Computational and Structural Biotechnology Journal 16 (2018) 307–315

COMPUTATIONAL AND STRUCTURAL BIOTECHNOLOGY JOURNAL

journal homepage: www.elsevier.com/locate/csbj

Intermediate Levels of Antibiotics May Increase Diversity of Colony Size Phenotype in Bacteria

Lewis Lee [a], Van M. Savage [a,b,c], Pamela J. Yeh [b,c,*]

[a] Department of Biomathematics, David Geffen School of Medicine, University of California, Los Angeles, USA
[b] Department of Ecology and Evolutionary Biology, University of California, Los Angeles, USA
[c] Santa Fe Institute, Santa Fe, NM, USA

ARTICLE INFO

Article history:
Received 21 May 2018
Received in revised form 21 August 2018
Accepted 22 August 2018
Available online 30 August 2018

Keywords:
Sub-lethal
Drug concentration
Variation
Genetic
Phenotypic
Selection
Disturbance

ABSTRACT

Antibiotics select for resistant bacteria whose existence and emergence is more likely in populations with high phenotypic and genetic diversity. Identifying the mechanisms that generate this diversity can thus have clinical consequences for drug-resistant pathogens. We show here that intermediate levels of antibiotics are associated with higher levels of phenotypic diversity in size of colony forming units (cfus), within a single bacterial population. We examine experimentally thousands of populations of bacteria subjected to different disturbance levels that are created by varying antibiotic concentrations. Based on colony sizes, we find that intermediate levels of antibiotics always result in the highest phenotypic variation of this trait. This result is supported across bacterial densities and in the presence of three different antibiotics with two different mechanisms of action. Our results suggest intermediate levels of a stressor (as opposed to very low or very high levels) could affect the phenotypic diversity of a population, at least with regards to the single trait measured here. While this study is limited to a single phenotypic trait within a single species, the results suggest examining phenotypic and genetic variation created by disturbances and stressors could be a promising way to understand and limit variation in pathogens.

1. Introduction

There is growing interest in using ecological and evolutionary principles to manage and treat a wide range of health problems [1], from cancer [2–5] to obesity [6] to infectious diseases [7–10]. Work on the evolution of drug resistant pathogens has primarily focused on drug dosages as a source of novel selection pressure. However, selection is only one factor in the rate of evolution of resistance. Fisher's fundamental theorem relates the rate of change in allele frequency to the product of the selection pressure and the genetic variance [11]. Thus, increases in the variance or selection coefficient can both have strong effects. Although variation is critical to the rate of evolution, it has not been as well studied in relation to drug resistance as selection pressure.

Here we ask how different levels of antibiotics affect phenotypic variation in bacteria. Because the amount of variation could affect the rate of evolution towards resistance, this knowledge could have profound consequences for slowing the evolution of drug resistance. Specifically, we assess how varying concentrations of antibiotics and varying population densities affects the variation in one phenotypic trait, the size of bacterial colonies. We chose to examine colony size and coefficient of variation (CV = standard deviation/mean) in colony size because we can take single "snapshots" in time of a large number of colonies within a petri dish, making these phenotypes easy to measure (Fig. 1 and Supplementary Fig. 1a-b). Colony size is also a useful phenotype to study because increases in its variation have been shown to be associated with increases in genetic diversity [12–15]. This correlation between phenotypic and genetic diversity is not surprising because experimental evidence points to colony size being a heritable trait with a genetic basis [14,15]. Due to its ease of measurement and its association with genetic changes, the distribution of colony sizes and its central moments could be excellent proxies for mutant diversity that are inexpensive and quick to obtain. These proxies could be one step towards enabling predictions for the likelihood of resistance in the clinic and the field based on specific drug dosages.

To obtain highly accurate measurements of the size of each colony in our digital images, we developed customized software, MeasureIt (Fig. 1) that extracts areas—measured by total number of pixels, not approximated from diameters of circles—of the colonies. Digital technology in the microbial lab is increasingly being used to track colonies with time-lapsed imaging for determining growth strategies and detecting rare phenotypes. Our automated quantitative measurement of colony sizes is able to detect small variation and irregular-sized

* Corresponding author at: Charles E. Young Dr South, Los Angeles, CA 90095, USA.
E-mail address: pamelayeh@ucla.edu (P.J. Yeh).

https://doi.org/10.1016/j.csbj.2018.08.004

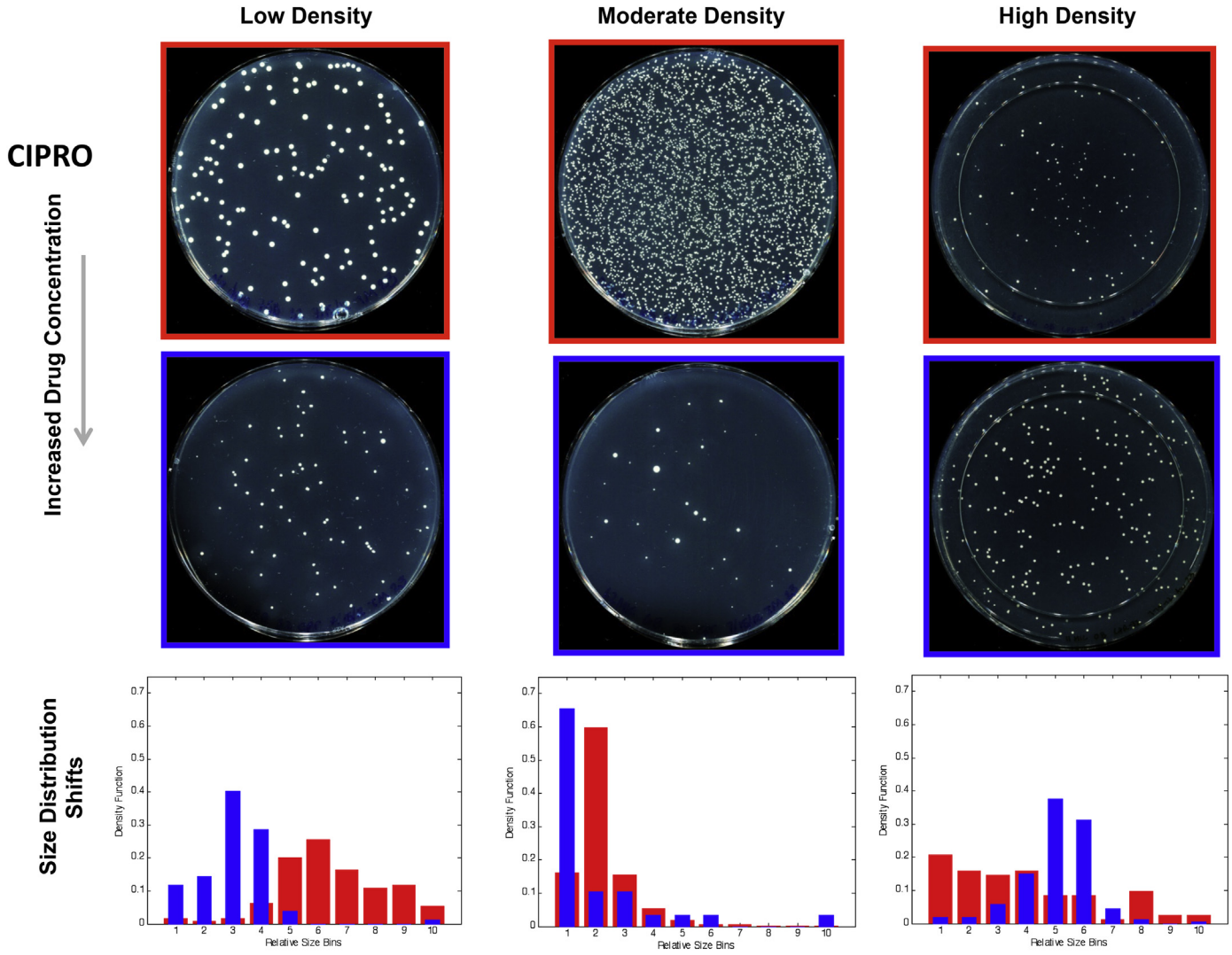

Fig. 1. Shape shifts in colony size distribution with increasing drug concentration and bacterial density. Images for *Staphylococcus* spp. grown in petri dishes across a range of bacterial densities (increasing from left to right) and in the presence of the drugs ciprofloxacin, amikacin, and streptomycin. Separate histograms are constructed at low, moderate, and high densities with each histogram using red bars to indicate data at low drug concentrations and blue bars at high drug concentrations. These images reveal how number of colonies and average size of a colony change, and the extracted data and the histograms quantify these changes. Drug concentrations were as follows: Low Density lower drug concentration: no drugs, Low Density higher drug concentration: 0.144 μg/ml; Moderate Density lower drug concentration: 0.072 μg/ml, Moderate Density higher drug concentration: 0.216 μg/ml; High Density lower drug concentration: 0.63 μg/ml, High Density higher drug concentration: 1.98 μg/ml.

colonies, which enables high-throughput processing to quickly obtain large datasets of colony size.

2. Methods

2.1.1. Laboratory

2.1.1.1. Bacterial strain. We used an environmental strain of Staphylococcus spp. cultured from the public transportation system in Portland, Oregon and identified via 16S rRNA subunit sequencing [16]. The bacteria falls within the "Epidermidis cluster group." Due to the uncertainty in exact strain identification, we call this strain *Staphylococcus* spp. here.

We streaked out colonies on a plate and used one single colony. We created a master tube of *S. epidermidis* from this colony, with several hundred aliquots made from this original master tube; both master tube and aliquots were kept frozen at −80 °C with 17% glycerol. We used one aliquot for each experiment. Strains were grown in Luria broth (LB) media to exponential growth phase at 37 °C with shaking via an incubated shaker (VWR model 1585), and these broths were then spun down and *S. epidermidis* was sampled and grown on agar plates to examine numbers and size variation of colonies. Thus, all experiments were conducted by starting with the same species, strain, and master tube from a single colony, enabling us to begin with minimal genetic variation.

Table 1
List of drugs used and the corresponding ranges of concentrations that yielded surviving colonies. Column 3 lists the drug concentration that resulted in the highest amount of variation in colony size. All concentrations are measured in MIC units.

Drug	Range of concentrations	Peak coefficient of variation
Ciprofloxacin	0-20MIC	8 MIC
Amikacin	0-64 MIC	23 MIC
Streptomycin	0-200 MIC	80 MIC

2.1.1.2. Antibiotics. We used three antibiotics: ciprofloxacin, amikacin, and streptomycin (Sigma-Aldrich) (see Table 1). Ciprofloxacin, a synthetic second-generation fluoroquinolone, disrupts DNA synthesis by inhibiting bacterial enzymes DNA gyrase and topoisomerase, both

of which are involved in the unwinding and supercoiling of DNA during DNA replication. Amikacin is a semi-synthetic aminoglycoside and affects protein synthesis by binding to the 30S ribosomal subunit, interfering with mRNA translation and thus the elongation of polypeptides. Streptomycin, like amikacin, is an aminoglycoside that inhibits protein synthesis. Drugs were stored as frozen stocks in a -20 °C freezer, and were thawed at 4 °C before use.

2.1.1.3. Experimental set-up. For each drug, we first determined the minimum inhibitory concentration (MIC) (minimum concentration of drug that inhibits wild type growth) and the mutant prevention concentration (MPC) of each drug [17,18](minimum concentration of drug that prevents any resistant mutants from surviving). MIC was measured by first conducting two-fold serial dilutions and then equidistant dilutions of the antibiotic in a 96 well plate. Two-fold serial dilutions typically start with a drug at 10× estimated MIC (obtained from literature), then diluted to 5× MIC, then 2.5× MIC and so on until the 11th well has approximately 0.0098× MIC. The 12th (last) well is used as a no-drug control. We then narrow down the MIC by doing equidistant dilutions by taking the narrowed range where the MIC should be based on the serial dilutions, and using equidistant drug concentrations between wells 1 and 11, again leaving well 12 as a no-drug control.

Approximately 10^3–10^4 cells were inoculated in each well and allowed to grow for 22 h while being shaken and incubated at 37 °C. Plates were read at OD600 using Victor X (Perkin Elmer), and the MIC was the lowest drug concentration that yielded no growth. Using LB plates containing dilutions of antibiotic, MPC was determined as the antibiotic concentration (in MIC units) that prevents the growth of any resistant mutants following an inoculum at very high densities of $>10^{10}$ cells. Bacteria were plated on petri dishes and dishes were incubated at 37 °C for 72–96 h. Viable cells, seen as colonies, were quantified manually and through the scanning and automated extraction methods (below).

Each experiment consisted of approximately 80 plates with a range of drug concentrations and bacterial densities. We poured plates with drugs mixed directly into the agar solution, ranging from 0.25 liquid MIC to approximately 5–10 MIC beyond the identified MPC drug concentration. For the lower concentrations, we examined 0.2 to 2.0 x liquid MIC at every 0.2 MIC interval, and thus were able to determine if liquid MIC matched with agar MIC. We plated a range of bacterial cells (potentially from ~100 cells to 10^{10} cells per plate) at each drug concentration. For lower drug concentrations, we typically plated 3 different bacterial densities. For higher drug concentrations, we typically plated only 1 bacterial density (the highest at 10^{10} cells). This is because at higher drug concentrations, very few cells survive, and thus only a very high density of initial cells would yield any countable results. We defined intermediate levels of antibiotic as any concentration that was not the highest nor the lowest antibiotic concentration used. After allowing bacteria to grow on the petri dishes for 72–96 h, we scanned each petri dish using a standard office scanner (Epson 1800; more details below). For each drug, we conducted 10–15 replicate experiments.

2.1.1.4. Digital image processing. Each petri dish was placed on a scanner with a fixed black felt background and then scanned at an 1100 × 1100 pixel resolution. The resulting image was stored as a standard jpg image file. We developed software that could identify individual colonies. Our method for digital recognition of individual colonies consists of serial application of (1) a circular Hough transform and Otsu's thresholding methods and (2) watershed segmentation. First, our software identifies the edge of the dish and also contiguous objects within that are above a chosen threshold for intensity. Next, the software categorizes the topology of these contiguous objects and imposes an approximate boundary to separate adjoining objects.

2.1.1.5. Optimization of measurement of colony sizes. Several steps were taken to review and optimize the quality of the image-extracted data. Our ERO (Extract-Review-Optimize) process facilitates quality review and verification of all processed images. First, plates that did not meet experimental quality due to observed contamination or plate defects (often identifiable by statistical anomalies) were documented and excluded from image processing. For the high-quality images that remained, we extracted data, and then reviewed the results by using the following criteria to assure correct identification of colonies: (i) the centroid location of the individual "extracted" colonies should be consistent with visual inspection, (ii) the counts of the colonies should match visual inspection for subsections of the dish that are countable, and (iii) Dust, bubbles in the medium, features of the dish, and scanning artifacts (e.g., light reflections) should not be counted or identified as colonies. We excluded these cases or anything indistinguishable from these. The selection of light intensity threshold in Otsu's method to convert colour to grayscale is crucial for filtering out these non-colony objects, and the correct threshold depends on the scanning parameters. Following these extract and review steps, we further optimized by removing edge reflections and "partial" colonies along the dish perimeter.

2.1.1.6. Extracted data of colony sizes. For each colony, we measure total area in terms of pixels, estimate the perimeter, and place a centroid in the middle of the colony. This allows us to identify each colony by a single point using the MATLAB image processing platform. Counting all of these centroids gives the total number of colonies in each dish. Extraction of colony data from petri dishes can be run in batch or individually using the software. A vector corresponding to each data element (e.g., colony size) is stored in a MATLAB cell array along with corresponding experimental information (e.g., bacterial density, drug concentration) for each plate. Standardized measures and statistics discussed below were also computed and stored in a matrix and exported in ASCII format for analysis in R. In this way, we automated the extraction of a size distribution of colonies from each dish.

2.2. Statistical analysis

To characterize how colony size distribution varies with bacterial density and antibiotic drug concentration, two analyses were conducted using statistics computed for the colony sizes from subsets of plates and experimental series. All statistical analyses were performed using the R statistical platform.

First, colony size variation was assessed and compared across bacterial densities in the absence of any antibiotic drug. Mean, standard deviation (SD), coefficient of variation (CV), skewness, kurtosis, and interquartile range were examined as a function of bacterial density. Tests for departure from normality were performed using both the Kolmogorov-Smirnov and the D'Agostino K^2 tests at 0.05 significance level to identify any shifts to non-normality as a function of bacterial density. Collective analysis of no-drug plates also provides an additional control for comparability and outliers in laboratory conditions.

Second, colony size variation was analyzed in escalating doses of single antibiotic drugs. Trends in measures of centrality and dispersion were similarly characterized both for a fixed bacterial density across a range of drug concentrations and for a fixed drug concentration across a range of bacterial densities. Non-linearity and rates of change in size variation, minimum and maximum colony size, and similarities between metrics for the distributions were found for each single-drug and compared with the no-drug results observed in (I). Statistical comparisons were also made for differences in dispersion parameters (e.g., coefficient of variation (CV)—standard deviation divided by the mean) between the single- and no-drug size distributions at the same bacterial densities.

3. Results

Bacterial populations that were exposed to intermediate levels of disturbance—created by intermediate amounts of antibiotics—exhibited

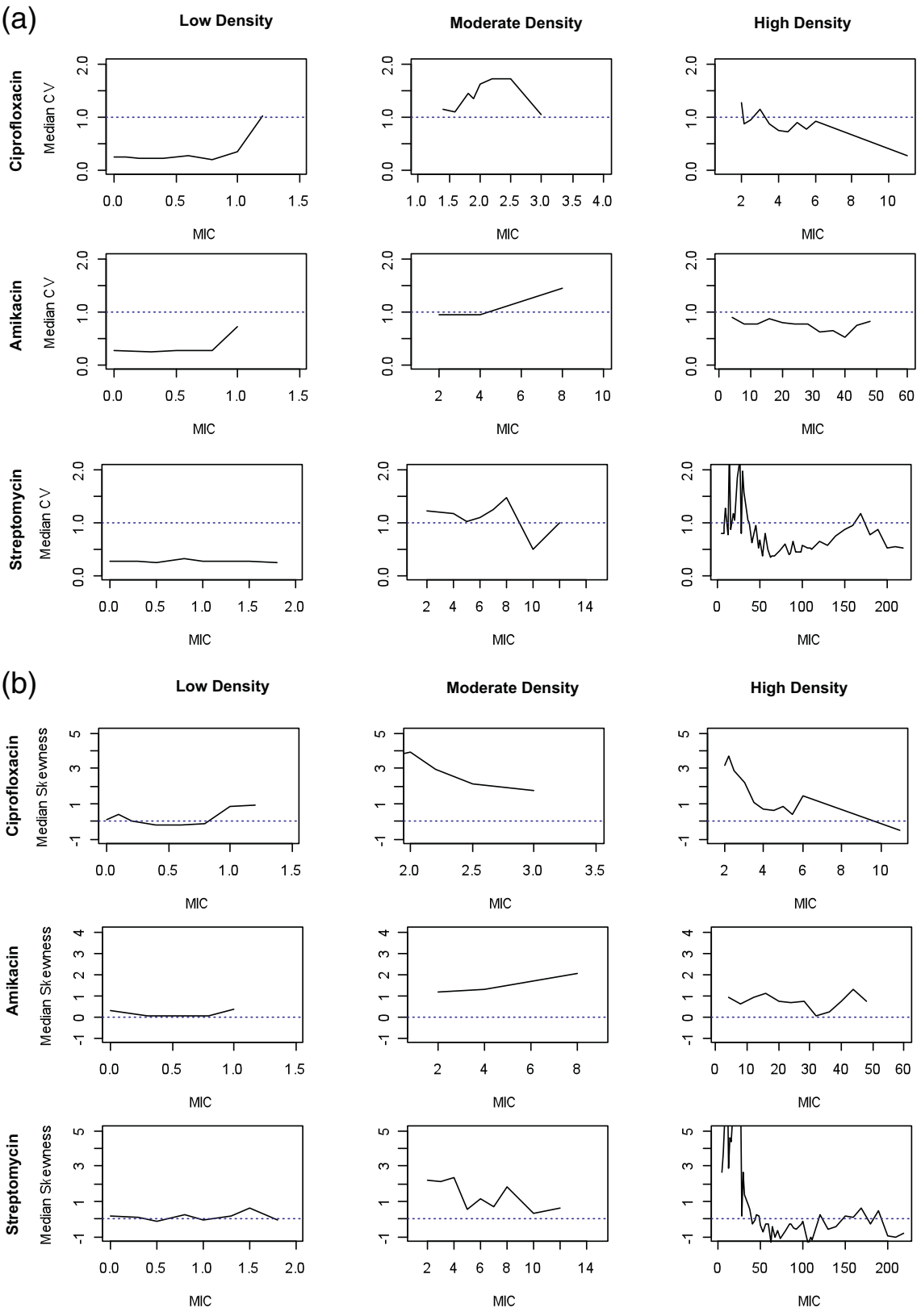

Fig. 2. Size variation and increasing concentration of three drugs with fixed bacterial density. Plots of the median Coefficient of Variation (CV = standard deviation/mean) (a), median skewness (b), and median kurtosis (c) in colony size versus drug concentration for three different drugs across a range of bacterial densities. Expectations for normal distribution are represented by dashed lines in Fig. 2b-c. The drug concentration is measured in units of the Minimum Inhibitory Concentration (MIC)—minimal drug concentration that inhibits wild-type growth—and has a different absolute level of concentration for each drug. The blue dashed horizontal line at CV = 1 indicates the mean and standard deviation are equal. High bacterial densities allow a larger range of drug concentrations because there is an increased chance that some cells (possibly mutants) will survive at high concentrations. An increase in CV is often observed as the drug concentration increases above 1 MIC. The largest values of CV, indicating the most variation, tend to occur at intermediate levels of both bacterial density and drug concentration.

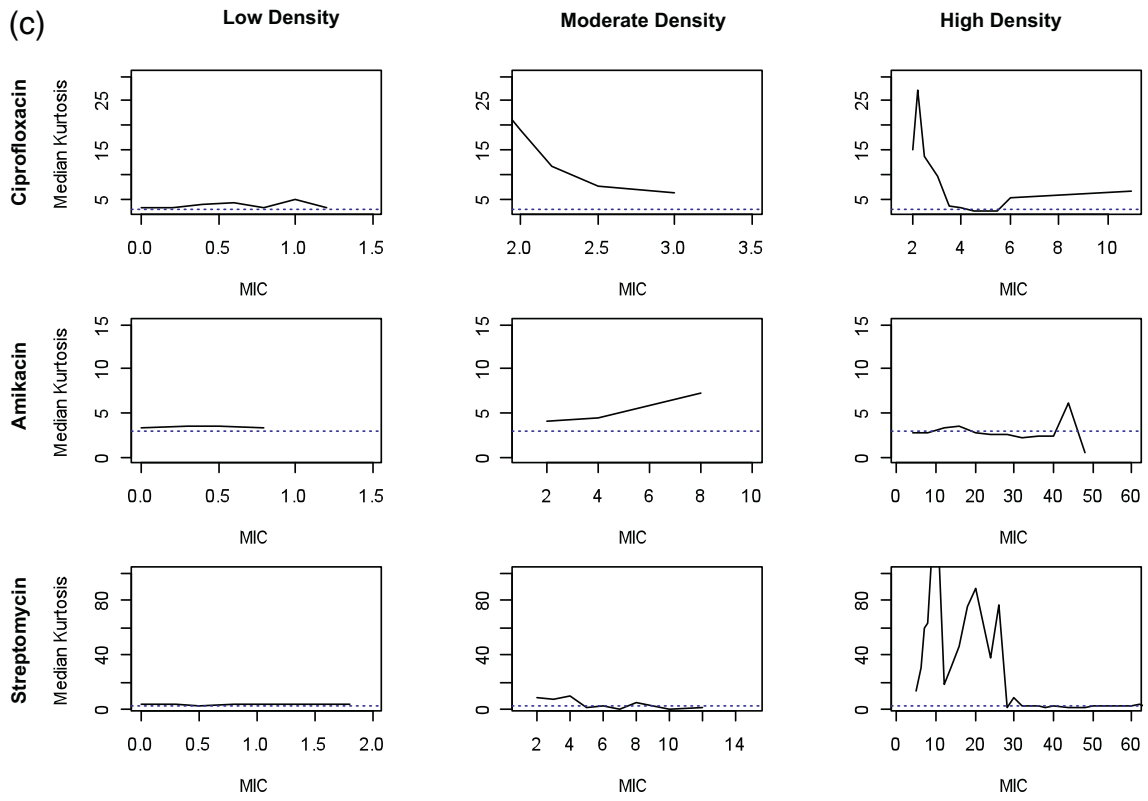

Fig. 2 (*continued*).

the highest CV (Fig. 2a). The CV stays approximately constant and with a value less than but close to 1 for antibiotic concentrations below MIC for each drug (Fig. 2a). However, the CV begins to change significantly as the concentration is increased above the MIC. Specifically, the median CV is always >1 at intermediate levels of antibiotics, while it is always <1 at low levels of antibiotics and almost always <1 at high levels of antibiotics. Plots of the mean CV lead to the same conclusions (Supplementary Fig. 2). This pattern held true for all three antibiotics tested.

Beyond the mean, variance, and CV, we can measure higher-order moments such as the skewness and kurtosis of the distribution of colony sizes (Fig. 2 and Supplementary Fig. 3). These moments can be used to measure how strongly a distribution deviates from normality (additive random process) or how well it matches other distributions such as log-normal (multiplicative random process), power law (self-similar process), binomial (random binary process), or Poisson (random number of hits model). For a normal distribution, the skewness and all odd-order moments are zero, indicating there is no asymmetry. All even-order moments, such as the kurtosis that measures rate of decrease from the peak, are well known for the normal distribution.

We also examined another stressor besides drugs: bacterial density. First, we examined bacterial density in the absence of drugs. While the CV increases with density in no-drug cases (Fig. 3), we find that intermediate densities lead to the highest CV in the presence of drugs (Fig. 2), although streptomycin shows the highest levels of CV in a high density, low-drug combination. Second, we combined our two stressors experimentally and analyzed three-dimensional plots of median CV versus both antibiotic concentration and bacterial density (Fig. 4 and Supplementary Fig. 4a-e). This three-dimensional plot enables the study of the combined effects of drug concentration and bacterial density, and also allows measures of CV at higher initial bacterial densities. The peak CV occurs in the middle of the three-dimensional plot, indicating that CV is highest when both drug concentration and bacterial density are at intermediate levels. We have now shown that intermediate levels of stressors yield the highest variation for: 1) three different drugs, 2) two distinct stressors—drugs and densities, and 3) two stressors combined.

For low densities and low drug concentrations (corresponding to wild-type distributions) we find skewness is zero and kurtosis is small, so the distribution of colony sizes is well approximated as normal. Intriguingly, these results change dramatically at intermediate to high levels of both density and drug concentrations. As observed in Fig. 2b, skewness is much larger than expected for a normal distribution for all three drugs we used. This result implies that the distribution of colony sizes is far from symmetric about the mean and deviates strongly from a normal distribution. Our computation of the kurtosis further confirms this finding because it is typically much larger than the expectation for a normal distribution, meaning that our distributions decay more quickly away from the mean than would be expected. A highly skewed distribution that frequently arises in biological distributions is the lognormal [19–24]—a distribution that is normal in logarithmic space.

Finally, a more mechanistic mathematical description of transitions in the shape of distribution would greatly aid predictions and analysis for future studies. Therefore, we tested a range of statistical models—combinations of linear, quadratic, and cubic terms of the drug concentration and bacterial density—fitted to empirical data for CV of colony size. The fit that is the best across all three drugs ($r^2 > 0.77$ in all cases) is also one of the most simple—linear term in drug concentration (MIC), linear term in bacterial density (BAC), and an interaction term that is the product of these two linear terms (BAC*MIC). The simple form of this equation suggests that simple mechanisms and principles (e.g., circle packing/space filling) may underlie the very general results reported here.

4. Discussion

Our findings of intermediate doses causing greater variance hold for three drugs with two different mechanisms of action. Here we defined

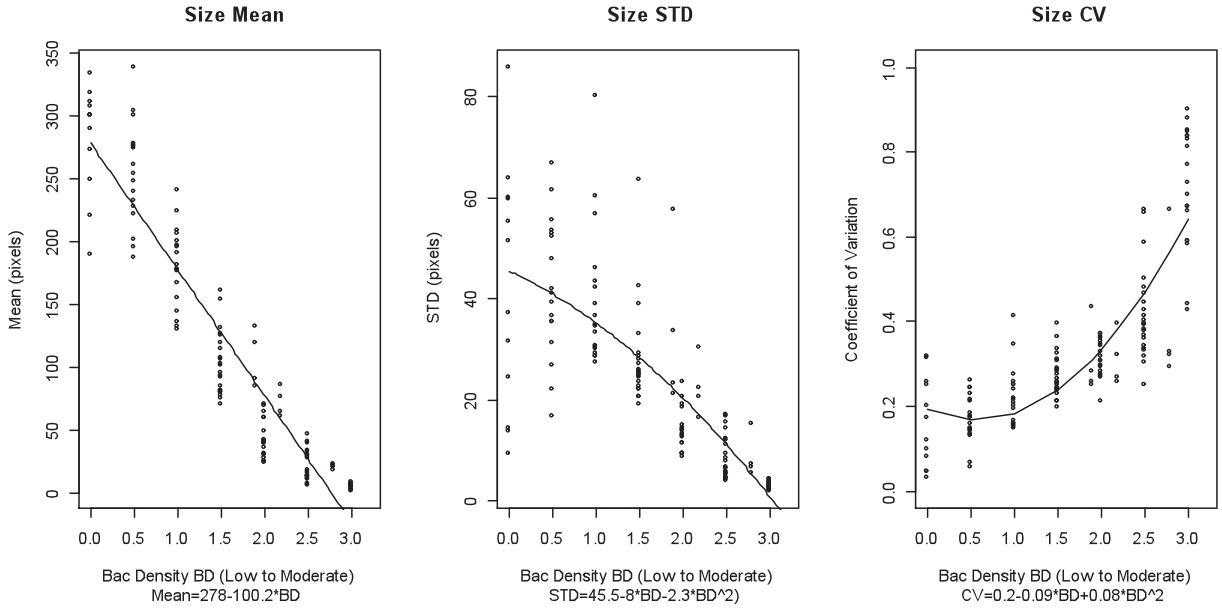

Fig. 3. Effect of bacterial density on colony size variation with no drug. Plots of the mean, standard deviation, and Coefficient of Variation for bacterial colony size versus bacterial density. No drug was used in these experiments. The best-fit equation based on linear regression is given at the bottom of each plot. The mean colony size and its standard deviation both decrease with density. The coefficient of variation (CV), ratio of the mean and standard deviation, exhibits a slight decrease initially but then increases with colony size, demonstrating that variation in colony size increases with the stress or disturbance caused by higher bacterial densities. For bacterial densities on the x-axis, "n" corresponds to approximately 10^n cells.

intermediate as anything that is not the lowest or highest concentrations of a drug. Intermediate levels of each drug are defined relative to their MIC to allow comparisons irrespective of absolute concentration and lethality of each drug. Indeed, the antibiotics have very different values for critical resistance phenotypes—MIC, MPC, and frequencies of resistance along the drug gradient. Despite functional and quantitative differences among the three antibiotics, the intermediate levels of antibiotics always yielded the most variation within single populations.

The most parsimonious explanation for this finding is that there exists a strong correlation between the variance in colony size and the genetic diversity of mutants. Indeed, our interpretation is consistent with findings from previous studies [12–15]. Drugs likely slow growth rate and interfere with cell-cell signaling, but these effects do not predict strong changes in the CV beginning at MIC = 1 or any other consistent MIC value. Changes in CV (relative variation or error) rarely occur in nature [25–27], so when changes do occur, they can help yield insights into underlying processes and mechanisms. The CV is constant when errors are proportional to the mean. For physical processes (e.g., catapulted objects), psychological judgments (Weber's law) [25,26], and biological traits (e.g., variation in body size) [27], constant CV has been observed even when comparing across systems with means and standard deviations that vary by orders of magnitude.

Beyond just the CV, we documented changes in the overall shape of the distribution. These changes in distribution shape may be due to random evolutionary processes through time, balancing mutations and selection by the antibiotic, or due to random spatial processes that influence the dispersal of bacteria and drugs across the plate. Much research has been done to understand which factors and mechanisms shape a distribution to be lognormal or normal [20,22,24]. A normal distribution corresponds to additive random processes and indicates no interaction between the growth of the colonies, whereas a lognormal distribution corresponds to multiplicative random processes and more likely indicates interactions among colonies [19–24]. Our findings show that disturbances or stressors may push the distribution for colony size from normal to a distribution similar to a lognormal. This highly controlled system and our automated measurements thus could enable the experimental and high-throughput exploration of transitions between distributions.

More generally, it is well understood that both selection and mutation are necessary for evolution, but the mutation component and associated variation has been far less studied for questions such as the evolution of antibiotic resistance. Instead, the main focus has been on antibiotics as a source of new selection pressures for bacteria. Notably, lower, sub-lethal levels of drugs have been shown to affect a wide range of bacterial physiology, genetics, and behavior (reviewed in [28]), including that sub-MIC levels of an antibiotic can exert selection pressure in bacterial populations [29,30].

Our findings can be considered an extension of the Intermediate Disturbance Hypothesis (IDH) in ecology, although there are some important caveats. The IDH states that species diversity is at its lowest when disturbances are very rare or very frequent. When disturbances are very rare, the environment is stable, so the selection pressures are effectively constant and the system is likely dominated by a few species with high fitness. When disturbances are very frequent, the environment is highly unstable, and it is likely that only a few species can survive these fluctuations. Consequently, the IDH states that diversity is highest at intermediate levels of disturbances that are infrequent enough that many species can survive the fluctuations but frequent enough that the selection pressures and species with highest fitness change with the disturbances. The IDH is supported by theory founded on ecological and evolutionary principles [31,32]. Tests of the IDH have focused on comparative analyses [33–36] with only a few smaller-scale experimental tests [37]. These studies have provided evidence both for [32,33,35,36] and against [34,36–39] the IDH.

There is intriguing evidence of the effects of intermediate intensities and rates of disturbance on bacterial populations. In one study which did not support the IDH, bacterial diversity was found to change with both increasing frequency of disturbance and with increasing intensity of disturbance [40]. Gibbons and colleagues showed that different rates of disturbance affect the diversity levels within a microbial community, and that a community can switch from a unimodal to a non-unimodal relationship between disturbance rates and diversity [41]. Another study showed that evolution of cooperation in biofilm bacteria peaks at intermediate levels of disturbances [42]. In addition, Kassen and colleagues [43] found that intermediate levels of productivity produced maximal species diversity in a community of bacteria where the environment was heterogeneous, but not in homogenous

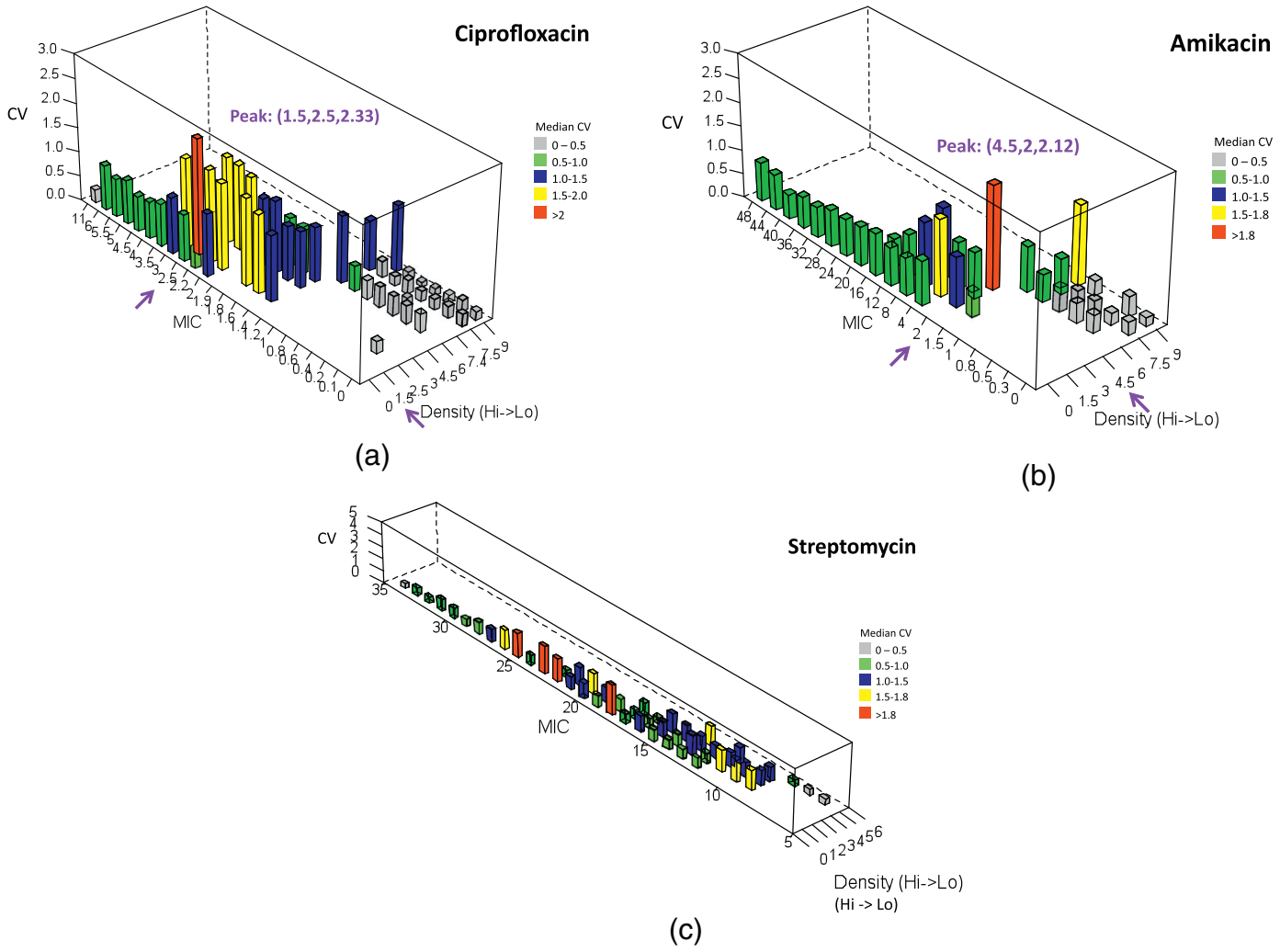

Fig. 4. Size of median CV as a joint function of bacterial density and drug concentration. Three-dimensional bar plots of the median Coefficient of Variation (CV) in colony size versus bacterial density (from high to low) and drug concentration (MIC; from high to low) for three drugs—amikacin, streptomycin, and ciprofloxacin. The peak value is indicated by the red bar, and its coordinate is given as (density, concentration, CV). The values of density and concentration at which the peak value occurs are also denoted by purple arrows, except for streptomycin where there are multiple equivalent peaks. For all drugs the CV is largest at intermediate values of the bacterial density and drug concentration, both of which can be considered disturbances or environmental stresses.

environments. Thus, several studies show intermediate levels of disturbances or productivity can achieve maximum diversity and cooperation.

Our results here focus on diversity as measured by variation within a single species, as opposed to diversity as measured by the number of species within a community. Similar mechanisms may be driving both within- and among-species diversity because we can think of divergence in bacterial populations as incipient differentiation. The underlying intermediate disturbances that allow different species to coexist may also allow greater variation within a single species to be maintained via coexistence of mutants. We expected this generalization might hold because variation within a single bacterial species can overlap and form a continuum with variation across species [44–47]. Indeed, mechanisms that drive variation within species can be similar to those that drive speciation [48]. Importantly, there is an important distinction between the IDH and the work we report here: the IDH is about response to *rates* of disturbances, rather than *levels* or *intensity* of a disturbance. Yet we can connect and extend the general idea of rates of disturbances to levels of disturbances. Our results suggest that more variation can be found in environmental conditions that allow for coexistence of a range of ecological strategies. For example, conditions with an intermediate level of antibiotics could allow the coexistence of both very resistant strains that grow poorly in the absence of antibiotics and less susceptible strains that grow well in the absence of antibiotics.

There are several key caveats to these results. First, high antibiotic concentrations were only able to be tested in high densities of cells; in lower or intermediate levels of bacteria, all cells would have died off. In addition, this study examined only a single species and used antibiotics representing only two different mechanisms of action. Future studies involving more bacterial traits, more species, and more antibiotics would allow us to determine whether the patterns found are pervasive in bacteria. Furthermore, it is possible that variation seen is a result of phenotypic plasticity, not genetics. We make a key assumption that diversity in colony sizes corresponds to diversity in genes. The variation could also be due to heterogeneity of antibiotic resistance. Finally, from a clinical perspective, our results are for a bacterial population within a petri dish and highly-controlled environment. Undoubtedly, life in the wild (or hospital) is more complicated, but using simple microbial model systems as part of the toolbox employed by researchers can expand our understanding of both medical and ecological questions.

We conclude that intermediate levels of disturbance—whether through antibiotic stress or density stress—correspond to an increased diversity of colony size phenotype. This is a pervasive pattern for how colony size in one species responds to three different antibiotics. Further exploration of stressor effects on phenotypic traits in bacteria could provide information on whether the intensity of a disturbance (such as that

wrought by antibiotics) could affect the genetic diversity of a bacterial population, which in turn could affect rates of evolution of resistance.

Ethics

No animal or human subjects were used in this study.

Data Accessibility

All data will be made available on the Savage Lab website upon publication.

Competing Interests

We have no competing interests.

Authors' Contributions

PJY and VMS designed the research, PJY conducted experiments, LL, VMS, and PJY analyzed the data, and LL, PJY and VMS wrote the manuscript.

Funding

We thank UCLA Faculty Career Development Award (PJY), Hellman Foundation (PJY), NIH/National Center for Advancing Translational Science (NCATS) UCLA CTSI Grant Number UL1TR001881 (a KL2 award to PJY), and NIH Systems and Integrative Biology training grant (LL).

Declarations of Interest

None

Acknowledgments

We thank A. Fetherston-Wright, N. George, R. Houser, J. Lee, K. Miller, T. Mortenson, S. Nair, L. Shultz, and J. Stobbe for assistance in the lab. We thank N. Singh, C. Henderson, C. White, R. Kishony, S. Pawar, and A. Dell for comments on the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2018.08.004.

References

[1] Stearns SC, Nesse RM, Govindaraju DR, Ellison PT. Evolutionary perspectives on health and medicine. Proc Natl Acad Sci 2010;107:1691–5. https://doi.org/10.1073/pnas.0914475107.
[2] Crespi B, Summers K. Evolutionary biology of cancer. Trends Ecol Evol 2005;20:545–52. https://doi.org/10.1016/j.tree.2005.07.007.
[3] Merlo LMF, Pepper JW, Reid BJ, Maley CC. Cancer as an evolutionary and ecological process. Nat Rev Cancer 2006;6:924–35. https://doi.org/10.1038/nrc2013.
[4] Greaves M, Maley CC. Clonal evolution in cancer. Nature 2012;481:306–13. https://doi.org/10.1038/nature10762.
[5] Aktipis CA, Nesse RM. Evolutionary foundations for cancer biology. Evol Appl 2013;6:144–59. https://doi.org/10.1111/eva.12034.
[6] Speakman JR. Thrifty genes for obesity, an attractive but flawed idea, and an alternative perspective: the "drifty gene" hypothesis. Int J Obes (Lond) 2008;32:1611–7. https://doi.org/10.1038/ijo.2008.161.
[7] Nesse RM, Stearns SC, Omenn GS. Medicine needs evolution. Science 2006;311:1071. https://doi.org/10.1126/science.1125956.
[8] Michel J-B, Yeh PJ, Chait R, Moellering RC, Kishony R. Drug interactions modulate the potential for evolution of resistance. Proc Natl Acad Sci 2008;105:14918–23. https://doi.org/10.1073/pnas.0800944105.
[9] Read AF, Lynch PA, Thomas MB. How to make evolution-proof insecticides for malaria control. PLoS Biol 2009;7:0001–00010. https://doi.org/10.1371/journal.pbio.1000058.
[10] Read AF, Day T, Huijben S. The evolution of drug resistance and the curious orthodoxy of aggressive chemotherapy. Proc Natl Acad Sci 2011;108:10871–7. https://doi.org/10.1073/pnas.1100299108.
[11] Rice SH. Evolutionary theory: Mathematical and conceptual foundations. Sinauer Associates; 2004. https://doi.org/10.1600/036364405775097789.
[12] Young KD. The selective value of bacterial shape. Microbiol Mol Biol Rev 2006;70:660–703. https://doi.org/10.1128/MMBR.00001-06.
[13] Justice SS, Hunstad DA, Cegelski L, Hultgren SJ. Morphological plasticity as a bacterial survival strategy. Nat Rev Microbiol 2008;6:162–8. https://doi.org/10.1038/nrmicro1820.
[14] Saxer G, Doebeli M, Travisano M. The repeatability of adaptive radiation during long-term experimental evolution of *Escherichia coli* in a multiple nutrient environment. PLoS One 2010;5:e14184. https://doi.org/10.1371/journal.pone.0014184.
[15] Kastbjerg VG, Hein-Kristensen L, Gram L. Triclosan-induced aminoglycoside-tolerant listeria monocytogenes isolates can appear as small-colony variants. Antimicrob Agents Chemother 2014;58:3124–32. https://doi.org/10.1128/AAC.02266-13.
[16] Yeh PJ, Simon DM, Millar JA, Alexander HF, Franklin D. A diversity of antibiotic-resistant *Staphylococcus* spp. in a public transportation system. Osong Public Heal Res Perspect 2011;2:202–9. https://doi.org/10.1016/j.phrp.2011.11.047.
[17] Dong Y, Zhao X, Domagala J, Drlica K. Effect of fluoroquinolone concentration on selection of resistant mutants of *Mycobacterium bovis* BCG and Staphylococcus aureus. Antimicrob Agents Chemother 1999;43:1756–8.
[18] 18. Drlica K. The mutant selection window and antimicrobial resistance. J Antimicrob Chemother 2003;52:11–7. https://doi.org/10.1093/jac/dkg269.
[19] Mosimann JE. Size allometry: size and shape variables with characterizations of the lognormal and generalized gamma distributions. J Am Stat Assoc 1970;65:930–45. https://doi.org/10.1080/01621459.1970.10481136.
[20] 20. Lawton JH. Species richness and population dynamics of animal assemblages. Patterns in body size: abundance space. Philos Trans R Soc B Biol Sci 1990;330:283–91. https://doi.org/10.1098/rstb.1990.0199.
[21] Blackburn TM, Gaston KJ. Animal body size distributions: patterns, mechanisms and implications. Trends Ecol Evol 1994;9:471–4. https://doi.org/10.1016/0169-5347(94)90311-5.
[22] Hubbell SP. The unified neutral theory of biodiversity and biogeography. Princeton University Press; 2012.
[23] Volkov I, Banavar JR, Hubbell SP, Maritan A. Neutral theory and relative species abundance in ecology. Nature 2003;424:1035–7. https://doi.org/10.1038/nature01883.
[24] Newman MEJ. Power laws, Pareto distributions and Zipf's law. Contemp Phys 2005;46:323–51. https://doi.org/10.1080/00107510500052444.
[25] Weber EH. Weber on the tactile senses. Hove, UK: Erlbaum, Taylor & Francis; 1996.
[26] Dehaene S. The neural basis of the Weber-Fechner law: a logarithmic mental number line. Trends Cogn Sci 2003;7:145–7. https://doi.org/10.1016/S1364-6613(03)00055-X.
[27] Savage VM. Improved approximations to scaling relationships for species, populations, and ecosystems across latitudinal and elevational gradients. J Theor Biol 2004;227:525–34. https://doi.org/10.1016/j.jtbi.2003.11.030.
[28] Andersson DI, Hughes D. Microbiological effects of sublethal levels of antibiotics. Nat Rev Microbiol 2014;12:465–78. https://doi.org/10.1038/nrmicro3270.
[29] Gullberg E, Cao S, Berg OG, Ilbäck C, Sandegren L, Hughes D, et al. Selection of resistant bacteria at very low antibiotic concentrations. PLoS Pathog 2011;7:e1002158. https://doi.org/10.1371/journal.ppat.1002158.
[30] Liu A, Fong A, Becket E, Yuan J, Tamae C, Medrano L, et al. Selective advantage of resistant strains at trace levels of antibiotics: a simple and ultrasensitive color test for detection of antibiotics and genotoxic agents. Antimicrob Agents Chemother 2011;55:1204–10. https://doi.org/10.1128/AAC.01182-10.
[31] Dial R, Roughgarden J. Theory of marine communities: the intermediate disturbance hypothesis. Ecology 1998;79:1412–24. https://doi.org/10.1890/0012-9658(1998)079[1412:TOMCTI]2.0.CO;2.
[32] Roxburgh SH, Shea K, Wilson JB. The intermediate disturbance hypothesis: Patch dynamics and mechanisms of species coexistence. Ecology 2004;85:359–71. https://doi.org/10.1890/03-0266.
[33] Tilman D. Resource competition and community structure, Vol. 17; 1982. https://doi.org/10.7861/clinmedicine.14-3-000.
[34] Hubbell SP, Foster RB, O'Brien ST, Harms KE, Condit R, Wechsler B, et al. Light-gap disturbances, recruitment limitation, and tree diversity in a neotropical forest. Science 1999;283:554–7. https://doi.org/10.1126/science.283.5401.554.
[35] Molino J, Sabatier D. Tree diversity in tropical rain forests: a validation of the intermediate disturbance hypothesis. Science 2001;294:1702–4. https://doi.org/10.1126/science.1060284.
[36] Bongers F, Poorter L, Hawthorne WD, Sheil D. The intermediate disturbance hypothesis applies to tropical forests, but disturbance contributes little to tree diversity. Ecol Lett 2009;12:798–805. https://doi.org/10.1111/j.1461-0248.2009.01329.x.
[37] Beckage B, Stout IJ. Effects of repeated burning on species richness in a Florida pine savanna: a test of the intermediate disturbance hypothesis. J Veg Sci 2000;11:113–22. https://doi.org/10.2307/3236782.
[38] 38. McGuinness KA. Disturbance and organisms on boulders. 2. Causes of patterns in diversity and abundance. Oecologia 1987;71:420–30. https://doi.org/10.2307/4218178.
[39] Fox JW. The intermediate disturbance hypothesis should be abandoned. Trends Ecol Evol 2013;28:86–92. https://doi.org/10.1016/j.tree.2012.08.014.
[40] Berga M, Székely AJ, Langenheder S. Effects of disturbance intensity and frequency on bacterial community composition and function. PLoS One 2012;7:e36959. https://doi.org/10.1371/journal.pone.0036959.
[41] Gibbons SM, Scholz M, Hutchison AL, Dinner AR, Gilbert JA, Colemana ML. Disturbance regimes predictably alter diversity in an ecologically complex bacterial system. MBio 2016;7:e01372–6. https://doi.org/10.1128/mBio.01372-16.
[42] Brockhurst MA, Buckling A, Gardner A. Cooperation peaks at intermediate disturbance. Curr Biol 2007;17:761–5. https://doi.org/10.1016/j.cub.2007.02.057.

[43] Kassen R, Buckling A, Bell G, Ralney PB. Diversity peaks at intermediate productivity in a laboratory microcosm. Nature 2000;406:508–12. https://doi.org/10.1038/35020060.
[44] Niklas KJ, Cobb ED, Niinemets U, Reich PB, Sellin A, Shipley B, et al. "Diminishing returns" in the scaling of functional leaf traits across and within species groups. Proc Natl Acad Sci 2007;104:8891–6. https://doi.org/10.1073/pnas.0701135104.
[45] Albert CH, Thuiller W, Yoccoz NG, Soudant A, Boucher F, Saccone P, et al. Intraspecific functional variability: Extent, structure and sources of variation. J Ecol 2010;98:604–13. https://doi.org/10.1111/j.1365-2745.2010.01651.x.
[46] Messier J, McGill BJ, Lechowicz MJ. How do traits vary across ecological scales? A case for trait-based ecology. Ecol Lett 2010;13:838–48. https://doi.org/10.1111/j.1461-0248.2010.01476.x.
[47] Violle C, Enquist BJ, McGill BJ, Jiang L, Albert CH, Hulshof C, et al. The return of the variance: Intraspecific variability in community ecology. Trends Ecol Evol 2012;27:244–52. https://doi.org/10.1016/j.tree.2011.11.014.
[48] Norberg J, Swaney DP, Dushoff J, Lin J, Casagrandi R, Levin SA. Phenotypic diversity and ecosystem functioning in changing environments: a theoretical framework. Proc Natl Acad Sci 2001;98:11376–81. https://doi.org/10.1073/pnas.171315998.