



Evaluation of Acquired Antibiotic Resistance in *Escherichia coli* Exposed to Long-Term Low-Shear Modeled Microgravity and Background Antibiotic Exposure

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ABSTRACT The long-term response of microbial communities to the microgravity environment of space is not yet fully understood. Of special interest is the possibility that members of these communities may acquire antibiotic resistance. In this study, *Escherichia coli* cells were grown under low-shear modeled microgravity (LSMMG) conditions for over 1,000 generations (1000G) using chloramphenicol treatment between cycles to prevent contamination. The results were compared with data from an earlier control study done under identical conditions using steam sterilization between cycles rather than chloramphenicol. The sensitivity of the final 1000G-adapted strain to a variety of antibiotics was determined using Vitek analysis. In addition to resistance to chloramphenicol, the adapted strain acquired resistance to cefalotin, cefuroxime, cefuroxime axetil, cefoxitin, and tetracycline. In fact, the resistance to chloramphenicol and cefalotin persisted for over 110 generations despite the removal of both LSMMG conditions and trace antibiotic exposure. Genome sequencing of the adapted strain revealed 22 major changes, including 3 transposon-mediated rearrangements (TMRs). Two TMRs disrupted coding genes (involved in bacterial adhesion), while the third resulted in the deletion of an entire segment (14,314 bp) of the genome, which includes 14 genes involved with motility and chemotaxis. These results are in stark contrast with data from our earlier control study in which cells grown under the identical conditions without antibiotic exposure never acquired antibiotic resistance. Overall, LSMMG does not appear to alter the antibiotic stress resistance seen in microbial ecosystems not exposed to microgravity.

IMPORTANCE Stress factors experienced during space include microgravity, sleep deprivation, radiation, isolation, and microbial contamination, all of which can promote immune suppression (1, 2). Under these conditions, the risk of infection from opportunistic pathogens increases significantly, particularly during long-term missions (3). If infection occurs, it is important that the infectious agent should not be antibiotic resistant. Minimizing the occurrence of antibiotic resistance is, therefore, highly desirable. To facilitate this, it is important to better understand the long-term response of bacteria to the microgravity environment. This study demonstrated that the use of antibiotics as a preventive measure could be counterproductive and would likely result in persistent resistance to that antibiotic. In addition, unintended resistance to other antimicrobials might also occur as well as permanent genome changes that might have other unanticipated and undesirable consequences.

KEYWORDS *Escherichia coli*, antibiotic resistance, microgravity

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The idea of long-term human space flight has gained increasing traction (4–6). The planned durations for missions range from around a month for lunar missions to 1 year on the International Space Station to 30 months on Mars (Design Reference Mission) (7, 8). Efforts to understand possible negative effects of the space environment on human physiology and immune function have been a high priority (3, 6, 9, 10). In particular, spaceflight may render astronauts increasingly prone to bacterial and viral infections (11–15). This, in turn, raises the issue of how the microorganisms themselves respond to the space environment.

Previous reports on the effects of microgravity or spaceflight on physiological properties such as biofilm formation, bacterial motility, acid stress resistance (AST), virulence, and antibiotic resistance (AR) have shown mixed results which vary from one organism to another (16–22). These include studies done on diverse organisms, including the pathogens *Pseudomonas aeruginosa* (23, 24), *Salmonella enterica* serovar Typhimurium (25), *Streptococcus mutans* (26), *Yersinia pestis* (21, 27), the yeast *Candida albicans* (28), *Serratia marcescens* (18), *Enterobacter cloacae* (18), *Enterococcus faecalis* (29), pathogenic *Escherichia coli* (30–32), nonpathogenic *E. coli* (33–38), and microbial isolates (opportunistic pathogens *P. fluorescens*, *Stenotrophomonas maltophilia*, and *Chryseobacterium* spp.) from water systems of the Mir Space Station (39, 40) or from the International Space Station (ISS) (*Enterobacter bugandensis* and staphylococcal and enterococcal strains) (41, 42) or on the space station MIR (31).

Acquisition of antibiotic resistance (AR) and its implications for human health are significant concerns from clinical and evolutionary perspectives (32, 43–47). AR studies performed under simulated microgravity and spaceflight conditions have yielded contrasting results. An *E. coli* strain sent into space onboard the Shenzhou-VIII spacecraft for 17 days showed increased AR (48). Microbial isolates, including staphylococcal and enterococcal strains (such as *Enterobacter bugandensis*) from the International Space Station (ISS), showed AR (41, 42). Spaceflight (33) and LSMMG (34, 49) enhanced antibiotic stress tolerance in *E. coli*. In a manned flight experiment, *Staphylococcus aureus* and *E. coli* exhibited enhanced antimicrobial resistance relative to ground controls (50). A study on *Staphylococcus epidermidis* cells flown aboard the ISS and compared to matched ground controls showed that the frequency of mutation to rifampin resistance (Rif^r) was significantly greater in the spaceflight samples (51). A similar study on *Bacillus subtilis* revealed significant differences in the spectrum of mutations in the stress response gene *rpoB*, leading to Rif^r differences between flight and ground control samples (52). In another example, spaceflight enhanced the production of the metabolite monorden (radicol) by the fungus *Humicola fuscoatra* WC5157 (53). LSMMG conditions enhanced resistance to gentamicin in stationary-phase uropathogenic *E. coli* (UPEC) (54) and upregulated antibiotic stress resistance in nonpathogenic *E. coli* (38). In contrast, studies on *Staphylococcus haemolyticus* (41) and on four other species of bacteria subjected to long-term exposure to microgravity for 4 months on the Space Station MIR showed increased bacterial susceptibility to antibiotics (31). In other studies, LSMMG did not affect antibiotic tolerance in *E. coli* (35, 36) or *Y. pestis* (21). With such contrasting observations, no clear consensus exists with respect to the effects of microgravity/space conditions on microbial antibiotic resistance properties. In the light of plans for future manned space missions, understanding and evaluating the response of microbial strains to antibiotics thus represent vital challenges.

In an earlier study, *E. coli* was grown under LSMMG conditions for over 1,000 generations spread over 6 months (35). These cells acquired an adaptive advantage, a portion of which was genomic and as a result was maintained when the strain was returned to a shake flask environment for 30 generations (3 cycles). Sensitivity to 20 antibiotics was evaluated by the antibiotic susceptibility testing (AST) feature (which uses prefabricated AST antibiotic cards) of the Vitek automated system studies. The strain failed to acquire resistance to any of the 20 antibiotics monitored by the Vitek system throughout the adaptation period (35). That earlier result serves as the key control for the current study. Here, the same strain of *E. coli* was again grown for over

1,000 generations under LSMMG conditions. The only difference was the use of chloramphenicol treatment rather than steam sterilization to prevent contamination between growth cycles.

RESULTS

Since the *E. coli* MG1655 lac plus strain did not possess any natural growth advantage over the lac minus strain under LSMMG conditions (35), the lac plus strain was grown for over 1,000 generations in high-aspect-ratio vessels (HARVs) cleaned by exposure to chloramphenicol and was stored. The resulting 1,000-generation chloramphenicol-exposed strain (designated 1000G-BA [1,000-generations/background levels of antibiotic]) was reactivated by 3 cycles (1 cycle refers to 10 generations, with 20 min for each generation) of growth under LSMMG conditions. The reactivated strain outcompeted the unadapted lac minus strain when they were grown together under LSMMG conditions in Luria broth (LB) medium with a lac plus/lac minus ratio of 2.71 ± 1.25 , whereas the original unadapted lac plus/lac minus ratio was 1:1, as reported earlier (35). When the 1000G-BA lac plus strain was first grown under shaker conditions over 1 cycle and then subjected to competition with the lac minus strain under LSMMG conditions, the lac plus/lac minus ratio decreased to 2.02 ± 0.46 (see Table S1 in the supplemental material).

Statistical analysis was performed to analyze differences in variance (if any). The data set from the competition between the 1000G-BA strain and the unadapted lac minus strain showed a variance value of 1.56, while the competition between the 1000G-BA strain grown under shake flask conditions for 10 generations (adaptation/memory "erasure") and the unadapted lac minus strain showed a variance value of 0.21 (see Table S1). Given the differences in variance values between the two data sets, we performed the *t* test, assuming unequal variances for the same. Despite the unequal variances, comparisons of the *t* test results between these two data sets showed that the two-tailed and one-tailed *P* values (0.03 and 0.02, respectively) were only slightly below the statistically significant threshold value of 0.05. Thus, the LSMMG adaptation of the 1000G-BA plus strain despite 10 generations of adaptation/memory erasure on shaker flasks was only partially lost (see Table S2).

Antibiotic susceptibility. Vitek studies on the 1000G-BA strain showed that resistance to the antibiotics cefalotin, cefuroxime, cefuroxime axetil, cefoxitin, and tetracycline had been acquired (Fig. 1; see also Table S3). The cells did, however, remain sensitive to ampicillin, amoxicillin-clavulanic acid, cefazolin, cefpodoxime, ceftazidime, ceftriaxone, cefepime, gentamicin, tobramycin, ciprofloxacin, levofloxacin, nitrofurantoin, and trimethoprim-sulfamethoxazole (see Fig. 3; see also Table S3). Following Vitek analysis, the 1000G-BA cells were grown in shaker flasks without any further antibiotic exposure for 11 cycles. Resistance to several of the antibiotics continued. In particular, the cephalosporin antibiotic cefalotin (55, 56) tested positive for resistance even after 11 cycles in shaker flasks without any antibiotic exposure (Fig. 1 and 2) (see also Table S3). It took 5 cycles of adaptation erasure to lose the resistance to the cephamycin antibiotic cefoxitin (57) and the broad-spectrum antibiotic tetracycline (58, 59) (see Table S3 and Fig. S1 in the supplemental material). The resistance of the 1000G-BA strain to the second-generation cephalosporins, namely, cefuroxime (60) and cefuroxime axetil (61), was lost after 20 generations of adaptation erasure (see Table S3 and Fig. S1).

Because the Vitek system does not include chloramphenicol in the test panel, separate studies for this antibiotic were undertaken. The initial lac plus (wild-type [WT]) strain served as a control. It was not resistant, whereas the final adapted 1000G-BA strain was (data not shown). When the 1000G-BA cells were grown in shake flasks in the absence of chloramphenicol, the resistance persisted for over 100 generations.

Genome resequencing. The genome of the 1000G-BA strain was resequenced to identify changes, if any, acquired as a result of 1,000 generations of growth under conditions of LSMMG and background antibiotic (chloramphenicol) exposure. A total of 17,801,713 reads were obtained with even coverage, showing a normal distribution of

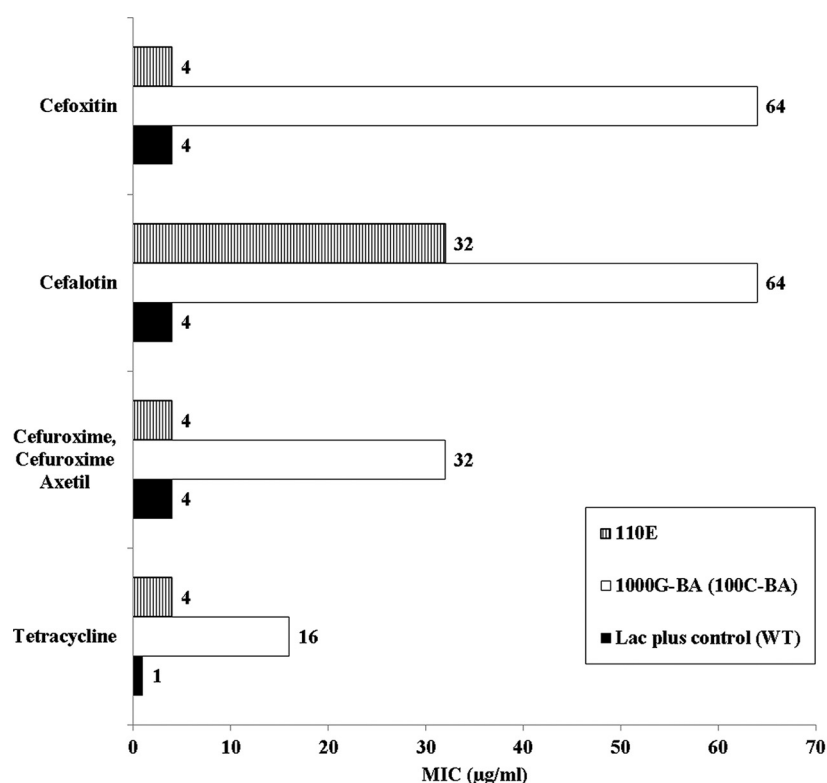


FIG 1 Resistance of the *E. coli* 1000G-BA strain to five antibiotics compared with that of the *E. coli* lac plus (WT) strain and the *E. coli* 1000G-BA strain exposed to nonantibiotic conditions over 110 generations (110E = 11 cycles) in shaker flasks.

read depths. Overall, 22 major changes were seen (Table 1). The changes included 14 point mutations. Eight of these occurred within (intragenic) coding regions (i.e., genes). Seven of these intragenic mutations were nonsynonymous mutations, occurring in genes involved in antibiotic resistance/drug transport (*acrB*, *marR*, *mdfA/cmr*), cell adhesion (*fimE*), transcription (*rpoC*), and general metabolism (*treB* and *chbF*). The single synonymous point mutation occurred in the *yadL* gene, which is involved in adhesion. In addition, two base changes were within pseudogenes, one of which is in the pseudogene of *lafU* (*mbhA*) (pseudogene of a flagellar system gene, motility). The remaining four point mutations occurred between genes (intergenic). Two of these were between genes involved in drug transport (*acrA* \leftrightarrow *acrR* and *ybjG* \leftrightarrow *mdfA*), one was between genes involved in adhesion (*fimE* \rightarrow *fimA*), and one was between genes involved in general metabolite transport across membrane (*gltS* \leftrightarrow *xanP*). In three cases, *acrA* \leftrightarrow *acrR*, *ybjG* \leftrightarrow *mdfA*, and *gltS* \leftrightarrow *xanP*, the change was clearly within the promoter region(s). In another instance, *fimE* \rightarrow *fimA*, the mutation occurred 3 bases upstream of the ATG start codon of the *fimA* gene (Table 1).

Base insertions and deletions. Among the remaining eight changes, one was a base insertion found in a pseudogene (*ylbE*). *glpR* (involved in transcriptional regulation) showed a single base deletion, while *ompF* (antibiotic/drug resistance) underwent a significant $\Delta 203$ -bp deletion (Table 1).

The remaining five changes represented transposon-mediated rearrangements (TMRs) associated with the IS1, IS5, and IS30 insertion sequences. Two of the TMRs were intergenic, mediated by IS5 and IS30. These occurred between genes involved in drug/peptide transport. The IS30 insertion occurred at a position very close to (8 bp away from) the PhosP regulator binding region and thus might affect the transcription of the downstream *ybjG* gene (Table 1; see also Fig. S2).

The remaining three TMRs were mediated by IS1. Two of these completely disrupted the *crl* (adhesion) and *yeaJ* (motility) genes. The third TMR associated with IS1 deleted

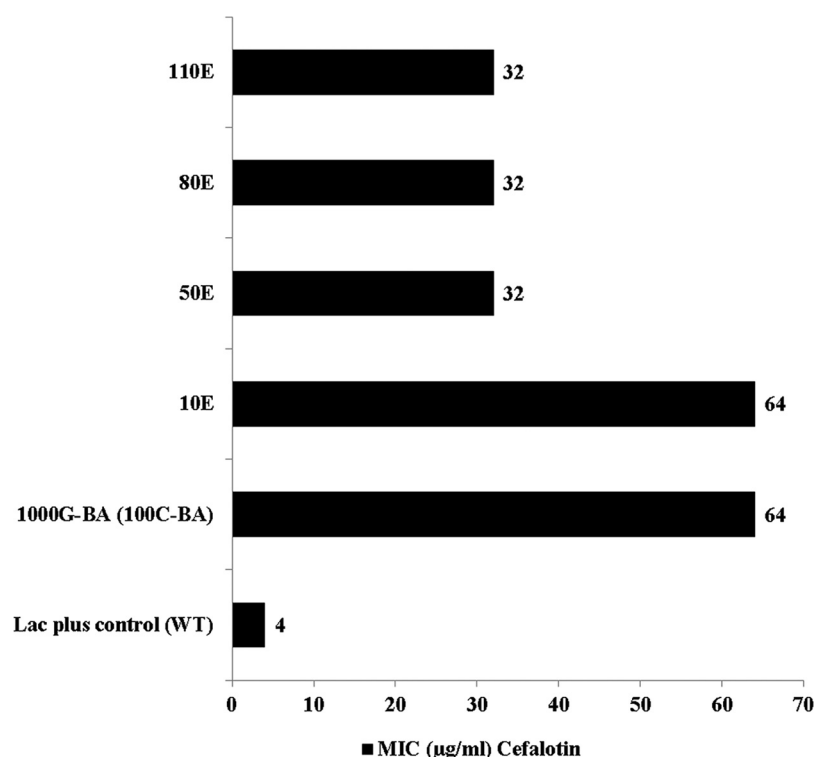


FIG 2 Persistence of antibiotic resistance of the *E. coli* 1000G-BA strain to the antibiotic cefalotin despite exposure to nonantibiotic conditions over 110 generations (110E = 11 cycles) in shaker flasks.

an entire segment of 14,314 bases. This included genes comprising the cluster of genes, viz., *flhA flhB* (flagellar biosynthesis) *cheZ cheY cheB cheR tap tar cheW cheA* (chemotaxis) *motB motA* (flagellar motor complex) *flhC flhD* (flagellar complex) (Table 1).

DISCUSSION

The results obtained in the current study are directly comparable to those obtained in the earlier control study (35) in which steam sterilization was used to prevent contamination between growth cycles. Genome resequencing experiments identified 25 changes in the current study, which is in contrast with the 17 changes seen when steam sterilization was used (35). With but one exception, none of the changes observed earlier were found again when chloramphenicol was used for sterilization. In addition, none of those earlier changes were strongly associated with AR. When chloramphenicol was used for sterilization, competition experiments revealed that the long-term 1000-BA strain lost only 25% of its advantage, thereby indicating that a significant portion of the adaptation was genomic.

In this study, genomic changes occurred in multiple genes known to be associated with AR. In particular, four of the genomic changes in the 1000G-BA strain occurred in key drug transport or AR genes, namely, *ompF*, *acrB*, *marR*, and *mdfA*. Both *ompF* and *acrB* (as part of the *acrAB* MDR efflux pump gene system) are controlled by the *marRAB* operon in response to tetracycline, chloramphenicol, and sodium salicylate stress (62–66). The *marRAB* operon encodes the autorepressor MarR (67) and the autoactivator MarA (68).

Mutations in such genes were anticipated given the fact that the 1000G-BA strain had acquired resistance to chloramphenicol in addition to resistance to other antibiotics. However, in general, one cannot deduce the effects of individual gene changes with certainty from sequence data alone. Simply put, in the absence of experimental verification, any such individual change might in fact be neutral or accidental. However, the finding that the strain itself has become chloramphenicol resistant and that many

TABLE 1 Mutations found in *E. coli* MG1655 (lac plus) after 1,000 generations of growth under LSMMG conditions with background exposure to chloramphenicol^a

Position	Type of change	Mutation type(s)	Annotation	Gene(s)	Usual product
151656	T→G	Base change	A192A (GCA→GCC)	<i>yadL</i> ←	Predicted fimbrial-like adhesin protein
483212	A→G	Base change	V139A (GTT→GCT)	<i>acrB</i> ←	Multidrug efflux system protein
883682	C→T	Base change	P263S (CCT→TCT)	<i>mdfA</i> →	Multidrug efflux system protein
4187022	C→G	Base change	P1217R (CCG→CGG)	<i>rpoC</i> →	RNA polymerase, beta prime subunit
4463866	A→T	Base change	V113E (GTG→GAG)	<i>treB</i> ←	Fused trehalose(maltose)-specific PTS enzyme: IIB component/IIC component
4540294	G→A	Base change	E79K (GAG→AAG)	<i>fimE</i> →	Tyrosine recombinase/inversion of on/off regulator of <i>fimA</i>
1617535	A→T	Base change	E131V (GAA→GTA)	<i>marR</i> →	DNA-binding transcriptional repressor of multiple antibiotic resistance
1816409	A→C	Base change	V39G (GTG→GGG)	<i>chbF</i> ←	Phosphochitobiase; general 6-phospho-beta-glucosidase activity
250390	A→G	Base change	Pseudogene (319/756 nt)	<i>lafU</i> →	Pseudogene, lateral flagellar motor protein fragment
547694	A→G	Base change	Pseudogene (114/252 nt)	<i>ylbE</i> →	Predicted protein, C-ter fragment (pseudogene)
547835	+G	Base insertion/ addition	Pseudogene (4/1,008 nt)	<i>ylbE</i> →	Predicted protein, C-ter fragment (pseudogene)
484938	A→G	Base change	Intergenic (95/47)	<i>acrA</i> ← / → <i>acrR</i>	Multidrug efflux system/DNA-binding transcriptional repressor
882870	G→A	Base change	Intergenic (259/26)	<i>ybjG</i> ← / → <i>mdfA</i>	Undecaprenyl pyrophosphate phosphatase/multidrug efflux system protein
3826853	T→C	Base change	Intergenic (165/115)	<i>gltS</i> ← / → <i>xanP</i>	Glutamate transporter/xanthine permease
4541135	A→C	Base change	Intergenic (+479/3)	<i>fimE</i> → / → <i>fimA</i>	Tyrosine recombinase/inversion of on/off regulator of <i>fimA</i> /major type 1 subunit fimbriae (pilin)
986125	Δ203 bp	Base deletion(s)	Coding	<i>ompF</i> ←	Outer membrane porin
3558478	Δ1 bp	Base deletion	Coding (151/759 nt)	<i>glpR</i> ←	DNA-binding transcriptional repressor
257900	IS1 (-) + 8 bp	TMR	Coding (7279/402 nt)	<i>crl</i> →	Sigma factor-binding protein (stimulates RNA polymerase holoenzyme formation)
882777	IS30 (+) + 2 bp	TMR	Intergenic (166/118)	<i>ybjG</i> ← / → <i>mdfA</i>	Undecaprenyl pyrophosphate phosphatase/multidrug efflux system protein
1298718	IS5 (+) + 4 bp	TMR	Intergenic (+250/485)	<i>yche</i> → / → <i>oppA</i>	Predicted inner membrane protein/oligopeptide transporter subunit
1871055	IS1 (-) + 9 bp	TMR	Coding (991999/ 1,491 nt)	<i>yeaJ</i> →	Predicted diguanylate cyclase
1962213	Δ14,314 bp	TMR	IS1 mediated	(<i>flhAB cheZYBR tap tarc cheWA motBA flhCD</i>) ←	Chemotaxis, flagellum, motility proteins

^a←, gene orientation on reverse strand; →, gene orientation on positive strand; ←/→, intergenic; Δ, deletion; C-ter, C-terminal; IS, insertion sequence; PTS, phosphotransferase system; TMR, transposon-mediated rearrangement.

genomic changes are in regions associated with such resistance makes it very likely that many of these changes are actually associated with the acquisition of resistance.

The *ompF* gene represents a clear, unambiguous link between the acquired AR and the sequencing data. In this case, a 1,089-bp section had been deleted such that this gene was clearly dysfunctional. OmpF is a major transmembrane channel porin regulating the permeability of the Gram-negative bacterial outer membranes and influencing AR (69–77). *E. coli* and *Serratia marcescens* lacking *ompF* were shown to be resistant to certain beta-lactam compounds (78–81). Deletion of *ompF* has also been shown to reduce the permeability of the cephamycin antibiotic cefoxitin (82, 83). In addition, there is a significant increase in antibiotic MIC values for beta-lactam drugs such as ampicillin and nitrofurantoin (besides cefoxitin) (79, 84). The resistance of the 1000G-BA strain to cefoxitin, while retaining sensitivity to ampicillin and nitrofurantoin (Fig. 1 and 3) (see also Table S3 in the supplemental material), suggests an alternate pathway for ampicillin and nitrofurantoin entering the cells.

Mutations in three other genes, *acrB*, *marR*, and *mdfA*, resulted in amino acid changes; those three genes are either directly implicated in AR or are in functional domains with established roles in resistance properties (85–96). Furthermore, bacterial exposure to low levels of antibiotics often results in resistance causing mutations in

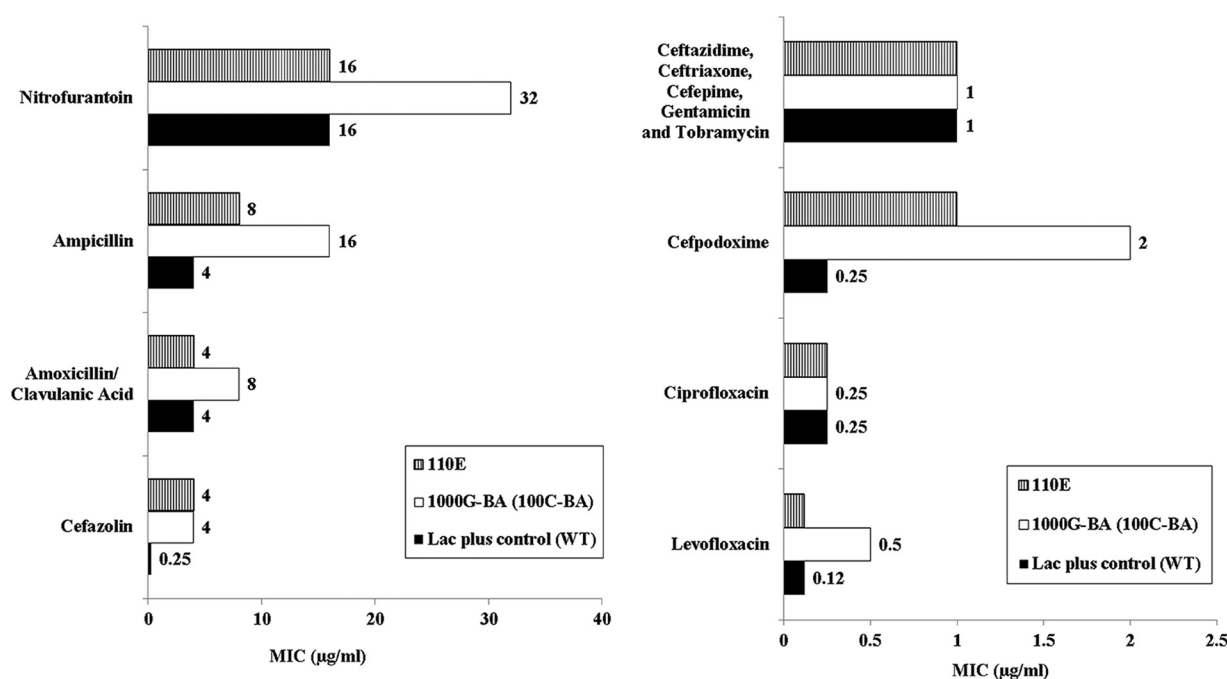


FIG 3 Nonresistance/susceptibility of the *E. coli* 1000G-BA strain to thirteen antibiotics, compared with that of the *E. coli* lac plus (WT) strain and the *E. coli* 1000G-BA strain exposed to nonantibiotic conditions over 110 generations (110E = 11 cycles) in shaker flasks. Data are presented in two columns for convenience of viewing.

genes not previously regarded as typical resistance genes. Such exposure to low-level antibiotics also leads to mutations in genes which are typically not affected under conditions of exposure to high doses (97). Similar changes in the 1000G-BA strain are detailed in Table 2 and illustrated in Fig. 4.

An important issue is whether LSMMG exposure significantly enhances AR adaptation rates to a greater extent than has been observed in other environments. Sustained exposure to low concentrations of antibiotics in non-LSMMG environments is known to result in the development of resistance to antibiotic at levels that are severalfold higher than the initial level to which the bacteria were exposed (97). For clinically important antibiotics (and nonantibiotic antimicrobials), concentrations that were several-hundred-fold below the MIC of susceptible bacteria not only enriched the numbers of resistant bacteria (98–100) but also resulted in cross-resistance across several classes of antibiotics (101–104). For example, exposure of *E. coli* to low concentrations of broad-spectrum antibiotics (tetracycline or chloramphenicol) resulted in an increased frequency of fluoroquinolone-resistant chromosomal multiple-antibiotic-resistant (Mar) mutants that was higher than that seen when *E. coli* was exposed to the fluoroquinolone norfloxacin directly (77).

Chloramphenicol exposure of *E. coli* MG1655 under LSMMG conditions resulted in similar cross-resistance to 5 antibiotics in our study (Fig. 1; see also Table S3). Despite 11 cycles of antibiotic adaptation erasure (under conditions of exposure to nonmicrogravity [non-LSMMG] and nonantibiotic conditions in shaker flasks), the 1000G-BA strain continued to demonstrate resistance to cefalotin (Fig. 2; see also Table S3). In fact, it required 5 cycles of adaptation erasure to lose resistance to ceftazidime and tetracycline (see Table S3 and Fig. S1 in the supplemental material). Overall, our findings strongly suggest that the responses observed in the LSMMG environment are very similar to those observed in non-LSMMG environments. Exposure to background levels of an antibiotic could lead to acquisition of resistance under microgravity conditions as well.

Ideally, growth in HARVs would be undertaken with horizontal rather than vertical rotation as a control in which LSMMG is eliminated. As was the case earlier (35), this non-LSMMG/nonantibiotic control is not available, a constraint resulting from HARV

TABLE 2 Description of mutations and their context in the genome of the *E. coli* 1000G-BA strain

Gene(s)/genomic location & function	Description or known function(s)	Effects of mutations (if any)
<i>yadL</i> ← (intragenic)	Adhesion and tissue tropism in <i>E. coli</i> (115, 116)	Unknown
<i>acrB</i> ← (intragenic)	Antibiotic/drug efflux (86, 117–120)	V139A is involved in tetracycline resistance in <i>E. coli</i> (81) and in substrate binding and carbapenem resistance (85)
<i>acrA</i> ← / → <i>acrR</i> (intergenic)	<i>acrR</i> regulates efflux (<i>acrAB</i>) pump operon, solvent tolerance (121), motility, flagellar and biofilm/pellicle formation, and pathogenesis (122, 123)	Mutation is in the promoter “ <i>acrRp</i> ,” upstream of <i>acrR</i> ; mutation effect unknown
<i>mdfA</i> → (intragenic)	Multidrug resistance, active exclusion of chloramphenicol (95, 96, 124, 125)	P263S; changes nonreactive proline to serine, is often found in protein functional centers, occurs in a transmembrane domain (125, 126), and is a new mutation and an addition to <i>mdfA</i> mutations as a response(s) to antibiotic stress
<i>ybjG</i> ← / → <i>mdfA</i> (intergenic)	<i>ybjG</i> —bacitracin resistance (127, 128); <i>mdfA</i> —multidrug resistance (85–88)	Mutation is in the promoter “ <i>cmrp</i> ,” upstream of <i>mdfA</i> ; effect unknown; IS30 insertion occurs only 8 bp away from a (PhosP) regulator binding region of <i>ybjG</i> ; the 3 changes (on <i>mdfA</i> and its promoter <i>cmrp</i> and in its immediate neighborhood) are additions to changes related to <i>mdfA</i> in response to antibiotic exposure
<i>rpoC</i> → (intragenic)	Encodes the RNA polymerase subunit β'; categorized as an essential gene for <i>E. coli</i> (129)	Unknown
<i>treB</i> ← (intragenic)	Encodes trehalose-specific PTS enzyme IIBC, linked with biofilms in <i>E. coli</i> (130)	Unknown
<i>gltS</i> ← / → <i>xanP</i> (intergenic)	Flanking genes encode metabolite transport proteins	Mutation is in the promoter “ <i>xanPp5</i> ” upstream of <i>xanP</i>
<i>fimE</i> → (intragenic)	<i>fimE</i> regulates adhesion protein coding gene <i>fimA</i>	Unknown
<i>fimE</i> → / → <i>fimA</i> (intergenic)	Flanking genes encode metabolite transport proteins	Mutation is in the 3rd base upstream of the start codon “ATG” of the <i>fimA</i> gene
<i>marR</i> → (intragenic)	Multidrug resistance (81)	E131V is in the DNA-binding domain involved in organic solvent tolerance (88) regulating a global network of 80 genes (89–91, 131); fluoroquinolone resistance (92–94) in clinically relevant <i>E. coli</i> strains
<i>ompF</i> ← (intragenic)	Encodes outer membrane porin involved in antibiotic resistance (78, 132, 133), acid stress response (ASR) (72, 134)	Dysfunctional <i>ompF</i> likely causes increased antibiotic resistance of 1000-BA
<i>crl</i> → (intragenic TMR)	Highly conserved in Gram-negative bacteria; encodes the thin, coiled aggregative surface filaments called curli (curli mediate adhesion [135–138] and wound colonization and interaction with the immune system [138, 139] and influence stress responses, quorum sensing [140], and biofilms [141] and resistance to gentamicin [54] and control ASR genes [142, 143])	Loss of the <i>crl</i> gene potentially results in a fitness cost of antibiotic resistance in 1000-BA
<i>yeaJ</i> → (intragenic TMR)	Encodes a diguanylate cyclase and regulates swimming motility and biofilm formation (144–146)	Loss of the <i>yeaJ</i> gene potentially results in a potential fitness cost of antibiotic resistance in 1000-BA
(<i>flhAB cheZYBR tap tar cheWA motBA flhCD</i>) ← (inter- and intragenic TMR)	14-gene cluster, central to chemotaxis and biofilm formation (144, 147); <i>cheR</i> , <i>cheW</i> (chemotaxis), and <i>motA</i> (motility) are considered essential for <i>E. coli</i> (129)	Loss of gene cluster potentially results in a fitness cost of antibiotic resistance in 1000-BA

availability. In lieu of this control, the unadapted lac plus strain was used as the control for comparisons of sequencing results, and the unadapted lac minus strain was used for the competition assay. Despite the absence of a 1000G non-LSMMG/nonantibiotic control, the antibiotic resistance of the adapted strain (1000G-BA) and its dominance over the unadapted lac minus strain under LSMMG conditions are indicators of the combined effects of the antibiotic and the LSMMG.

The retention of AR as observed in the 1000G-BA strain suggests that similar persistence of microbial AR could also occur in other microorganisms. This is of particular concern with respect to the use of antibiotics as cleaning agents to reduce

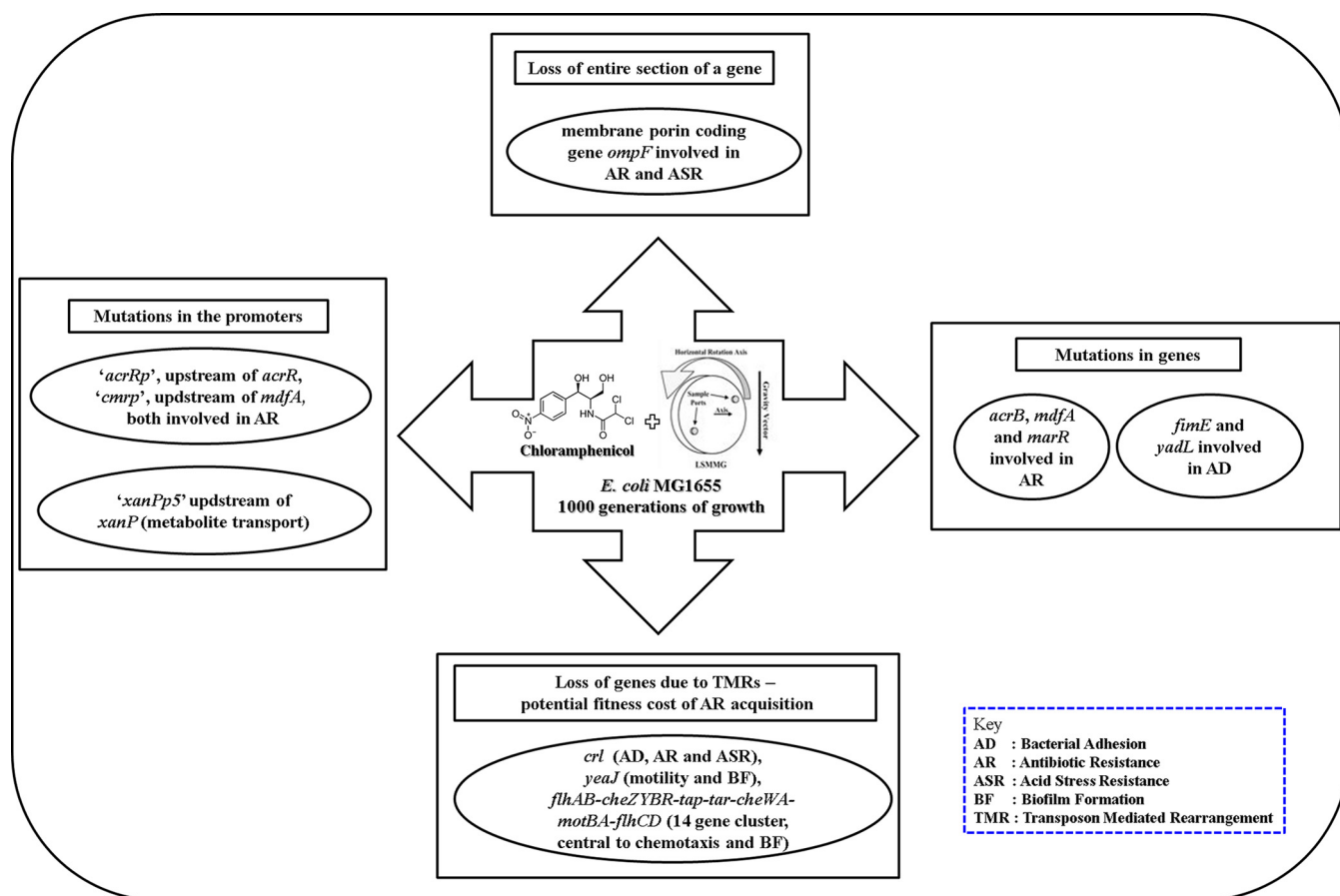


FIG 4 Putative mechanisms, viz., genomic changes contributing to or representing the consequences of the antibiotic resistance of the *E. coli* 1000G-BA strain.

the bioburden of microbes in the confined spaces of manned space flight missions. This is most likely to happen independently of the microgravity component. An overall scheme representing how a combination of various genome changes resulted in AR is shown in Fig. 4.

Space and microgravity represent a unique environment. Microorganisms can survive even the combination of disintegration of the space craft, heat of reentry, and impact (105). Given their resilience, understanding how bacteria evolve and adapt over the long term to space conditions is even more important now with the imminent increase in human space exploration (105). Long-term evolution studies performed on the Space Station, in low Earth orbit projects (54), or through CUBESAT and related projects (106–109) are critical to understanding how the spaceflight environment may influence microbial dynamics within the spacecraft with respect to antibiotics and other biocidal agents. This study was restricted to just one Gram-negative nonpathogenic strain, namely, *E. coli* MG1655. Such long-term studies further exploring AR of a human's (the astronaut's) gut microbiome, of which enterobacteria (such as *E. coli*) (110) as well as Gram-positive organisms are major components, are of utmost importance.

MATERIALS AND METHODS

Bacterial strains. An isogenic pair of *E. coli* strains was used. One was a lac minus strain derived from MG1655 (in which the entire lac operon was deleted) and the other a lac plus strain (MG1655; CGSC 6300) (111). Both strains were obtained from the *E. coli* Genetic Stock Center at Yale University (112). The two strains are distinguishable on MacConkey agar media, with the lac plus strain producing red/pink colonies and the lac minus strain producing white colonies (113). The growth and maintenance

conditions used were as described previously (35). In the work described here, the lac plus strain is referred to as the wild-type (WT) strain (see Table S5 in the supplemental material).

Preparation of HARVs. To obtain background antibiotic exposure, HARVs were assembled and each chamber was filled with a saturated solution of the broad-spectrum antibiotic chloramphenicol (Amresco; USP grade) (500 to 600 mg/ml) in a UV hood and then left to rotate for approximately 2 h. The HARVs were then emptied and repeatedly rinsed with sterile water to remove all traces of the antibiotic and then used for growth. These HARVs are designated HARV-BA.

Extended growth. Two HARV-BAs were used. While one was in use, the other was dismantled and prepared for reuse. The *E. coli* MG1655 lac plus strain (WT) strain was inoculated into a HARV-BA in 50 ml of LB medium at 37°C, followed by successive transfers into fresh HARV-BAs such that growth reached 1,000 generations. A 500- μ l volume of the resulting *E. coli* MG1655 lac plus strain was added to 500 μ l of 50% glycerol in a 2 ml screw top tube, mixed, and stored at minus 70°C as glycerol stocks. This is referred to here as the 1000G-BA strain.

Competition growth studies. The 1000G-BA strain was reactivated by growth in HARV-BAs and then coinoculated in LB medium in a HARV-BA with an equal amount of the lac minus strain (grown in LB medium in a flask at 37°C overnight). At growth saturation, the ratio of the 1000G-BA strain (producing pink colonies) to the lac minus strain (white colonies) was determined as described earlier (35).

Adaptation erasure experiment. The 1000G-BA-adapted strain and the unadapted lac minus strain were grown in LB medium in two separate flasks without any antibiotic under rotary conditions at 37°C overnight as described previously (35). The 1000G-BA strain grown in the absence of chloramphenicol for 10E = 1 cycle (10 generations) was (i) streaked on MacConkey agar plates, (ii) coinoculated with the unadapted lac minus strain under LSMMG conditions at 37°C, and (iii) subcultured into flasks without any antibiotic(s) over several cycles to generate a total of 11 cycles of adaptation erasure (11E = 110 generations of adaptation erasure), with streaking on plates performed after each cycle. This competition assay was analyzed by calculating the ratio of the lac plus strain to the lac minus strain. Antibiotic sensitivity assays were performed using a Vitek 2 Compact instrument and Vitek 2 PC software (bioMérieux, Inc., Hazelwood, MO) as described earlier (35). Vitek (AST) cards containing selected antimicrobials at various concentrations were used. The antibiotics included ampicillin (2 μ g/ml to 32 μ g/ml), amoxicillin/clavulanic acid (1 μ g/ml to 16 μ g/ml), cefalotin (2 μ g/ml to 64 μ g/ml), cefazolin (4 μ g/ml to 64 μ g/ml), cefuroxime (1 μ g/ml to 64 μ g/ml), cefuroxime axetil (1 μ g/ml to 64 μ g/ml), ceftazidime (1 μ g/ml to 64 μ g/ml), ceftazidime (1 μ g/ml to 64 μ g/ml), ceftriaxone (1 μ g/ml to 64 μ g/ml), cefepime (1 μ g/ml to 64 μ g/ml), gentamicin (1 μ g/ml to 16 μ g/ml), tobramycin (1 μ g/ml to 16 μ g/ml), ciprofloxacin (0.25 μ g/ml to 4 μ g/ml), levofloxacin (0.2 μ g/ml to 8 μ g/ml), tetracycline (1 μ g/ml to 16 μ g/ml), nitrofurantoin (16 μ g/ml to 64 μ g/ml), and trimethoprim-sulfamethoxazole (20 μ g/ml to 320 μ g/ml).

Chloramphenicol resistance/susceptibility testing. LB medium (pH adjusted to 7 with NaOH) to which (1.5%) agar was added was prepared, melted, and then autoclaved. The autoclaved LB agar medium was cooled under sterile conditions, chloramphenicol was added to reach a final concentration of 100 μ g/ml, and then mixing was performed. The LB agar-chloramphenicol medium was poured onto petri dish plates at 20 ml per plate. *E. coli* cultures were spread on these plates and incubated at 37°C overnight. Growth was observed visually.

Genome sequencing. The genome of the 1000G-BA strain was sequenced as described previously (35) and compared with the genome of the lac plus WT strain to identify genomic changes. The promoter sequences were identified using the database RegulonDB (114).

Data availability. The genome data set is available as follows: BioProject accession identifier (ID) PRJNA498488 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA498488>); <https://www.ncbi.nlm.nih.gov/biosample/10290157>.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.02637-18>.

FIG S1, TIF file, 0.01 MB.

FIG S2, TIF file, 0.03 MB.

TABLE S1, PDF file, 0.03 MB.

TABLE S2, PDF file, 0.03 MB.

TABLE S3, PDF file, 0.05 MB.

TABLE S4, PDF file, 0.05 MB.

TABLE S5, PDF file, 0.1 MB.

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