# High incidence of multiple antibiotic resistant cells in cultures of in enterohemorrhagic *Escherichia coli* O157:H7

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## Abstract

The spontaneous incidence of chloramphenicol (Cam) resistant mutant bacteria is at least ten-fold higher in cultures of enterohemorrhagic *E. coli* O157:H7 strain EDL933 than in *E. coli* K-12. It is at least 100-fold higher in the *dam* (DNA adenine methyltransferase) derivative of EDL933, compared to the *dam* strain of *E. coli* K-12, thereby preventing the use of Cam resistance as a marker in gene replacement technology. Genome sequencing of Cam-resistant isolates of EDL933 and its *dam* derivatives showed that the *marR* (multiple antibiotic resistance) gene was mutated in every case but not in the Cam-sensitive parental strains. As expected from mutation in the *marR* gene, the Cam-resistant bacteria were also found to be resistant to tetracycline and nalidixic acid. The *marR* gene in strain EDL933 is annotated as a shorter open reading frame than that in *E. coli* K-12 but the longer *marR*$^+$ open reading frame was more efficient at complementing the *marR* antibiotic-resistance phenotype of strain EDL933. Beta-lactamase-tolerant derivatives were present at frequencies 10–100 times greater in cultures of *marR* derivatives of strain EDL933 than the parent strain. Spontaneous mutation frequency to rifampicin, spectinomycin and streptomycin resistance was the same in *E. coli* O157:H7 and *E. coli* K-12 strains.

### Keywords

*Escherichia coli* O157; multiple antibiotic resistance; mutation; genes; repressor protein; DNA

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**Conflict of interest**

The authors have no conflicts of interests to declare.

## 1. Introduction

Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 are frequently the cause of human food-borne illness in the U.S., Europe, and Japan (for review, see [1–3]). Ingestion of only 100–200 organisms can produce debilitating diarrheal disease. Potentially fatal hemolytic uremic syndrome occurs in approximately 2–7% of cases and is due to the production of Shiga toxin. In addition to their effects on human health, these infections also have an economic consequence in lost sick days and medical costs. Furthermore, when found in human food such as tainted meat or bagged vegetables, EHEC contamination leads to large-scale recalls with the potential of bankrupting established commercial enterprises.

In addition to the four normal bases, the DNA of *E. coli* O157:H7 and *E. coli* K-12 also contains N6-methyladenine. This modified base is produced by the postreplication action of a DNA methyltransferase, encoded by the *dam* (DNA adenine methyltransferase) gene, at GATC sequences in double-stranded DNA. Studies in *E. coli* K-12 have shown that N6-methyladenine is involved in (a) controlling the frequency of initiation of chromosome replication at *oriC* (b) regulation of transcription at promoters containing GATC sequences and (c) strand discrimination during post-replicative mismatch repair [4–6]. The misdirection of mismatch repair in *dam* mutant bacteria leads to a mutator phenotype; that is, mutations are introduced into the parental DNA strand due to lack of direction normally imparted by methylation. Dam methylation has also been shown to affect the levels of virulence gene products in several pathogenic bacterial species [7].

Resistance to antibiotics can occur by mutation using a variety of mechanisms [8–11]. Among these is the acquisition of multiple antibiotic resistance conferred by mutation of the *marRAB* (multiple antibiotic resistance) genes [10, 12, 13]. The *marR* gene encodes a repressor while *marB* produces a probable periplasmic protein of unknown function [13–15]. The *marA* product is a transcription factor that activates or represses at least 60 chromosomal genes [16–19]. The inactivation of MarR results in an increased level of MarA, which upregulates the expression of *acrAB* and *tolC* resulting in increased number of AcrAB-TolC efflux pumps thereby leading to drug-resistance [20, 21]. The increased level of MarA also upregulates the *micF* gene, which produces a small regulatory antisense RNA that ultimately reduces expression of the *ompF* gene, thereby increasing permeability to antibiotics [22]. As a result, loss of a functional *marR* gene leads to broad spectrum antibiotic resistance in a number of bacteria including *Salmonella, Shigella, Klebsiella* and *Yersinia* species [23]. Here, we report that the higher than expected presence of chloramphenicol-resistant (Cam[R]) variants in cultures of *E. coli* O157:H7 is due to either an increased mutation rate in the *marR* gene or to a normal mutation rate plus selection due to a high level of MarA. Antibiotic resistance not associated with *marR*, does not occur at high frequency.

## 2. Materials and methods

### 2.1 Bacterial strains and plasmids

*E. coli* O157:H7 strain EDL933 and KM69 have been described [24, 25]. It was previously demonstrated that deletion of the *dam* gene in EDL933 (KM69) resulted in loss of the 933W

prophage [25]. The *dam* gene was restored to the chromosome of this construct, generating strain KM80, as follows: The *dam* gene of EHEC was cloned by gap repair following transformation of pKM210 into cells expressing the phage lambda Red recombineering functions. Plasmid pKM210 has been described before [24] and contains a NotI site flanked by upstream (1.3 kb) and downstream (1.2 kb) regions of the EHEC *dam* gene; the cassette is flanked by SacI and SphI sites. A NotI digest of pKM210 was electroporated into EDL933 containing pKM200, a λ Red-producing Cam[R] plasmid [26]. After electroporation, the cells were grown for 1.5 hours in Luria broth and dilutions of the transformants were plated in Luria broth plates containing ampicillin (100 μg/ml). Gap repair of pKM210 resulted in levitation of the *dam* gene from the EDL933 chromosome at high efficiency, as most of the colonies tested positive for the EHEC *dam* gene. The resulting construct (pKM263) was digested with SacI and SphI, to liberate the recombineering fragment, and electroporated into KM68 ( *dam::cat-sacB*) containing pKM208, a λ Red-producing Amp[R] plasmid [26]. Following electroporation, the cells were diluted into 5 ml L and allowed to grow overnight at 37°C. Dilutions of the culture were plated on L plates containing 10% sucrose and colonies were allowed to grow overnight. The next day, colonies were re-streaked on Cam plates. A total of nine out of 63 Suc[R] colonies were found to be Cam[S], suggesting loss of the *cat-sacB* cassette ; 4/4 of these candidates tested positive by PCR for the restoration of the *dam* gene to the EHEC chromosome. All four strains were also Amp[S], suggesting they did not contain an intact pKM263, and had spontaneously lost pKM208. These last points were verified by the absence of both pKM263 and pKM208 in a minilysate preparation of KM80. The *dam*[+] phenotype of KM80 was verified by the restoration of wild type levels of spontaneous mutation resistance to rifampicin (data not shown).

Cam-resistant derivatives of KM80 and KM69 were isolated as spontaneous colonies arising on L broth plates with 10 μg/ml Cam after plating aliquots of overnight cultures. Individual colonies were purified by streaking three more times on the same medium.

*E. coli* K-12 strains AG100 (wildtype) and AG102 (*marR1*) [27, 28] were kindly provided by Dr. J.L Rosner (NIDDK, NIH, Bethesda, MD USA).

Plasmids pMQ583 and pMQ584, containing the short (378 bp) and long (435 bp) ORFs of *marR* respectively, were constructed by PCR amplification from EDL933 and cloning into the EcoRI and HindIII sites of pBAD18 [29]. The plasmids were introduced into the *marR* mutant bacteria by electroporation [26] and transformants were grown in L broth with carbenicillin and spotted onto L broth-carbenicillin plates containing Cam (10 μg/ml) or tetracycline (10 μg/ml) or nalidixic acid (25 μg/ml). The plates were incubated at 37°C overnight.

Ampicillin-resistant plasmids, pMQ315 (*mutS*), pMQ350 (*mutL*), pRH71-17 (*mutH*) have been described previously [30–32] and were introduced into KM69 by electroporation. Ten transformants of each plasmid were grown to saturation in L broth with carbenicillin and 10 ul spotted onto L broth plates containing 25 μg nalidixic acid and 50 μg carbenicillin per ml. The plates were incubated at 37°C overnight.

*E. coli* K-12 strain MM294 [33] was used as a recipient in an attempt to clone Cam-resistance genes from EDL933.

## 2.2 Media

Bacterial strains were cultivated in either L broth (10 g/l tryptone, 5 g/l yeast extract, 0.5 g/l NaCl) or Difo Brain Heart Infusion (20 g/l) and solidified, when required, with 16 g Difco Agar per l. Media were supplemented with ampicillin, carbenicillin, tetracycline, Cam, nalidixic acid and rifampicin at 100, 50, 10, 10, 25 and 100 μg/ml respectively.

## 2.3 Deep sequencing of bacterial strains

DNA was isolated from seven strains of *E. coli* by lysis in 1% SDS and 1mg/ml Proteinase K overnight at 55°C followed by 25:24:1 phenol:chloroform:isoamyl alcohol extraction and ethanol precipitation. Ten μg of purified DNA was sheared with a Covaris M220 ultrasonicator to a median size range of 250 bp. Deep sequencing libraries were prepared as per standard Illumina protocols (http://www.illumina.com). Single read 36 bp sequences were generated for strains GM7275, GM9236, and GM9237 on Illumina GAII sequencer and 100 bp sequences were generated for strains GM7274, GM9241, GM9242, and GM9243 on Illumina HiSeq 2000 sequencer with a mean coverage of 42 times. Perfectly matching reads were mapped to to *E. coli* O157:H7 EDL933 genome (Genebank AE005174.2) using the Bowtie software package [34]. Locations of single nucleotide polymorphisms and indels were determined using Seqmonk (www.bioinformatics.babraham.ac.uk) by identifying regions which contain no mapping sequences.

## 2.4 DNA fragment synthesis and sequencing

The *marR* gene was synthesized using the polymerase chain reaction (PCR) with Herculase II DNA polymerase (Agilent Technologies) and primers GCTATGAATGGTAATAGCGTCAG and GACTTATACTTGCCTGGGCA as described by the manufacturer except that 2 μl of cells from an overnight culture were included. The DNA fragment was gel-purified using Qiagen columns and reagents and sequenced by GeneWiz Corporation.

## 2.5 Minimal Inhibitory Concentrations (MICs) and Fitness

The MIC of a strain was determined by adding about 10,000 cells to 1 ml of L broth containing two-fold dilutions of Cam and incubating the tubes overnight at 37°C. To measure fitness, about 1000 wildtype and *marR* cells were introduced together into 1 ml of L broth and after overnight incubation dilutions were spread on L broth plates, with and without 10 μg/ml Cam, to assay for viability.

# 3. Results

## 3.1 Strain construction

We reported previously that *dam* mutants from *E. coli* O157:H7 strain EDL933 were generated at a very low frequency using bacteriophage lambda Red recombination of DNA

fragments into the bacterial chromosome ("recombineering") [25]. The few *dam* mutant strains that were isolated from EDL933 also were deleted for the 933W prophage. In order to use an isogenic wildtype control strain in the experiments described here, KM69 ( *dam* 933W) was converted to *dam*+ as follows to yield strain KM80 ( 933W). The *dam*+ gene from EDL933 was levitated into a suitable vector, cut with restriction enzymes and electroporated into *dam::cat-sacB* mutant bacteria expressing bacteriophage lambda Red recombination enzymes. Recombinant cells, in which the *dam*+ gene has replaced the *dam::cat-sacB* allele, should be resistant to sucrose since the *cat-sacB* fusion results in sucrose-sensitivity. Strain KM80 ( 933W) was isolated as such a sucrose-resistant clone and confirmed to have an intact *dam* gene.

### 3.2 Mutation frequency to chloramphenicol resistance

The spontaneous mutation frequencies of KM69 (*dam*) to various drug resistances are shown in Table 1. The result for Cam was surprising because the high level of spontaneous resistance was not encountered with *E. coli* K-12 *dam* strains. Furthermore, the frequency of spontaneous Cam^R mutant bacteria from the isogenic wildtype strain, KM80 ( 933W), was also higher than expected compared to *E. coli* K-12 (Table 1). This spontaneous frequency to Cam^R during recombineering of a 933W derivative of EDL933 was observed previously (Murphy and Campellone, unpublished observations). Re-streaking on fresh Cam plates identified recombinants in which the Cam^R gene was recombined into the chromosome, as the spontaneous Cam^R mutants (not recombined) grew poorly upon re-streaking. In this study, authentic recombinant colonies of KM80 could also be found by re-streaking (data not shown). However, with KM69 (*dam*), the number of spontaneous Cam^R colonies far outnumbered true recombinants, so that additional streaking of the colonies (or changing the Cam concentration) could not reliably distinguish between spontaneous versus recombinant Cam^R colonies following recombineering. For further study, and characterization of the source of this increased frequency to Cam^R, five independently-isolated Cam^R strains were obtained from each of KM69 (designated GM9236-9240) and KM80 (GM9241-9245).

### 3.3 Attempted cloning of chloramphenicol resistance

In order to identify the mechanism of Cam-resistance in EDL933, DNA was isolated from GM9236, digested with either EcoRI or HindIII and ligated to pBAD18 vector DNA. The ligation mixtures yielded many ampicillin-resistant transformants of strain MM294, but no colonies that were both Cam^R and ampicillin-resistant were obtained. The failure to clone the drug-resistance was due to the responsible gene being recessive to its wildtype allele in the cloning host (see Discussion).

### 3.4 Whole genome sequencing

The failure to clone Cam^R from GM9236 provoked us to use whole genome sequencing of representative Cam-sensitive (KM69, KM80) and resistant (GM9236, GM9237, GM9241, GM9242 and GM9243) bacteria. Chromosomal DNA libraries were constructed from each strain and subjected to Illumina sequencing technology. The DNA sequences were aligned to the EDL933 genome. Every Cam^R mutant bacterial clone had a mutation in the *marR* gene while the Cam^S parental strains did not.

### 3.5 Sequencing of the *marR* gene

Identification of a mutated *marR* gene as the cause for Cam[R] in the whole genome sequenced strains, prompted us to sequence this gene in all Cam[R] mutant and parental bacteria using PCR cloning and sequencing. The results are shown in Table 2. As expected, the *marR* mutation identification by sequencing of PCR products from these strains confirmed those obtained by whole genome sequencing.

The *marR* mutations identified from Cam[R] bacteria derived from the wildtype strain (KM80) comprised an IS (insertion sequence) element insertion, two deletions, a transition and a transversion (Table 2). In contrast, with one exception, the mutations in *marR* from the *dam* mutant derivative (KM69) occur in runs of iterated bases and are due to transition mutations (AT to GC and GC to AT). These mutation spectra in wildtype and *dam* mutant strains are precisely those previously described using other mutational targets [35, 36].

The N-terminal 41 amino acids of the MarR protein have been proposed to be responsible for dimerization and amino acids 31–132 as a DNA binding winged helix-turn-helix (HTH) domain [37]. Mutant L33P should affect the oligomeric state while all other mutations listed in Table 2 should prevent DNA binding.

### 3.6 Multiple antibiotic resistance

The identification of *marR* as the basis for Cam-resistance in EDL933 wildtype and *dam* mutant strains indicated that there should also be resistance to other antibiotics. This indeed is the case and all Cam[R] bacteria were also resistant to tetracycline (10 μg/ml) and the quinolone antibiotic, nalidixic acid (25 μg/ml) (Fig. 1).

### 3.7 Complementation of *marR*

The *marR* gene in *E. coli* K-12 is annotated to encode an additional 19 N-terminal amino acids compared with *marR* in EDL933. We constructed plasmids pMQ583 and pMQ584 containing the shorter (378 bp) and longer (435 bp) predicted open reading frames respectively of the EDL933 *marR* sequence under the control of the arabinose BAD promoter. Strain GM9242 (Mar⁻) was transformed with each *marR* plasmid and the vector control and tested for resistance/sensitivity to Cam, tetracycline and nalidixic acid. Expression of *marR*⁺ in GM9242, in the presence of arabinose, reversed the antibiotic resistance to sensitivity for all three antibiotics (Fig. 1). For Cam and tetracycline, the reduction of viability was greater than $10^5$-fold but less for nalidixic acid. Even in the absence of arabinose (and the presence of glucose), there was antibiotic sensitivity indicating that the level of basal transcription in the absence of inducer was sufficient to allow some MarR production and reversal of phenotype. All the other EDL933 Cam[R] strains were transformed with the longer *marR* open reading frame plasmid and shown to become antibiotic sensitive (data not shown).

The plasmid containing the longer *marR* open reading frame was more efficient at reversing the antibiotic resistance phenotype than the shorter version. We suggest that the annotation of *marR* in EDL933 be changed in view of these results.

### 3.8 High frequency carbenicillin resistance in *marR* mutant bacteria

During the *marR* complementation experiments described above, it was noticed that there were a substantial number of carbenicillin-(or ampicillin) tolerant colonies on the control plates during transformation of the *marR* mutant strains. This feature was not observed with the wildtype strains (KM69, KM80) transformed with the same plasmid (data not shown). Further experiments showed that, indeed, the *marR* cultures had a high frequency of carbenicillin-tolerant variants relative to the wildtype parent (Table 1).

The carbenicillin-tolerant colonies were extremely heterogeneous in colony size and properties. At one end of the spectrum, colonies from the primary plate grew well when re-streaked on agar with carbenicillin and retained carbenicillin-resistance even after growth without the drug. At the other end of the spectrum were colonies that failed to grow when re-streaked on carbenicillin medium. Between these two extremes were colonies that retained carbenicillin-tolerance for a random number of passages on carbenicillin medium. Other colonies lost resistance to 50 μg carbenicillin per ml but were able to grow in lower concentrations of the drug (data not shown). It was also found that carbenicillin-tolerant colonies can be isolated from an *E. coli* K-12 *marR* strain but the frequency is at least 100-fold less than for *E. coli* O157:H7 *marR* bacteria (data not shown). The properties described above are remarkably similar to those described by George and Levy [27, 28] for *marR*-mediated drug-resistance in *E. coli* K-12 suggesting a similar mechanistic basis (i.e., increased MarA level).

The carbenicillin-tolerant phenotype of *E. coli* O157:H7 *marR* bacteria was completely suppressed by expression of MarR from the pMQ584 plasmid (data not shown). Beta-lactamase resistance in *E. coli* is often caused by increased *ampC* promoter activity [38] but no such alterations were found in several of the carbenicillin-tolerant *marR* mutant strains (data not shown).

An increased MarA level is a consequence of loss of MarR repressor activity. We conclude that in a sub-set of the cells in the population, MarA promotes carbenicillin-tolerance.

### 3.9 Expression of multicopy mismatch repair genes in KM69 (  *dam*)

*E. coli* K-12 *dam* mutants contain single- and double-strand breaks in their genomes that are produced by misdirected mismatch repair in which mutations are introduced into the parental strand [4, 7]. Jacquelin et al [39] have shown that multicopy mismatch repair genes (*mutH, mutL,* or *mutS*) in *E. coli* K-12 *dam* bacteria reduce the mutation frequency relative to the control. We repeated this experiment in KM69 and the results shown in Fig. 2 indicate that the presence of multicopy *mutS* and *mutL*, but not *mutH*, dramatically reduced the formation of nalidixic acidresistant colonies We conclude that mismatch repair is responsible for the increased multiple antibiotic resistance frequency in KM69 (  *dam*) by introducing the "correction" into the parental DNA strand of a *dam* mutant strain.

### 3.10 Mutant frequency to streptomycin, spectinomycin and rifampicin resistance

In order to test if the increase in spontaneous *marR* mutability is a general feature of *E. coli* O157:H7, we compared the frequencies of streptomycin, spectinomycin and rifampicin

resistance in *E. coli* K-12 and KM80 strains. The streptomycin and spectinomycin mutant frequencies were the same in both strains – $1.2 \times 10^{-9}$ and for rifampicin resistance, $1.4 \times 10^{-8}$ for both.

### 3.11 Minimal Inhibitory Concentration (MIC) and fitness

The MIC for Cam$^R$ of KM80 and AG100, the wildtype EHEC and K-12 strains respectively, in our L broth is 0.6 – 1.2 μg/ml and the MIC of the *marR* mutant strains GM4292 and AG102 is 20 – 25 μg/ml. The doubling time for both EHEC and K-12 wildtype and *marR* strains was the same (about 27 min).

To study fitness, we inoculated about 1000 wildtype and *marR* cells together into L broth and grew the cells until the culture was saturated. Dilutions were spread on L broth plates with and without 10 μg/ml Cam. For both EHEC and K-12, the number of colonies on the Cam plates was about one-third that of the control plates. We conclude that the fitness for both EHEC and K-12 *marR* strains is the same.

## 4. Discussion

There is clearly something different about MarR in EHEC versus *E. coli* K-12. This difference could be at the gene or protein level. The EHEC MarR protein has two amino acid changes compared with that in K-12 which might alter the affinity of the protein for its recognition sequence. Alternatively, the absolute level of the MarR protein may be different in the two strains leading to the changes we have found. In addition, it may be that when MarR is inactive, the MarA levels are different in the two strains leading to an alternate activation/repression program of chromosomal genes. This alternate program might permit cells to survive antibiotic challenge more forcefully in EHEC than K-12. At present, we do not know which of these alternatives, if any, is the correct one.

At the gene level, the increased numbers of *marR* mutant bacteria in cultures of *E. coli* O157:H7 versus *E. coli* K-12 could be due to an increase in mutation frequency or to a normal mutation frequency plus a selective advantage during growth of the culture or to both of these possibilities. The mutant frequencies to streptomycin, spectinomycin and rifampicin resistance are similar in both EHEC and K-12 strains suggesting that the increase in the number of *marR* variants in EHEC may be due to a selective advantage during growth. However, we found that differences in doubling time or fitness could not explain the increase. That the MICs of the wildtype and *marR* strains was the same for both EHEC and K-12 indicates that there is no difference in intrinsic resistance when selection was at 10 μg/ml Cam. In wildtype KM80, the increased *marR* mutant frequency is unlikely to reflect an increase in a specific mutational pathway or a specific DNA repair system (including the SOS response) because the increased spontaneous mutant frequency is non-specific in the types of mutations recovered. The *marR* genes are located in the same chromosomal surroundings (synteny) in the two strains thereby excluding the possibility of alternate chromosomal location as an explanation.

An alternative explanation that the EHEC *marR* locus is more mutable than *rpsL, rpsE* and *rpoB*, is more problematical because it is expected that genes should be equally mutable

irrespective of their location. It has been reported that mutability is correlated with the level of gene expression [40, 41]. This explanation is rendered unlikely for our results because the *rpsL* and *rpsE* genes are more highly expressed than *marR* in *E. coli* O157:H7 [24] but the mutant frequency for the ribosomal genes is at the same level in both EHEC and K-12. A recent study found that the frequency of spontaneous mutation in *E. coli* K-12 was more closely correlated with chromosome supercoiling than gene expression [42]. The K-12 chromosome is organized into about 400 domains with dynamic boundaries and this number correlates with the average length of supercoiled loops (see [43] for review). In both K-12 and EHEC, the *marR, rpoB* and the two ribosomal genes would be in separate supercoiled loops. If, however, the extent of supercoiling in the *marR* loop is different in EHEC and K-12, then a difference in spontaneous mutagenesis might result. It should be noted that the MarR mutability phenotype is too variable to allow Luria-Delbruck fluctuation analysis.

The *marR* mutants arise during growth in L broth before exposure to Cam. It is possible that stress responses, such as those mediated by LexA (SOS) [44] or RpoE [45] or RpoS [46], in EHEC are of a greater magnitude in either duration and/or strength than those in K-12. If so, there would be a greater survival of potential Cam$^R$ cells and an apparent increase in mutation frequency.

At present, we cannot distinguish between the possibilities of an increase in mutation frequency or to a normal mutation frequency plus a selective advantage or to both. Whatever the mechanism, the high background of *marR* cells is a hindrance for recombineering experiments in EHEC strains. Our current working hypothesis is that mutation in the *marR* gene activates MarA but that the cascade of genes activated or repressed is different in EHEC and K-12, which may lead to a selective advantage to the former upon exposure to antibiotics. If the mutation rate of *marR* is indeed higher due to supercoiling effects it would amplify this process.

Although the sample size of the *marR* sequenced strains is small, it is interesting that no mutation was recovered twice. This result suggests that there is no mutational "hotspot" in the EDL933 *marR* gene and that the gene can be inactivated at a large number of sites, at least between amino acid residues 33–108.

The initial attempt to identify the cause of increased mutagenesis in strain EDL933 by random cloning of DNA fragments encoding Cam resistance was not successful. In retrospect, this is consistent with the mutagenic target being the MarR repressor. Since the plasmid-encoded *marR*$^−$ allele is recessive to host-encoded *marR*$^+$, drug-resistant colonies cannot be formed.

The crystal structure of *E. coli* K-12 MarR protein (144 amino acids) has been solved at 2.3 A resolution using the 435 bp long open reading frame [37]. The EDL933 *marR* sequence is 98% identical but is annotated as a shorter 378 bp reading frame missing the N-terminal 19 amino acids. This truncation removes part of the first alpha helix of the protein and, as we show in Fig. 1, is not as effective in complementing the *marR* defect in GM9242 as is the longer reading frame. Clearly, the longer open reading frame is the correct sequence for *E.*

*coli* O157:H7 *marR* gene based on our results and those for *E. coli* K-12 and the structural data of the protein.

The *E. coli* K-12 and annotated EHEC *marR* gene sequences are shown in Fig. 3. The EHEC sequence has the 57 bp 5' truncation and seven other differences. Five of these are conserved changes that do not alter the encoded amino acid. Two sequence differences result in changes Gly103Ser and Tyr137His but these still allow the MarR protein to be fully functional (Fig. 1). We have not yet tested if these two amino acid changes in the *marR* gene affect spontaneous mutation frequency to Cam-resistance. However, none of the mutations in the *marR* gene identified in Table 2 map to the codons for these two amino acids.

We assume that a computer program searching for an open reading frame beginning with an ATG and ending with a nonsense codon generated the annotation for the EHEC *marR* gene. We have found no experimental evidence in the literature to support this annotation. The computer-derived annotation would explain why the structural and genetic data in K-12 were not taken into account. The actual start codon for *marR* gene is a GTG.

Over-expression of MutS and MutL in KM69 reduced the frequency of nalidixic acid-resistant mutant colonies. A similar observation (but also including MutH) has been made in an *E. coli* K-12 *dam* mutant strain. Jacquelin et al [39] argued on the basis of their experimental results, that the reduction in mutant frequency in *E. coli* K-12 was due to mismatch repair-dependent cell killing. That is, increasing the level of mismatch repair proteins would, in turn, increase the number of DNA double-strand breaks thereby resulting in an increased number of fatalities in the bacterial population and eliminating those cells from the population. This interpretation is consistent with the results obtained in Fig. 2 for *E. coli* O157:H7.

An unexpected result was the high frequency of carbenicillin-tolerant bacteria in cultures of *marR* strains. Although promoter-up mutations in the *ampC* promoter are frequently the cause of such resistance, this is not the case in beta-lactam-tolerant *marR* mutants (data not shown). The suppression of beta-lactam resistance by episomal *marR*+ argues that the simplest explanation for their existence is that the high level of MarA promotes changes in the cell envelope that, in a presumably stochastic manner, can render tolerance to beta-lactam antibiotics. In *Enterobacter aerogenes*, for example, a high level of MarR promotes resistance to beta-lactams by overexpression of OmpX [47, 48]. A similar rationale is probably the basis for the observations of beta-lactam resistance in *E. coli* that we have made in this report.

*E. coli* O157:H7 *marR* mutant strains were first described by Yaron et al [49] who isolated a spontaneous *E. coli* O157:H7 Cam^R strain from cattle feces and showed that it had a G to T transversion in *marR* leading to Glu10Ochre change in the protein. In general, the mutant strain had the same growth characteristics in a variety of media and cultural conditions as the wildtype but was resistant to nalidixic acid, tetracycline and ciprofloxacin in addition to Cam. DNA sequencing of the *mar* operon indicated that it was 99% identical to that in *E. coli* K-12. The resistance to antibiotics could be complemented in the mutant strain [50]

(although the data were not shown) by an *E. coli* K-12 *marR* plasmid constructed by Alekshun and Levy [51].

The relative increase in *marR* variants in cultures of *E. coli* O157:H7 and the propensity of such mutant strains to also become carbenicillin-tolerant, indicates how easily such pathogenic bacteria can become resistant to antibiotics. This is in contrast to the laboratory adapted *E. coli* K-12 strain which has a lower incidence of *marR*-mediated antibiotic resistance. In the external environment, it may be desirable for bacteria to rapidly select for antibiotic resistant variants especially in niches where antibiotic-producing organisms are present. In addition, the widespread use of antibiotics in veterinary and medical use, would allow rapid selection of antibiotic-resistant variants.

## Acknowledgements

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- *marR* gene mutations occur at a higher frequency in *E. coli* O157:H7 than *E. coli* K-12

- *rpsL, rpsE* and *rpoB* mutations occur at the same frequency in both *E. coli* strains

- *E. coli* O157:H7 *marR* mutant strains can become tolerant to beta-lactam antibiotics
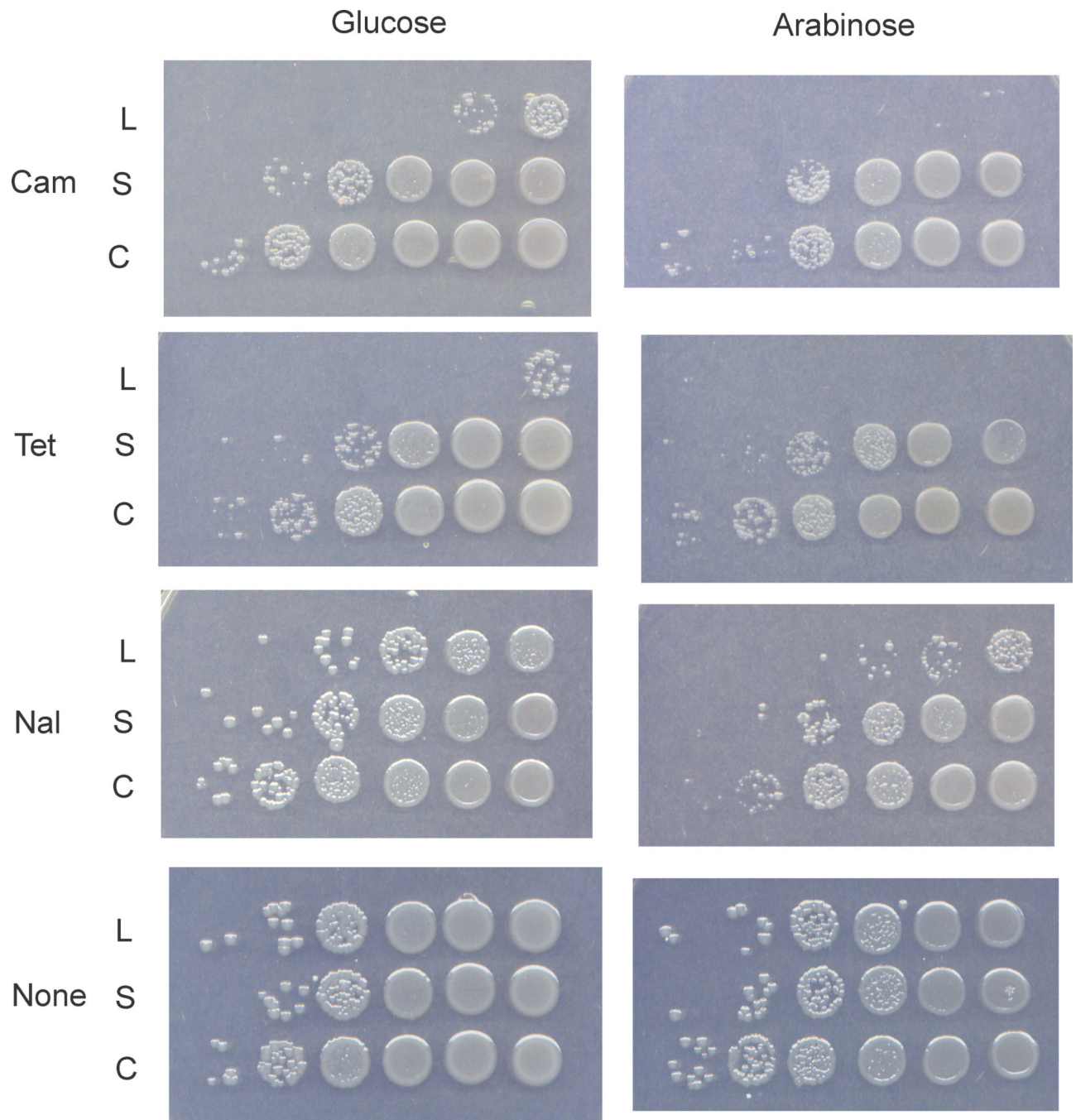
**Fig 1.**

*marR*+ complementation of GM9242 (*marR*). The *marR* gene was cloned into pBAD18 in which expression of the gene is reduced when the cell containing it is grown with glucose and expression is induced when the cell is grown in the presence of arabinose. Two constructs were used: the long *marR* ORF (L) as annotated in *E. coli* K-12 and the short ORF (S) as annotated in *E. coli* O157::H7 EDL933. The control strain (C) contained the vector plasmid. Cells grown overnight were diluted in ten-fold increments and 10 µl drops

placed on appropriate medium. The plates were incubated overnight at 37°C. Cam = chloramphenicol, Tet = Tetracycline, Nal = Nalidixic acid.

**Fig 2.**
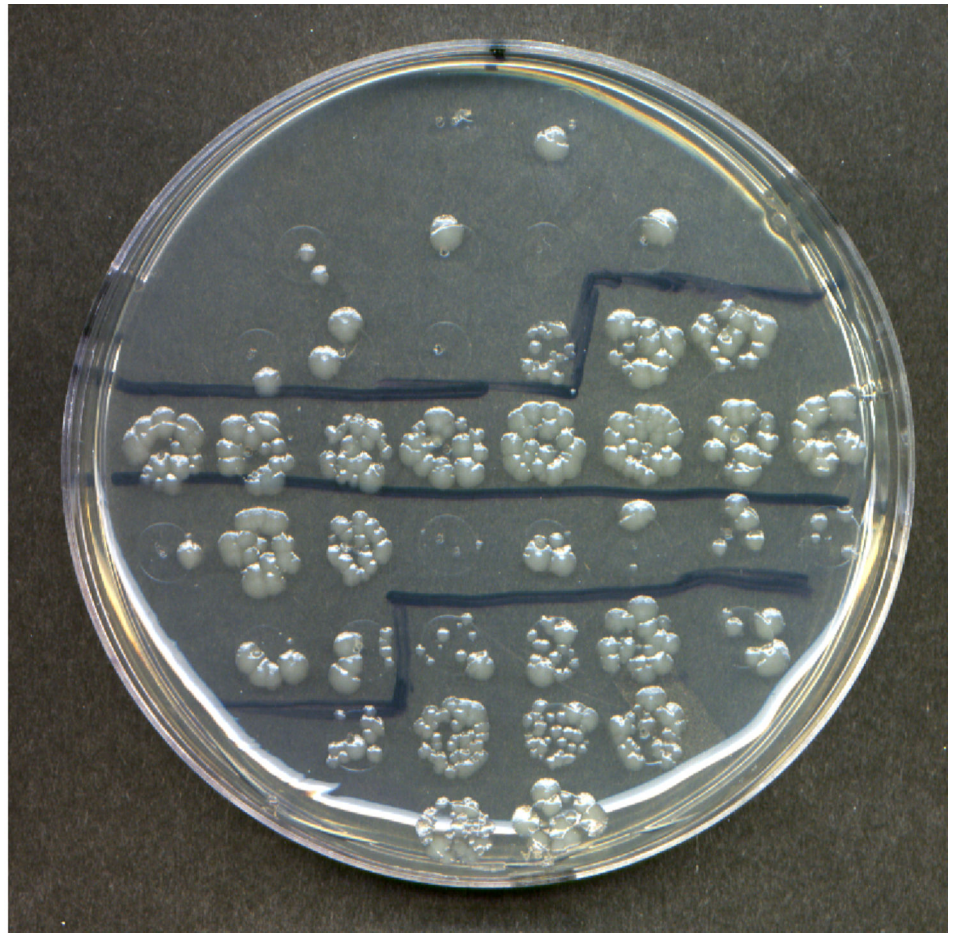Effect of overproduction of mismatch repair proteins on mutant frequency to nalidixic acid in KM69 ( dam). Ten independent cultures of strain KM69 with multicopy plasmids encoding *mutS, mutL, mutH* and the vector were grown to saturation in L broth and 10 ul from each culture was spotted on an L broth plate containing 25 μl nalidixic acid per ml. The plate was incubated overnight at 37°C.

```
K       1   GTGAAAAGTACCAGCGATCTGTTCAATGAAATTATTCCATTGGGTCGCTTAATCCAT      57

E


K      58   ATGGTTAATCAGAAGAAAGATCGCCTGCTTAACGAGTATCTGTCTCCGCTGGATATTACC    117
            |||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
E       1   ATGGTTAATCAGAAGAAAGATCGTCTGCTTAACGAGTATCTGTCTCCGCTGGATATTACC     60


K     118   GCGGCACAGTTTAAGGTGCTCTGCTCTATCCGCTGCGCGGCGTGTATTACTCCGGTTGAA    177
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
E      61   GCGGCACAGTTTAAGGTGCTCTGCTCTATCCGCTGCGCGGCGTGTATTACTCCGGTTGAA    120


K     178   CTGAAAAAGGTATTGTCGGTCGACCTGGGAGCACTGACCCGTATGCTGGATCGCCTGGTC    237
            ||||||||| ||| ||||||||||||||||||||||||||||||||||||||||||||||
E     121   CTGAAAAAGTGTTGTCGGTCGACCTGGGAGCACTGACCCGTATGCTGGATCGCCTGGTC    180


K     238   TGTAAAGGCTGGGTGGAAAGGTTGCCGAACCCGAATGACAAGCGCGGCGTACTGGTAAAA    297
            ||||||||||||| |||||||||||||||||||||||| |||||||||||||||||||||
E     181   TGTAAAGGCTGGGTAGAAAGGTTGCCGAACCCGAATGATAAGCGCGGCGTACTGGTAAAA    240


K     298   CTTACCACCGGCGGCGCGGCAATATGTGAACAATGCCATCAATTAGTTGGCCAGGACCTG    357
            |||||||||| |||||||||||||||||||||||||||||||||||||||||||||||||
E     241   CTTACCACCAGCGGCGCGGCAATATGTGAACAATGCCATCAATTAGTTGGCCAGGACCTG    300


K     358   CACCAAGAATTAACAAAAAACCTGACGGCGGACGAAGTGGCAACACTTGAGTATTTGCTT    417
            ||  |||||||||||||||||||||||||||||||||||||||||||||||| ||||||||
E     301   CATCAAGAATTAACAAAAAACCTGACGGCGGACGAAGTGGCAACACTTGAGCATTTGCTT    360


K     418   AAGAAAGTCCTGCCGTAA    435
            ||||||||||||||||||
E     361   AAGAAAGTCCTGCCGTAA    378
```

**Fig. 3.**

Comparison of the *marR* gene sequence of *E. coli* K-12 (K) and the annotated *E. coli* O157:H7 *marR* sequence (E). The top line is the sequence present in the K-12 *marR* gene but not in the annotated EHEC sequence.

**Table 1**

Frequencies of *E. coli* K-12 and EHEC to antibiotic resistance.

| Strain | Background | Genotype | Cam$^R$ Frequency | Nal$^R$ Mutant Frequency | Tet$^R$ Mutant Frequency | Carb$^R$ Frequency |
|---|---|---|---|---|---|---|
| AB1157 | K-12 | wild | $<10^{-8}$ | $2 \times 10^{-8}$ | $<10^{-8}$ | $<10^{-8}$ |
| GM3819 | K-12 | *dam-16* | $<10^{-8}$ | $51 \times 10^{-8}$ | $<10^{-8}$ | $<10^{-8}$ |
| KM80 | EDL933 | wild | $1 - 10 \times 10^{-8}$ | $3 \times 10^{-8}$ | $2 \times 10^{-8}$ | $1 - 4 \times 10^{-8}$ |
| KM69 | EDL933 | *dam* | $208 - 1500 \times 10^{-8}$ | $152 \times 10^{-8}$ | $105 \times 10^{-8}$ | $2 - 12 \times 10^{-8}$ |
| GM9241 | EDL933 | *marR* | R | R | R | $24 - 558 \times 10^{-8}$ |

Mutant frequency is expressed as the number of antibiotic resistant colonies per $10^8$ cells. The numbers for Nal and Tet are the average of ten independent cultures. For Cam and Carb, the range of values from ten independent cultures are shown. R = resistant; Carb= carbenicillin, Nal = nalidixic acid, Tet = tetracycline

**Table 2**

Type and location of mutations in the *marR* gene.

| Strain | Parental genotype | Mutation in *marR* | Location in chromosome | Amino acid change |
|--------|-------------------|--------------------|------------------------|-------------------|
| GM9236 | *dam* | GGG to GAG | 1,944,735 | G69R |
| GM9237 | *dam* | AT to GC | 1,944,803 | L46P |
| GM9238 | *dam* | TTT to TT | 1,944,811–813 | F43 fs |
| GM9239 | *dam* | GGG to GGA | 1,944,734 | G69E |
| GM9240 | *dam* | CGCC to CACC | 1,944,710 | R77Q |
| GM9241 | wt | IS629 | 1,944,792–3 | |
| GM9242 | wt | Deletion | 1,944,603–618 | C108 |
| GM9243 | wt | AT to CT | 1,944,707 | L78R |
| GM9244 | wt | AT to GC | 1,944,842 | L33P |
| GM9245 | wt | Deletion | 1,944,698–716 | R73P |

The amino acid sequence numbering is that of the annotated E. *coli* K-12 gene. fs = frameshift, wt = wildtype.