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Inactivation of mismatch repair increases the diversity of *Vibrio parahaemolyticus*

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Summary

Inactivation of mismatch repair (MMR) has been shown to increase the accumulation of spontaneous mutations and frequency of recombination for diverse pathogenic bacteria. Currently, little is known regarding the role of mutator phenotypes for the diversification of natural populations of opportunistic human pathogens in marine environments. In this study, a higher frequency of mutators was detected among *V. parahaemolyticus* strains obtained from environmental sources compared with clinical sources. Inactivation of the MMR gene *mutS* caused increased antibiotic resistance and phase variation resulting in translucent colony morphologies. Increased nucleotide diversity in *mutS* and *rpoB* alleles from mutator compared with wild-type strains indicated a significant contribution of the mutator phenotype to the evolution of select genes. The results of this study indicate that the inactivation of MMR in *V. parahaemolyticus* leads to increased genetic and phenotypic diversity. This study is the first to report a higher frequency of natural mutators among *Vibrio* environmental strains and to provide evidence that inactivation of MMR increases the diversity of *V. parahaemolyticus*.

Introduction

Vibrio parahaemolyticus is a halophilic γ-proteobacterium that occurs in sediments, is associated with particles and zooplankton, and is found in high densities in molluscs residing in coastal environments (DePaola *et al*., 2003; Thompson *et al*., 2004a,b). *Vibrio parahaemolyticus* can cause gastro-enteritis and less commonly wound infections in humans from either consumption of contaminated shellfish or infection of broken skin upon exposure to seawater. An increase in the frequency and the geographical range of *V. parahaemolyticus*-associated food-poisoning outbreaks has occurred in the United States in recent years (Daniels *et al*., 2000; McLaughlin *et al*., 2005) and emphasizes the need to study the distribution and ecology of *V. parahaemolyticus* in coastal environments. *Vibrio parahaemolyticus* isolates obtained from environmental samples have been shown to have considerable genetic and phenotypic diversity (Martinez-Urtaza *et al*., 2004; 2006; González-Escalona *et al*., 2008). Pulsed-field gel electrophoresis analysis of *V. parahaemolyticus* has demonstrated the clonal nature of clinical strains while environmental strains exhibit greater diversity based on DNA restriction pattern analysis (Martinez-Urtaza *et al*., 2004; Parsons *et al*., 2007). In addition, a recent multilocus sequence typing (MLST) study that analysed the nucleotide diversity of seven housekeeping genes (*recA*, *gyrB*, *pntA*, *dtdS*, *pyrC*, *dnaE* and *tnaA*) further emphasized the clonality of the pandemic clinical strains while non-pandemic clinical strains and environmental strains had greater diversity in allelic profiles (González-Escalona *et al*., 2008). Although *V. parahaemolyticus* strains were shown to have considerable genetic diversity, surprisingly little is known about the mechanisms and conditions that contribute to this observed diversity. Determining the mechanisms that increase the diversity of *V. parahaemolyticus* is important for understanding the conditions that contribute to the genetic diversification of environmental strains and the ability of these strains to acquire novel phenotypes for survival and adaptation during changing environmental conditions or infection of a host.

Inactivation of methyl-directed mismatch repair (MMR) is one of several stress-induced mechanisms that increases genetic diversity (Giraud *et al*., 2001; Kivisaar, 2003). The loss of function of MMR leads to the emergence of mutator strains characterized by high spontaneous mutation frequencies (Kivisaar, 2003; Denamur and Matic, 2006). The MMR proteins, MutS and MutL, reduce the number of errors that accumulate during DNA replication by recognizing and removing mismatches respectively (Schofield and Hsieh, 2003). In addition, MMR limits the recombination of divergent sequences and a loss of function of MMR would lower barriers to recombination that would have otherwise restricted horizontal gene transfer (Matic *et al*., 1995). For example, MMR-deficient strains of *Pseudomonas stutzeri* had increased recombination of divergent *rpoB* alleles

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(Meier and Wackernagel, 2005). Inactivation of MMR was also shown to increase phenotypic diversity resulting in phase variation of *Neisseria meningitidis* (Richardson and Stojiljkovic, 2001; Richardson *et al*., 2002) and *Pseudomonas aeruginosa* (Lújan *et al*., 2007) thereby increasing the adaptive abilities of these strains. Mutators have been characterized for diverse pathogenic bacteria including *Escherichia coli* (Rayssiguier, 1989; LeClerc *et al*., 1996), *P. aeruginosa* (Oliver *et al*., 2000; 2002), *Haemophilus influenzae* (Watson *et al*., 2004), *N. meningitidis* (Richardson and Stojiljkovic, 2001), *Salmonella* spp. (Zahrt *et al*., 1999), *Staphylococcus aureus* (Prunier and Leclercq, 2005) and *Bacillus anthracis* (Zeibell *et al*., 2007). Many of these studies focused on the role of mutator strains for increased antibiotic resistance especially in regard to treating chronic infections. In contrast, few studies have examined the role of inactivation of MMR in increasing the diversity of bacteria in the environment.

The objective of this study was to determine whether *V. parahaemolyticus* strains of clinical or environmental origin exhibited mutator phenotypes. In addition, we determined whether inactivation of MMR increases the diversity of *V. parahaemolyticus*. The nucleotide sequences of several conserved housekeeping genes from mutator and wild-type strains were analysed to determine whether inactivation of MMR increases genetic diversity. This study provides the first characterization of natural mutators in environmental populations of *V. parahaemolyticus* and demonstrates a contribution of inactivation of MMR to the diversity of a marine microorganism.

Results

Vibrio parahaemolyticus *MMR homologues and organization of the* mutS-rpoS *region*

To determine if homologues to known MMR proteins were present in *V. parahaemolyticus*, we examined the sequenced genome of the pandemic O3:K6 *V. parahaemolyticus* RIMD2210633. A BLAST search of the genome of RIMD2210633 using MMR proteins of *E. coli* revealed *V. parahaemolyticus* possessed homologues of all the major MMR proteins (Table 1). *Vibrio parahaemolyticus* RIMD2210633 did not have a homologue of the *Vibrio cholerae* MMR protein MutK, which to date has only been identified in *V. cholerae* (Bhakat *et al*., 1999). In *E. coli* K12 and other Gram-negative bacteria, *mutS* is located adjacent to the stationary phase regulator *rpoS* (Ferenci, 2003). The *mutS* of Gram-positive bacteria such as *S. aureus* is organized as a *mutSL* operon (Prunier and Leclercq, 2005) rather than the *mutS-rpoS* organization characterized for most Gram-negative bacteria. In addition, the *mutS-rpoS* intergenic region of *E. coli* was previously characterized as a site for recombinational insertion of DNA segments of 8.5–14.5 kb (Herbelin *et al*., 2000). Although the *V. parahaemolyticus* RIMD2210633 genome had a similar genetic organization of *mutS* to that of other Gram-negative bacteria, this strain lacked insertions in the intergenic region as previously reported for *E. coli* (Herbelin *et al*., 2000) (data not shown). To determine whether there were insertions within the *mutS-rpoS* intergenic region among our collection of clinical and environmental strains, we designed primers to amplify the *mutS-rpoS* intergenic region. A PCR screen of the nine clinical and environmental strains using primers designed in this study indicated there were no detectable insertions within this intergenic region (data not shown).

Table 1. Mismatch repair homologues of *V. parahaemolyticus* RIMD2210633.

Gene[a]	Gene ID	Coordinates (Chr)[b]
mutS	VP2552	2695120–2697681 (I)
mutK	–	–
mutL	VP2819	2984301–2982292 (I)
mutH	VP0518	535688–535008 (I)
mutU/uvrD	VP0041	47155–49170 (I)
	VP1083	1135576–1138995 (I)
	VP3013	3216340–3218514(I)
mutT	VP0468	789046–789504 (II)
	VPA0758	473012–473410 (I)

a. MMR homologues were identified by BLAST (Schäffer *et al*., 2001) analysis of *E. coli* K12 proteins and *V. cholerae* MutK to the *V. parahaemolyticus* RIMD2210633 genome.
b. Indicates whether the gene is located on chromosome I or II.

Identification of natural mutators among V. parahaemolyticus *environmental strains*

Vibrio parahaemolyticus strains from clinical and environmental sources ($n = 142$) were examined for possible mutator phenotypes that may have arisen due to inactivation of MMR. Of the 78 environmental and 64 clinical *V. parahaemolyticus* strains tested, four environmental *V. parahaemolyticus* strains exhibited at least 10-fold greater mutation frequency to rifampin (Rif) than the wild-type strain *V. parahaemolyticus* ATCC 17802 (Table 2). The majority of the *V. parahaemolyticus* clinical strains were isolated from different outbreaks that occurred in the USA from 1997 to 2007 (Table S1). Among the clinical strains are 20 with the clonal pandemic serotypes O3:K6 and O4:K12 while the remaining clinical strains represent diverse serotypes. Surprisingly, mutator phenotypes were not identified among any of the *V. parahaemolyticus* clinical strains tested. The environmental strains were isolated from sediment and water associated with the salt marsh plants *Juncus* spp. of the high intertidal zone and *Spartina* spp. from the middle to low intertidal zone. All of the *V. parahaemolyticus* mutator strains were isolated from the rhizosphere of the salt marsh plant *Juncus* spp. from

Table 2. Frequency of spontaneous mutations conferring antibiotic resistance among wild-type and mutator strains.

	Rifampin				Ciprofloxacin		
Strain[a]	Avg ± SD[b]	Fold difference[c]	Complemented with wild-type *mutS*	Fold change[d]	Avg ± SD[b]	Fold difference[c]	Complemented with wild-type *mutS*
17802	$7.7 \pm 6.5 \times 10^{-9}$	–	$6.7 \pm 4.1 \times 10^{-9}$	–0.13	$9.1 \pm 2.8 \times 10^{-7}$	–	–
17802 Δ*mutS*	$\mathbf{8.4 \pm 2.1 \times 10^{-7}}$	**108.1**	$\mathbf{7.6 \pm 6.4 \times 10^{-8}}$	**–10.05**	$\mathbf{9.3 \pm 3.4 \times 10^{-5}}$	**101.2**	$\mathbf{5.0 \pm 4.1 \times 10^{-6}}$
RIMD	$1.0 \pm 0.8 \times 10^{-8}$	0.3	$7.7 \pm 5.4 \times 10^{-9}$	–0.30	$2.1 \pm 0.9 \times 10^{-7}$	–0.8	ND
K1223	$1.0 \pm 0.6 \times 10^{-8}$	0.3	$6.0 \pm 4.1 \times 10^{-9}$	–0.67	$1.2 \pm 0.6 \times 10^{-6}$	0.3	ND
22702	$1.0 \pm 0.9 \times 10^{-8}$	0.3	$9.7 \pm 9.9 \times 10^{-9}$	–0.03	$1.5 \pm 0.4 \times 10^{-7}$	–0.8	ND
J-C1-5	$\mathbf{6.6 \pm 1.7 \times 10^{-7}}$	**84.7**	$\mathbf{9.4 \pm 3.8 \times 10^{-9}}$	**–69.21**	$\mathbf{3.0 \pm 3.0 \times 10^{-5}}$	**32.0**	ND
J-C1-39	$\mathbf{1.3 \pm 0.9 \times 10^{-6}}$	**167.8**	$\mathbf{6.3 \pm 3.9 \times 10^{-9}}$	**–205.35**	$\mathbf{3.2 \pm 3.4 \times 10^{-7}}$	**–0.6**	ND
J-C2-27	$\mathbf{6.2 \pm 4.8 \times 10^{-7}}$	**79.5**	$\mathbf{3.8 \pm 2.7 \times 10^{-9}}$	**–162.16**	$\mathbf{4.1 \pm 3.9 \times 10^{-7}}$	**–0.5**	ND
J-C2-29	$5.0 \pm 3.0 \times 10^{-9}$	–0.4	$12.4 \pm 6.2 \times 10^{-9}$	–1.48	$3.0 \pm 1.3 \times 10^{-7}$	–0.7	ND
J-C2-34	$\mathbf{1.5 \pm 0.8 \times 10^{-6}}$	**193.8**	$\mathbf{2.5 \pm 1.1 \times 10^{-6}}$	**0.67**	$\mathbf{6.9 \pm 5.7 \times 10^{-7}}$	**–0.2**	ND
SG358	$\mathbf{6.3 \pm 4.2 \times 10^{-7}}$	**80.8**	$\mathbf{4.9 \pm 2.8 \times 10^{-7}}$	**–0.29**	$\mathbf{4.3 \pm 0.9 \times 10^{-5}}$	**46.3**	ND

a. Mutators are identified in bold. All are *V. parahaemolyticus* except SG358, which is a *V. campbellii* strain.
b. Averages represent five independent replicates ± the standard deviation (SD).
c. Fold difference is calculated relative to the wild-type strain 17802.
d. Fold change is calculated relative to the values determined for each strain before complementation.
ND, not determined.

North Inlet, North Carolina. There was an additional mutator (strain SG358; Table 2) most closely related to *Vibrio campbellii* that was isolated from the water column of a *Spartina*-dominated salt marsh in Georgia. Sequence analysis of partial 16S rDNA, *rpoA* and *recA* alleles from this strain revealed to 100%, 99% and 98% nucleotide identity to *V. campbellii* respectively.

In addition to determining whether *V. parahaemolyticus* strains had increased mutations conferring resistance to Rif, we determined whether they also had increased resistance to ciprofloxacin (Cip) (Table 2). One of the *V. parahaemolyticus* mutator strains (J-C1-5) and the *V. campbellii* mutator strain SG358 had higher resistance to Cip (3.0×10^{-5} and 4.3×10^{-5} respectively; Table 2) relative to 17802.

Deletion of mutS *increased resistance while complementation of natural mutator strains with wild-type* mutS *restored wild-type sensitivities*

The role of inactivation of *mutS* for increased accumulation of mutation conferring resistance to Rif and Cip was determined by construction of a 17802 Δ*mutS* strain. Deletion of *mutS* in 17802 resulted in a 108-fold higher mutation frequency than the wild-type strain conferring Rif^r (Table 2). Likewise, the Δ*mutS* strain had a 101-fold higher mutation frequency conferring resistance to Cip (Table 2). To determine if inactivation of *mutS* was a possible cause for the spontaneous mutation frequencies detected in the mutator strains each mutator was complemented with the wild-type *mutS* from 17802. The Δ*mutS* strain and three of the mutator strains were restored to wild-type mutation frequencies following complementation with wild-type *mutS* (Table 2). In contrast, the mutator strain J-C2-34 exhibited little change in Rif^r when complemented with the wild-type *mutS* (Table 2). In order to determine whether inactivation of other MMR genes may be the cause of the Rif^r in J-C2-34 we complemented this strain with *mutH* and *mutL* from 17802. Complementation of J-C2-34 with *mutH* and *mutL* resulted in no change in the spontaneous mutation frequency to Rif^r (data not shown). This result suggests that other MMR genes such as *uvrD* may have been inactivated or multiple MMR genes may be simultaneously defective. In addition, the *V. campbellii* mutator strain SG358 was not complemented with the *mutS* from *V. parahaemolyticus* 17802 (Table 2). The MMR gene *mutH* of *V. cholerae* was previously shown to restore wild-type function to an *E. coli mutH* mutant indicating the conserved function of these proteins (Friedhoff *et al*., 2002). The lack of complementation of *V. campbellii* SG358 in this study indicates that *mutS* of *V. parahaemolyticus* may not be transferable to closely related *Vibrios* or that other MMR genes may be defective in SG358.

Increased diversity of mutS *and* rpoB *alleles from mutator strains*

A neighbour-joining tree was constructed from a concatenation of the housekeeping gene nucleotide sequences to examine the diversity of the *V. parahaemolyticus* wild-type and mutator strains characterized in this study. The *V. parahaemolyticus* strains formed a monophyletic group that consisted of separate clusters of environmental and clinical strains (Fig. 1) with the exception of the environmental strain 22702. *Vibrio parahaemolyticus* 22702 was previously isolated from a salt marsh environment and is able to fix nitrogen (Criminger *et al*., 2007) yet clustered

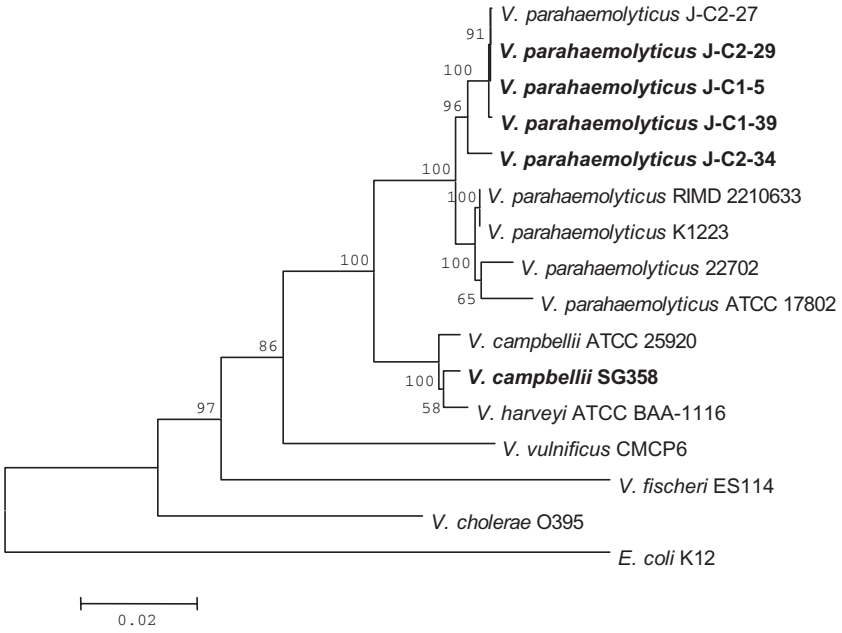

Fig. 1. Neighbour-joining tree of a concatenation of 16S rDNA (1412 bp), *recA* (732 bp) and *rpoA* (723 bp) nucleotide sequences from *V. parahaemolyticus* clinical and environmental strains compared with other *Vibrios*. The tree was constructed with the Kimura 2-parameter model and values shown represent 1000 bootstrap replications. The scale bar represents 0.02 nucleotide substitution per site. Mutator strains are indicated in bold.

with the strains of clinical origin (Fig. 1). The two *V. parahaemolyticus* clusters were distinct from the closely related *V. alginolyticus*, *V. harveyi* and *V. campbellii* sequences indicating that they formed separate clusters within the *V. parahaemolyticus* group (Fig. 1). Likewise, a phylogenetic tree of the *mutS* nucleotide sequences had a monophyletic organization for *V. parahaemolyticus* similar to that observed with the concatenated tree (Fig. S1). In addition, the *mutS* sequences from three of the mutator strains formed a distinct group (Fig. S1).

To compare the level of genetic diversity of wild-type and mutator strains, we analysed sequence diversity of the following four loci: *recA*, *rpoA*, *rpoB* and *mutS*. We sequenced five and four alleles from the wild-type and mutator strains respectively. The lengths of the nucleotide sequences analysed were 729, 723, 1539 and 2487 bp for *recA, rpoA, rpoB,* and *mutS* respectively (Table 3). Also, the MutS coding region was divided into smaller regions that encompassed the MutS I (pfam01624) and MutS II (pfam05188) protein domains (nt 22–759), MutS III protein domain (nt 787–1689) and the MutS ATPase domain (1732–2376) (Table 3). The nucleotide diversity within each domain was assessed independently to determine whether there are regions within the *mutS* gene that are undergoing increased mutation and/or recombination. We analysed the level of diversity at the nucleotide level using two commonly used measures, the nucleotide diversity (π) (Nei, 1987) and Watterson's theta (θ_W) (Watterson, 1975). The level of nucleotide diversity for non-synonymous and synonymous sites was examined separately to determine whether different neutral and selective forces are influencing these two types of sites (Table 3).

The nucleotide diversities of *rpoA* and *recA* loci were similar between the mutator compared with the wild-type strains (Table 3). In contrast, the nucleotide diversity was greatly elevated in mutator strains compared with wild-type strains for *mutS* and *rpoB.* This was evident for both synonymous and non-synonymous sites (Table 3), suggesting that there is an overall increase of mutation rates in *mutS* and *rpoB* of mutator strains. The *mutS* ATPase domain showed the largest increase of nucleotide diversity in mutator compared with wild-type strains (Table 3). Synonymous sites of the mutator strains were approximately 10-fold more variable than those of wild-type strains in this locus. Visual inspection of the *mutS* alleles did not indicate any frameshift or nonsense mutations; however, there were numerous non-synonymous changes (data not shown).

Despite the general increase of the nucleotide variability in mutator compared with wild-type strains, mutator alleles appear to be under strong selective constraint. One method to examine the presence of selective constraint in protein-coding sequences is by comparing the ratio of non-synonymous to synonymous site changes. Here we examined the ratio of non-synonymous to synonymous changes in the four loci at two levels. First, we examined the selective constraint acting on the population by analysing levels of nucleotide diversity (π), which measures within population diversity. Second, we examined the ratio of non-synonymous to synonymous substitutions to a *V. harveyi* allele (K_a/K_s). The K_a/K_s relative to *V. harveyi* is indicative of selective constraint on an evolutionary timescale since the divergence of *V. harveyi* and *V. parahaemolyticus.* The ratios of nucleotide diversity for

Table 3. Nucleotide diversity of housekeeping genes from *V. parahaemolyticus* wild-type and mutator strains.

Gene	Length (bp)	No. polymorphic sites	θ_W Total[a]	π[a]			Divergence from *V. harveyi*[a]	Z-test *P* value[a]	K_a/K_s[a]	SSCF (*P* value)[b]	SSUF (*P* value)[b]	No. of recombination events[a]
				Synonymous	Non-synonymous	Total						
recA	729	40	0.02116 (0.01352)	0.07796 (0.04857)	0.00073 (0.00091)	0.01928 (0.01240)	0.10100 (0.10202)	0.00 (0.00)	0.004 (0.006)	1 879 (0.0006)	1 027 829 (0.6481)	2
rpoA	723	10	0.00465 (0.00302)	0.01454 (0.00839)	0.00074 (0.00092)	0.00415 (0.00277)	0.03714 (0.03758)	0.02 (0.12)	0.014 (0.015)	276 (0.5058)	3 436 349 (0.4983)	0
rpoB	1539	53	0.00998 (0.01240)	0.03571 (0.03940)	0.00069 (0.00257)	0.00916 (0.01148)	0.06060 (0.06247)	0.00 (0.00)	0.071 (0.066)	5 543 (0.0026)	9 779 819 (0.0232)	5
mutS	2487	198	0.01255 (0.03816)	0.04468 (0.14112)	0.00132 (0.00291)	0.01174 (0.03612)	0.21185 (0.21519)	0.00 (0.00)	0.023 (0.024)	95 500 (0.0000)	20 156 770 (0.0000)	8
mutS I & II	720	44	0.01333 (0.02879)	0.04387 (0.10227)	0.00147 (0.00184)	0.01181 (0.02639)	0.20364 (0.19521)	0.00 (0.00)	0.012 (0.014)	3 306 (0.0000)	1 059 640 (0.0556)	3
mutS III	903	54	0.01435 (0.02537)	0.05485 (0.09878)	0.00058 (0.00363)	0.01351 (0.02621)	0.23383 (0.23929)	0.00 (0.00)	0.028 (0.031)	7 762 (0.0000)	3 355 901 (0.0003)	2
mutS ATPase	645	74	0.00670 (0.05751)	0.02445 (0.20889)	0.00082 (0.00306)	0.00651 (0.05271)	0.17760 (0.19399)	0.01 (0.00)	0.018 (0.017)	8 506 (0.5871)	750 742 (0.9949)	0

a. Values of mutator alleles are reported in parentheses under values for wild-type alleles.
b. The sum of squares of condensed fragments (SSCF) and uncondensed fragments (SSUF) of Sawyer's Test.

non-synonymous to synonymous sites (π_{nonsyn}/π_{syn}) for mutator alleles were all below 1 and comparable to those in wild-type alleles, indicating the presence of strong purifying selection at the population level. For example, even though overall nucleotide diversity for the *mutS* ATPase is almost 10-fold greater in mutator strains than in wild-type strains, π_{nonsyn}/π_{syn} in the mutator strains is even lower than that in the wild-type strains (0.0146 versus 0.0335). This pattern is concordant with the long-term selective constraint measured by the ratio of non-synonymous to synonymous substitutions (K_a/K_s). For all four of the genes analysed, the K_a/K_s (compared with a *V. harveyi* allele) ratios are all below 1, indicating the presence of strong purifying selection (Table 3). The *Z*-test for neutral selection relative to *V. harveyi* also confirmed that both the mutator and wild-type alleles were under purifying selection for each gene ($P < 0.05$; Table 3).

Recombination was detected using two tests. First, we used the Sawyer's test, implemented in the DnaSP program (Rozas *et al*., 2003). This test examines whether there are more consecutive identical polymorphic sites than expected, by calculating the sum of squared lengths of condensed fragments (SSCF) and the sum of squared lengths of uncondensed fragments (SSUF) (Sawyer, 1989). The significance of the SSCF and SSUF scores were obtained by means of 10 000 random simulations. It is known that Sawyer's test may be too stringent in determining recombination events (Scally *et al*., 2005). Thus, we used an additional test, the four-gametic test of Hudson and Kaplan (1985) to detect recombination. Both tests indicated there was evidence of recombination in *recA, rpoB* and *mutS* (Table 3). The MutS I, II and III domains had undergone recombination while the MutS ATPase domain had not (Table 3). When we do not separate the MutS locus into three domains, the number of total recombination events in this locus increases to 8. This suggests that we may have lost some power to detect recombination events between domains by separating the MutS coding region into three domains (Table 3).

Increased transition mutations in rpoB *of Rif*^{r} *mutator strains*

Partial sequences of *rpoB* from Rif^r colonies were examined from the wild-type strain 17802, the 17802 Δ*mutS* strain, mutator strain J-C1-5, and wild-type strain J-C2-29 to determine whether inactivation of MMR increased the frequency of transition mutations among mutator strains. Sequence analysis of a 995 bp region of *rpoB* from 10 Rif^r colonies each of 17802, Δ*mutS,* J-C1-5 and J-C2-29 revealed six potential sites that are hotspots of mutation for *V. parahaemolyticus* (Table 4). The nucleotide locations are indicated relative to the start of the *V.*

Table 4. Mutations in *rpoB* of Rif^r colonies from *V. parahaemolyticus* wild-type and mutator strains.

Mutation type	Nucleotide[a]	Amino acid change	17802	Δ***mutS***	**J-C1-5**	J-C2-29
G-A	1586	R529H	1	**7**	**0**	0
A-G	1538	Q513R	0	**0**	**2**	0
	1577	H526R	0	**0**	**6**	1
	1703	N586S	0	**0**	**2**	0
C-T	1576	H526Y	0	**0**	**2**	2
	1592	S531F	0	**3**	**0**	1
Total transitions			1	**10**	**12**	4
A-T	1538	Q513L	8	**0**	**0**	5
A-C	1538	Q513P	0	**0**	**0**	1
C-A	1592	S531Y	1	**0**	**0**	0
Total transversions			9	**0**	**0**	6
Total mutations			10	**10**	**12**	10
% transitions			10	**100**	**100**	40

a. All nucleotide locations are designated according to the start of the RIMD2210633 *rpoB* coding region.
Mutator strains are indicated in bold.

parahaemolyticus-coding region (Table 4). All of the mutation sites resulted in an amino acid change (Table 4). The percentage of nucleotide changes that were transitions represented 100% of the overall nucleotide differences for the mutator strains compared with only 10% and 40% for the wild-type strains (Table 4).

Phenotypic variation of V. parahaemolyticus *clinical and environmental strains*

Vibrio parahaemolyticus strains have been shown to exhibit natural phenotypic variability such as phase variation, which is the transition from opaque (OP) to translucent (TR) colony morphologies (McCarter, 1998). Following deletion of *mutS* in strain 17802 there was a significant change in colony phenotype from OP colony morphologies of the wild-type to all TR colonies for the Δ*mutS* strain (Table 5). Among the 64 clinical and 78 environmental strains examined for increased spontaneous mutation, 18 environmental strains (23%) and one clinical strain (1.2%) had a significant number of TR colonies compared with OP colonies (≥ 10% TR cfu ml^{-1}) (Table S2). Among the environmental strains exhibiting phase variation were all of the natural mutator strains with percentages of TR cfu ml^{-1} ranging from 28% to 100% (Table 5). The additional 14 environmental strains exhibiting phase variation had between 10% and 88% TR cfu ml^{-1} with an average of 47% TR cfu ml^{-1} (Table S2). The colonies of the Δ*mutS* strain had visibly less opacity than other TR colonies (data not shown). Four of the environmental strains exhibited three distinct colony morphologies, OP and TR, and a more translucent version of TR (Table S2).

Discussion

The loss of DNA repair mechanisms such as MMR may increase rates of bacterial diversification and facilitate more rapid niche expansion (Giraud *et al*., 2001; Kivisaar, 2003). In this study, we detected a greater occurrence of mutators among *V. parahaemolyticus* environmental isolates compared with clinical strains. The finding of a higher frequency of mutators among environmental strains is in surprising contrast to previous reports for other bacteria that showed a greater frequency of mutators among clinical strains (LeClerc *et al*., 1996; Oliver *et al*., 2000; Bjedov *et al*., 2003). The frequency of mutators identified among the *Vibrio* environmental strains in this study was approximately 7%, which was higher than the 1% identified for non-pathogenic *E. coli* strains (Bjedov *et al*., 2003). Mutators are usually identified in pathogenic populations, representing 2.6% of *E. coli* and *Salmonella enterica* pathogens (LeClerc *et al*., 1996) and 19.5% of *P. aeruginosa* pathogens (Oliver *et al*., 2000), while typically only 1% of non-pathogenic strains. An exception are the *E. coli* commensal strains that had higher mutation frequencies than *E. coli* clinical strains

Table 5. Percentage of TR colonies of *V. parahaemolyticus* clinical and environmental strains.

Strain[a]	% TR cfu ml^{-1} (SD)[b]
RIMD	0.0 (0.0)
17802	0.5 (0.0)
17802 Δ*mutS*	**99.5 (0.0)**
K1223	0.0 (0.0)
F7979	18.3 (0.1)
22702	49.1 (0.1)
J-C1-5	**100 (0.0)**
J-C1-39	**28.9 (0.2)**
J-C2-27	**74.6 (0.4)**
J-C2-29	0.0 (0.0)
J-C2-34	**28.9 (0.1)**
SG358	**26.4 (0.1)**

a. Mutator strains are indicated in bold. F7979 is a clinical strain.
b. Values represent the percentage of cfu ml^{-1} that are translucent (TR) determined from five or more independent replicates with the standard deviation (SD) indicated in parentheses.

(Bjedov *et al*., 2003). The greater frequency of mutators observed among clinical bacteria is often linked to use of antibiotics for treatment of chronic infections. For example, cystic fibrosis patients have been shown to be infected with not only *P. aeruginosa* mutators, but also *H. influenzae* mutators (Watson *et al*., 2004). This suggests that the stress of certain pathogenic niches or the prolonged use of antibiotics to treat chronic infections may induce mutations and loss of MMR in diverse bacteria. A possible explanation for the lack of mutators identified among the *V. parahaemolyticus* clinical strains in this study may be the self-limiting nature of *V. parahaemolyticus* infections and the infrequent use of antibiotics for treatment of these infections (Daniels *et al*., 2000). *Vibrios* isolated from oysters exhibit resistance to numerous antibiotics including Rif, ampicillin, nalidixic acid and Cip (Han *et al*., 2007). The increase in resistance to Cip demonstrated for the Δ*mutS* strain (Table 3) indicates a role of inactivation of MMR for increased resistance to an antibiotic used to treat severe *Vibrio* infections (Tang *et al*., 2002). A previous study has reported that mutations in *gyrA* or *parC* of *V. parahaemolyticus* can confer low-level resistance to Cip (Okuda *et al*., 1999), while mutations in both *gyrA* and *parC* resulted in higher levels of resistance (Okuda *et al*., 1999). The increased resistance to Cip we report for the Δ*mutS* strain, one of the *V. parahaemolyticus* mutator strains, and the *V. campbellii* mutator strain may have resulted from the accumulation of mutations in both *gyrA* and *parC* while the remaining mutator strains may not have mutations in both genes. The occurrence of mutators in *V. parahaemolyticus* populations from environmental sources may be one possible explanation for the increased observation of antibiotic-resistant *Vibrios* in the environment such as that recently reported for isolates cultured from oysters (Han *et al*., 2007). In addition, the stress of the environmental niche may play a role in the emergence of *Vibrio* natural mutator strains. The majority of the *Vibrio* mutators in this study were isolated from sediment of the rhizosphere of *Juncus* spp. occurring in the high intertidal zone (Bagwell *et al*., 1998), which may undergo greater fluctuations in salinity, temperature, and UV exposure. In contrast, the *V. campbellii* mutator was isolated from water associated with *Spartina* spp. from the middle to low intertidal zones. The greater frequency of mutators associated with *Juncus* spp. suggests that the level of environmental stress may increase the occurrence of mutator strains in certain environmental niches. Further studies are necessary to investigate the role of environmental stress for the emergence of *Vibrio* mutators.

A previous study has reported the horizontal transfer and recombination of MMR genes as evidence of the inactivation and reacquisition of functional MMR genes in *E. coli* (Denamur *et al*., 2000). *Oenococcus oeni* and *Oenococcus kitaharae* were shown to lack *mutS* and *mutL*, which may account for their more rapid evolution relative to other *mutSL*-containing *Lactobacillales* as determined by 16S phylogenetic analysis (Marcobal *et al*., 2008). Inactivation of MMR increased the frequency of transition mutations in *rpoB* from Rif^r colonies of *Oenococcus* spp. (Marcobal *et al*., 2008), *B. anthracis* (Zeibell *et al*., 2007) and *E. coli* (Choy and Fowler, 1985). Our results also showed that inactivation of *V. parahaemolyticus* MMR increased the frequency of transition mutations in *rpoB.* In addition, we found evidence of a role of mutator phenotypes for increased nucleotide diversity in select genes (*mutS* and *rpoB*). In contrast, variability at *recA* and *rpoA* were similar in the mutator and wild-type strains. The increase of nucleotide diversity of select genes in mutator compared with wild-type *V. parahaemolyticus* strains could instead reflect the diversity of environmental compared with clinical strains that has been previously reported (Martinez-Urtaza *et al*., 2004). However, the fact that the diversities of *recA* and *rpoA* alleles of mutator and wild-type strains were similar to each other indicates that the increased nucleotide diversity in *mutS* and *rpoB* alleles could be directly attributed to the increase in spontaneous mutation or recombination associated with the mutator phenotype. Overall, the levels of selective constraint observed in *V. parahaemolyticus* variability (π_{nonsyn}/π_{syn}) and nucleotide substitution compared with *V. harveyi* (K_a/K_s) indicates that the genes examined here are all under strong selective constraint. The increase of nucleotide variability in *mutS* and *rpoB* thus probably reflects increases in underlying mutation rates rather than an increase of loss-of-function mutations. Although there were no frameshift mutations or nonsense mutations identified in the mutator *mutS* sequences, alleles from several strains had non-synonymous substitutions that could have altered the function of the MutS protein. Likewise, the absence of frameshifts and nonsense mutations in the mutator *mutS* alleles was reported in a previous study that examined the inactivation of *mutS* and *mutL* of *S. aureus* (Prunier and Leclercq, 2005). Several of the *S. aureus* mutator MMR genes had only a few non-synonymous substitutions while other alleles had no substitutions at all (Prunier and Leclercq, 2005).

Further analysis of the housekeeping genes revealed there was strong evidence of recombination events in *recA*, *rpoB* and *mutS*. Multilocus sequence typing of *V. parahaemolyticus* showed that while the pandemic strains are clonal they may have originated from diverse strains that had undergone high levels of recombination (González-Escalona *et al*., 2008). The MLST study also showed that many of the non-synonymous changes in the housekeeping genes were the result of recombination rather than mutation. Of the seven housekeeping genes examined in the MLST study, *recA* had alleles with varia-

tion resulting from both mutational and recombinational changes (González-Escalona *et al*., 2008). We have previously shown that *recA* may undergo horizontal transfer mediated by mobile genetic elements such as plasmids (Hazen *et al*., 2007). We identified a nearly complete *recA* allele on a plasmid isolated from an environmental *Vibrio* strain most closely related to *V. mediterranei* (Hazen *et al*., 2007). Thus, MMR-deficient strains may have increased likelihood of recombination of horizontally acquired alleles such as a plasmid-encoded *recA* from other *Vibrio* spp. Although we have demonstrated in this study that the spontaneous mutation of *V. parahaemolyticus* mutators does not increase the selective pressure acting on housekeeping genes, inactivation of MMR has been previously shown to increase the frequency of recombination events (Meier and Wackernagel, 2005; Prunier and Leclercq, 2005).

Recent studies have shown the genetic diversity of environmental *Vibrios* may correlate with the niche they occupy (Hunt *et al*., 2008). A comparative genome hybridization of *V. cholerae* environmental strains revealed there were distinct populations that varied relative to changing environmental parameters such as nutrient composition, salinity and temperature (Keymer *et al*., 2007). Mechanisms influencing the diversification of *V. parahaemolyticus* environmental strains may depend on abiotic and biotic factors of their environmental niche such as nutrient availability, oxidative stress, temperature fluctuations, competitive interactions and the presence of mutator strains. For example, mutations could accumulate in genes such as *opaR*, which encodes a quorum-sensing transcriptional regulator (McCarter, 1998). OpaR is one of several proteins that controls the production of capsular polysaccharide that gives a colony an opaque appearance (Güvener and McCarter, 2003). *Vibrio parahaemolyticus* strains with mutations in *opaR* exhibit delayed attachment and produce biofilms that have more distinct microcolonies and channels than observed in wild-type biofilms (Enos-Berlage *et al*., 2005). OpaR mutants have been shown to naturally occur (McCarter, 1998); however, the frequency of mutation of OpaR among *V. parahaemolyticus* clinical and environmental strains and the mechanisms and conditions contributing to the inactivation of OpaR have not been described. Inactivation of MMR has previously been shown to increase the frequency of mutation of the quorum-sensing regulator, LasR, of *P. aeruginosa* (Lújan *et al*., 2007) and we postulate a similar possibility for OpaR. In addition, *N. meningitidis* serogroup A isolates were shown to have increased mutation frequencies and phase variation (Richardson and Stojiljkovic, 2001; Richardson *et al*., 2002). *Vibrio vulnificus* strains reported to have environmental genotypes were shown to have greater phase variation from OP to TR colony morphologies compared with strains with genotypes denoted as clinical (Hilton *et al*., 2006). *Vibrio vulnificus* phase variation was independent of quorum sensing and due instead to the disruption of the stationary phase regulator *rpoS* (Hilton *et al*., 2006). Further investigation is underway to characterize the role of inactivation of MMR for accumulation of mutations in genes such as *rpoS* or *opaR* that could increase phenotypic diversity of *V. parahaemolyticus*.

In summary, we report that inactivation of the MMR gene *mutS* of *V. parahaemolyticus* increases the accumulation of spontaneous mutations conferring resistance to Rif and Cip. The higher frequency of transition mutations among mutator strains and increased nucleotide diversity in mutator *mutS* and *rpoB* alleles relative to wild-type alleles indicates a contribution of mutator phenotypes to the diversity of *V. parahaemolyticus*. Each of the housekeeping genes examined was under strong purifying selection; however, *mutS* and *rpoB* alleles of mutator strains had significantly more synonymous and non-synonymous nucleotide changes compared with the wild-type alleles. Inactivation of MMR may also contribute to increased diversity of natural populations of *V. parahaemolyticus* by lowering barriers to recombination. The greater frequency of natural mutators among the *V. parahaemolyticus* environmental strains examined in this study may result from significant fluctuations in nutrients, salinity and temperature these strains would experience in their environmental niche. Our study provides the first characterization of a higher frequency of mutators among *Vibrio* environmental strains and provides evidence that inactivation of MMR increases the diversity of *V. parahaemolyticus*. Further research is necessary to determine whether environmental stress increases the frequency of natural mutator strains and the role of natural mutators for niche adaptation and diversification in the environment. In addition, studies are underway to determine the role of inactivation of MMR for lowered barriers to recombination among *Vibrios* and to understand the extent of gene transfer among *Vibrio* pathogens and environmental *Vibrio* spp. Understanding the contribution of mutator phenotypes to the dissemination of genes associated with these strains is especially important for examining mechanisms and environmental conditions increasing horizontal transfer of genes for niche expansion and the emergence of *Vibrio* pathogens in coastal marine environments.

Experimental procedures

Bacterial strains, media and antibiotics

Vibrio parahaemolyticus clinical strains used in this study were obtained from samples associated with human illnesses and were provided by the Centers for Disease Control and Prevention (CDC; Atlanta, GA) and characterized as previously described (Meador *et al*., 2007). *Vibrio parahaemolyti-*

cus environmental strains were isolated from sediment, water and oysters collected in September 2006 from Skidaway Island, Georgia ($n = 44$) and Apalachicola Bay, Florida ($n = 13$) (Table S1). Additional environmental strains were isolated from North Inlet, NC ($n = 21$) as previously described (Bagwell *et al*., 1998) (Table S1). The *V. parahaemolyticus* environmental strains were isolated by plating sediment, water or oyster samples on thiosulfate citrate bile salts sucrose agar. The environmental strains from Georgia were isolated primarily from sediment and water associated with the plant *Spartina* spp. in the middle to low intertidal zones. Strains from Florida were isolated from sandy to muddy sediments and oysters collected from a seafood market. Environmental strains isolated from North Carolina were from sediments of the rhizosphere of *Juncus* spp. or *Spartina* spp. in a *Spartina*-dominated salt marsh (Bagwell *et al*., 1998).

Marine strains were grown at 30°C in M10 broth (0.4% tryptone, 0.25% yeast extract, artificial seawater; 0.6 M NaCl, 0.02 M KCl, 0.1 M $MgSO_4$, 0.02 M $CaCl_2$) or on M10 plates with 1.8% agar. *Escherichia coli* strains were grown at 37°C in Luria–Bertani (LB) broth with 1% NaCl or on LB plates with 1.5% agar. Phase variation was examined using heart infusion (HI) broth supplemented with 2% agar for plates (McCarter, 1998). All strains used in this study are listed in Table S1. The antibiotics Rif, Cip and chloramphenicol were used at 100 μg ml^{-1}, 8 μg ml^{-1} and 20 μg ml^{-1}, respectively, unless otherwise noted.

Spontaneous mutation

Spontaneous mutation frequencies to Rif were determined as previously described (Oliver *et al*., 2002; Meier and Wackernagel, 2005) with modifications for testing marine bacteria. Specifically, strains were grown overnight in M10 followed by serial dilutions and plating on M10 and M10Rif. The mutation frequency was calculated as the cfu ml^{-1} on M10Rif plates divided by cfu ml^{-1} on M10 without antibiotic. Plates were incubated overnight at 30°C. Each mutation frequency represents the average of five replicates. Mutator strains were characterized as those having at least a 10-fold elevated mutation frequency compared with the wild-type strain *V. parahaemolyticus* 17802. Mutation frequencies to Cip were determined in the same manner as described for Rif.

DNA amplification and sequencing

The 16S rDNA, *recA* and *rpoA* nucleotide sequences for phylogenetic identification of the environmental strains were obtained as previously described (Criminger *et al*., 2007; Hazen *et al*., 2007). All additional primers are listed in Table 6. We PCR-amplified and sequenced the predicted 2613 bp coding region of *mutS* using primers 71F and 2710R (Table 6). Internal primers listed in Table 6 were used for sequencing. Primers to screen for insertions within the *mutS-rpoS* intergenic region are listed in Table 6. All PCR amplifications were performed using Bio-Rad iproof HF polymerase with the GC buffer (Bio-Rad Laboratories; Hercules, CA) and standard reaction and cycle conditions. PCR amplicons were separated on a 0.7% agarose gel and DNA was extracted using the GenElute gel extraction kit (Sigma-Aldrich; St Louis, MO). Sequencing was performed at the University of Nevada, Reno Genomics Facility and the Georgia Institute of Technology School of Biology Genome Center. Sequences were trimmed and contigs assembled using BioEdit (Hall, 1999).

Sequence and phylogenetic analysis

Phylogenetic analysis was conducted to compare sequence variation of a concatenation of 16S rDNA, *recA* and *rpoA* nucleotide sequences as previously described (Criminger *et al*., 2007; Hazen *et al*., 2007). The nucleotide sequence lengths of 16S rDNA, *recA* and *rpoA* used to construct the phylogenetic tree were 1412, 732 and 723 bp respectively. A neighbour-joining tree was constructed for the nucleotide sequences of the concatenated housekeeping genes in MEGA version 4.0 (Kumar *et al*., 2004) using the Kimura 2-parameter model with 1000 bootstrap replications. Alignments were performed in BioEdit (Hall, 1999) and MEGA (Kumar *et al*., 2004) and all phylogenetic analyses were conducted in MEGA (Kumar *et al*., 2004). The nucleotide diversity (π), nucleotide divergence (θ_W) and the K_a/K_s ratio were determined using DnaSP version 4.5 (Rozas *et al*., 2003) relative to *V. harveyi* as an outgroup. Sawyer's test in MEGA (Kumar *et al*., 2004) and DnaSP (Rozas *et al*., 2003) were used to test for evidence of recombination.

Construction of a ΔmutS *strain*

Deletion of > 90% of the predicted coding region of *mutS* was performed using methods previously developed (J.L. Burns and T.J. DiChristina, submitted for publication) with adaptations for use with *V. parahaemolyticus*. In order to increase the efficiency of in-frame deletions in *V. parahaemolyticus,* the suicide vector pKO2.0 was modified using inverse PCR to remove the gentamicin-resistance cassette and attach the restriction sites AscI and BspHI to the remaining vector using the primers listed in Table 6. The chloramphenicol acetyltransferase (CAT) from pBBR1MCS (Kovach *et al*., 1994) was PCR-amplified and restriction sites AscI and BspHI were added for ligation into the inverse PCR-amplified pKO2.0. The CAT amplicon and pKO2.0 were digested with AscI and BspHI then gel-purified and ligated together using the Fast-Link DNA ligation kit (Epicentre, Madison, WI). The assembled vector was electroporated into *E. coli* EC100 and isolated using the Qiagen mini-prep kit (Qiagen, Valencia, CA) followed by restriction endonuclease digestion with AscI and BspHI to confirm the ligation.

The *mutS* deletion was performed in *V. parahaemolyticus* ATCC 17802, which is an O3:K6 strain (Fujino *et al*., 1965). Sequence analysis of *mutS* and adjacent DNA in 17802 showed this region was identical to that of the completed genome of *V. parahaemolyticus* RIMD2210633 (Makino *et al*., 2003). The 17802 and RIMD sequence data were used to design all primers. Following modification of pKO2.0 we used primers to amplify 894 and 1003 bp flanking regions adjacent to *mutS* in 17802 and attach restriction sites SacI and ApaI. These amplicons were fused in a PCR reaction using the external primers D1 and D4. Fusion products were digested with ApaI and SacI then ligated into

Table 6. Primers used in this study.

Primer	Source	T_m	Expected amplicon (bp)	Sequence (5′–3′)
mutS sequence analysis				
*mutS*71F	This study	54	2781	CGCTACGGAAATAAACAAAAGAACT
*mutS*124F	This study	57	–	AAACGAGCGTCTCAACTGCT
*mutS*169F	This study	54	–	GCCGCTACGGAAATAAACAA
*mutS*492R	This study	53	–	CATCATGGCTTCTTCGGTTT
*mutS*771F	This study	55	–	TGATCGCCAAGATCACTCTG
*mutS*875R	This study	57	–	AGAACTGCGGCGAGTGTATT
*mutS*1259F	This study	55	1451	ATGGTGGTGTGATTGCAGAA
*mutS*1478R	This study	54	1507	TTCTGCAATCACACCACCAT
*mutS*1629F	This study	56	–	CCTAGCTTCTGCCGTTTCAC
*mutS*2440R	This study	58	–	TGCTACATCCACAGTGCTTGG
*mutS*2710R	This study	56	2781	TGGATAACCATAGCCCTTTTCTGT
Intergenic region screen				
mutS-rpoS F	This study	54	2100	AAGCAAATCATACGGCTTGG
mutS-rpoS R	This study	53	2100	CCATTCGCTTGCCTATTCAT
Modification of pKO2.0				
pKO2.0IPCRAscI	This study	71	4400	GACT GGCGCGCC TTCGTTCAAGCCGAGATCGGCTTC
pKO2.0IPCRBspHI	This study	65	4400	GACT TCATGA AACATGAAACATCGACCCACGGCG
chlAscI	This study	70	1195	GACTGGCGCGCCAGCCGGAAGCATAAAGTGTAAAGC
chlBspHI	This study	61	1195	GACTTCATGATTTGGCGAAAATGAGACGTTGATC
Constructing *mutS* strain				
*mutS*D1ApaI	This study	66	914	GACTGGGCCCTATCACCAAGGCGCATGATA
*mutS*D2fusion	This study	68	914	CGATACAGCTCTTCAAGGGCCTGCATCATCGGAGTGTGTTT
*mutS*D3fusion	This study	68	1024	AAACACACTCCGATGATGCAGGCCCTTGAAGAGCTGTATCG
*mutS*D4SacI	This study	65	1024	GACTGAGCTCAAGAGTTTGACGCGAGCAGT
Confirming *mutS* deletion				
*mutS*D0	This study	56	4200	GTGCAGCAGCGATAAGCTC
*mutS*D5	This study	56	4200	GACAATGCGTCAGAGACGAC
Complementation				
*mutS*C1ApaI	This study	70	2900	GACTGGGCCCGTCCCGCAAGTAACACACCT
*mutS*C2XhoI	This study	64	2900	GACTCTCGAGGAAGTGGGACGAGAGATTGG
Phylogenetic markers				
*recA*33F	Criminger *et al.* (2007)	53	975	TGCGCTAGGTCAAATTGAAA
*recA*622R	This study	55	–	TCGTTTCAGGGTTACCGAAC
*recA*1008R	This study	58	975	AGCAGGTGCTTCTGGTTGAG
*rpoA*58F	This study	57	900	AGCTCGACTCACGCAAAAGT
*rpoA*958R	This study	57	900	CTAGGCGCATACCCAGAGAC
Sequence analysis of *rpoB*				
*rpoB*458F	This study	60	1647	AGGCGTGTTCTTCGACAGCGATAA
*rpoB*1110F	Tarr *et al.* (2007)	50	963	GTAGAAATCTACCGCATGATG
*rpoB*2105R	This study	59	1647	CGGCTACGTTACGTTCGATACCAG

pKO2.0 as described above. The ligation was then transformed into *E. coli* EC100 (Table 6) and confirmed by restriction endonuclease digestion. The assembled suicide vector pKO2.0mutS was transformed into *E. coli* β2155 (Dehio and Meyer, 1997) (Table 6) and plated on LB supplemented with 20 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} diaminopimelic acid. pKO2.0mutS was transferred from β2155 to 17802 by biparental mating. Colonies that were resistant to chloramphenicol and grew without diaminopimelic acid were screened for a single recombination event using primers D0 and D4 and primers D1 and D5 (Table 6). A confirmed single-cross was grown overnight in LB with 0.25% NaCl and plated on 10% sucrose 0.25% NaCl LB plates. *Vibrio parahaemolyticus* as well as other *Vibrios* are unable to grow without NaCl, and including NaCl in the sucrose plates reduces the efficiency of using *sacB* as counterselection. As previously reported for *V. anguillarum,* the *V. parahaemolyticus* colonies containing the vector initially grew then exhibited delayed sucrose sensitivity (Milton *et al.*, 1996). Strains containing the suicide vector were plated on sucrose and incubated for 36–48 h. Colonies were then screened for wild-type morphology as an indication that the suicide vector had been lost as previously described (Milton *et al.*, 1996). Colonies that exhibited a wild-type morphology and were sensitive to chloramphenicol were examined for deletion of *mutS* using primers D0 and D5 (Table 6). This PCR generated an approximately 1.9 kb amplicon compared with the 4.2 kb wild-type amplicon. The amplicon from the mutant was sequenced to confirm that the deletion was in-frame. The deletion of *mutS* was also verified by an inability to PCR-amplify using the internal primers 1259F and 2440R (Table 6).

Complementation with wild-type mutS

The Δ*mutS* strain was confirmed to have a *mutS* deletion by complementation with wild-type *mutS* PCR amplified from 17802 with primers *mutS*C1 and *mutS*C2 (Table 6). The gel-purified product was restriction digested with XhoI and ApaI and ligated into XhoI and ApaI-digested and purified pBBR1MCS (Table 6). The ligation was transformed into *E. coli* EC100. The DNA was recovered and transformed into *E. coli* β2155 then transferred to each of the mutator and wild-type strains by biparental mating as described above. Five chloramphenicol-resistant colonies for each strain were assayed for changes in spontaneous mutation to Rif as described above. J-C2-34 was also complemented with *mutH* and *mutL* PCR-amplified using primers *mutH*C1 and *mutH*C2 and primers *mutL*C1 and *mutL*C2 (Table 6).

Analysis of rpoB *from* Rif^r *colonies*

Sequence analysis of *rpoB* from Rif^r colonies was performed by PCR amplification of partial *rpoB* regions from colonies that had grown overnight on Rif. The DNA used for PCR-amplification was isolated directly from Rif^r colonies and amplified using conditions described above with primers *rpoB*458F and *rpoB*2105R listed in Table 6. A total of 10 colonies were examined for each of the strains 17802, Δ*mutS*, J-C1-5 and J-C2-29. Sequences were aligned as described above and the types of mutations were determined by visual inspection.

Percentage of TR colonies

The frequency of phase variation was examined by plating strains on HI agar (Difco), which was previously used to distinguish between OP and TR colony phenotypes (McCarter, 1998) to monitor opacity. Strains were grown overnight in HI broth at 30°C with shaking and plated on HI agar. The percentage of TR cfu ml^{-1} was determined by dividing the TR cfu ml^{-1} by the combined total of TR and OP cfu ml^{-1} on HI agar. Values represent the averages of five to seven independent replicates for each strain.

Accession numbers

All *mutS*, Rif *rpoB*, *rpoA*, *recA* and 16S rDNA sequences generated in this study are deposited in GenBank under the accession numbers EU652246–EU652314. The *rpoB* sequences obtained from colonies not under Rif selection are deposited under the accession numbers EU909181–EU909188. The 16S rDNA, *recA* and *rpoA* sequences of 22702 were previously deposited in GenBank under the accession numbers EF203421, EU018456 and EU018455 respectively (Criminger *et al*., 2007).

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Neighbour-joining tree of the *mutS* nucleotide sequences (2490 bp) from *V. parahaemolyticus* clinical and environmental strains compared with sequences available in GenBank for other *Vibrios*. The tree was constructed with the Kimura 2-parameter model and values represent 1000 bootstrap replications. The scale bar represents 0.05 nucleotide substitutions per site. Mutator strains are indicated in bold.
Table S1. Bacterial strains used in this study.
Table S2. Percentage of translucent (TR) colonies of *V. parahaemolyticus* clinical and environmental strains.

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