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The Evolution of Heteroresistance via Small Colony Variants in *Escherichia coli* Following Long Term Exposure to Bacteriostatic Antibiotics

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Abstract

Traditionally, bacteriostatic antibiotics are agents able to arrest bacterial growth. Despite being unable to kill bacterial cells, when they are used clinically the outcome of these drugs is frequently as effective as when a bactericidal drug is used. We explore the dynamics of *Escherichia coli* after exposure to two ribosome-targeting bacteriostatic antibiotics, chloramphenicol and azithromycin, for thirty days. The results of our experiments provide evidence that bacteria exposed to these drugs replicate, evolve, and generate a sub-population of small colony variants (SCVs) which are resistant to multiple drugs. These SCVs contribute to the evolution of heteroresistance and rapidly revert to a susceptible state once the antibiotic is removed. Stated another way, exposure to bacteriostatic drugs selects for the evolution of heteroresistance in populations previously lacking this trait. More generally, our results question the definition of bacteriostasis as populations exposed to bacteriostatic drugs are replicating despite the lack of net growth.

Introduction

Antibiotics can be broadly classified as being bactericidal or bacteriostatic based on whether they kill bacteria or simply arrest their growth [1]. Intuitively, it would make sense to treat an infection with drugs that kill the infecting bacteria, the bactericidal drugs, and thereby eliminate the reliance on the host's immune system to clear the infection, as would be the case with bacteriostatic antibiotics. For this reason, bacteriostatic drugs have been

considered "weaker" than bactericidal drugs and are not recommended for the treatment of severe infections or infections in immunodeficient patients [2,3].

This distinction between bactericidal and bacteriostatic drugs is manifest in quantitative experimental studies of the pharmacodynamics (PD) of antibiotics and bacteria. These studies focus on the rates and dynamics of the drug's ability to kill exposed populations of bacteria [4-7]. Many of the studies concerning why antibiotics fail to control bacterial infections have focused on phenomena solely belonging to bactericidal antibiotics such as persistence and tolerance [6,8-10]. With much of the clinical application of antibiotics focusing on bactericidal drugs and the majority of the research on the PD also focusing on bactericidal drugs, PD research on bacteriostatic drugs has been relatively neglected.

However, in recent years, clinicians have given less importance to the antibiotic's ability to kill bacteria *in vitro* and instead have focused on the outcome of treatment with these drugs. By this criterion, in many cases bacteriostatic antibiotics are as effective as bactericidal even in severe infections, with the possible exception of immunosuppressed patients [2,11]. The increase in use of bacteriostatic antibiotics could reduce the selection pressure for resistance to bactericidal agents used in these critical infections. However, the shift to using bacteriostatic antibiotics requires the development of quantitative measures of the PD of antibiotics that arrest the growth of, rather than kill, bacteria [12]. One cannot solely characterize the PD of bacteriostatic antibiotics by the minimum concentration required to prevent the replication of exposed bacteria, the MIC. Furthermore, the evolution of genomic resistance for these agents is rare and the resistant traits tolerance or persistence that occur in bactericidal antibiotics are difficult to define and perhaps impossible to detect with these bacteriostatic agents. This raises questions about the population and evolutionary dynamics of bacteria confronted with these drugs. If the bacteria exposed to bacteriostatic antibiotics are not replicating, one would not expect them to evolve. Therefore, more considerations of the pharmaco-, population, and evolutionary dynamics of bacteriostatic antibiotics are needed both academically and clinically.

In this study, we explore the pharmaco-, population, and evolutionary dynamics of *Escherichia coli* exposed to two bacteriostatic antibiotics of different classes, chloramphenicol (CHL) and azithromycin (AZM), over 30 days. The results of our experiments provide evidence that: (i) long term exposure to these ribosome-targeting bacteriostatic antibiotics does not change the absolute density of exposed populations, (ii) despite the fact that the population's net density does not change, bacteria exposed to these drugs replicate, evolve, and generate small colony variants, and (iii) the selective pressure mediated by these drugs favors the evolution of heteroresistant populations, i.e. the emergence of resistant minority populations, in a strain previously lacking this trait.

Results

Long Term Exposure to Bacteriostatic Antibiotics

We begin our investigation into the effects of long term exposure to ribosome-targeting bacteriostatic drugs by evaluating the impact that these agents have on bacterial survival over 30 days. We exposed four independent cultures of ~10^5 CFU/mL of *E. coli* MG1655 in glucose-limited minimal media to super-MIC (Minimum Inhibitory Concentration) concentrations of CHL and AZM for 30 days (Figure 1). The MIC of CHL and AZM with MG1655 were estimated by broth microdilution in this glucose-limited minimal media and found to be 6.25 ug/mL for both drugs [13]. Super-MIC concentrations of each drug that were shown to be bacteriostatic with minimal killing and/or growth were used in the experiment (Supplemental Figure 1). Cultures exposed to these drugs were sampled every 5 days. We observed that over the course of the experiment, the control cultures containing no drugs reached its maximum stationary phase density of approximately 10^9 CFU/mL and then went down by approximately two logs over the course of 30 days, while the densities in the cultures containing the drugs remained stable, with at most a half-log change in density in the drug-treated populations. Notably, in drug-treated cultures where the bacteria were not lost, two distinct colony morphologies emerged. The colonies were either similar to the ancestral wild-type *E. coli* or were much smaller bacterial colonies, small colony variants (SCVs). This evolution occurred while under strong selective pressure from these drugs. There was no change in colony size in the drug-free controls. To assess the maintenance of the activity of the antibiotics after 30 days, bacteria resistant to the antibiotic in each culture was added at approximately 10^6 CFU/mL and over the course of 24 hours each culture grew approximately three orders of magnitude (Supplemental Figure 2). This residual growth indicates that at 30 days the antibiotic is still at a super-MIC concentration and thereby is the limiting factor for growth in the long term experiments.

To determine how evolution is occurring in the apparent absence of net growth in the presence of the antibiotic, we performed a long term experiment using a conditionally non-replicative plasmid [14] to identify if growth is occurring at the same rate as death (Figure 2). After 10 days the plasmid frequency decreased by 100-fold, and after 20 days the plasmid frequency was near the limit of detection. That means that the plasmid containing cells are progressively diluted (as each cell division gives rise to a plasmid-free descendant) and indicates that the population is growing and replicating at least once a day and dying at the same rate.

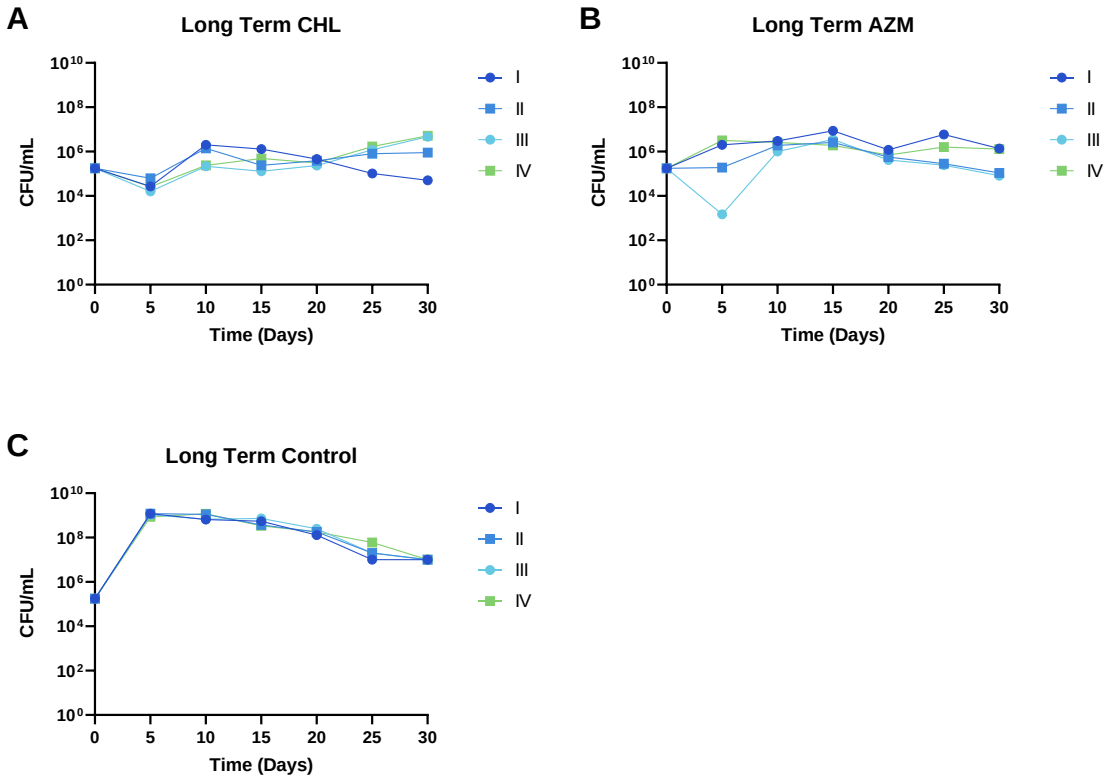

Fig 1. Long term exposure of *E. coli* to bacteriostatic drugs. Density in CFU/mL of *E. coli* MG1655 measured every 5 days for 30 days of 4 independent biological replicas (I-IV). **(A)** *E. coli* exposed to 4x MIC CHL; **(B)** *E. coli* exposed to 3x MIC AZM; **(C)** Drug-free control.

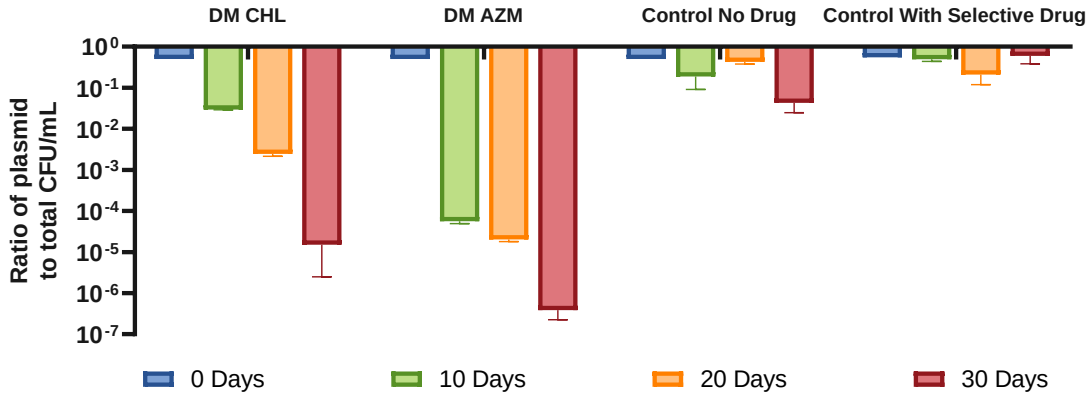

Fig 2. Long term experiment with a non-replicative plasmid. Ratio of the plasmid-containing cells to the total number of cells in CFU/mL of *E. coli* MG1655 with the non-replicative plasmid pAM34 which bears an ampicillin resistance cassette. The total cell density and density of cells bearing the plasmid were measured every 10 days for 30 days of 4 independent biological replicas in four conditions: i) minimal media with CHL, ii)

minimal media with AZM, iii) minimal media with no antibiotic, iv) minimal media with ampicillin.

Small Colony Variants Characterization

To determine what these SCVs are, we isolated 6 independently generated SCVs of MG1655 and characterized them phenotypically and genotypically. Firstly, to determine if the SCVs are a form of resistance that has emerged over the long term experiment, we determined their MIC to the drugs they were previously exposed to (Figure 3). Each SCV showed at least a 5-fold change over the ancestral MG1655's MIC. Each SCV has a distinct antibiotic sensitivity profile in terms of collateral sensitivities and cross-resistances (Supplemental Figure 3).

Notably, although these SCVs are resistant to the bacteriostatic antibiotic to which they were exposed, there is very little growth in the long term culture over the course of 30 days. To determine why a marked increase in density does not occur despite the evolution of resistance, we performed OD-based growth experiments of the SCVs with different concentrations of antibiotics. We found that even though these mutants are resistant, their growth rates and the maximum optical densities decreased proportionally to the drug concentration and their lag time was substantially increased (Supplemental Figure 4). This result is consistent with previous observations [15].

The small colonies obtained from both CHL and AZM cultures appear unstable, that is, when streaked on LB plates without the drug both small colonies and normal sized colonies appear. After the genotypic characterization of these SCVs we do find genetic differences in most of them (Supplemental Table 1), but we could not find a clear mechanism that would explain this resistance.

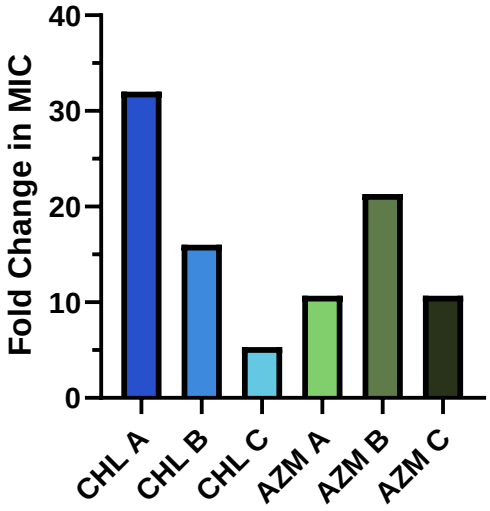

Fig 3. MIC of the SCVs to their respective drugs. Three SCVs were isolated from each condition from day 30 of the long term experiment, grown up in 1.5x *E. coli* MG1655's MIC for these respective drug, and then E-tested.

Heteroresistance

Antibiotic heteroresistance (HR) is defined as, "a phenotype in which a bacterial isolate contains subpopulations of cells that show a substantial reduction in antibiotic susceptibility compared with the main population", and is detected via a population analysis profile (PAP) test [16]. The revertant populations obtained from these SCVs have a lower MIC than those of their small colony ancestor. These revertant populations are capable of rapidly regenerating the SCVs, which have a higher MIC – meeting the definition of antibiotic HR. The ancestral *E. coli* MG1655 is not capable of generating resistant subpopulations, as shown via PAP test (Supplemental Figure 5 Panels A and B). In Figure 4, we show PAP tests of a CHL SCV (Panel A) and an AZM SCV (Panel B). Both SCVs are shown to be heteroresistant based on the above criteria. Moreover, highly resistant colonies isolated from these PAP tests were found to be unstable and revert to the initial SCV state in 15 days. As shown in Supplemental Figure 5 Panels C and D, all SCVs obtained from the long term experiment meet the criteria for HR.

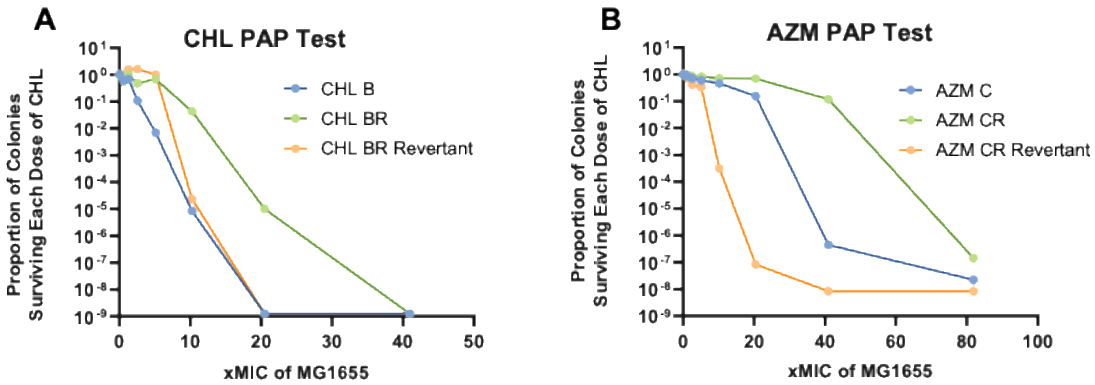

Fig 4. PAP tests of a CHL and an AZM SCV. (A) PAP tests of a CHL SCV (blue line), the most resistant isolate of this clone (green line), and the most resistant isolate after being grown without antibiotic pressure for 15 days (orange line). **(B)** PAP tests of an AZM SCV (blue line), the most resistant isolate of this clone (green line), and the most resistant isolate after being grown without antibiotic pressure for 15 days (orange line).

Mathematical Model and Computer Simulations

To explore the generality of our experimental results, we constructed a mathematical and computer-simulation model. In Figure 5 we depict the model graphically and in the Supplemental Text we describe the model and its equations.

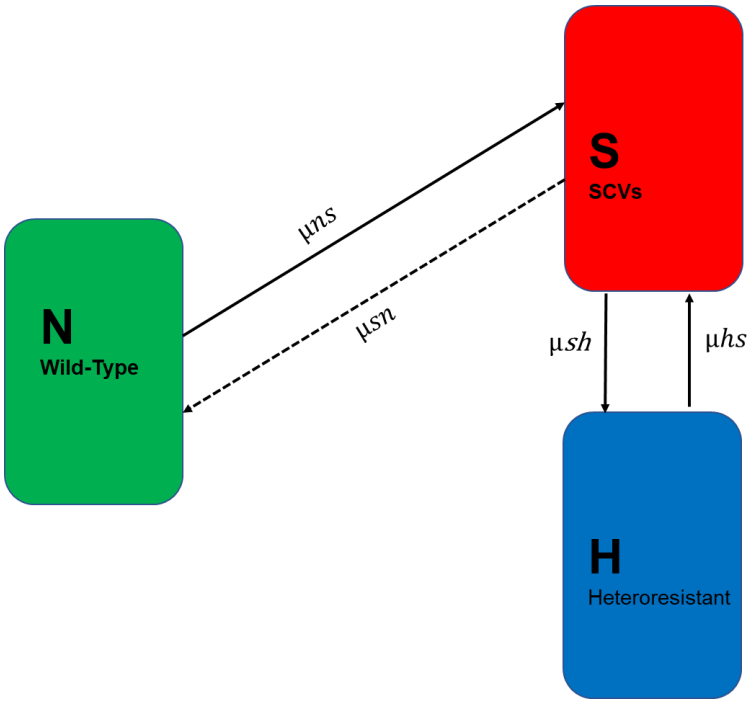

Fig 5. Diagram of a semi-stochastic model of the evolution of HR. The variables N, S, and H are, respectively, the wildtype *E. coli* MG1655, SCVs, and the heteroresistant bacteria in cells/mL. The parameters μ_{ns}, μ_{sn}, μ_{sh} and μ_{hs} are the transition rates, per cell per hour, between the different states.

In Supplemental Figure 6A we illustrate how the presence of an antibiotic selects for the emergence and ascent of resistant SCVs and a heteroresistant population from an initially wild-type population. In Supplemental Figure 6B, we show that an initial population of SCVs when grown without antibiotics will rapidly transition and give rise to a heteroresistant population which ascends and becomes limited by the resource. In Supplemental Figure 6C, we show that exposing the heteroresitant population to the drug more rapidly selects for the emergence and dominance of reistant SCVs than when exposing the wild type population to the same concentraton of the drug. In Supplemental Figure 6D, we show the changes in average MIC for the three scenarios depicted in panels A, B, and C.

Discussion

The canonical distinction between bacteriostatic and bactericidal antibiotics has deeply influenced their clinical usage. Traditionally, bacteriostatic antibiotics were considered "weaker drugs", but this traditional view is questionable. Drugs which are classified as bacteriostatic can and do kill bacteria in a concentration dependent manner [17]. Moreover, meta-analysis studies do not demonstrate differences in the clinical success of therapy with either types of drugs even in severe infections [11]. Indeed, bactericidal agents could be reserved for life-threatening infections, particularly in immunocompromised patients or those suffering from chronic infections. A more extended use of bacteriostatic drugs could

be beneficial to spare the use and overuse of bactericidal antibiotics which fosters resistance. A limitation to progress in the extended use of bacteriostatic drugs is the shortage of pharmacodynamic (PD) data with these drugs.

The distinction between bacteriostatic and bactericidal antibiotics is confounded by another factor: The primary cellular and molecular targets do not necessarily differ between these classes of drugs. Several bacteriostatic antibiotics have mechanisms of action that one would anticipate being bactericidal: mecillinam and cefsulodin inhibit cell wall synthesis [18]; novobiocin inhibits DNA gyrase [19]; and rifampin inhibits the DNA-dependent RNA polymerase which is bacteriostatic for *E. coli* and bactericidal for *Mycobacterium* [20,21]. Most notably, drugs which target the ribosome and inhibit protein synthesis can be either bactericidal (such as gentamicin or tobramycin) or bacteriostatic (such as the macrolides, phenicols, tetracyclines, oxazolidinones, and spectinomycin) [22]. Interestingly, the potential bactericidal effect of one of the ribosome-targeting drugs, chloramphenicol, is prevented by the production of (p)ppGpp in the exposed cells [23]. This suggests that the difference between a bacteriostatic drug and a bactericidal one is a property of the treated cell rather than the antibiotic [24]. All the above examples illustrate the need for a better understanding of the PD, population biology, and evolutionary biology of treatment with bacteriostatic antibiotics.

In this study, we present evidence that bacteriostatic drugs of two different classes (the phenicols and macrolides) inhibit the growth of *E. coli* for extended periods, i.e. 30 days, and moreover maintain the culture in a kind of stationary phase where the density of viable bacterial cells is stable. Although, unlike stationary phase we found there to be an abundance of the limiting resource, implying that the cultures remain drug-limited even after a month. Most interestingly, despite the fact that the bacteria in the population appears to not be replicating due to the lack of net growth, evolution still occurred. A population of small colony variants (SCVs) emerged and ascended to become the dominant population of bacteria. We attribute this evolution to the fact that even though the population at large was neither increasing nor decreasing, the population was replicating at a rate roughly equal to that at which it was dying. This finding is unanticipated and inconsistent with the common perception that bacteriostatic drugs simply arrest bacterial growth. This result questions the definition of bacteriostasis.

These SCVs were found to be highly resistant not only to the challenging agents, but even to some types of bactericidal agents, such as aminoglycosides and rifampin. Curiously, the resistance of the SCVs to chloramphenicol (CHL) and azithromycin (AZM) are not due to the canonical resistance mechanisms [25,26]. SCVs have been implicated in treatment failure, primarily in *Staphylococcus aureus*, but there are limited reports of SCVs being associated with treatment failure in *E. coli* [27,28]. We have yet to determine the genetic and molecular basis of the observed SCVs, but they appear to be distinct from the previously described mechanisms [29-33]. Certain mutations observed here (Supplemental Table 1) might account for the SCV phenotype. For instance, in the case of AZM-induced SCVs, missense variants of the *citG* gene, encoding the 2-(5''-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase, were consistently found. This mutation might alter members of the GntR family of transcriptional regulators which could influence the DNA-binding properties of the

regulator resulting in repression or activation of transcription, or could directly impact ATP synthesis [34], leading to the generation of the SCVs. Additionally, mutations in either *acrAB* which interferes with AZM membrane transport [35] or mutations in the gene encoding the 50S ribosomal protein L22, a known mechanism of macrolide resistance [36], account for the increase in the MIC to AZM but does not account for the emergence of the SCVs. This is also true in the case of CHL-induced SCVs in which we found mutations in the gene encoding the 50S ribosomal protein L4, a CHL binding site [37]. We found a mutation in a CHL-induced SCV in the gene encoding the Tyrosine-tRNA ligase. This mutation could account for the generation of SCVs, since inhibitors of this ligase strongly decrease bacterial growth, but this mutation was only found in one of the six SCVs [38]. Most interestingly, we were unable to find any SNPs in one of the isolated CHL SCVs.

Unexpectedly, the antibiotic resistance observed here is transient, as would be anticipated for heteroresistance (HR), suggesting a high fitness cost of the mutations detected. In support of this HR hypothesis we found that these SCVs meet all the criteria set forth for HR: there are subpopulations present at a frequency greater than 10^{-7}, with an MIC higher than 8x that of the main population, and reversion of the resistant subpopulation occurs in short order [39]. To our knowledge, this is the first report of both the spontaneous evolution of HR as well as HR to bacteriostatic drugs.

Materials and Methods

Bacterial Strains. *E. coli* MG1655 was obtained from the Levin Lab bacterial collection [40]. pAM34 with the origin of replication under control of an IPTG promoter and an ampicillin resistance cassette was obtained from Calin Guet from IST Austria [14].

Growth Media. LB (Lysogeny Broth) (244620) was obtained from BD. The DM (Davis Minimal) minimal base without dextrose (15758-500G-F) was obtained from Sigma Aldrich (7 g/L dipotassium phosphate, 2 g/L monopotassium phosphate, 0.5 g/L sodium citrate, 0.1 g/L magnesium sulfate, 1 g/L ammonium sulfate). MHII plates were made from MHII broth (212322) obtained from BD. Glucose (41095-5000) was obtained from Acros. LB agar (244510) for plates was obtained from BD.

Growth Conditions. Unless otherwise stated, all experiments were conducted at 37°C with shaking.

Sampling bacterial densities. The densities of bacteria were estimated by serial dilution in 0.85% saline and plating. The total density of bacteria was estimated on LB agar plates.

Antibiotics. Chloramphenicol (23660) was obtained from United States Pharmacopeia. Azithromycin (3771) was obtained from TOCRIS. Ampicillin (A9518-25G) was obtained from Sigma Aldrich. Isopropyl β-D-1-thiogalactopyranoside (IPTG; I56000-5.0) was obtained from Research Products International. All E-tests were obtained from bioMérieux.

Estimating Minimal Inhibitory Concentrations. Antibiotic MICs were estimated using E-tests on MHII plates or via broth microdilution [41,42].

Long Term Experiments. Flasks containing 10 mL of DM with 1000 μg/mL of glucose and an initial density of 10^5 CFU/mL cells were grown at 37ºC with shaking for 30 days. *E. coli* MG1655 was grown with either no drug, 4x MIC of CHL or 3x MIC of AZM. Samples were taken every 5 days and plated on LB agar plates.

Long Term Experiments with non-replicative plasmid. Flasks containing 10 mL of DM with 1000 μg/mL of glucose and an initial density of 10^5 CFU/mL cells were grown at 37ºC with shaking for 30 days. *E. coli* MG1655 pAM34 was grown with either no drug, 4x MIC of CHL or 3x MIC of AZM. Samples were taken every 5 days and plated on LB agar plates as well as 100 μg/mL Ampicillin and 0.5 mM IPTG LB agar plates.

Sequencing. Complete genomes were obtained with hybrid Illumina/Nanopore sequencing by SeqCenter. Samples were extracted from single colonies using Zymo Quick-DNA HMW MagBead Kit. Oxford Nanopore Sequencing library prep was performed with PCR-free ligation library prep using ONT's V14 chemistry. Long read sequencing was performd using R10.4.1 flowcells on a GridION with basecalling performed by Guppy in Super High Accuracy mode. Illumina libraries were prepared and sequenced per SeqCenter's standards. Quality control and adapter trimming was performed with bcl-convert and porechop for Illumina and ONT sequencing respectively. Hybrid assembly with Illumina and ONT reads was performed with Unicycler [43]. Assembly statistics were recorded with QUAST [44]. Assembly annotation was performed with Prokka [45].

Growth Rate Estimation. Exponential growth rates were estimated from changes in optical density (OD600) in a Bioscreen C. For this, 24-hours stationary phase cultures were diluted in LB or glucose-limited liquid media to an initial density of approximately 105 cells per mL. Five replicas were made for each estimate by adding 300μl of the suspensions to the wells of the Bioscreen plates. The plates were incubated at 37°C and shaken continuously. Estimates of the OD (600nm) were made every five minutes for 24 hours in LB and 48 hours in glucose-limited medium. Normalization, replicate means and error, growth rate, lag and maximum OD were found using a novel R Bioscreen C analysis tool accessible at https://josheclf.shinyapps.io/bioscreen_app.

Residual Growth. After 30 days the cultures were centrifuged and filtered through a 0.22 μm filter. Strain resistant for CHL (Strain 1012 from the US Center for Disease Control's MuGSI Isolate Bank which is *cmlA5* positive), AZM (Strain 1007 from the US Center for Disease Control's MuGSI Isolate Bank which is *mph(A)* positive), or just MG1655 were added to the supernatants and allowed to grow for 24h.

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Data Availability Statement

All the data generated are available in this manuscript and its supporting supplemental material. Copies of the genomes sequence here have been made available under the NCBI BioProject ID PRJNA1032893.

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