate the larval tail.

The pattern of *Manx* expression suggests that the gene might function in tail development. To determine whether *Manx* mRNA was up-regulated in hybrids, we probed Northern blots of RNA from *M. oculata*, *M. occulta*, and hybrid embryos with *Manx* antisense RNA (*14*). Hybrid embryos had increased amounts of both *Manx* transcripts (Fig. 2A). The spatial distribution of *Manx* mRNA in hybrid embryos was determined by in situ hybridization (*14*). *Manx* transcripts were present during gastrulation and neurulation in *M. oculata* (Fig. 2, B and E) and were not detected in *M. occulta* embryos (Fig. 2, C and F). The temporal and spatial accumulation of *Manx* mRNA was the same in hybrids and *M. oculata* embryos; transcripts were present in the ectoderm, notochord, and muscle cells during gastrulation (Fig. 2D) and in the neural folds, notochord, and posterior ectoderm cells during neurulation (Fig. 2G). Therefore, the ancestral timing and pattern of zygotic *Manx* expression were restored in hybrid embryos.

Because *Manx* transcripts are primarily of zygotic origin in hybrid embryos, we used antisense procedures to investigate the role of *Manx* during gastrulation and tail formation. Antisense phosphorothioate oligodeoxynucleotides (ODNs) were designed corresponding to three different regions in the

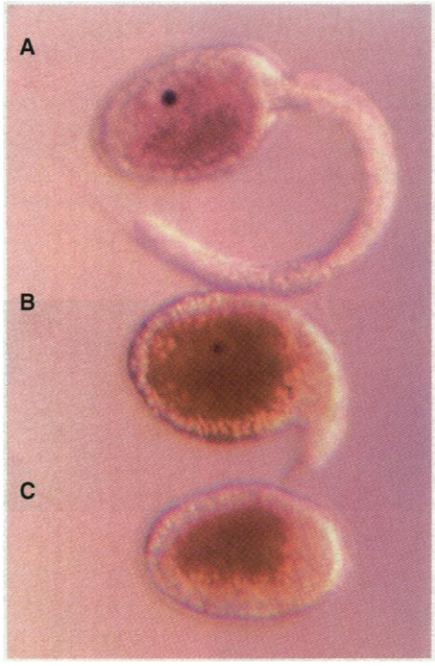

Fig. 1. *Molgula oculata*, hybrid, and *M. occulta* larvae. (**A**) An *M. oculata* larva exhibiting an otolith (dark pigment spot in head) and a tail with a notochord and flanking muscle cells. (**B**) A hybrid larva, exhibiting an otolith and a short tail containing a notochord, which developed from an *M. occulta* egg fertilized with an *M. oculata* sperm. (**C**) An *M. occulta* larva lacking the otolith, tail, notochord, and muscle cells.

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Manx cDNA sequence (*15*). Phosphorothioate ODNs are resistant to degradation and are capable of duplex formation with complementary target molecules, and the hybrids are sensitive to hydrolysis by ribonuclease H (*16*). Gene expression in chick and mouse embryos, explants, and embryonic cells has been specifically inhibited by the addition of phosphorothioate ODNs to the culture medium (*17*). We used rhodamine-labeled ODNs to determine whether this approach was feasible in ascidian embryos (*18*). Antisense and sense ODNs were taken up by hybrid embryos, distributed between the embryonic cells, and retained throughout development (Fig. 3, A through D).

Embryos of both *Molgula* species were treated with antisense ODNs, but because antisense will not inhibit the oogenic synthesis of maternal protein in the tailed species (*M. oculata*), the effects on tail and otolith development were variable. In contrast, embryos of the tailless species (*M. occulta*) developed normally and metamorphosed in the presence of antisense ODNs. Therefore, we studied the effect of loss of *Manx* in hybrid embryos, which only express zygotic *Manx* transcripts. Hybrid embryos were treated with ODNs at first cleavage, cultured in the presence of the ODNs until hatching, and then assayed for phenotypic effects (*15*). The effect of antisense ODNs on *Manx* mRNA accumulation was investigated with in situ hybridization. *Manx* transcripts were not detected in antisense ODN–treated hybrid embryos (Fig. 2H), but zygotic *Manx* expression was normal in sense ODN–treated embryos (Fig. 2I). Thus, antisense ODNs reduced the titer of *Manx* mRNA to below the level of detection by in situ hybridization. In contrast, antisense *Manx* ODNs had no effect on muscle actin mRNA, another zygotic transcript that is up-regulated in hybrid embryos (Fig. 2, J and K) (*19*). Furthermore, ODN treatment did not affect acetylcholinesterase (AChE) expression in the primary muscle cells, which is dependent on new zygotic transcription (*20*). These results show that antisense ODNs specifically inhibit *Manx* gene expression in hybrid embryos.

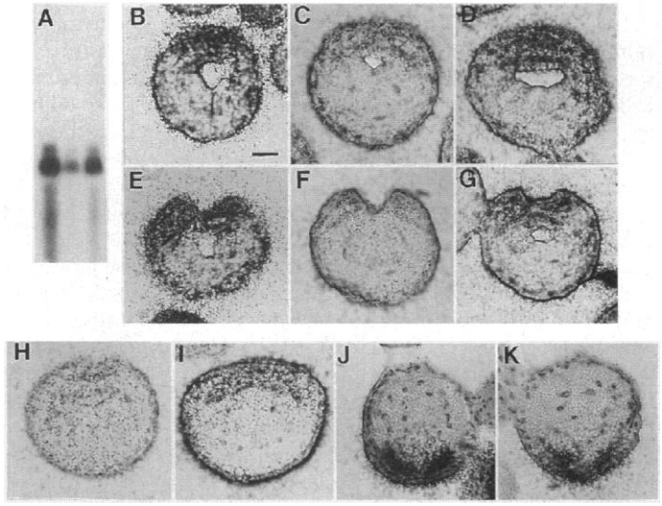

Fig. 2. Expression of *Manx* mRNA in *M. oculata*, *M. occulta*, and hybrid embryos, and expression of *Manx* and muscle actin mRNA in ODN-treated hybrid embryos. (**A**) Northern hybridization showing the accumulation of 2.0- and 2.3-kb *Manx* transcripts in *M. oculata* (left), *M. occulta* (center), and hybrid (right) gastrulae. In situ hybridizations showing *Manx* mRNA distribution in *M. oculata* (left), *M. occulta* (center), and hybrid (right) gastrulae (**B** through **D**) and neurulae (**E** through **G**), respectively. In situ hybridizations showing *Manx* mRNA accumulation in hybrid gastrulae treated with antisense (**H**) and sense (**I**) ODN-1. In situ hybridizations showing the distribution of muscle actin mRNA in hybrid gastrulae treated with antisense (**J**) and sense (**K**) ODN-1. Cross sections through the dorsoventral axis of gastrulae or neurulae with the dorsal pole at the top are shown in (B) through (I). Frontal sections through the anteroposterior axis of gastrulae with the anterior pole at the top are shown in (J) through (K). Scale bar, 20 μm.

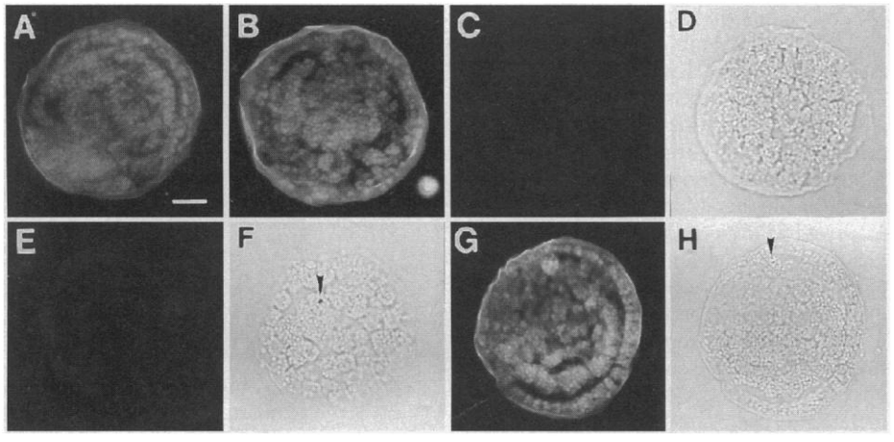

Fig. 3. Uptake and distribution of rhodamine-labeled ODNs in hybrid embryos. Fluorescence micrographs of embryos treated with antisense (**A**) and sense (**B**) ODN-1. Fluorescence (**C**) and bright-field (**D**) micrographs of an untreated embryo. Fluorescence (**E** and **G**) and bright-field (**F** and **H**) micrographs of an embryo lacking (E and F) and containing (G and H) rhodamine-labeled ODN-1. The melanocyte in the restored sensory organ is shown by the arrowhead in (F). The arrowhead in (H) shows the brain lacking a melanocyte. The exposure times for each dark-field photograph were the same. Scale bar, 20 μm.

The effects of antisense ODNs on the hybrid larval phenotype is shown in Fig. 4. Although the ODNs did not affect cleavage, gastrulation, neurulation, or developmental timing, antisense but not sense ODNs abolished the sensory organ and tail (Fig. 4, A and B). The ODN-treated larvae were fixed and sectioned to determine their internal morphology (*14*). *Molgula occulta* embryos contain a small number of vestigial notochord-lineage cells organized in a posterior placode (*7*, *8*). In hybrid embryos, the ability of the placode to differentiate into a notochord is restored, and a short tail is generated. Although the placode did not produce a notochord in antisense ODN–treated hybrids (Fig. 4C), notochord formation was not affected by sense ODNs (Fig. 4E). Differentiation of the cerebral vesicle and melanocyte in the brain sensory organ was also inhibited in antisense ODN– but not sense ODN–treated embryos, although the antisense ODN–treated hybrids developed a brain (Fig. 4, C and D). Thus, the *Manx* gene is required for sensory organ but not brain development.

The same results were obtained when embryos were treated with antisense ODNs that correspond to other regions of *Manx* mRNA or when different *Manx* antisense ODNs were used in combination. Figure 4F summarizes experiments in which different clutches of hybrid embryos were subjected to ODN treatment. Although the proportion of larvae with restored urodele features was significantly reduced by antisense ODNs, the extent of inhibition varied quantitatively among different hybrid clutches. Repetition of these experiments with rhodamine-labeled ODNs showed that the antisense ODN–treated hybrids with urodele features lacked rhodamine fluores-

cence, whereas those without these features showed fluorescence (Fig. 3, E through H). The results suggest that the residual urodele features are the result of differential ODN uptake in hybrid clutches (*21*). Because *Manx* mRNA is expressed transiently in hybrid embryos, antisense ODNs should inhibit urodele features only when added before or during the *Manx* expression period. Figure 4G shows the results of experiments in which antisense ODN treatment was initiated at different times during embryogenesis. Antisense ODNs were effective only when added to embryos before or during the brief *Manx* expression period. In summary, the antisense experiments show that zygotic expression of the *Manx* gene is necessary for restoration of the brain sensory organ, notochord, and tail in hybrid embryos.

The ascidian tadpole larva contains two types of muscle cells with different origins and mechanisms of determination (*22*). The primary muscle cells (PMCs) are specified by cytoplasmic determinants, and the secondary muscle cells (SMCs) are specified by inductive cell interactions. *Molgula oculata* larvae have about 40 AChE-positive PMCs and SMCs, and *M. occulta* larvae have about 20 PMCs. In hybrid embryos, the urodele complement of 40 PMCs and SMCs is restored. Hybrid embryos treated with ODNs and assayed for AChE expression (*23*) exhibited only about 20 AChE-positive PMCs, and sense ODNs had no effect on the number of AChE-positive PMCs and SMCs. Thus, *Manx* expression is required for AChE expression in the SMCs but not the PMCs. The differential inhibition of AChE expression in the PMCs and SMCs is strong evidence that the effects of antisense ODNs are specific and not due to general toxicity.

We conclude that expression of the *Manx* gene is required for the restoration of ancestral chordate features in a tailless ascidian larva. When *Manx* expression was inhibited with antisense ODNs, the hybrid larvae did not exhibit chordate features. The expression of *Manx* in embryonic cells that generate the tailed larva suggests that *Manx* may function early during gastrulation, possibly by activating a hierarchy of genes leading to tail development. The transient expression of *Manx* in the mesoderm, ectoderm, and neural tube, which undergo concerted changes in cell shape and migration, suggests that *Manx* may regulate tail development by coordinating morphogenetic movements. Although cell migration also occurs in the developing nervous system, the requirement of *Manx* for sensory organ differentiation may be a secondary effect of an inductive signal originating from the prospective notochord or spinal cord (*24*). It will be important to identify other genes that function in the *Manx* pathway and to determine whether the same or different modifications have occurred in ascidian species that have evolved tailless larvae independently (*6*). Analysis of genes such as *Manx*, which have been modified during the evolutionary regression of chordate features, may provide clues as to how these features originated in the ancestral chordate.

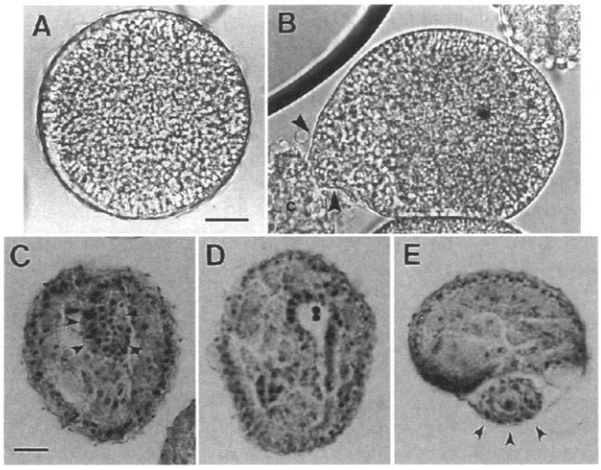

Fig. 4. The effect of ODN treatment on the restoration of urodele features in hybrid embryos. Larvae developed from embryos treated with antisense (**A**) and sense (**B**) ODN-1. The larva treated with sense ODN-1 formed a short tail (arrowheads) adjacent to the discarded chorion (c) and melanized brain sensory organ (black spot), whereas these structures are absent in larva treated with antisense ODN-1. Scale bar in (A), 20 μm; magnification is the same in (B). Sections were made of larvae that developed from embryos treated with antisense (**C**) and sense (**D** and **E**) ODN-1. Larvae treated with sense ODN-1 each have a brain sensory organ, with one or two melanocytes and a cerebral vesicle (D), and a tail containing a differentiated notochord (arrowheads in E). In contrast, the larva treated with antisense ODN-1 does not have a differentiated notochord or sensory organ, although a brain is present (arrowheads in C). Frontal sections are shown through the dorsal (C and D) or ventral (E) regions of the larvae. Scale bar in (C), 20 μm. Magnification is the same in (D) and (E). (**F**) A histogram showing the effect of antisense (solid bar), sense (hatched bar), and control (open bar) ODN treatments on the restoration of urodele features. The results of nine experiments with antisense and sense ODN-1 and three experiments with the control ODN are shown. (**G**) A histogram showing the effect of antisense (solid bars) ODN-1 treatment beginning at 1, 3, and 6 hours after fertilization on the restoration of urodele features. The results of three experiments are shown. The approximate timing of *Manx* expression is shown by the horizontal bar at the top of the histogram. The bars and SEMs in (F) and (G) represent the mean and standard error.

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14. Gametes were obtained by dissection from gonads of *M. oculata* and *M. occulta* collected in Roscoff, France. Insemination and embryo culture was carried out as described (*7, 8*). Interspecific hybrids were produced according to the procedure of Swalla and Jeffery (*7, 8*). RNA was isolated from gastrula stage embryos [C. J. March *et al.*, *Nature* **315**, 641 (1985)]. For Northern blot analysis, gel lanes were loaded with 10 μg of total gastrula RNA, and blots were hybridized with a ^{32}P–adenosine triphosphate (ATP)–labeled (1300 Ci/mmol; New England Nuclear, Boston, MA) antisense RNA probe synthesized from the *Manx* cDNA insert. The blots were checked for equivalent loading by determining the amounts of 18*S* ribosomal RNA in each lane. Northern hybridizations and washes were conducted as described (*11*). In situ hybridization was carried out on sections of paraplast-embedded embryos (*11*) with a ^{35}S-labeled ATP and ^{35}S-labeled uridine triphosphate (1300 Ci/mmol; New England Nuclear)–labeled

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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23. Larvae were fixed with 5% formalin in sea water for 30 min at 4°C. AChE activity was determined as described [M. J. Karnovsky and L. Roots, *J. Histochem. Cytochem.* **12**, 219 (1964)]. After the assay was complete, the embryos were removed to microscope slides in a small amount of fluid, a cover slip was applied, and the number of AChE-positive cells was counted as described (*8*).
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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.).

30 July 1996; accepted 12 September 1996

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)