Supporting Information for

The Evolution of Heteroresistance and Small Colony Variants in *Escherichia coli* Following Long Term Bacteriostatic Drug Exposure

Teresa Gil-Gil*, Brandon A. Berryhill*, Joshua A. Manuel, Andrew P. Smith, Ingrid C. McCall, Fernando Baquero, Bruce R. Levin*

***Co-first authors**

Mathematical Model Description

In the absence of antibiotics, the maximum growth rates of these populations are, respectively, v_{maxN}, v_{maxS}, and v_{maxH} (>0 per cell per hour). In the presence of antibiotics, the minimum growth rate (maximum death rates) of these populations are v_{minN}, v_{minS}, and v_{minH} (<0 per cell per hour), and the respective MICs of these populations are MIC_N, MIC_S, and MIC_H μg/mL. The net rates of growth of these three populations are proportional to the concentration of the resource, *r* μg/mL and the concentration of the antibiotic, *A* μg/mL [1,2]. The equations below are given in the general form where *X* is either N, S, or H.

$$\Pi_X(A,r) = v_{maxX} - \left[\frac{(v_{maxX}-v_{minX})\cdot\left(\frac{A}{MIC_X}\right)^{Kx}}{\left(\frac{A}{MIC_X}\right)^{Kx}-\left(\frac{v_{minX}}{v_{maxX}}\right)}\right]\cdot \psi x(r) \quad \textbf{Eq.1}$$

$$yx(r) = \frac{r}{(r+k_x)} \quad \textbf{Eq.2}$$

K_x, the Hill coefficient [1], is a shape parameter where the greater the value of K_x the more acute the function. The parameter k_x, the Monod constant, is the concentration of the resource when the growth rate is half its maximum value [3].

With the above definitions and assumptions, the rates of change in the densities of the populations of bacteria and the change in resource and antibiotic concentrations are given by the below set of coupled differential equations (Eq. 3-7).

$$\frac{dN}{dt} = \Pi_N(A,r)\cdot(N + N\cdot(\mu_{sn}-\mu_{ns})) \quad \textbf{Eq. 3}$$

$$\frac{dS}{dt} = \Pi_S(A,r)\cdot(S + S\cdot(\mu_{ns}-\mu_{sn}+\mu_{hs}-\mu_{sh})) \quad \textbf{Eq. 4}$$

$$\frac{dH}{dt} = \Pi_H(A,r)\cdot(H + H\cdot(\mu_{sh}-\mu_{hs})) \quad \textbf{Eq. 5}$$

$$\frac{dr}{dt} = -e\cdot(N\cdot v_{maxN} + S\cdot v_{maxS} + H\cdot v_{maxH}) \quad \textbf{Eq. 6}$$

$$\frac{dA}{dt} = -da\cdot A \quad \textbf{Eq. 7}$$

The conversion efficiency, e μg [4], is the amount of the limiting resource needed to produce a new cell (Eq. 6) and the parameter da is the hourly rate of decline in the concentration of the antibiotic in μg/hour (Eq. 7).

We use Berkeley Madonna and the Euler method to generate numerical solutions to these differential equations. In these simulations the changes in the densities of these populations and concentrations of the limiting resource are deterministic. The generation of mutants, however, is stochastic and simulated by a Monte Carlo process [5]. At each time interval, t to dt (where dt is the step size), a random number ($0 \leq z \leq 1$) from a rectangular distribution is generated. If the random number is less than the probability in Equation 8, we add 1/dt to the X population. For Equation 8, Vol is the volume of the vessel which we simulate as 10 mL and X is the density of the respective population.

$$P(\mu) = \mu_X \cdot X \cdot dt \cdot \text{Vol} \quad \textbf{Eq. 8}$$

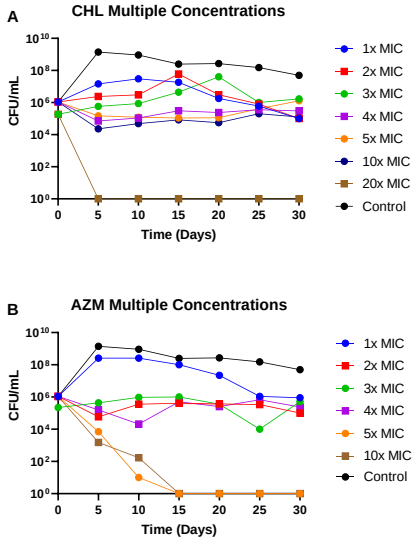

Fig S1. Selection of bacteriostatic concentrations of CHL and AZM. CFU/mL of *E. coli* MG1655 in cultures with varying concentrations of **(A)** CHL or **(B)** AZM for 30 days.

Blue line- 1x MIC, Red line- 2x MIC, Green line- 3x MIC, Purple line- 4x MIC, Orange line- 5x MIC, Brown line- 10x MIC, Black line- Drug-free control.

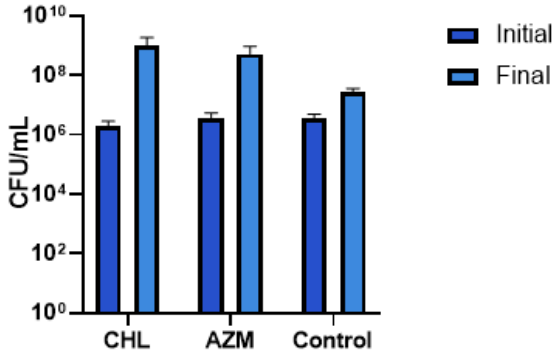

Fig S2. Growth of *E. coli* strains in supernatants from day 30 of the long term experiments. $5x10^6$ CFU/mL of AZM-resistant, CHL-resistant, or *E. coli* MG1655 strains when inoculated in the cell-free supernatants of the 30-day time point, in which the corresponding antibiotic was present. Error bars represent the standard deviation of 4

biological replicates. Dark blue- Initial (Time= 0 hours), Light blue- Final (Time= 24 hours).

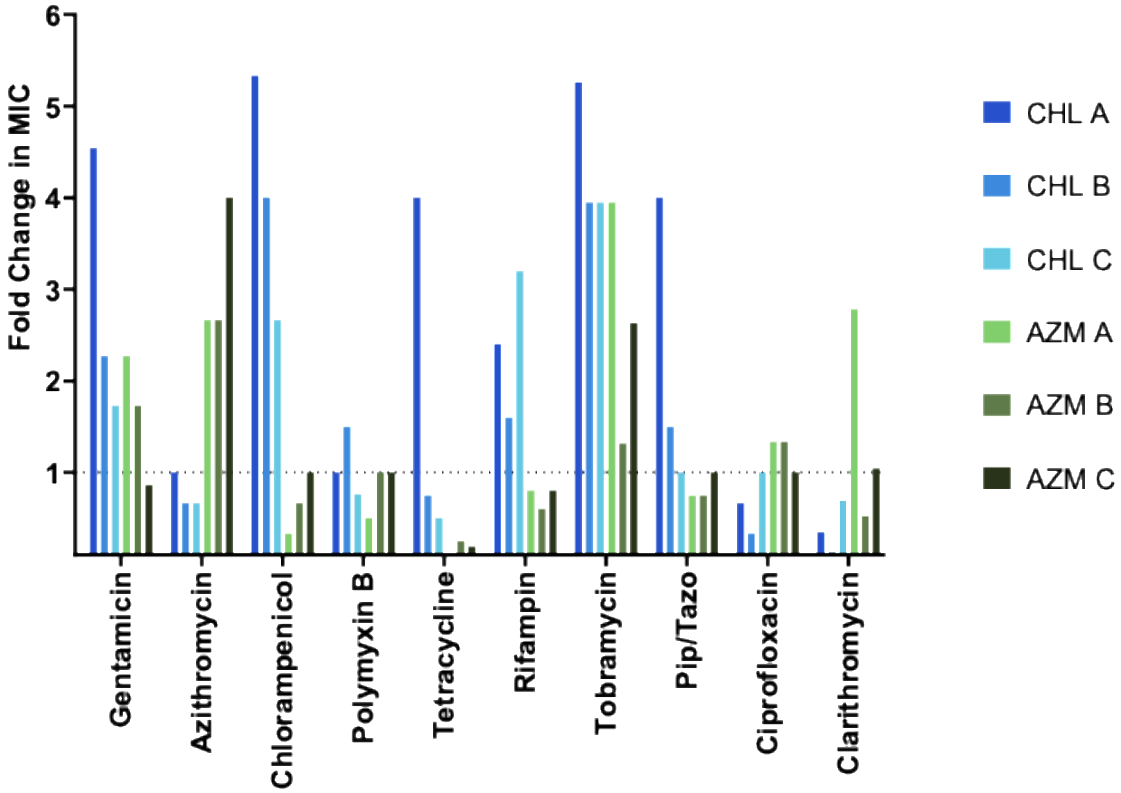

Fig S3. SCV collateral sensitivities and cross-resistances. Ratio of the MICs of the 6 SCVs compared to the ancestral *E. coli* MG1655 strain measured by E-test. Pip/Tazo stands for piperacillin/tazobactam.

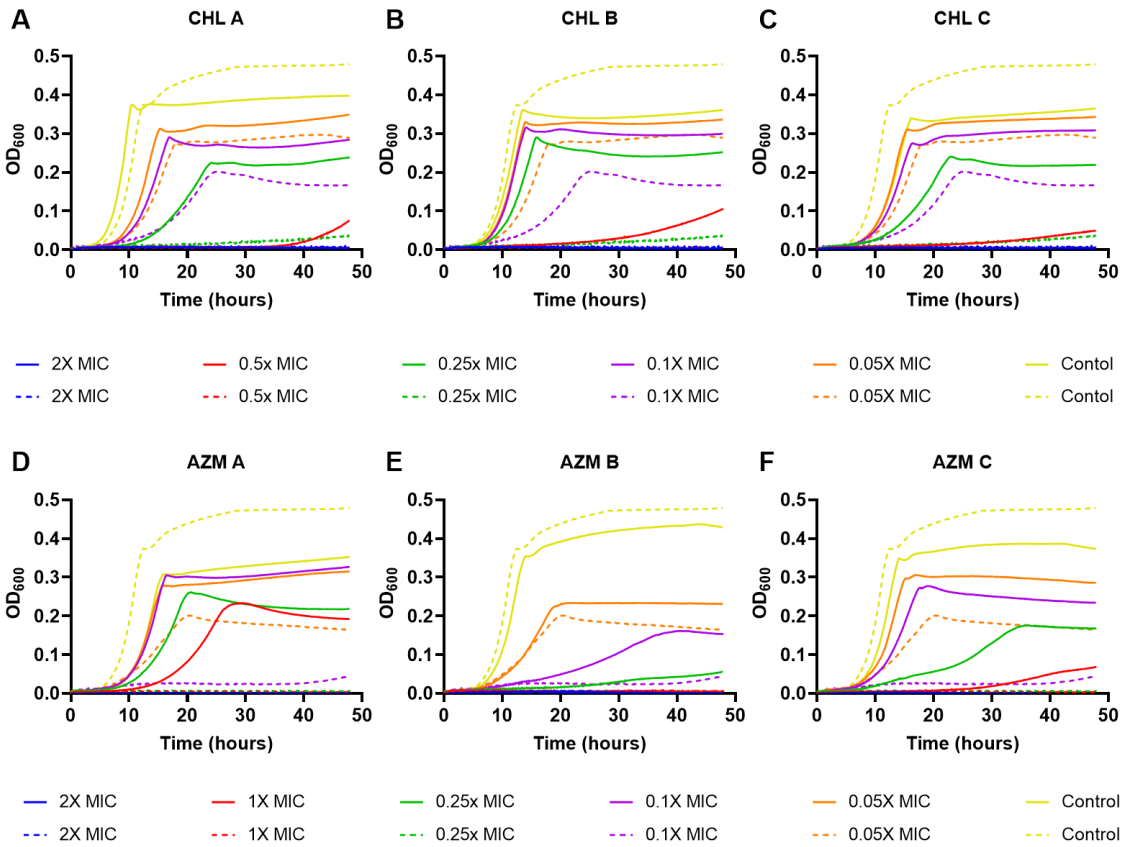

Fig S4. Growth dynamics of CHL SCVs (solid lines A-C), AZM SCVs (solid lines D-F) and *E. coli* MG1655 (dashed lines A-F). Changes in optical density (600nm) of *E. coli* MG1655 exposed to five different concentrations of both drugs for 48 hours in minimal media. Lines are representative of the average of five technical replicas and normalized to

the time zero optical density. Each concentration is shown as a fraction of the MIC for the noted drug, 6.25 μg/mL.

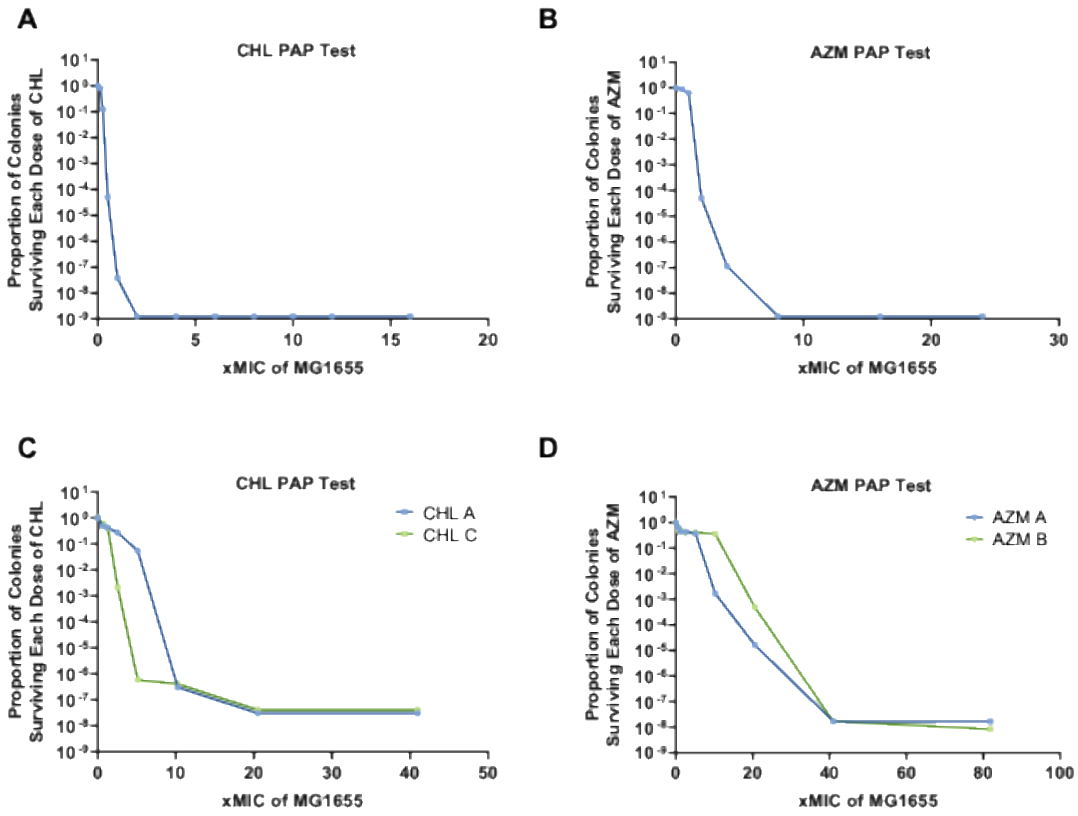

Fig S5. **PAP tests. (A)** PAP test of *E. coli* MG1655 with CHL. **(B)** PAP test of *E. coli* MG1655 with AZM. **(C)** PAP test of CHL SCVs. **(D)** PAP test of AZM SCVs.

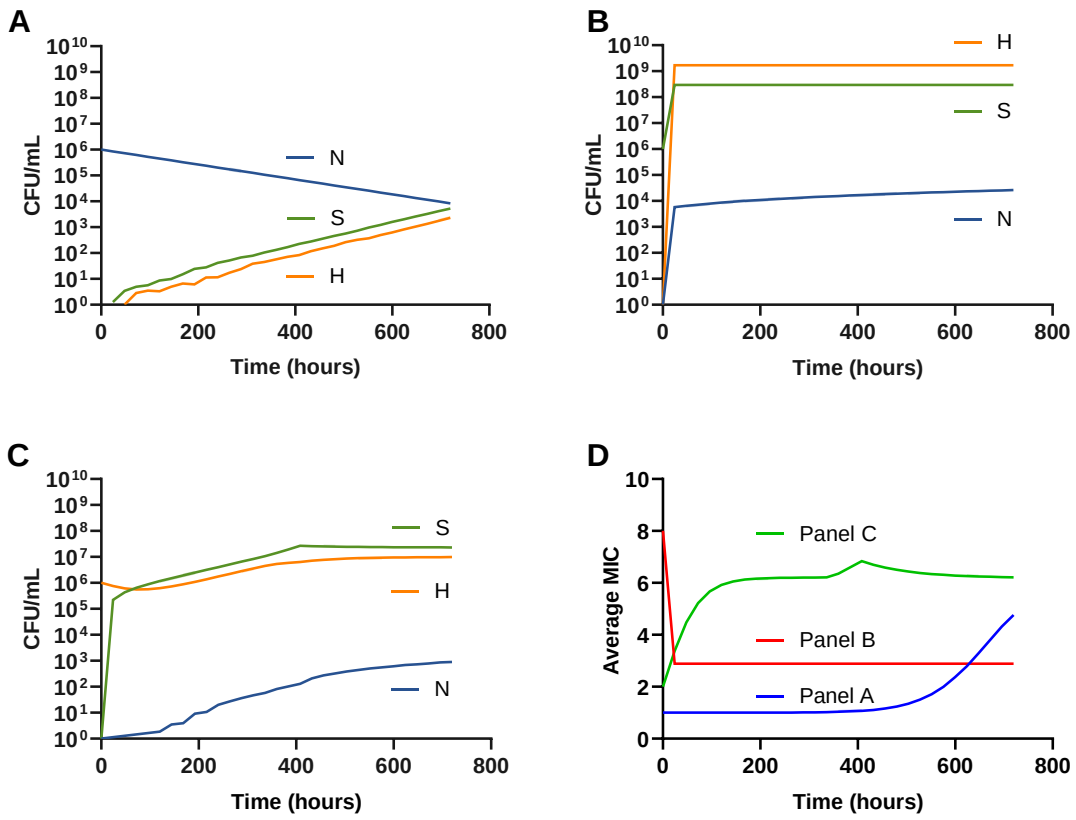

Fig S6. Computer simulations of the proposed model of evolved HR. Parameters used for these simulations are e= 5x10^{-7} µg/cell; v_{maxN} = 1.0, v_{maxS} = 0.5, v_{maxH} = 1.0 per cell per hour; v_{min} = -0.01 per cell per hour; K=1; k=1; MIC$_N$=1.0, MIC$_S$=8.0, MIC$_H$=2.0 µg/ml; da= 0 µg/hour; μ_{ns}=1x10^{-8}, μ_{sn}=1x10^{-8}, μ_{sh}=1x10^{-3}, μ_{hs}= 1x10^{-3} per cell per hour. **(A)** H and S selection from an initial sensitive population. Parameters are N=1x10^6 cells/mL; A=3 µg/mL. **(B)** H selection from an initial SCV population in the absence of the drug. Parameters are S=1x10^6 cells/mL; A=0 µg/mL. **(C)** S selection from an initial H population in the presence of the drug. H=1x10^6 cells/mL; A=3 µg/mL. **(D)** Changes in the average MIC of the system over the simulations shown (A, B, and C).

Supplemental Table 1. SCV Genomic Changes

SCV	GENE	NUCLEOTIDE POSITION	TYPE	NUCLEOTIDE CHANGE	EFFECT	PRODUCT
CHLB	*tyrS*	2166796	Ins	T → TTAACGG	Conservative in-frame insertion Asn387→Gly388dup	Tyrosine-tRNA ligase
CHLC	*rplD*	431632	SNP	A → G	Missense variant Lys63Arg	50S ribosomal protein L4
		3178132	SNP	C → A		Unannotated region
AZMA	*rplV*	433741	Del	ATGAAGCGCATTAT GCCGCGTGCAAAAG GTCGTGCAGATCGC ATCC → A	Disruptive in-frame deletion Ile85Arg99del	50S ribosomal protein L22
	lon_1	2864809	SNP	C → A	Stop gained Ser422	Lon protease
AZMB	*citG*	3235035	SNP	A → G	Missense variant Glu234Gly	2-(5"-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase
	citG	3235041	SNP	G → T	Missense variant Gly236Val	2-(5"-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase
AZMC	*citG*	3235035	SNP	A → G	Missense variant Glu234Gly	2-(5"-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase
	citG	3235041	SNP	G → T	Missense variant Gly236Val	2-(5"-triphosphoribosyl)-3'-dephosphocoenzyme-A synthase
	acrB_2	3399923	SNP	G → T	Missense variant Gly236Val	Multidrug efflux pump subunit AcrB

Ins: insertion; SNP: Single Nucleotide Polymorphism; Del: deletion

SI References

1 Regoes, R. R. *et al.* Pharmacodynamic functions: a multiparameter approach to the design of antibiotic treatment regimens. *Antimicrob Agents Chemother* **48**, 3670-3676, doi:10.1128/aac.48.10.3670-3676.2004 (2004).

2 Berryhill, B. A. *et al.* What's the Matter with MICs: Bacterial Nutrition, Limiting Resources, and Antibiotic Pharmacodynamics. *Microbiology Spectrum* **11**, e04091-04022, doi:doi:10.1128/spectrum.04091-22 (2023).

3 Monod, J. THE GROWTH OF BACTERIAL CULTURES. *Annual Review of Microbiology* **3**, 371-394, doi:10.1146/annurev.mi.03.100149.002103 (1949).

4 Stewart, F. M. & Levin, B. R. Partitioning of Resources and the Outcome of Interspecific Competition: A Model and Some General Considerations. *The American Naturalist* **107**, 171-198, doi:10.1086/282825 (1973).

5 Metropolis, N. & Ulam, S. The Monte Carlo Method. *Journal of the American Statistical Association* **44**, 335-341, doi:10.1080/01621459.1949.10483310 (1949).