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

Madhan R. Tirumalai,[a] Fathi Karouia,[b] Quyen Tran,[a] Victor G. Stepanov,[a] Rebekah J. Bruce,[c] C. Mark Ott,[c] Duane L. Pierson,[c] George E. Fox[a]

[a]Department of Biology and Biochemistry, University of Houston, Houston, Texas, USA
[b]NASA Ames Research Center, Moffett Field, California, USA
[c]NASA Lyndon B. Johnson Space Center, Houston, Texas, USA

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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Address correspondence to George E. Fox, fox@uh.edu.

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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 (radicicol) 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 $\pm$ 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 $\pm$ 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 1000-BAstrain 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 identity 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

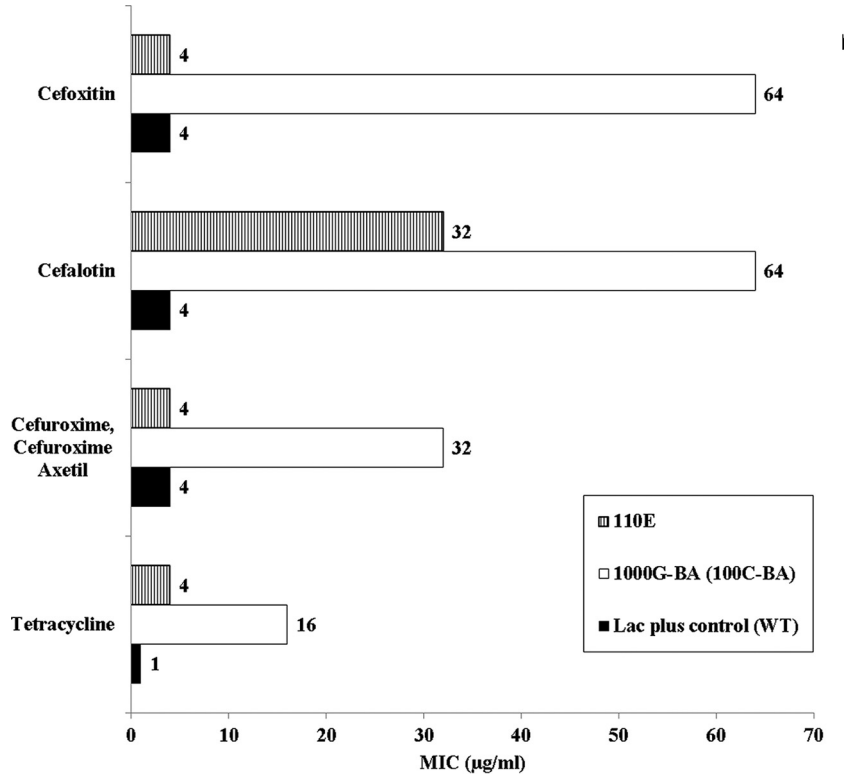

FIG 1 Resistance of the *E. coli* 1000G-BA strain to five antibiotics compared with that of the *E. coli* lac plus (WT) strain and the *E. coli* 1000G-BA strain exposed to nonantibiotic conditions over 110 generations (110E = 11 cycles) in shaker flasks.

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* ←/→ *acrR* and *ybjG* ←/→ *mdfA*), one was between genes involved in adhesion (*fimE* →/→ *fimA*), and one was between genes involved in general metabolite transport across membrane (*gltS* ←/→ *xanP*). In three cases, *acrA* ←/→ *acrR*, *ybjG* ←/→ *mdfA*, and *gltS* ←/→ *xanP*, the change was clearly within the promoter region(s). In another instance, *fimE* →/→ *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 Δ203-bp deletion (Table 1).

The remaining five changes represented transposon-mediated rearrangements (TMRs) associated with the IS*1*, IS*5*, and IS*30* insertion sequences. Two of the TMRs were intergenic, mediated by IS*5* and IS*30*. These occurred between genes involved in drug/peptide transport. The IS*30* 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 IS*1*. Two of these completely disrupted the *crl* (adhesion) and *yeaJ* (motility) genes. The third TMR associated with IS*1* deleted

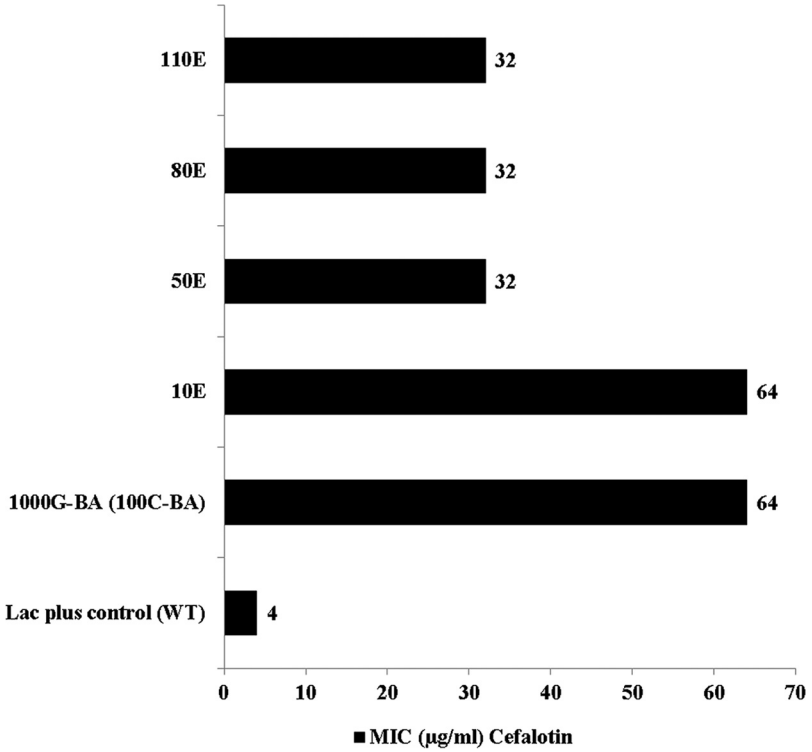

FIG 2 Persistence of antibiotic resistance of the *E. coli* 1000G-BA strain to the antibiotic cefalotin despite exposure to nonantibiotic conditions over 110 generations (110E = 11 cycles) in shaker flasks.

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)	*yadL* ←	Predicted fimbrial-like adhesin protein
483212	A→G	Base change	V139A (GTT→GCT)	*acrB* ←	Multidrug efflux system protein
883682	C→T	Base change	P263S (CCT→TCT)	*mdfA* →	Multidrug efflux system protein
4187022	C→G	Base change	P1217R (CCG→CGG)	*rpoC* →	RNA polymerase, beta prime subunit
4463866	A→T	Base change	V113E (GTG→GAG)	*treB* ←	Fused trehalose(maltose)-specific PTS enzyme: IIB component/IIC component
4540294	G→A	Base change	E79K (GAG→AAG)	*fimE* →	Tyrosine recombinase/inversion of on/off regulator of *fimA*
1617535	A→T	Base change	E131V (GAA→GTA)	*marR* →	DNA-binding transcriptional repressor of multiple antibiotic resistance
1816409	A→C	Base change	V39G (GTG→GGG)	*chbF* ←	Phosphochitobiase; general 6-phospho-beta-glucosidase activity
250390	A→G	Base change	Pseudogene (319/756 nt)	*lafU* →	Pseudogene, lateral flagellar motor protein fragment
547694	A→G	Base change	Pseudogene (114/252 nt)	*ylbE* →	Predicted protein, C-ter fragment (pseudogene)
547835	+G	Base insertion/ addition	Pseudogene (4/1,008 nt)	*ylbE* →	Predicted protein, C-ter fragment (pseudogene)
484938	A→G	Base change	Intergenic (95/47)	*acrA* ← / → *acrR*	Multidrug efflux system/DNA-binding transcriptional repressor
882870	G→A	Base change	Intergenic (259/26)	*ybjG* ← / → *mdfA*	Undecaprenyl pyrophosphate phosphatase/multidrug efflux system protein
3826853	T→C	Base change	Intergenic (165/115)	*gltS* ← / → *xanP*	Glutamate transporter/xanthine permease
4541135	A→C	Base change	Intergenic (+479/3)	*fimE* → / → *fimA*	Tyrosine recombinase/inversion of on/off regulator of *fimA*/major type 1 subunit fimbrin (pilin)
986125	Δ203 bp	Base deletion(s)	Coding	*ompF* ←	Outer membrane porin
3558478	Δ1 bp	Base deletion	Coding (151/759 nt)	*glpR* ←	DNA-binding transcriptional repressor
257900	IS*1* (–) + 8 bp	TMR	Coding (7279/402 nt)	*crl* →	Sigma factor-binding protein (stimulates RNA polymerase holoenzyme formation)
882777	IS*30* (+) + 2 bp	TMR	Intergenic (166/118)	*ybjG* ← / → *mdfA*	Undecaprenyl pyrophosphate phosphatase/multidrug efflux system protein
1298718	IS*5* (+) + 4 bp	TMR	Intergenic (+250/485)	*ychE* → / → *oppA*	Predicted inner membrane protein/oligopeptide transporter subunit
1871055	IS*1* (–) + 9 bp	TMR	Coding (991999/ 1,491 nt)	*yeaJ* →	Predicted diguanylate cyclase
1962213	Δ14,314 bp	TMR	IS*1* mediated	(*flhAB cheZYBR tap tarc cheWA motBA flhCD*) ←	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 permeativity 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

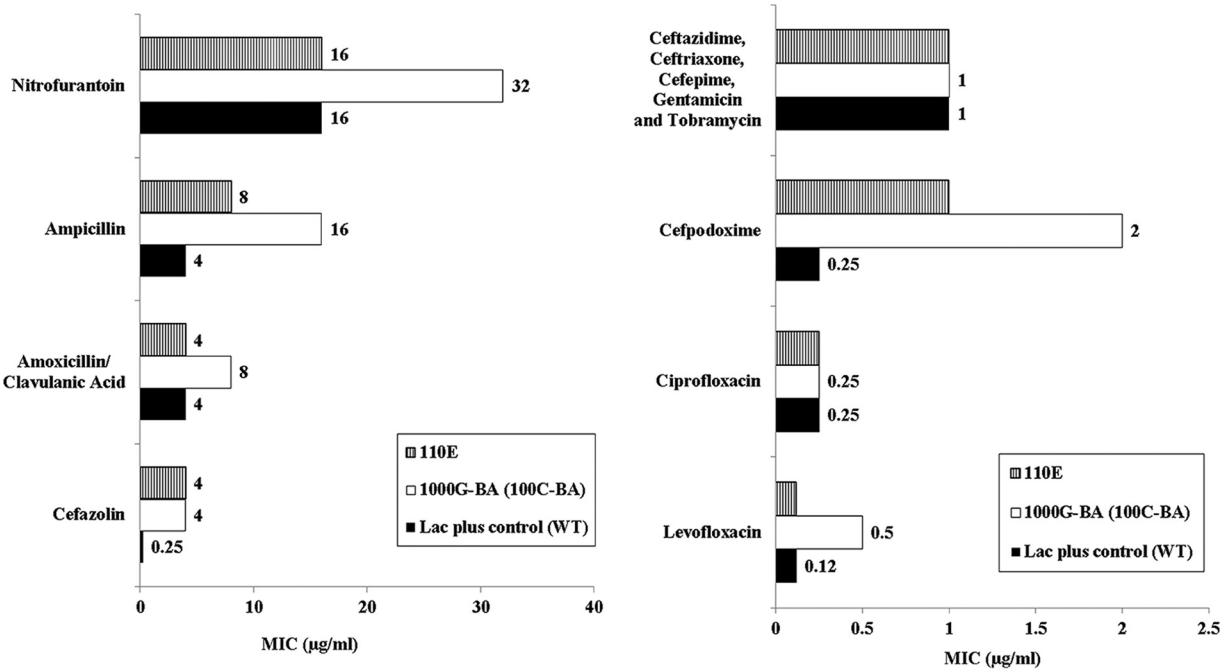

FIG 3 Nonresistance/susceptibility of the *E. coli* 1000G-BA strain to thirteen antibiotics, compared with that of the *E. coli* lac plus (WT) strain and the *E. coli* 1000G-BA strain exposed to nonantibiotic conditions over 110 generations (110E = 11 cycles) in shaker flasks. Data are presented in two 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 cefoxitin 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)
yadL ← (intragenic)	Adhesion and tissue tropism in *E. coli* (115, 116)	Unknown
acrB ←(intragenic)	Antibiotic/drug efflux (86, 117–120)	V139A is involved in tetracycline resistance in *E. coli* (81) and in substrate binding and carbapenem resistance (85)
acrA ← / → *acrR* (intergenic)	*acrR* regulates efflux (*acrAB*) pump operon, solvent tolerance (121), motility, flagellar and biofilm/pellicle formation, and pathogenesis (122, 123)	Mutation is in the promoter "*acrRp*," upstream of *acrR*; mutation effect unknown
mdfA → (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 *mdfA* mutations as a response(s) to antibiotic stress
ybjG ← / → *mdfA* (intergenic)	*ybjG*—bacitracin resistance (127, 128); *mdfA*—multidrug resistance (85–88)	Mutation is in the promoter "*cmrp*," updstream of *mdfA*; effect unknown; IS*30* insertion occurs only 8 bp away from a (PhosP) regulator binding region of *ybjG*.; the 3 changes (on *mdfA* and its promoter *cmrp* and in its immediate neighborhood) are additions to changes related to *mdfA* in response to antibiotic exposure
rpoC → (intragenic)	Encodes the RNA polymerase subunit β'; categorized as an essential gene for *E. coli* (129)	Unknown
treB ← (intragenic)	Encodes trehalose-specific PTS enzyme IIBC, linked with biofilms in *E. coli* (130)	Unknown
gltS ← / → *xanP* (intergenic)	Flanking genes encode metabolite transport proteins	Mutation is in the promoter "*xanPp5*" upstream of *xanP*
fimE → (intragenic)	*fimE* regulates adhesion protein coding gene *fimA*	Unknown
fimE → / → *fimA* (intergenic)	Flanking genes encode metabolite transport proteins	Mutation is in the 3rd base upstream of the start codon "ATG" of the *fimA* gene
marR → (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 *E. coli* strains
ompF ← (intragenic)	Encodes outer membrane porin involved in antibiotic resistance (78, 132, 133), acid stress response (ASR) (72, 134)	Dysfunctional *ompF* likely causes increased antibiotic resistance of 1000-BA
crl → (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 *crl* gene potentially results in a fitness cost of antibiotic resistance in 1000-BA
yeaJ → (intragenic TMR)	Encodes a diguanylate cyclase and regulates swimming motility and biofilm formation (144–146)	Loss of the *yeaJ* gene potentially results in a potential fitness cost of antibiotic resistance in 1000-BA
(*flhAB cheZYBR tap tar cheWA motBA flhCD*) ← (inter- and intragenic TMR)	14-gene cluster, central to chemotaxis and biofilm formation (144, 147); *cheR*, *cheW* (chemotaxis), and *motA* (motility) are considered essential for *E. coli* (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

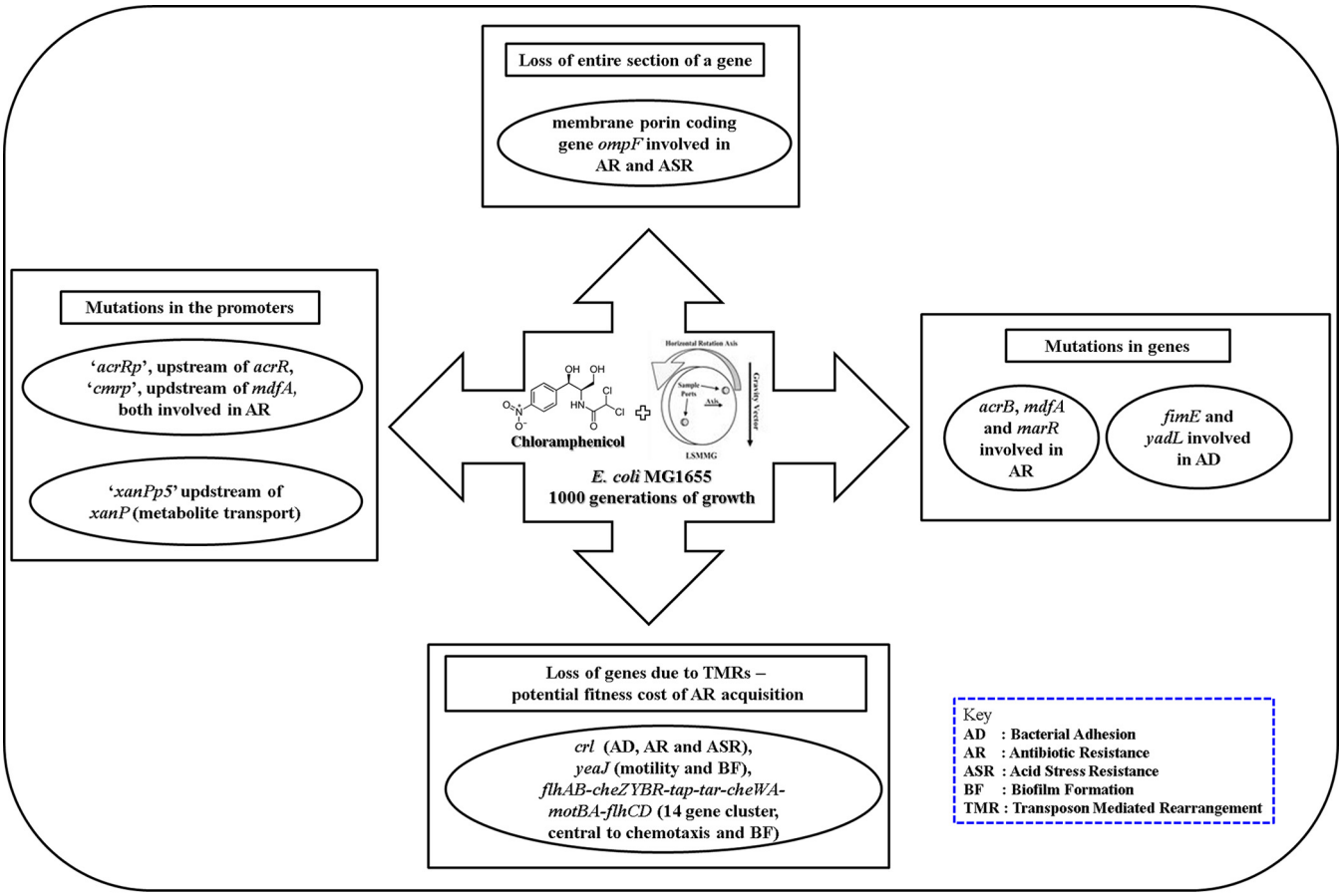

FIG 4 Putative mechanisms, *viz.*, genomic changes contributing to or representing the consequences of the antibiotic resistance of the *E. coli* 1000G-BA strain.

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-μl volume of the resulting *E. coli* MG1655 lac plus strain was added to 500 μl 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 μg/ml to 32 μg/ml), amoxicillin/clavulanic acid (1 μg/ml to 16 μg/ml), cefalotin (2 μg/ml to 64 μg/ml), cefazolin (4 μg/ml to 64 μg/ml), cefuroxime (1 μg/ml to 64 μg/ml), cefuroxime axetil (1 μg/ml to 64 μg/ml), cefoxitin (4 μg/ml to 64 μg/ml), cefpodoxime (0.25 μg/ml to 8 μg/ml), ceftazidime (1 μg/ml to 64 μg/ml), ceftriaxone (1 μg/ml to 64 μg/ml), cefepime (1 μg/ml to 64 μg/ml), gentamicin (1 μg/ml to 16 μg/ml), tobramycin (1 μg/ml to 16 μg/ml), ciprofloxacin (0.25 μg/ml to 4 μg/ml), levofloxacin (0.2 μg/ml to 8 μg/ml), tetracycline (1 μg/ml to 16 μg/ml), nitrofurantoin (16 μg/ml to 64 μg/ml), and trimethoprim-sulfamethoxazole (20 μg/ml to 320 μg/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 μg/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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REFERENCES

1. Aponte VM, Finch DS, Klaus DM. 2006. Considerations for non-invasive in-flight monitoring of astronaut immune status with potential use of MEMS and NEMS devices. Life Sci 79:1317–1333. https://doi.org/10.1016/j.lfs.2006.04.007.
2. Rykova MP, Antropova EN, Larina IM, Morukov BV. 2008. Humoral and cellular immunity in cosmonauts after the ISS missions. Acta Astronaut 63:697–705. https://doi.org/10.1016/j.actaastro.2008.03.016.
3. Cervantes JL, Hong BY. 2016. Dysbiosis and immune dysregulation in outer space. Int Rev Immunol 35:67–82. https://doi.org/10.3109/08830185.2015.1027821.
4. Reichert M. 2001. The future of human spaceflight. Acta Astronaut 49:495–522.
5. Williams DR, Turnock M. 2011. Human space exploration the next fifty years. McGill J Med 13:76.
6. Council NR. 2014. Pathways to exploration: rationales and approaches for a U.S. program of human space exploration. The National Academies Press, Washington, DC.
7. Baisden DL, Beven GE, Campbell MR, Charles JB, Dervay JP, Foster E, Gray GW, Hamilton DR, Holland DA, Jennings RT, Johnston SL, Jones JA, Kerwin JP, Locke J, Polk JD, Scarpa PJ, Sipes W, Stepanek J, Webb JT. 2008. Human health and performance for long-duration spaceflight. Aviat Space Environ Med 79:629–635.
8. Ball JR, Evans CH, Jr (ed). 2001. Safe passage: astronaut care for exploration missions. National Academy Press, Washington, DC.
9. Gueguinou N, Huin-Schohn C, Bascove M, Bueb JL, Tschirhart E, Legrand-Frossi C, Frippiat JP. 2009. Could spaceflight-associated immune system weakening preclude the expansion of human presence beyond Earth's orbit? J Leukoc Biol 86:1027–1038. https://doi.org/10.1189/jlb.0309167.
10. Fischer CL, Gill C, Daniels JC, Cobb EK, Berry CA, Ritzmann SE. 1972. Effects of the space flight environment on man's immune system. I. Serum proteins and immunoglobulins. Aerosp Med 43:856–859.
11. Taylor PW, Sommer AP. 2005. Towards rational treatment of bacterial infections during extended space travel. Int J Antimicrob Agents 26:183–187. https://doi.org/10.1016/j.ijantimicag.2005.06.002.
12. Nefedov YG, Shilov VM, Konstantinova IV, Zaloguyev SN. 1971. Microbiological and immunological aspects of extended manned space flights. Life Sci Space Res 9:11–16.
13. Mermel LA. 2013. Infection prevention and control during prolonged human space travel. Clin Infect Dis 56:123–130. https://doi.org/10.1093/cid/cis861.
14. Riley LK. 2006. Bacterial meningitis exposure during an international flight: lessons for communicable pathogens. Aviat Space Environ Med 77:758–760.
15. Ilyin VK. 2005. Microbiological status of cosmonauts during orbital spaceflights on Salyut and Mir orbital stations. Acta Astronaut 56:839–850.
16. Taylor PW. 2015. Impact of space flight on bacterial virulence and antibiotic susceptibility. Infect Drug Resist 8:249–262. https://doi.org/10.2147/IDR.S67275.
17. Yang J, Barrila J, Roland KL, Ott CM, Nickerson CA. 2016. Physiological fluid shear alters the virulence potential of invasive multidrug-resistant non-typhoidal Salmonella Typhimurium D23580. NPJ Microgravity 2:16021. https://doi.org/10.1038/npjmgrav.2016.21.
18. Soni A, O'Sullivan L, Quick LN, Ott CM, Nickerson CA, Wilson JW. 2014. Conservation of the low-shear modeled microgravity response in Enterobacteriaceae and analysis of the trp genes in this response. Open Microbiol J 8:51–58. https://doi.org/10.2174/1874285801408010051.
19. Nickerson CA, Ott CM, Wilson JW, Ramamurthy R, Pierson DL. 2004. Microbial responses to microgravity and other low-shear environments. Microbiol Mol Biol Rev 68:345–361. https://doi.org/10.1128/MMBR.68.2.345-361.2004.
20. Nickerson CA, Ott CM, Wilson JW, Ramamurthy R, LeBlanc CL, Honer zu Bentrup K, Hammond T, Pierson DL. 2003. Low-shear modeled microgravity: a global environmental regulatory signal affecting bacterial gene expression, physiology, and pathogenesis. J Microbiol Methods 54:1–11.
21. Lawal A, Kirtley ML, van Lier CJ, Erova TE, Kozlova EV, Sha J, Chopra AK, Rosenzweig JA. 2013. The effects of modeled microgravity on growth kinetics, antibiotic susceptibility, cold growth, and the virulence potential of a Yersinia pestis ymoA-deficient mutant and its isogenic parental strain. Astrobiology 13:821–832. https://doi.org/10.1089/ast.2013.0968.
22. Rosenzweig JA, Abogunde O, Thomas K, Lawal A, Nguyen YU, Sodipe A, Jejelowo O. 2010. Spaceflight and modeled microgravity effects on microbial growth and virulence. Appl Microbiol Biotechnol 85:885–891. https://doi.org/10.1007/s00253-009-2237-8.
23. Crabb A, De Boever P, Van Houdt R, Moors H, Mergeay M, Cornelis P. 2008. Use of the rotating wall vessel technology to study the effect of shear stress on growth behaviour of Pseudomonas aeruginosa PA01. Environ Microbiol 10:2098–2110. https://doi.org/10.1111/j.1462-2920.2008.01631.x.
24. McLean RJ, Cassanto JM, Barnes MB, Koo JH. 2001. Bacterial biofilm formation under microgravity conditions. FEMS Microbiol Lett 195:115–119. https://doi.org/10.1111/j.1574-6968.2001.tb10507.x.
25. Wilson JW, Ott CM, Honer zu Bentrup K, Ramamurthy R, Quick L, Porwollik S, Cheng P, McClelland M, Tsaprailis G, Radabaugh T, Hunt A, Fernandez D, Richter E, Shah M, Kilcoyne M, Joshi L, Nelman-Gonzalez M, Hing S, Parra M, Dumars P, Norwood K, Bober R, Devich J, Ruggles A, Goulart C, Rupert M, Stodieck L, Stafford P, Catella L, Schurr MJ, Buchanan K, Morici L, McCracken J, Allen P, Baker-Coleman C, Hammond T, Vogel J, Nelson R, Pierson DL, Stefanyshyn-Piper HM, Nickerson CA. 2007. Space flight alters bacterial gene expression and virulence and reveals a role for global regulator Hfq. Proc Natl Acad Sci U S A 104:16299–16304. https://doi.org/10.1073/pnas.0707155104.
26. Orsini SS, Lewis AM, Rice KC. 2017. Investigation of simulated microgravity effects on Streptococcus mutans physiology and global gene expression. NPJ Microgravity 3:4. https://doi.org/10.1038/s41526-016-0006-4.
27. Lawal A, Jejelowo OA, Rosenzweig JA. 2010. The effects of low-shear mechanical stress on Yersinia pestis virulence. Astrobiology 10:881–888. https://doi.org/10.1089/ast.2010.0493.
28. Searles SC, Woolley CM, Petersen RA, Hyman LE, Nielsen-Preiss SM. 2011. Modeled microgravity increases filamentation, biofilm formation, phenotypic switching, and antimicrobial resistance in Candida albicans. Astrobiology 11:825–836. https://doi.org/10.1089/ast.2011.0664.
29. Hammond TG, Stodieck L, Birdsall HH, Becker JL, Koenig P, Hammond JS, Gunter MA, Allen PL. 2013. Effects of microgravity on the virulence of Listeria monocytogenes, Enterococcus faecalis, Candida albicans, and methicillin-resistant Staphylococcus aureus. Astrobiology 13:1081–1090. https://doi.org/10.1089/ast.2013.0986.
30. Rosenzweig JA, Ahmed S, Eunson J, Jr, Chopra AK. 2014. Low-shear force associated with modeled microgravity and spaceflight does not similarly impact the virulence of notable bacterial pathogens. Appl Microbiol Biotechnol 98:8797–8807. https://doi.org/10.1007/s00253-014-6025-8.
31. Juergensmeyer MA, Juergensmeyer EA, Guikema JA. 1999. Long-term exposure to spaceflight conditions affects bacterial response to antibiotics. Microgravity Sci Technol 12:41–47.
32. Bell G, MacLean C. 2018. The search for 'evolution-proof' antibiotics. Trends Microbiol 26:471–483. https://doi.org/10.1016/j.tim.2017.11.005.
33. Aunins TR, Erickson KE, Prasad N, Levy SE, Jones A, Shrestha S, Mastracchio R, Stodieck L, Klaus D, Zea L, Chatterjee A. 2018. Spaceflight modifies Escherichia coli gene expression in response to antibiotic exposure and reveals role of oxidative stress response. Front Microbiol 9:310. https://doi.org/10.3389/fmicb.2018.00310.
34. Lynch SV, Mukundakrishnan K, Benoit MR, Ayyaswamy PS, Matin A. 2006. Escherichia coli biofilms formed under low-shear modeled microgravity in a ground-based system. Appl Environ Microbiol 72:7701–7710. https://doi.org/10.1128/AEM.01294-06.
35. Tirumalai MR, Karouia F, Tran Q, Stepanov VG, Bruce RJ, Ott CM, Pierson DL, Fox GE. 2017. The adaptation of Escherichia coli cells grown in simulated microgravity for an extended period is both phenotypic and genomic. NPJ Microgravity 3:15. https://doi.org/10.1038/s41526-017-0020-1.
36. Tucker DL, Ott CM, Huff S, Fofanov Y, Pierson DL, Willson RC, Fox GE. 2007. Characterization of Escherichia coli MG1655 grown in a low-shear modeled microgravity environment. BMC Microbiol 7:15. https://doi.org/10.1186/1471-2180-7-15.
37. Karouia FTM, Nelman-Gonzalez MA, Sams CF, Ott MC, Pierson DL, Fofanov Y, Willson RC, Fox GE. Transcriptional and physiological char-

acterization of Escherichia coli K12 MG1655 grown under low shear simulated microgravity for 1000 generation. International Astronautical Federation, Paris, France.
38. Arunasri K, Adil M, Venu Charan K, Suvro C, Himabindu Reddy S, Shivaji S. 2013. Effect of simulated microgravity on E. coli K12 MG1655 growth and gene expression. PLoS One 8:e57860. https://doi.org/10.1371/journal.pone.0057860.
39. Baker PW, Leff LG. 2005. Attachment to stainless steel by Mir Space Station bacteria growing under modeled reduced gravity at varying nutrient concentrations. Biofilms 2:1–7. https://doi.org/10.1017/S1479050504001437.
40. Song B, Leff LG. 2005. Identification and characterization of bacterial isolates from the Mir space station. Microbiol Res 160:111–117. https://doi.org/10.1016/j.micres.2004.10.005.
41. Urbaniak C, Sielaff AC, Frey KG, Allen JE, Singh N, Jaing C, Wheeler K, Venkateswaran K. 2018. Detection of antimicrobial resistance genes associated with the International Space Station environmental surfaces. Sci Rep 8:814. https://doi.org/10.1038/s41598-017-18506-4.
42. Schiwon K, Arends K, Rogowski KM, Fürch S, Prescha K, Sakinc T, Van Houdt R, Werner G, Grohmann E. 2013. Comparison of antibiotic resistance, biofilm formation and conjugative transfer of Staphylococcus and Enterococcus isolates from International Space Station and Antarctic Research Station Concordia. Microb Ecol 65:638–651. https://doi.org/10.1007/s00248-013-0193-4.
43. Martinez JL. 2014. General principles of antibiotic resistance in bacteria. Drug Discov Today Technol 11:33–39. https://doi.org/10.1016/j.ddtec.2014.02.001.
44. Martinez JL, Baquero F. 2014. Emergence and spread of antibiotic resistance: setting a parameter space. Ups J Med Sci 119:68–77. https://doi.org/10.3109/03009734.2014.901444.
45. Sundqvist M. 2014. Reversibility of antibiotic resistance. Ups J Med Sci 119:142–148. https://doi.org/10.3109/03009734.2014.903323.
46. Ventola CL. 2015. The antibiotic resistance crisis: part 2: management strategies and new agents. P T 40:344–352.
47. Ventola CL. 2015. The antibiotic resistance crisis: part 1: causes and threats. P T 40:277–283.
48. Zhang D, Chang D, Zhang X, Yu Y, Guo Y, Wang J, Li T, Xu G, Dai W, Liu C. 6 March 2014. Genome sequence of Escherichia coli strain LCT-EC52, which acquired changes in antibiotic resistance properties after the Shenzhou-VIII mission. Genome Announc https://doi.org/10.1128/genomeA.00081-14.
49. Matin A, Lynch SV, Benoit MR. 2006. Increased bacterial resistance and virulence in simulated microgravity and its molecular basis. Gravit Space Biol Bull 19:31–41.
50. Tixador R, Richoilley G, Gasset G, Templier J, Bes JC, Moatti N, Lapchine L. 1985. Study of minimal inhibitory concentration of antibiotics on bacteria cultivated in vitro in space (Cytos 2 experiment). Aviat Space Environ Med 56:748–751.
51. Fajardo-Cavazos P, Nicholson WL. 2016. Cultivation of Staphylococcus epidermidis in the human spaceflight environment leads to alterations in the frequency and spectrum of spontaneous rifampicin-resistance mutations in the rpoB gene. Front Microbiol 7:999. https://doi.org/10.3389/fmicb.2016.00999.
52. Fajardo-Cavazos P, Leehan JD, Nicholson WL. 2018. Alterations in the spectrum of spontaneous rifampicin-resistance mutations in the Bacillus subtilis rpoB gene after cultivation in the human spaceflight environment. Front Microbiol 9:192. https://doi.org/10.3389/fmicb.2018.00192.
53. Lam KS, Mamber SW, Pack EJ, Forenza S, Fernandes PB, Klaus DM. 1998. The effects of space flight on the production of monorden by Humicola fuscoatra WC5157 in solid-state fermentation. Appl Microbiol Biotechnol 49:579–583.
54. Matin AC, Wang JH, Keyhan M, Singh R, Benoit M, Parra MP, Padgen MR, Ricco AJ, Chin M, Friedericks CR, Chinn TN, Cohen A, Henschke MB, Snyder TV, Lera MP, Ross SS, Mayberry CM, Choi S, Wu DT, Tan MX, Boone TD, Beasley CC, Piccini ME, Spremo SM. 2017. Payload hardware and experimental protocol development to enable future testing of the effect of space microgravity on the resistance to gentamicin of uropathogenic Escherichia coli and its sigma(s)-deficient mutant. Life Sci Space Res (Amst) 15:1–10. https://doi.org/10.1016/j.lssr.2017.05.001.
55. Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, Lago BA, Dave BM, Pereira S, Sharma AN, Doshi S, Courtot M, Lo R, Williams LE, Frye JG, Elsayegh T, Sardar D, Westman EL, Pawlowski AC, Johnson TA, Brinkman FS, Wright GD, McArthur AG. 2017. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. Nucleic Acids Res 45:D566–D573. https://doi.org/10.1093/nar/gkw1004.
56. Petersen SW, Rosin E. 1995. Cephalothin and cefazolin in vitro antibacterial activity and pharmacokinetics in dogs. Vet Surg 24:347–351. https://doi.org/10.1111/j.1532-950X.1995.tb01341.x.
57. Faraci WS, Pratt RF. 1986. Mechanism of inhibition of RTEM-2 beta-lactamase by cephamycins: relative importance of the 7 alpha-methoxy group and the 3′ leaving group. Biochemistry 25:2934–2941.
58. Smilack JD. 1999. The tetracyclines. Mayo Clin Proc 74:727–729. https://doi.org/10.4065/74.7.727.
59. Schnappinger D, Hillen W. 1996. Tetracyclines: antibiotic action, uptake, and resistance mechanisms. Arch Microbiol 165:359–369.
60. Ryan DM, O'Callaghan C, Muggleton PW. 1976. Cefuroxime, a new cephalosporin antibiotic: activity in vivo. Antimicrob Agents Chemother 9:520–525.
61. Dellamonica P. 1994. Cefuroxime axetil. Int J Antimicrob Agents 4:23–36.
62. Cohen SP, McMurry LM, Levy SB. 1988. marA locus causes decreased expression of OmpF porin in multiple-antibiotic-resistant (Mar) mutants of Escherichia coli. J Bacteriol 170:5416–5422.
63. Maira-Litran T, Allison DG, Gilbert P. 2000. An evaluation of the potential of the multiple antibiotic resistance operon (mar) and the multidrug efflux pump acrAB to moderate resistance towards ciprofloxacin in Escherichia coli biofilms. J Antimicrob Chemother 45:789–795.
64. Okusu H, Ma D, Nikaido H. 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of Escherichia coli multiple-antibiotic-resistance (Mar) mutants. J Bacteriol 178:306–308. https://doi.org/10.1128/jb.178.1.306-308.1996.
65. Ruiz C, Levy SB. 2010. Many chromosomal genes modulate MarA-mediated multidrug resistance in Escherichia coli. Antimicrob Agents Chemother 54:2125–2134. https://doi.org/10.1128/AAC.01420-09.
66. Ruiz C, Levy SB. 2014. Regulation of acrAB expression by cellular metabolites in Escherichia coli. J Antimicrob Chemother 69:390–399. https://doi.org/10.1093/jac/dkt352.
67. Ariza RR, Cohen SP, Bachhawat N, Levy SB, Demple B. 1994. Repressor mutations in the marRAB operon that activate oxidative stress genes and multiple antibiotic resistance in Escherichia coli. J Bacteriol 176:143–148.
68. Cohen SP, Hachler H, Levy SB. 1993. Genetic and functional analysis of the multiple antibiotic resistance (mar) locus in Escherichia coli. J Bacteriol 175:1484–1492.
69. Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited. Microbiol Mol Biol Rev 67:593–656.
70. Nikaido H, Vaara M. 1985. Molecular basis of bacterial outer membrane permeability. Microbiol Rev 49:1–32.
71. Benz R. 1988. Structure and function of porins from gram-negative bacteria. Annu Rev Microbiol 42:359–393. https://doi.org/10.1146/annurev.mi.42.100188.002043.
72. Schnaitman CA, McDonald GA. 1984. Regulation of outer membrane protein synthesis in Escherichia coli K-12: deletion of ompC affects expression of the OmpF protein. J Bacteriol 159:555–563.
73. Yoshida T, Qin L, Egger LA, Inouye M. 2006. Transcription regulation of ompF and ompC by a single transcription factor, OmpR. J Biol Chem 281:17114–17123. https://doi.org/10.1074/jbc.M602112200.
74. Nikaido H, Rosenberg EY. 1983. Porin channels in Escherichia coli: studies with liposomes reconstituted from purified proteins. J Bacteriol 153:241–252.
75. Nikaido H, Rosenberg EY, Foulds J. 1983. Porin channels in Escherichia coli: studies with beta-lactams in intact cells. J Bacteriol 153:232–240.
76. Pages JM, James CE, Winterhalter M. 2008. The porin and the permeating antibiotic: a selective diffusion barrier in Gram-negative bacteria. Nat Rev Microbiol 6:893–903. https://doi.org/10.1038/nrmicro1994.
77. Cohen SP, McMurry LM, Hooper DC, Wolfson JS, Levy SB. 1989. Cross-resistance to fluoroquinolones in multiple-antibiotic-resistant (Mar) Escherichia coli selected by tetracycline or chloramphenicol: decreased drug accumulation associated with membrane changes in addition to OmpF reduction. Antimicrob Agents Chemother 33:1318–1325.
78. Begic S, Worobec EA. 2006. Regulation of Serratia marcescens ompF and ompC porin genes in response to osmotic stress, salicylate, temperature and pH. Microbiology 152:485–491. https://doi.org/10.1099/mic.0.28428-0.
79. Moya-Torres A, Mulvey MR, Kumar A, Oresnik IJ, Brassinga AK. 2014. The lack of OmpF, but not OmpC, contributes to increased antibiotic resis-

tance in Serratia marcescens. Microbiology 160:1882–1892. https://doi.org/10.1099/mic.0.081166-0.
80. Harder KJ, Nikaido H, Matsuhashi M. 1981. Mutants of Escherichia coli that are resistant to certain beta-lactam compounds lack the ompF porin. Antimicrob Agents Chemother 20:549–552.
81. Jahn LJ, Munck C, Ellabaan MMH, Sommer MOA. 2017. Adaptive laboratory evolution of antibiotic resistance using different selection regimes lead to similar phenotypes and genotypes. Front Microbiol 8:816. https://doi.org/10.3389/fmicb.2017.00816.
82. Mortimer PG, Piddock LJ. 1993. The accumulation of five antibacterial agents in porin-deficient mutants of Escherichia coli. J Antimicrob Chemother 32:195–213.
83. Clarke B, Hiltz M, Musgrave H, Forward KR. 2003. Cephamycin resistance in clinical isolates and laboratory-derived strains of Escherichia coli, Nova Scotia, Canada. Emerg Infect Dis 9:1254–1259. https://doi.org/10.3201/eid0910.030093.
84. Delcour AH. 2009. Outer membrane permeability and antibiotic resistance. Biochim Biophys Acta 1794:808–816. https://doi.org/10.1016/j.bbapap.2008.11.005.
85. Adler M, Anjum M, Andersson DI, Sandegren L. 2016. Combinations of mutations in envZ, ftsI, mrdA, acrB and acrR can cause high-level carbapenem resistance in Escherichia coli. J Antimicrob Chemother 71:1188–1198. https://doi.org/10.1093/jac/dkv475.
86. Elkins CA, Nikaido H. 2002. Substrate specificity of the RND-type multidrug efflux pumps AcrB and AcrD of Escherichia coli is determined predominantly by two large periplasmic loops. J Bacteriol 184:6490–6498.
87. Vargiu AV, Nikaido H. 2012. Multidrug binding properties of the AcrB efflux pump characterized by molecular dynamics simulations. Proc Natl Acad Sci U S A 109:20637–20642. https://doi.org/10.1073/pnas.1218348109.
88. Watanabe R, Doukyu N. 2012. Contributions of mutations in acrR and marR genes to organic solvent tolerance in Escherichia coli. AMB Express 2:58. https://doi.org/10.1186/2191-0855-2-58.
89. Alekshun MN, Levy SB. 1997. Regulation of chromosomally mediated multiple antibiotic resistance: the mar regulon. Antimicrob Agents Chemother 41:2067–2075.
90. Alekshun MN, Levy SB. 1999. The mar regulon: multiple resistance to antibiotics and other toxic chemicals. Trends Microbiol 7:410–413.
91. Alekshun MN, Levy SB, Mealy TR, Seaton BA, Head JF. 2001. The crystal structure of MarR, a regulator of multiple antibiotic resistance, at 2.3 A resolution. Nat Struct Biol 8:710–714. https://doi.org/10.1038/90429.
92. Praski Alzrigat L, Huseby DL, Brandis G, Hughes D. 2017. Fitness cost constrains the spectrum of marR mutations in ciprofloxacin-resistant Escherichia coli. J Antimicrob Chemother 72:3016–3024. https://doi.org/10.1093/jac/dkx270.
93. Webber MA, Piddock LJ. 2001. Absence of mutations in marRAB or soxRS in acrB-overexpressing fluoroquinolone-resistant clinical and veterinary isolates of Escherichia coli. Antimicrob Agents Chemother 45:1550–1552. https://doi.org/10.1128/AAC.45.5.1550-1552.2001.
94. Knopp M, Andersson DI. 2018. Predictable phenotypes of antibiotic resistance mutations. mBio 9:e00770-18. https://doi.org/10.1128/mBio.00770-18.
95. Bibi E, Adler J, Lewinson O, Edgar R. 2001. MdfA, an interesting model protein for studying multidrug transport. J Mol Microbiol Biotechnol 3:171–177.
96. Nilsen IW, Bakke I, Vader A, Olsvik O, El-Gewely MR. 1996. Isolation of cmr, a novel Escherichia coli chloramphenicol resistance gene encoding a putative efflux pump. J Bacteriol 178:3188–3193.
97. Wistrand-Yuen E, Knopp M, Hjort K, Koskiniemi S, Berg OG, Andersson DI. 2018. Evolution of high-level resistance during low-level antibiotic exposure. Nat Commun 9:1599. https://doi.org/10.1038/s41467-018-04059-1.
98. Gullberg E, Cao S, Berg OG, Ilback C, Sandegren L, Hughes D, Andersson DI. 2011. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 7:e1002158. https://doi.org/10.1371/journal.ppat.1002158.
99. Andersson DI, Hughes D. 2012. Evolution of antibiotic resistance at non-lethal drug concentrations. Drug Resist Updat 15:162–172. https://doi.org/10.1016/j.drup.2012.03.005.
100. Hughes D, Andersson DI. 2012. Selection of resistance at lethal and non-lethal antibiotic concentrations. Curr Opin Microbiol 15:555–560. https://doi.org/10.1016/j.mib.2012.07.005.
101. Lu J, Jin M, Nguyen SH, Mao L, Li J, Coin LJM, Yuan Z, Guo J. 2018. Non-antibiotic antimicrobial triclosan induces multiple antibiotic resistance through genetic mutation. Environ Int 118:257–265. https://doi.org/10.1016/j.envint.2018.06.004.
102. Hoeksema M, Brul S, Ter Kuile BH. 25 May 2018. Influence of reactive oxygen species on de novo acquisition of resistance to bactericidal antibiotics. Antimicrob Agents Chemother https://doi.org/10.1128/AAC.02354-17.
103. Bhattacharya G, Dey D, Das S, Banerjee A. 2017. Exposure to subinhibitory concentrations of gentamicin, ciprofloxacin and cefotaxime induces multidrug resistance and reactive oxygen species generation in meticillin-sensitive Staphylococcus aureus. J Med Microbiol 66:762–769. https://doi.org/10.1099/jmm.0.000492.
104. Kohanski MA, DePristo MA, Collins JJ. 2010. Sublethal antibiotic treatment leads to multidrug resistance via radical-induced mutagenesis. Mol Cell 37:311–320. https://doi.org/10.1016/j.molcel.2010.01.003.
105. McLean RJ, Welsh AK, Casasanto VA. 2006. Microbial survival in space shuttle crash. Icarus 181:323–325. https://doi.org/10.1016/j.icarus.2005.12.002.
106. Jones N. 2014. Mini satellites prove their scientific power. Nature 508:300–301. https://doi.org/10.1038/508300a.
107. Asphaug E, Thangavelautham J, Klesh A, Chandra A, Nallapu R, Raura L, Herreras-Martinez M, Schwartz S. 2017. A cubesat centrifuge for long duration milligravity research. NPJ Microgravity 3:16. https://doi.org/10.1038/s41526-017-0021-0.
108. Cottin H, Kotler JM, Billi D, Cockell C, Demets R, Ehrenfreund P, Elsaesser A, d'Hendecourt L, van Loon JJWA, Martins Z, Onofri S, Quinn RC, Rabbow E, Rettberg P, Ricco AJ, Slenzka K, de la Torre R, de Vera J-P, Westall F, Carrasco N, Fresneau A, Kawaguchi Y, Kebukawa Y, Nguyen D, Poch O, Saiagh K, Stalport F, Yamagishi A, Yano H, Klamm BA. 2017. Space as a tool for astrobiology: review and recommendations for experimentations in Earth orbit and beyond. Space Sci Rev 209:83–181. https://doi.org/10.1007/s11214-017-0365-5.
109. Nicholson WL, Ricco AJ, Agasid E, Beasley C, Diaz-Aguado M, Ehrenfreund P, Friedericks C, Ghassemieh S, Henschke M, Hines JW, Kitts C, Luzzi E, Ly D, Mai N, Mancinelli R, McIntyre M, Minelli G, Neumann M, Parra M, Piccini M, Rasay RM, Ricks R, Santos O, Schooley A, Squires D, Timucin L, Yost B, Young A. 2011. The O/OREOS mission: first science data from the Space Environment Survivability of Living Organisms (SESLO) payload. Astrobiology 11:951–958. https://doi.org/10.1089/ast.2011.0714.
110. Cresci GA, Bawden E. 2015. Gut microbiome: what we do and don't know. Nutr Clin Pract 30:734–746.
111. Jensen KF. 1993. The Escherichia coli K-12 "wild types" W3110 and MG1655 have an rph frameshift mutation that leads to pyrimidine starvation due to low pyrE expression levels. J Bacteriol 175:3401–3407.
112. Berlyn MB, Letovsky S. 1992. Genome-related datasets within the E. coli Genetic Stock Center database. Nucleic Acids Res 20:6143–6151. https://doi.org/10.1093/nar/20.23.6143.
113. Macconkey A. 1905. Lactose-fermenting bacteria in faeces. J Hyg (Lond) 5:333–379.
114. Gama-Castro S, Salgado H, Santos-Zavaleta A, Ledezma-Tejeida D, Muñiz-Rascado L, García-Sotelo JS, Alquicira-Hernández K, Martínez-Flores I, Pannier L, Castro-Mondragón JA, Medina-Rivera A, Solano-Lira H, Bonavides-Martínez C, Pérez-Rueda E, Alquicira-Hernández S, Porrón-Sotelo L, López-Fuentes A, Hernández-Koutoucheva A, Del Moral-Chávez V, Rinaldi F, Collado-Vides J. 2016. RegulonDB version 9.0: high-level integration of gene regulation, coexpression, motif clustering and beyond. Nucleic Acids Res 44:D133–D143. https://doi.org/10.1093/nar/gkv1156.
115. Korea CG, Badouraly R, Prevost MC, Ghigo JM, Beloin C. 2010. Escherichia coli K-12 possesses multiple cryptic but functional chaperone-usher fimbriae with distinct surface specificities. Environ Microbiol 12:1957–1977. https://doi.org/10.1111/j.1462-2920.2010.02202.x.
116. Korea CG, Ghigo JM, Beloin C. 2011. The sweet connection: solving the riddle of multiple sugar-binding fimbrial adhesins in Escherichia coli: multiple E. coli fimbriae form a versatile arsenal of sugar-binding lectins potentially involved in surface-colonisation and tissue tropism. BioEssays 33:300–311. https://doi.org/10.1002/bies.201000121.
117. Li XZ, Nikaido H. 2009. Efflux-mediated drug resistance in bacteria: an update. Drugs 69:1555–1623. https://doi.org/10.2165/11317030-000000000-00000.
118. Ma D, Cook DN, Alberti M, Pon NG, Nikaido H, Hearst JE. 1995. Genes acrA and acrB encode a stress-induced efflux system of Escherichia coli. Mol Microbiol 16:45–55.

119. Nikaido H, Pagès JM. 2012. Broad-specificity efflux pumps and their role in multidrug resistance of Gram-negative bacteria. FEMS Microbiol Rev 36:340–363. https://doi.org/10.1111/j.1574-6976.2011.00290.x.
120. Nikaido H. 1996. Multidrug efflux pumps of gram-negative bacteria. J Bacteriol 178:5853–5859.
121. Lee JO, Cho KS, Kim OB. 2014. Overproduction of AcrR increases organic solvent tolerance mediated by modulation of SoxS regulon in Escherichia coli. Appl Microbiol Biotechnol 98:8763–8773. https://doi.org/10.1007/s00253-014-6024-9.
122. Kim YJ, Im SY, Lee JO, Kim OB. 2016. Potential swimming motility variation by AcrR in Escherichia coli. J Microbiol Biotechnol 26:1824–1828. https://doi.org/10.4014/jmb.1607.07058.
123. Subhadra B. 2018. Local repressor AcrR regulates AcrAB efflux pump required for biofilm formation and virulence in Acinetobacter nosocomialis. Front Cell Infect Microbiol 8:270. https://doi.org/10.3389/fcimb.2018.00270.
124. Mine T, Morita Y, Kataoka A, Mizushima T, Tsuchiya T. 1998. Evidence for chloramphenicol/H+ antiport in Cmr (MdfA) system of Escherichia coli and properties of the antiporter. J Biochem 124:187–193.
125. Edgar R, Bibi E. 1997. MdfA, an Escherichia coli multidrug resistance protein with an extraordinarily broad spectrum of drug recognition. J Bacteriol 179:2274–2280.
126. Edgar R, Bibi E. 1999. A single membrane-embedded negative charge is critical for recognizing positively charged drugs by the Escherichia coli multidrug resistance protein MdfA. EMBO J 18:822–832. https://doi.org/10.1093/emboj/18.4.822.
127. Harel YM, Bailone A, Bibi E. 1999. Resistance to bacitracin as modulated by an Escherichia coli homologue of the bacitracin ABC transporter BcrC subunit from Bacillus licheniformis. J Bacteriol 181:6176–6178.
128. El Ghachi M, Derbise A, Bouhss A, Mengin-Lecreulx D. 2005. Identification of multiple genes encoding membrane proteins with undecaprenyl pyrophosphate phosphatase (UppP) activity in Escherichia coli. J Biol Chem 280:18689–18695. https://doi.org/10.1074/jbc.M412277200.
129. Schembri MA, Kjaergaard K, Klemm P. 2003. Global gene expression in Escherichia coli biofilms. Mol Microbiol 48:253–267.
130. Gerdes SY, Scholle MD, Campbell JW, Balazsi G, Ravasz E, Daugherty MD, Somera AL, Kyrpides NC, Anderson I, Gelfand MS, Bhattacharya A, Kapatral V, D'Souza M, Baev MV, Grechkin Y, Mseeh F, Fonstein MY, Overbeek R, Barabasi AL, Oltvai ZN, Osterman AL. 2003. Experimental determination and system level analysis of essential genes in Escherichia coli MG1655. J Bacteriol 185:5673–5684. https://doi.org/10.1128/JB.185.19.5673-5684.2003.
131. Pomposiello PJ, Bennik MH, Demple B. 2001. Genome-wide transcriptional profiling of the Escherichia coli responses to superoxide stress and sodium salicylate. J Bacteriol 183:3890–3902. https://doi.org/10.1128/JB.183.13.3890-3902.2001.
132. Fernandez L, Hancock RE. 2012. Adaptive and mutational resistance: role of porins and efflux pumps in drug resistance. Clin Microbiol Rev 25:661–681. https://doi.org/10.1128/CMR.00043-12.
133. Masi M, Pages JM. 2013. Structure, function and regulation of outer membrane proteins Involved in drug transport in Enterobactericeae: the OmpF/C - TolC case. Open Microbiol J 7:22–33. https://doi.org/10.2174/1874285801307010022.
134. Schnaitman CA. 1974. Outer membrane proteins of Escherichia coli. IV. Differences in outer membrane proteins due to strain and cultural differences. J Bacteriol 118:454–464.
135. Bougdour A, Lelong C, Geiselmann J. 2004. Crl, a low temperature-induced protein in Escherichia coli that binds directly to the stationary phase sigma subunit of RNA polymerase. J Biol Chem 279:19540–19550. https://doi.org/10.1074/jbc.M314145200.
136. Arnqvist A, Olsen A, Pfeifer J, Russell DG, Normark S. 1992. The Crl protein activates cryptic genes for curli formation and fibronectin binding in Escherichia coli HB101. Mol Microbiol 6:2443–2452.
137. Olsen A, Jonsson A, Normark S. 1989. Fibronectin binding mediated by a novel class of surface organelles on Escherichia coli. Nature 338:652–655. https://doi.org/10.1038/338652a0.
138. Olsén A, Wick MJ, Mörgelin M, Björck L. 1998. Curli, fibrous surface proteins of Escherichia coli, interact with major histocompatibility complex class I molecules. Infect Immun 66:944–949.
139. Romling U, Sierralta WD, Eriksson K, Normark S. 1998. Multicellular and aggregative behaviour of Salmonella typhimurium strains is controlled by mutations in the agfD promoter. Mol Microbiol 28:249–264.
140. Lelong C, Aguiluz K, Luche S, Kuhn L, Garin J, Rabilloud T, Geiselmann J. 2007. The Crl-RpoS regulon of Escherichia coli. Mol Cell Proteomics 6:648–659. https://doi.org/10.1074/mcp.M600191-MCP200.
141. Gonzalez Barrios AF, Zuo R, Hashimoto Y, Yang L, Bentley WE, Wood TK. 2006. Autoinducer 2 controls biofilm formation in Escherichia coli through a novel motility quorum-sensing regulator (MqsR, B3022). J Bacteriol 188:305–316. https://doi.org/10.1128/JB.188.1.305-316.2006.
142. Dudin O, Lacour S, Geiselmann J. 2013. Expression dynamics of RpoS/Crl-dependent genes in Escherichia coli. Res Microbiol 164:838–847. https://doi.org/10.1016/j.resmic.2013.07.002.
143. Gut H, Pennacchietti E, John RA, Bossa F, Capitani G, De Biase D, Grütter MG. 2006. Escherichia coli acid resistance: pH-sensing, activation by chloride and autoinhibition in GadB. EMBO J 25:2643–2651. https://doi.org/10.1038/sj.emboj.7601107.
144. Amores GR, de Las Heras A, Sanches-Medeiros A, Elfick A, Silva-Rocha R. 2017. Systematic identification of novel regulatory interactions controlling biofilm formation in the bacterium Escherichia coli. Sci Rep 7:16768. https://doi.org/10.1038/s41598-017-17114-6.
145. Sommerfeldt N, Possling A, Becker G, Pesavento C, Tschowri N, Hengge R. 2009. Gene expression patterns and differential input into curli fimbriae regulation of all GGDEF/EAL domain proteins in Escherichia coli. Microbiology 155:1318–1331. https://doi.org/10.1099/mic.0.024257-0.
146. Sanchez-Torres V, Hu H, Wood TK. 2011. GGDEF proteins YeaI, YedQ, and YfiN reduce early biofilm formation and swimming motility in Escherichia coli. Appl Microbiol Biotechnol 90:651–658. https://doi.org/10.1007/s00253-010-3074-5.
147. Niba ET, Naka Y, Nagase M, Mori H, Kitakawa M. 2008. A genome-wide approach to identify the genes involved in biofilm formation in E. coli. DNA Res 14:237–246. https://doi.org/10.1093/dnares/dsm024.