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

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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

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

2. Methods

2.1. Laboratory

2.1.1. 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.

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

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

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: 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.
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 10^4–10^5 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 allowed to grow for 22 h while being shaken and incubated at 37 °C.

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 (1). 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.
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—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 ($BAC\times MIC$). 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
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. 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 10^9 cells.
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 of antibiotic stress) might drive variation. These findings highlight the importance of considering the full spectrum of environmental conditions within which bacterial populations are exposed.

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.
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.

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Declarations of Interest

None

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Appendix A. Supplementary data

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

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