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

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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

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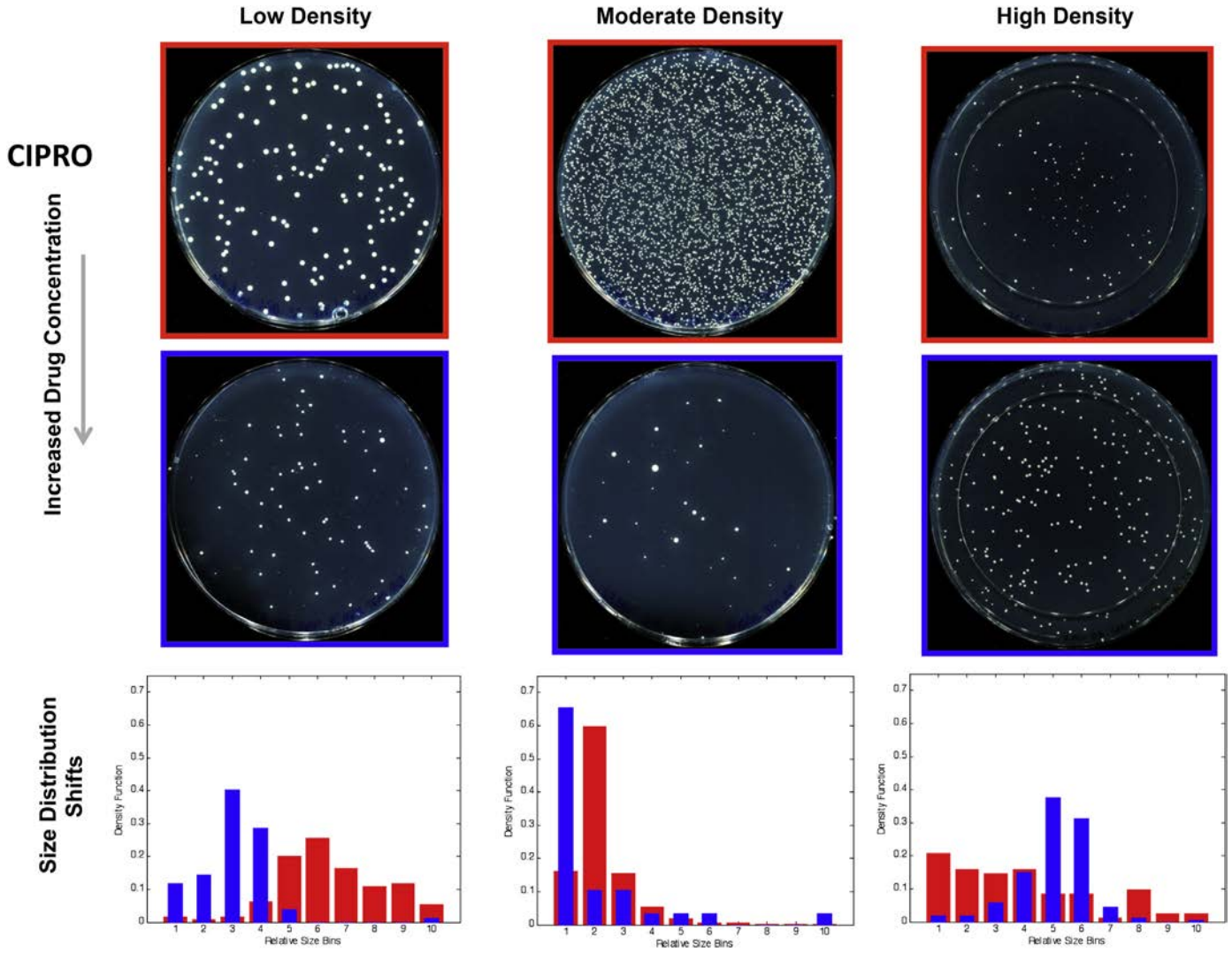

Fig. 1. Shape shifts in colony size distribution with increasing drug concentration and bacterial density. Images for *Staphylococcus* spp. grown in petri dishes across a range of bacterial densities (increasing from left to right) and in the presence of the drugs ciprofloxacin, amikacin, and streptomycin. 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. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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