NOTE TO USERS

Page(s) not included in the original manuscript are unavailable from the author or university. The manuscript was microfilmed as received

Pages 37-38

This reproduction is the best copy available.

UMI[®]

Interactions in the Bacterial World: An Exploration of Antibiotic Resistance

by

Tim Boland

A thesis submitted to the Department of Biology

In conformity with the requirements for

the degree of Masters of Biology

Carleton University

Ottawa, Ontario, Canada

(May, 2009)

Copyright © Tim Boland, 2009

Library and Archives
Canada

Published Heritage
Branch

395 Wellington Street
Ottawa ON K1A 0N4
Canada

Bibliothèque et
Archives Canada

Direction du
Patrimoine de l'édition

395, rue Wellington
Ottawa ON K1A 0N4
Canada

Your file Votre référence
ISBN: 978-0-494-64437-9
Our file Notre référence
ISBN: 978-0-494-64437-9

NOTICE:

The author has granted a non-exclusive license allowing Library and Archives Canada to reproduce, publish, archive, preserve, conserve, communicate to the public by telecommunication or on the Internet, loan, distribute and sell theses worldwide, for commercial or non-commercial purposes, in microform, paper, electronic and/or any other formats.

The author retains copyright ownership and moral rights in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

AVIS:

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque et Archives Canada de reproduire, publier, archiver, sauvegarder, conserver, transmettre au public par télécommunication ou par l'Internet, prêter, distribuer et vendre des thèses partout dans le monde, à des fins commerciales ou autres, sur support microforme, papier, électronique et/ou autres formats.

L'auteur conserve la propriété du droit d'auteur et des droits moraux qui protègent cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

In compliance with the Canadian Privacy Act some supporting forms may have been removed from this thesis.

While these forms may be included in the document page count, their removal does not represent any loss of content from the thesis.

Conformément à la loi canadienne sur la protection de la vie privée, quelques formulaires secondaires ont été enlevés de cette thèse.

Bien que ces formulaires aient inclus dans la pagination, il n'y aura aucun contenu manquant.

■ ■ ■
Canada

Abstract

How antibiotics influence the evolution of bacterial population interactions is the central tenet examined in this study. To test antibiotic influences I used two populations of *Escherichia coli*; one resistant to β -lactam antibiotics, and one sensitive. For both populations I vary the initial growing conditions (the frequencies of each population and the total population density), and environmental structure (homogeneous versus heterogeneous media). By using a GLM design I determined the influence of frequency and population density on the relative fitness of the sensitive bacteria in two environments. I found a significant effect of both frequency and population density dependence on the relative fitness of the sensitives within both environments but only between the environments when density was studied. These results suggest that changing the frequencies of the populations do not have an effect on population fitness levels to the same effect that population density does between different environmental structures.

Acknowledgements

I would like to thank to Dr. Tom Sherratt and Dr. Rees Kassen for their valuable guidance, and Dr. Nigel Waltho for his invaluable writing suggestions. Thanks also go to Dr. Root Gorelick and Dr. Lenore Fahrig for all their valuable comments. To all the members of both the Kassen and Sherratt labs for valuable input and idea bouncing, my deep gratitude is given. For his valuable editing suggestions, I would like to thank Steven Lusk. Finally, I would offer my most sincere thanks to my wife Isabelle for her unwavering support and patience, and last but not least, to the newest addition to our family, Sascha for the motivation to finish. Thank you everyone!

Table of Contents

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
List of Figures	v
List of Tables	ix
List of Appendices	xiv
Chapter 1 Introduction	1
Chapter 2 The Role of Frequency and Density Dependence in a Liquid Environment....	11
Chapter 3 The Role of Frequency and Density Dependence in a Solid Environment and a Comparison between the two Environments	46
Chapter 4 General Conclusions	84
Appendix A - Modelling Population Interactions in a Liquid Environment	89
Appendix B - Tables from Statistical Analyses	99
Appendix C - Raw Data Graphs	104
Appendix D - Glossary	106
References	108

List of Figures

Figure 1.1: Simple representation of the hydrolytic deactivation of the β -lactam ring (represented in red) of penicillin by β -lactamase.....	5
Figure 1.2: Theoretical slope of the effect that antibiotic sensitive bacteria have on the relative fitness of the sensitive bacteria as the sensitive bacteria frequencies increase and show negative frequency dependence.....	8
Figure 1.3: Theoretical slope of the effect that bacterial population density has on the relative fitness of the sensitive bacteria as the population density increase and show positive density dependence.	8
Figure 1.4: The growth curves of pure culture <i>E. coli</i> DH5 α grown in Luria-Bertani (LB) broth. Both the resistant and sensitive strains were grown independently of each other and in the absence of antibiotics in a shaken liquid environment.....	25
Figure 1.5: The growth curves of <i>E. coli</i> DH5 α grown in Luria-Bertani broth. Both the resistant and sensitive strains were grown together and in the absence of antibiotics in a shaken liquid environment.	27
Figure 1.6: Growth curves of <i>E. coli</i> DH5 α grown separately in both Luria-Bertani liquid solid media in three different concentrations of ampicillin (0, 16, and 128 mg/l and corresponding colours dark red, orange, and light green respectively). Diamond symbols correspond to the antibiotic resistant strain and triangle symbols correspond to the antibiotic sensitive strain.....	29
Figure 1.7: The effect of the frequency of the sensitives on the selection coefficient of the sensitives (s) over the entirety of the experiment at different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the liquid environment. For each trend line, the R^2 values are in the following order (from top to bottom): 0, 16, 128 mg/l ampicillin concentration and are exclusively for the fitted lines (not the overall model). The p -values for each regression line are 0.0426, 0.0534, and 0.0859 for the 0, 16, and 128 mg/l lines respectively.	31
Figure 1.8: The effect of the frequency of the sensitives on the selection coefficient of the sensitives (s) at three sampling points during the experiment at three different	

ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the liquid environment. All R^2 values correspond with the fitted ampicillin concentration lines. The p -values for each regression line for Time=24 are 0.9762, 0.4594, and 0.0755 for the 0, 16, and 128 mg/l lines respectively. For Time=48, the p -values (in the same order as in the previous sentence) are 0.0393, 0.2554, and 0.7136. For Time=72, the p -values (in the same order as in the Time=24 sentence) are 0.1362, 0.1678, and 0.2586. 33

Figure 1.9: The effect of the population density of the sensitives on s over the entirety of the experiment at different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the liquid environment. For each trend line, the R^2 values are in the following order (from top to bottom): 0, 16, 128 mg/l ampicillin concentration. The p -values for each regression line are 0.0017, 0.9295, and 0.1173 for the 0, 16, and 128 mg/l lines respectively. 35

Figure 1.10: The effect of the final population density of the sensitives on the selection coefficient of the sensitives (s) at three sampling points during the experiment at three different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the liquid environment. The p -values for each regression line for Time=24 are 0.0996, 0.2493, and 0.0415 for the 0, 16, and 128 mg/l lines respectively. For Time=48, the p -values (in the same order as in the previous sentence) are 0.0873, 0.7575, and 0.1579. For Time=72, the p -values (in the same order as in the Time=24 sentence) are 0.1639, 0.1342, and 0.1982. 36

Figure 2.1: The effect of the frequency of the sensitives on s over the entirety of the experiment at different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the solid environment. For each trend line, the R^2 values are in the following order (from top to bottom): 0, 16, 128 mg/l ampicillin concentration were calculated for the regression lines. The p -values for each regression line are 0.5440, 0.4432, and 0.3581 for the 0, 16, and 128 mg/l lines respectively. 53

Figure 2.2: The effect of the frequency of the sensitives on the selection coefficient of the sensitives (s) at three sampling points during the experiment at three different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend

lines in the solid environment. The R^2 values correspond to the 0, 16, and 128 mg/l ampicillin trend lines from top to bottom respectively. The p -values for each regression line for Time=24 are 0.6066, 0.4434, and 0.1593 for the 0, 16, and 128 mg/l lines respectively. For Time=48, the p -values (in the same order as in the previous sentence) are 0.6823, 0.7240, and 0.9265. For Time=72, the p -values (in the same order as in the Time=24 sentence) are 0.5284, 0.0585, and 0.0938. 54

Figure 2.3: The effect of the population density of the sensitives on s over the entirety of the experiment at different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the solid environment. For each trend line, the R^2 values are in the following order (from top to bottom): 0, 16, 128 mg/l ampicillin concentration. The p -values for each regression line are 0.3334, 0.0002, and 0.0297 for the 0, 16, and 128 mg/l lines respectively. 57

Figure 2.4: The effect of the population density of the sensitives on the selection coefficient of the sensitives (s) at three sampling points during the experiment at three different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the solid environment. The R^2 values correspond to the fitted lines only and represent (from top to bottom): 0, 16, and 128 mg/l ampicillin. The p -values for each regression line for Time=24 are 0.0141, 0.1987, and 0.2870 for the 0, 16, and 128 mg/l lines respectively. For Time=48, the p -values (in the same order as in the previous sentence) are 0.0303, 0.0150, and 0.1104. For Time=72, the p -values (in the same order as in the Time=24 sentence) are 0.2833, 0.8283, and 0.3413. 59

Figure 2.5: Comparison of the effect of the relative frequency of the sensitives (to the resistants) on the selection coefficient of the sensitives (s) in the two different environments (Liquid environment – purple, Solid environment – Orange). The p -values for each regression line are 0.0013 and 0.6086 for the liquid and solid environment regression lines respectively. 62

Figure 2.6: The effect of the frequency of sensitive *E.coli* on the selection coefficient s in the two different environments (liquid and solid) examined in this study. The examinations are split according to the concentrations of ampicillin present in the environment. R^2 values are for the fitted lines only. The p -values for each regression

line are 0.0426 and 0.5440, 0.0534 and 0.0859, and 0.4432 and 0.3581 each for the liquid and solid environment regression lines in the 0 mg/l, 16 mg/l, and 128 mg/l ampicillin conditions respectively.....	65
Figure 2.7: Comparison of the effect of the population density of the sensitives on the selection coefficient of the sensitives (s) in the two different environments (Liquid environment – purple, Solid environment – Orange). R^2 values are for the fitted lines only. The p -values for each regression line are 0.1764 and <0.0001 each for the liquid and solid environment respectively.....	68
Figure 2.8: The effect of the population density of sensitive <i>E.coli</i> on the selection coefficient s in the two different environments (liquid and solid) examined in this study. The examinations are split according to the concentrations of ampicillin present in the environment. R^2 values are for the fitted lines only. The p -values for each regression line are 0.0017 and 0.3334, 0.9295 and 0.0002, and 0.0951 and 0.0297 each for the liquid and solid environment regression lines in the 0 mg/l, 16 mg/l, and 128 mg/l ampicillin conditions respectively.....	71
Figure A.1: Simulation results of the effects of the frequency of the sensitives and time on the selection coefficient s in three different ampicillin concentrations: a) 0 mg/l; b) 16 mg/l; and c) 128 mg/l. Model equations are found in Appendix A.	97
Figure A.2: Simulation results of the effects of population density and time on the selection coefficient s in three different ampicillin concentrations: a) 0 mg/l; b) 16 mg/l; and c) 128 mg/l. Model equations are found in Appendix A.....	98
Figure C.1: Raw data of the growth data in both environments for the frequency experiments for both the resistants and sensitives. Included are a representative sample of the volume ratios used (sensitive to resistant) in the experiments and the three different ampicillin concentrations used (0 mg/l, 16 mg/l, 128 mg/l).	104
Figure C.2: Raw data of the growth data in both environments for the density experiments for both the resistants and sensitives. Included are the inoculate volumes (μ l) used in the experiments and the three different ampicillin concentrations used (0 mg/l, 16 mg/l, 128 mg/l).	105

List of Tables

Table 2.1: Experimental setup of the liquid portion of this experiment examining frequency dependence and density dependence. ✖ signifies an experimental condition that was not investigated and ✓ signifies an experimental condition that was investigated. Each test was performed in triplicate. Note that for the frequency experiments, the volume of the inoculate is constant at 20 µl (right most column) and the density experiments only consist of the 50:50 initial by volume Sensitive to Resistant ratio in three different inoculate volumes (2, 10, and 20 µl).	21
Table 2.2: ANOVA results examining the effects of Time (15 time points over the course of the experiment) and Strain (resistant or sensitive bacterial populations) on the dependent variable (natural log transformed bacterial population size) to determine whether the resistant and sensitive growth curves (in the absence of ampicillin) are significantly different from each other in a liquid environment. Both Time and Strain are fixed factors. Both populations were grown independently of each other. Items in bold are statistically significant ($\alpha=0.05$).	24
Table 2.3: ANOVA results examining the effects of Time (11 time points (hours zero through sixteen) and Strain (resistant or sensitive bacterial populations) on the dependent variable (natural log transformed bacterial population size) to determine whether the resistant and sensitive growth curves (in the absence of ampicillin) are significantly different from each other during the exponential growth phase in a liquid environment. Both Time and Strain are fixed factors. Both populations were grown independently of each other. Items in bold are statistically significant ($\alpha=0.05$).	24
Table 2.4: ANOVA results examining the effects of Time (4 time points over the course of the experiment) and Strain (resistant or sensitive bacterial populations) on the dependent variable (natural log transformed bacterial population size) to determine whether the resistant and sensitive growth curves (in the absence of ampicillin) are significantly different from each other. Both Time and Strain are fixed factors. Both populations were grown in the presence of each other. Items in bold are statistically significant ($\alpha=0.05$).	26

Table 2.5: ANOVA results examining the effects of Time (2 time points (zero and twenty-four)) and Strain (resistant or sensitive bacterial populations) on the dependent variable (natural log transformed bacterial population size) to determine whether the resistant and sensitive growth curves (in the absence of ampicillin) are significantly different from each other during the exponential growth phase of the experiment. Both Time and Strain are fixed factors. Both populations were grown in the presence of each other. Items in bold are statistically significant ($\alpha=0.05$).	26
Table 2.6: ANOVA results examining the effects of Time (11 time points over the course of the experiment) and Strain (resistant or sensitive bacterial populations) on the dependent variable (natural log transformed bacterial population size) to determine whether the resistant and sensitive growth curves (in the absence and presence of ampicillin) grown in three different ampicillin concentrations, Ampicillin (0, 16, and 128 mg/l) are significantly different from each other when grown independently of each other. Time, Ampicillin and Strain are fixed factors. Both populations were grown in the presence of each other. Items in bold are statistically significant ($\alpha=0.05$).	28
Table 2.7: Full factorial ANOVA results table for the full frequency dependent analysis in the shaken liquid environment. The model includes the effect of Time (three time points during the experiment, categorical variable), Ampicillin (three ampicillin concentrations, categorical variable), and Frequency (relative frequency of the sensitives, covariate) on the selection coefficient of the sensitives, s (dependent variable). Items in bold are statistically significant ($\alpha=0.05$).	30
Table 2.8: Full factorial ANOVA results table for the full density dependent analysis in the shaken liquid environment. The model includes the effect of Time (three time points during the experiment, categorical variable), Ampicillin (three ampicillin concentrations, categorical variable), and Density (population density of the sensitives, covariate) on the selection coefficient of the sensitives, s (dependent variable). Items in bold are statistically significant ($\alpha=0.05$).	34
Table 3.1: Experimental setup of the solid portion of this experiment examining frequency dependence and density dependence. * signifies an unexamined experimental	

condition and ✓ signifies an examined experimental condition. Each test was performed in triplicate.	51
Table 3.2: Full factorial ANOVA results table for the full frequency dependent analysis in the solid media environment. The model includes the effect of Time (three time points during the experiment, categorical variable), Ampicillin (three ampicillin concentrations, categorical variable), and Frequency (relative frequency of the sensitives, covariate) on the selection coefficient of the sensitives, s (dependent variable). Items in bold are statistically significant ($\alpha=0.05$).	52
Table 3.3: Full factorial ANOVA results table for the full density dependent analysis in the solid environment. The model includes the effect of Time (three time points during the experiment, categorical variable), Ampicillin (three ampicillin concentrations, categorical variable), and Density (population density of the sensitives, covariate) on the selection coefficient of the sensitives, s (dependent variable). Items in bold are statistically significant ($\alpha=0.05$).	55
Table 3.4: Factorial ANOVA results examining the effect of the independent variables, including the frequency of the sensitive bacteria (Frequency), sampling points (Time), ampicillin concentrations (Ampicillin), and the environmental structure (Environment), on the dependent selection coefficient variable (s). Time, Ampicillin, and Environment are all categorical variable and Frequency is a continuous variable. Items in bold are statistically significant ($\alpha=0.05$).	61
Table 3.5: Factorial ANOVA analysis examining the effect of Environment (liquid or solid environment, categorical variable), Time (time, categorical variable), and Frequency (frequency of the sensitives, covariate) on the selection coefficient s (dependent variable) in three different ampicillin concentrations. Items in bold are statistically significant ($\alpha=0.05$).	64
Table 3.6: Factorial ANOVA analysis examining the effect of Environment (liquid or solid environment, categorical variable), Time (time, categorical variable), and Density (population density of the sensitives, covariate) on the selection coefficient s (dependent variable) in three different ampicillin concentrations. Items in bold are statistically significant ($\alpha=0.05$).	67

Table 3.7: Factorial ANOVA analysis examining the effect of Environment (liquid or solid environment, categorical variable), Time (time, categorical variable), and Density (population density of the sensitives, covariate) on the selection coefficient s (dependent variable) in three different ampicillin concentrations. For the purpose of clarity, only the main effect of Frequency is included within this table since it is the only significant effect present in the analysis. Items in bold are statistically significant ($\alpha=0.05$).....	70
Table B1. 1: Abridged parameter estimates from the full model ANOVA presented in Table 2.2. Included are the parameter estimates of only the strain (resistant or sensitive bacterial population) parameter.	99
Table B2.1: Abridged parameter estimates from the full model factorial ANOVA presented in Table 2.7. Included are the parameter estimates of only the significant effects in the full frequency analysis in the liquid environment.	99
Table B2.2: Abridged parameter estimates from the full model factorial ANOVA presented in Table 2.8. Included are the parameter estimates of only the significant effects in the full density analysis in the liquid environment.	100
Table B2.3: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.2. Included are the parameter estimates of only the significant effects in the full frequency analysis in the solid environment.....	100
Table B2.4: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.3. Included are the parameter estimates of only the significant effects in the full density analysis in the solid environment.	101
Table B3.1: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.4. Included are the parameter estimates of only the significant effects in the full frequency analysis in both environments.	101
Table B3.2: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.5. Included are the parameter estimates of only the significant effects in the full frequency analysis in both environments separated by ampicillin concentration.	102
Table B3.3: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.6. Included are the parameter estimates of only the significant	

effects in the full frequency analysis in both environments separated by ampicillin concentration. Not included within this table is the Time, Ampicillin, Environment, and Density interaction term as all parameter values are very small (but positive).	102
Table B3.4: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.7. Included are the parameter estimates of only the significant effects in the full density analysis in both environments separated by ampicillin concentration and with less than three interaction terms.	103

List of Appendices

Appendix A - Modelling Population Interactions in a Liquid Environment	89
Appendix B - Tables from Statistical Analyses	99
Appendix C - Raw Data Graphs	104
Appendix D - Glossary	106

Chapter 1

Introduction

Bacterial populations when grown in isolation show density-dependent growth rates (Greig and Travisano, 2008; Kaymak and Haas, 2008). When two populations of the same species but different phenotypes are grown together (sympatrically) the influence of one population on the other may interact. Such population-population interactions may be beneficial, neutral, or antagonistic to either one or both of the populations' growth rates (Wiener, 2000; Vulic and Kolter, 2001; Velicer, 2003). The consequence of these interactions is difficult to model because even slight variances in the initial conditions can lead to opposing outcomes (Velicer, 2003; Levin and Bull, 2004). Variables of interest that identify initial conditions include population frequencies, differences in initial population densities, and differences in environmental structure.

Variance in the initial or starting population frequencies of two bacterial populations grown together can affect the eventual fitness of each population (Andersson and Levin, 1999; Bryan *et al.*, 2004). Differing starting frequencies can lead to either a more stable limit population structure, or the competitive exclusion of one population by the second population (Andersson and Levin, 1999). This change in relative fitness is a result of frequency dependent selection and can be classified as either positive or negative frequency dependence. In positive frequency dependent selection, a population's fitness *increases* as it becomes more common (relative to a second population); in negative

frequency dependent selection a population's fitness *decreases* as its population becomes more common. Consequently positive frequency dependent selection promotes competitive exclusion, whereas negative frequency dependent selection promotes stable limits and increased population/species diversity (Dugatkin *et al.*, 2005b).

Frequency dependent selection can be seen in experiments performed by Levin *et al* (2000), who examined the effect of increasing mutant frequencies on a population non-mutant *Escherichia coli*. Levin *et al.* show that rare mutants can successfully invade a population with relatively high numbers of non-mutants. Similarly, using populations of iron-scavenging siderophore producing and non-producing populations of *Pseudomonas aeruginosa* Ross-Gillespie *et al.* (2007) show that the productivity of mixed cultures are significantly negatively related to the non-producers located within the mix. As a third example, Dugatkin *et al* (2005b) using *E. coli* and β -lactamase-mediated resistance to ampicillin determined that antibiotic sensitive bacteria are maintained within a population despite the levels of antibiotics present. These three examples collectively show that frequency dependent selection plays a significant role in population-population interactions.

Initial conditions can also vary within starting population densities. With everything else being the same if two experimental designs differed only in starting population densities the resulting fitnesses from the two experimental designs could be significantly different from each other. Any difference in fitness that can be attributed to initial differences in population densities can be undergoing density dependent selection. In this context, density dependent selection is the change in a population's relative fitness

as the numbers of individuals of that population change relative to all populations present. Such density dependence is observable in microaerophilic bacteria such as *Methanobacterium* sp. In *Methanobacterium* sp. where a minimum cell density is needed to reduce local oxygen levels to more habitable levels (Zehnder and Wuhrmann, 1977).

Further examples of density dependence in bacterial growth include those by Greig and Travisano (2008), and Kaymak and Haas (2008). Greig and Travisano examined the ability of rare types of the yeast *Saccharomyces cerevisiae* to invade populations of common types and found that at low population densities rare types cannot invade. In another example, Kaymak and Haas (2008) examined the effect of the disinfectant monochloramine on differing population densities of *E. coli*. Kaymak and Haas show that higher initial densities had a significantly higher effect on disinfection than at lower initial densities. These examples show density dependent population interactions can influence the trajectory of population-population interactions.

Variation in habitat structure is the third variable that may influence population interactions and subsequently the relative fitness of the bacterial populations grown together (Gordo and Campos, 2006; Perfeito *et al.*, 2008). For instance, in a liquid growth media that is constantly agitated (a homogenised environment), resources will be evenly disbursed throughout the media and will be available in equal quantities to any bacteria present. Conversely, the behaviour of bacteria in other environmental structures, e.g., a solid growth media (a petri dish), is likely to be different from a liquid growth media. In the case of a solid growth media, nutrients will be initially evenly distributed but as bacteria interact with the environmental nutrients will be consumed and not

replaced. This consumption of resources will lead to a shortfall of nutrients unless bacterial colonies expand their area of influence and thereby increasing the nutrients available to them.

Some examples of how spatial structure influences population fitness can be found in Gordo and Campos (2006), Habets *et al.* (2006), and Perfeito *et al.* (2008). Gordo and Campos demonstrated that the difference (of the substitution rate of beneficial mutations) between structured and unstructured environments as adaptive mutation rate increases in a hypothetical model. Gordo and Campos found that the difference between a structured population and an unstructured population increases as the adaptive mutation rate increase. As a second example, Habets *et al.* examined the role of spatial structure on adaptive radiation in *E. coli* and show that only the populations evolving in structured environments diversified. In a final example, Perfeito *et al.* also used *E. coli* but examined the affect that spatial structure has on adaptation and show that is slower on structure environments.

Collectively these studies illustrate how variances in initial phenotypic frequencies, population densities, and habitat structure may influence the fitness of phenotypically distinct populations grown together. Initial conditions are not only important to identify, but are necessary to understand because of our ever increasing reliance of antibiotic treatments. Antibiotic effectiveness, for example, may be more dependent on the initial phenotypic frequencies of resistant to non-resistant bacteria as much as the dose and treatment plan recommended (Ross-Gillespie *et al.*, 2007). Because resistant bacteria can transfer resistance to non-resistant bacteria (i.e., sensitives)

initial conditions and subsequent density dependent selection is important understand so as to minimize antibiotic resistance (Stewart and Costerton, 2001; McDermott *et al.*, 2003).

To examine the significance of varying initial conditions and density dependence selection on bacterial growth I use two bacterial populations that differ in their resistance to β -lactam antibiotics. Both populations are of *Escherichia coli* DH5 α but one population is resistant to β -lactam antibiotics, and one population is sensitive. Antibiotic resistance in *Escherichia coli* DH5 α is acquired through the incorporation of the genetic material located within the plasmid pBR322. pBR322 carries the *bla* gene (Horwitz *et al.*, 1980). This gene confers resistance to β -lactam antibiotics and tetracycline by coding for β -lactamase, an enzyme that degrades antibiotics by cleaving the β -lactam ring found in β -lactam antibiotics (Walsh, 2000; Babic *et al.*, 2006)...

Figure 1.1: Simple representation of the hydrolytic deactivation of the β -lactam ring (represented in red) of penicillin by β -lactamase.

β -lactamase is produced and is concentrated within the periplasmic space (the space between the lipid bi-layer in the cellular membrane) where it breaks down any β -lactam antibiotics diffusing through the cellular membrane. Some of the β -lactamase subsequently leaks through the outer lipid layer into the surrounding extra-cellular space (Sanders *et al.*, 1987). Once outside of the cell, β -lactamase breaks down antibiotics in

the same fashion previously mentioned and as a result, the concentration of environmental β -lactam is decreased. As a result of the decreased β -lactam antibiotic levels, non-resistant sensitive bacteria can take advantage of this opportunity and increase their abundances. The subsequent change in the numbers of the sensitives can be measured and relative fitness calculated.

However, before such work can be performed I need to test an initial assumption, that is, there is an associated fitness cost incurred by the resistant strain (Chapter 2). It is necessary to test this assumption because the lack of a fitness cost would be indicative of two identical populations (or populations that behave in the same manner within the same environment). Two identical populations would not differ in their responses to similar stimuli and therefore would not be indistinguishable from each other. Hence, the validation of the initial assumption (that there is a fitness cost associated with maintaining antibiotic resistance), if shown true, will result in a measureable difference between the resistant and sensitive bacterial populations.

I test the assumption of an associated fitness cost incurred by the resistant strain by growing the resistant and sensitive populations independently and in the absence of antibiotics. The null hypothesis is that there would be no difference in fitness cost between the resistant and sensitive strains. However, I expect to see a reduced rate of growth or a lower carrying capacity for the resistant bacteria due to a potential cost of maintaining resistance. Once the initial assumption is validated I then plan to test the major hypothesis of this thesis. The major hypothesis for this thesis states that there will

be a difference in the relative fitness of the antibiotic sensitive bacteria is dependent on habitat structure.

I aim to show such dependence through three independent experiments. The first experiment (Chapter 2) will examine both frequency and density dependent effects in a shaken liquid environment. In this first experiment, I will control for the relative fitness of the sensitives while varying the initial starting sensitive-resistant frequencies and total population densities in a liquid environment. In this experiment I hope to show that initial population frequencies and densities effects on the relative fitness of the sensitive bacteria change over the course of the experiment (72 hours). The null hypothesis for this experiment states that the initial conditions have no affect on the relative fitness of the sensitives. I expect to see that there is indeed a difference in the relative fitness of the sensitives when initial population frequencies and densities are varied. Specifically, I expect to see negative frequency dependence for the sensitive bacteria since there are fewer resistants when sensitive are common (Figure 1.2). With the density dependence experiments, I would expect to see positive density dependence since any increase in population density will result in a more favourable environment for the sensitives due to the increasing numbers of resistants (Figure 1.3). A difference in relative fitness would suggest that frequency and density dependent affects do have a significant role in how the sensitive and resistant populations interact.

Figure 1.2: Theoretical slope of the effect that antibiotic sensitive bacteria have on the relative fitness of the sensitive bacteria as the sensitive bacteria frequencies increase and show negative frequency dependence.

Figure 1.3: Theoretical slope of the effect that bacterial population density has on the relative fitness of the sensitive bacteria as the population density increase and show positive density dependence.

The second experiment (Chapter 3) will examine frequency and density dependent effects in a solid environment. Here, I will control for the relative fitness of the sensitives while varying the initial starting sensitive-resistant frequencies and total population densities in a solid environment. In this second experiment, I will attempt to demonstrate that initial population frequencies and population densities do have an affect on the relative fitness of the sensitives in a solid environment. The null hypothesis for this experiment will be that there is no difference in frequency and density dependence. Here, I expect that there will be a difference in both frequency and density dependence in the same patterns as discussed in the previous paragraph. That is, negative frequency

dependence is expected for the sensitive bacteria since higher frequencies of sensitives cannot take advantage of the low β -lactamase being leaked by the relatively low proportion of resistant bacteria (Figure 1.2). Expected for the density dependent experiments is positive density dependence (for the sensitive bacteria) since lower population densities will inherently have lower β -lactamase levels due to the low densities of resistant bacteria (Figure 1.3).

The third experiment (Chapter 3) will examine the main hypothesis of this thesis. This experiment entails an examination of the differences of both frequency and density dependence on the relative fitness of the sensitive bacteria between the two environmental structure types. In this experiment I hope to demonstrate that the liquid and solid environments influence the relative fitness of the sensitive bacteria. The null hypothesis is that there is no influence of the environment on the relative fitness of the sensitives from the structure of the environment. I do expect to see a difference (in the relative fitness of the sensitives) between the environments, with the liquid environment having a greater affect on fitness than the solid environment. This difference is expected since the sensitive bacteria in the liquid environment will be affected by any β -lactamase (leaked from the resistant bacteria) within the environment. Conversely, within the solid environment any β -lactamase leaked from resistant colonies will not disperse evenly throughout the environment but rather immediately surrounding resistant colonies. In turn, any sensitive bacteria within the solid environment will only have access to β -lactamase influenced media by being spatially close to resistant colonies. Any difference

between the environments would suggest that environmental structure plays an important role in the fitness of the sensitive bacteria.

Following the experimental work I provide a synthesis (Chapter 4) that not only summarizes the key experimental observations made, but integrates their meaning into the larger context of how environmental conditions can influence fitness in bacteria. Here I address the key hypothesis for the paper and what the ramifications based on the experimental results mean. I will also present a model to attempt to help explain the behaviour of the sensitive bacteria within the liquid environment (Appendix A).

Chapter 2 The Role of Frequency and Density Dependence in a Liquid Environment

Introduction

Studying how two populations of the same species interact with one another, given that one of the populations can change the fitness of the other in some way (through manipulations of its environment) has been looked at in recent literature (Ross-Gillespie *et al.*, 2007). Such interactions between the two populations can involve the effect of the frequency of the phenotype being examined relative to another phenotype on the former phenotype's relative fitness. This interaction is known as frequency dependency and is well observed and understood (Dugatkin *et al.*, 2005b; Ellis *et al.*, 2007). Another well understood population interaction involves the change in fitness as a phenotype changes in absolute numbers and is known as density dependence. Both frequency and density interactions could involve cooperative or competitive behaviours between populations and may be dependent on the type of environment the populations inhabit (Greig and Travisano, 2008).

This chapter will examine a shaken liquid environment inoculated with *Escherichia coli* DH5 α , a common laboratory strain. The *E. coli* within the shaken environment will be used to examine frequency and density dependent effects that are not possible under more structured environments. For instance, a carbon food source for bacteria can be introduced into the media where it will be available to all bacteria while in a more structured environment access to resources is limited as the mobility of resources are removed or at least severely limited. Other experiments can include the

examination of cooperative behaviour under stressful conditions, such as those involving antibiotics, on certain strains or species of bacteria. To test the roles of frequency and density dependence on selection, two bacterial populations, one that is not resistant to antibiotics and one that is resistant, were used.

Antibiotic resistance commonly arises in a population that undergoes strong selection in the presence of antibiotics (Mindlin *et al.*, 2006; Courvalin, 2008). That is, in the presence of antibiotics only those bacteria that have a higher relative resistance (compared to those who do not have resistance) will have an increased chance of surviving to reproduce for the next generation. Therefore, the resulting selection will cause sensitive individuals to die off and the survivors (likely resistant individuals) to take their place. If the pressure exerted on the population from the antibiotics is persistent then the numbers of resistant individuals will increase to a point where the resistant individuals will become the dominate phenotype in the population. Through the pressure exerted by antibiotics (both in natural and medical conditions a number of antibiotic resistance mechanisms, ranging from the production of enzymes capable of denaturing antibiotics (McDermott *et al.*, 2003) to changes in the cellular membrane which prevents target antibiotics penetrating the membrane (Normark and Normark, 2002) have evolved.

In the case of this study, the resistance conferred to bacteria is due to the production of an enzyme able to break down antibiotics within the environment. If the enzyme is produced by a resistant phenotype then it is conceivable that varying concentrations of antibiotics will result in varying frequencies of resistant to sensitive

bacteria. Such a production of enzymes is present in pBR322, an artificial plasmid created in 1977 by Bolivar and Rodriguez that contains genes that code for resistance to the antibiotics ampicillin and tetracycline by producing β -lactamase, an enzyme that degrades both antibiotics (Bolivar *et al.*, 1977). β -lactamase degrades β -lactam antibiotics such as penicillin and ampicillin (and also the non β -lactam tetracycline) by cleaving the β -lactam ring found in β -lactam antibiotics (Walsh, 2000; Babic *et al.*, 2006) and thereby reducing the antibiotic's ability to regulate its cellular membrane during cell division (and thereby destroying the cell when division is attempted) (Babic *et al.*, 2006). β -lactamase is then subsequently transported from within the cellular space to the periplasmic space (the space within the cellular membrane) where it can break down any ampicillin or tetracycline entering the cell. Once β -lactamase is within the periplasmic space, it has been shown to diffuse through the cellular membrane into the extra-cellular space and degrade any β -lactam antibiotics encountered externally (Feizabadi *et al.*, 2006).

The production of β -lactamase, through the gene *bla* found in pBR322's genetic code, can potentially be costly to the cell due to the cost of maintaining and running the machinery associated with the extra genetic material incorporated from the plasmid (Wijngaarden *et al.*, 2005; Handel *et al.*, 2006). The cost of β -lactamase production can manifest itself in a number of ways such as a slower growth rate or a lower carrying capacity. A slower growth rate would result in a greater competitive advantage for the individuals not paying the cost, at least in the presence of antibiotics. A lower carrying capacity would result in the population paying the metabolic cost of maintaining

resistance to have a lower carrying capacity relative to the population not paying the same cost. The cost of resistance would be due to the energetic cost of maintaining the machinery of pBR322 since the plasmid itself can be present in many copies within the genome thereby creating a larger bacterial genome. A larger genome would have higher energetic demands due to the extra genetic material that would likely result in a larger bacteria. The larger bacteria in turn will take up more physical space and could account for any drop in carrying capacity.

The resulting behaviour of β -lactamase and its effect on the environment can have pronounced effects on how various frequencies of sensitive to resistant bacteria and how initial population densities of both strains can have an effect on the relative fitness of the bacteria at different points of the experiment. This thesis will add to the knowledge of both of these frequency and density dependent effects and will help understand of how these effects may affect other natural or experimental systems. For instance, does the frequency of sensitive to resistant bacteria change the relative fitness of the sensitive bacteria? When the sensitive bacteria are common relative to the resistant bacteria do they have a higher fitness due to the increased amount of β -lactamase being leaked out of the resistants, or vice versa? Also, when sensitive and resistant populations are in equal proportions, does the increase or decrease in the density of the mixed population change the relative fitness of the sensitive bacteria since with increasing population densities an increased amount of β -lactamase is available?

With the previously discussed questions from the preceding paragraph in mind, specific hypotheses have been proposed. First, for the subsequent experiments to be

meaningful, it must be determined if the resistant bacteria actually pay a cost to maintain this antibiotic resistance. With no cost of resistance, there will be no method to measure the fitness of any sensitive bacteria. To address this cost of resistance, the following hypothesis will be addressed:

- **Cost of Resistance Hypothesis:**

- *NH1: There is no cost to resistance*
- *AH1: The resistants pay an energetic/metabolic cost to producing β -lactamase*

The role of both frequency and density dependence in an unstructured liquid media is important to set as a guideline for comparison with the structured solid media (which will be examined in Chapter 3). To understand the role of the frequency and density population interactions the following two hypotheses will be tested (these hypotheses are introduced in further detail in Chapter 1):

- **Frequency Dependence Hypothesis:**

- *NH2: There is no frequency dependent selection*
- *AH2: The sensitives have a relatively higher fitness than the resistants at low frequencies due to antibiotic breakdown by the β -lactamase leaked by the resistants and decreases as the frequencies of the sensitives increase*

- **Density Dependence Hypothesis:**

- *NH3: There is no density dependence*
- *AH3: The sensitives will have a lower relative fitness compared to the resistants when at a low total population density due to the lack of*

accessibility of the β -lactamase and will increase as the total population densities increase

In addition to examining these three hypotheses, I have also developed a mathematical model to understand the experimental results obtained in this study. The model is described in detail and a comparison of this chapter's results and the subsequent results of the model will be undertaken. The comparison and model are found in Appendix A.

Methods and Materials

(a) Species used

The species used in this study was *Escherichia coli* DH5 α in two distinct populations: one population containing the plasmid pBR322 (resistant strain), the other population does not contain the plasmid (sensitive strain). The *E. coli* strains were kindly provided by Dr. Paul Rainey of the New Zealand Institute for Advanced Study, College of Sciences, Massey University and Dr. Andrew Spiers of the SIMBIOS Centre, University of Abertay Dundee, Scotland. When plated on both standard Luria-Bertani (LB) and LB + 100 mg/l tetracycline (*tet*) (Sigma-Aldrich T7660) media the strains appear an off-white-yellow colour.

(b) Basic setup

All populations were derived from single isolate clones (either resistant or sensitive) that were grown from stock solutions in 40 ml tubes with 6 ml of LB broth in

each tube. The resulting isolate clone cultures were then shaken at 150 rpm at 37°C in an incubated shaker (New Brunswick C25 Shaker) for 24 hours. The resistant strain media contained *tet* at a concentration of 64 mg/l to maintain the plasmid while the sensitive media did not have any antibiotics within the media. Once grown for 24 hours the resulting resistant and sensitive solutions were placed in a centrifuge and spun at 12000 rpm for 1.5 minutes using a Thermo IEC Micromax centrifuge. After removing the resulting supernatant, the pellet was suspended in a M9 minimal salts solution and starved for two hours at 37°C. The resulting cultures (in 20 µl aliquots and depending on experimental conditions (see Methods sections (d) and (e))) were then dispensed into 24 wells (in a 24 well plate (Costar 3524)) each containing 2 ml of M9 minimal liquid media supplemented with 20% glucose (MM + Glucose) and shaken at 150 rpm at 37°C over the course of the experiment. At hours 0, 4, 8, 12, 16, 24, 36, 48, 60, and 72, one millilitre of the resulting culture was then frozen down with a 50% glycerol solution (to a 60:40 (culture: glycerol) ratio) in a -80°C freezer (Thermo 900 Series Forma -86C ULT) for later use. Each experiment was performed in triplicate. With the exception of the experiments testing for a cost of resistance, all experimental conditions were performed in three different ampicillin (Sigma-Aldrich A0166) concentrations: 0 mg/l, 16 mg/l, and 128 mg/l. It should be noted that this ampicillin is stable for 72 hours at 37°C (Perlman *et al.*, 1979), which is the duration of the experiment.

(c) Plating Protocol and Data Collection:

i. Testing the cost of resistance

To create the growth curves, pure cultures (of both the resistant and sensitive strains) were grown in MM + glucose and sampled at 15 time points (at hours 0, 2, 4, 6, 8, 10, 12, 13, 14, 15, 16, 24, 32, 48, and 72). At each sampling point, each sample was diluted down from frozen to manageable numbers (between 100 and 200 colonies per plate) and plated onto LB agar plates in 20 μ l aliquots. The resulting plates were then grown for 24 hours at 37°C and the colony forming units (CFUs) were determined by counting the resulting colonies, using a colony counter (Reichert Quebec Darkfield Colony Counter Model#13332500).

ii. Main experimental data collection

To distinguish the difference between the resistant and sensitive bacteria, the culture obtained from the initial conditions (Methods section (b)) for both the frequency (Methods section (d)) and density (Methods section (e)) dependence experiments were sampled at hours 0, 24, 48, and 72. The samples were plated into a standard LB plate and incubated for 24 hours at 37°C. The resulting colonies were transferred to a control plate containing LB media only and another plate containing LB media plus 100 mg/l of *tet* and grown for another 24 hours at 37°C. Transfers were performed using a custom piece of equipment (a metal ‘stamper’ consisting of a round metal plate with numerous thick pins) manufactured by the University of Ottawa. Colonies were counted for the LB only plate and the LB + *tet*. To obtain the number of resistants, the colonies on the LB + *tet* plates were counted and the resulting number was subtracted from the total number of colonies counted from the LB only plate.

(d) *Creation of different starting frequencies*

To create the initial starting frequencies volumes the mixtures of the resistant and sensitive population were summed up to one millilitre. These frequencies are set up as sensitive to resistant mixtures and include the following ratios: 0.5:99.5, 1:99, 5:95, 10:90, 50:50, 90:10, and 95:5. Each ratio is measured by volume (summing to one millilitre) in order to obtain a large spread of starting frequencies with emphasis on the low sensitive ratios. The numbers of both the resistant and sensitive populations were initially highly variable and lead to the lack of symmetry towards the low end of the spread of the sensitive frequencies. The low frequencies of the sensitives are to ensure that the sensitives were rare when compared to the resistants. At high frequencies the sensitives are expected to behave in a similar pattern where, when ampicillin is present, a high sensitive mortality is expected. 20 µl of each resulting culture were added to each of the experimental conditions mentioned in Methods section (b) and is summarised in Table 2.1.

Actual starting frequencies were assayed at the beginning of each experiment at Time=0 to obtain more accurate starting frequencies than those determined by volume. The assays of the starting frequencies were performed since a large variation in starting frequencies was detected early in the experiments. The reason for the large variation in the frequency of the sensitives is due to possible small variations in the growth media where the bacteria were grown in the initial experimental setup (Methods section (b)). The variations in the environment at the beginning of bacterial growth can have large

effects at the end of the curve due to possible stochastic differences in the media. These stochastic differences in the media will provide slightly different starting conditions that could affect the future growth trajectories of the bacteria.

(e) Creation of different starting densities

To create the initial starting densities in the liquid environment, 2 μ l, 10 μ l, and 20 μ l each of the 50:50 frequency condition were used and used to acquire a spread of starting densities as the numbers of both populations were variable. To maintain consistent inoculate volume, the 10 μ l and the 2 μ l conditions were placed in 10 μ l and 18 μ l of M9 minimal salt media respectively. 20 μ l of each resulting culture were added to each of the experimental conditions mentioned in Methods section (b) and summarised in Table 2.1.

		Volume of Inoculate (μ l)			
		2	10	20	
Initial Sensitive:Resistant Ratio (Frequency Dependent Conditions) by Volume (μ l)	0.5:99.5	x	x	✓	0
		x	x	✓	16
		x	x	✓	128
	1:99	x	x	✓	0
		x	x	✓	16
		x	x	✓	128
	5:95	x	x	✓	0
		x	x	✓	16
		x	x	✓	128
	10:90	x	x	✓	0
		x	x	✓	16
		x	x	✓	128
	50:50	✓	✓	✓	0
		✓	✓	✓	16
		✓	✓	✓	128
	90:10	x	x	✓	0
		x	x	✓	16
		x	x	✓	128
	95:5	x	x	✓	0
		x	x	✓	16
		x	x	✓	128

Table 2.1: Experimental setup of the **liquid** portion of this experiment examining frequency dependence and density dependence. x signifies an experimental condition that was not investigated and ✓ signifies an experimental condition that was investigated. Each test was performed in triplicate. Note that for the frequency experiments, the volume of the inoculate is constant at 20 μ l (right most column) and the density experiments only consist of the 50:50 initial by volume Sensitive to Resistant ratio in three different inoculate volumes (2, 10, and 20 μ l).

(f) *Measurement used*

The measurement used in this study is a calculated selection coefficient (denoted in this study as s). An example of a calculated selection coefficient is Lenski's r (Lenski *et al.*, 1991). This measurement of selection was calculated with the following formula:

Equation 2.1: Selection coefficient (s) equation

$$s = \frac{\ln\left(\frac{S_t}{S_0}\right) - \ln\left(\frac{R_t}{R_0}\right)}{t}$$

where S_t and R_t are the population densities of both the sensitive and resistant populations, respectively, at time t , and S_0 and R_0 are densities of the same respective populations at time 0. Equation 2.1 measures the changes in population densities of the sensitive bacteria relative to the resistant bacteria and can therefore be used as a measure of relative fitness. For example, when $s > 0$, the sensitives grow at a higher rate than the resistants and therefore have a higher relative fitness. Alternatively, $s < 0$ indicates that the sensitives are growing at a rate lower than the resistants which leads to a decreased relative fitness for the sensitives. Finally, $s = 0$ indicates no change between the growth of both the sensitives and the resistants which in turn implies neutral relative fitness of the sensitives.

Frequencies of the sensitive bacteria were calculated with the following equation:

Equation 2.2: Sensitive frequency equation

$$frequency_{S_t} = \frac{S_t}{S_t + R_t}$$

where S_t and R_t are the population densities of both the sensitive and resistant populations, respectively, at time t . Equation 2.2 was used to calculate the frequency of the sensitives at each sampling point.

(g) *Statistical analysis*

SPSS v.15 (SPSS, 2006) was the statistical software package used in all analyses for this work. For the examination of the frequency and density dependent data, SPSS was utilised to fit a general linear model (GLM) to examine the effects of initial densities, frequencies, ampicillin concentrations, and time on the calculated s value. Multiple hypothesis testing (such as the Bonferroni multiple hypothesis test) was not performed given most tests were conservative in their predictions.

Results

(a) *Testing the cost of resistance*

There is evidence that shows that there is a cost associated with maintaining resistance when the resistants are not in direct competition with the sensitives (Figure 2.1). The cost of resistance manifests itself in the form of a lower carrying capacity as shown in Figure 2.1 where the interaction effect of time and strain (resistant or sensitive bacterial populations) on the number of cells is highly significant as is the main effects of both time and strain (Table 2.2). Further evidence to support that the sensitive bacteria achieve higher population densities than the resistants can be found in Table B1. 1 that shows the parameter estimates of the ANOVA results in Table 2.2. The parameter

estimates show that the resistants do indeed have a cost associated with maintaining resistance when compared to the sensitives. It is also shown that there is a significant difference within the exponential growth phase of the experiment when examining the interaction effect between the strain of the bacteria and time (Table 2.3) that could indicate a cost associated in the exponential growth phase of the growth curve.

Table 2.2: ANOVA results examining the effects of Time (15 time points over the course of the experiment) and Strain (resistant or sensitive bacterial populations) on the dependent variable (natural log transformed bacterial population size) to determine whether the resistant and sensitive growth curves (in the absence of ampicillin) are significantly different from each other in a liquid environment. Both Time and Strain are fixed factors. Both populations were grown independently of each other. Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	P
Time	1.7948x10 ¹⁸	14	1.2820x10 ¹⁷	176.9016	< 0.0001
Strain	4.0551x10 ¹⁶	1	4.0551x10 ¹⁶	55.9573	< 0.0001
Time * Strain	5.3207x10 ¹⁶	14	3.8005x10 ¹⁵	5.2444	< 0.0001
Error	1.0870x10 ¹⁷	150	7.2468x10 ¹⁴		
Total	1.9972x10 ¹⁸	179			

Table 2.3: ANOVA results examining the effects of Time (11 time points (hours zero through sixteen) and Strain (resistant or sensitive bacterial populations) on the dependent variable (natural log transformed bacterial population size) to determine whether the resistant and sensitive growth curves (in the absence of ampicillin) are significantly different from each other during the exponential growth phase in a liquid environment. Both Time and Strain are fixed factors. Both populations were grown independently of each other. Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	P
Time	2.2448x10 ¹⁷	10	2.2448x10 ¹⁶	62.2473	< 0.0001
Strain	1.0224x10 ¹⁶	1	1.0224x10 ¹⁶	28.3506	< 0.0001
Time * Strain	1.7967x10 ¹⁶	10	1.7967x10 ¹⁵	4.9823	< 0.0001
Error	3.9668x10 ¹⁶	110	3.6062x10 ¹⁴		
Total	2.9234x10 ¹⁷	131

Figure 2.1: The growth curves of pure culture *E. coli* DH5 α grown in Luria-Bertani (LB) broth. Both the resistant and sensitive strains were grown independently of each other and in the absence of antibiotics in a shaken liquid environment.

When both strains are in direct competition with each other in the absence of antibiotics the sensitives and resistants have similar growth patterns (as seen in Figure 2.2) to Figure 2.1. Very similar patterns also suggest a cost associated with maintaining resistance (Figure 2.2). The cost of resistance is shown to be significant when looking at the effect of the resistants versus the sensitives (Strain) and the effect of time (Time) on the bacterial population sizes (Table 2.4). Significant results are also present when examining the first portion of the growth curve (between hours zero and twenty-four) (Table 2.5).

Table 2.4: ANOVA results examining the effects of Time (4 time points over the course of the experiment) and Strain (resistant or sensitive bacterial populations) on the dependent variable (natural log transformed bacterial population size) to determine whether the resistant and sensitive growth curves (in the absence of ampicillin) are significantly different from each other. Both Time and Strain are fixed factors. Both populations were grown in the presence of each other. Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	P
Strain	3.7566	1	3.7566	21.8247	0.0003
Time	24.5540	3	8.1847	47.5506	< 0.0001
Time * Strain	0.6691	3	0.2230	1.2957	0.3101
Error	2.7540	16	0.1721		
Total	31.7336	23			

Table 2.5: ANOVA results examining the effects of Time (2 time points (zero and twenty-four)) and Strain (resistant or sensitive bacterial populations) on the dependent variable (natural log transformed bacterial population size) to determine whether the resistant and sensitive growth curves (in the absence of ampicillin) are significantly different from each other during the exponential growth phase of the experiment. Both Time and Strain are fixed factors. Both populations were grown in the presence of each other. Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	P
Strain	1.6233	1	1.6233	7.0932	0.0287
Time	15.7580	1	15.7580	68.8548	< 0.0001
Time * Strain	0.0007	1	0.0007	0.0029	0.9582
Error	1.8309	8	0.2289		
Total	19.2129	11

Figure 2.2: The growth curves of *E. coli* DH5 α grown in Luria-Bertani broth. Both the resistant and sensitive strains were grown together and in the absence of antibiotics in a shaken liquid environment.

The growth of both the sensitive and resistant bacterial populations when grown in isolation from each other and in varying levels of antibiotics (0, 16, and 128 mg/l ampicillin) can be found in Figure 2.3. Figure 2.3 shows that the sensitives die off quickly when in the presence of ampicillin and grow to population sizes greater than those seen in all the resistant conditions when ampicillin is absent. All the conditions with the resistant bacteria have very similar growth trajectories that are not significantly different from each other (Table 2.6).

Table 2.6: ANOVA results examining the effects of Time (11 time points over the course of the experiment) and Strain (resistant or sensitive bacterial populations) on the dependent variable (natural log transformed bacterial population size) to determine whether the resistant and sensitive growth curves (in the absence and presence of ampicillin) grown in three different ampicillin concentrations, Ampicillin (0, 16, and 128 mg/l) are significantly different from each other when grown independently of each other. Time, Ampicillin and Strain are fixed factors. Both populations were grown in the presence of each other. Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	P
Time	121.1072	9	13.4564	80.4888	< 0.0001
Ampicillin	17.3171	2	8.6586	51.7909	< 0.0001
Strain	0.0701	1	0.0701	0.4191	0.5191
Time * Ampicillin	15.4902	18	0.8606	5.1474	< 0.0001
Time * Strain	6.7325	9	0.7481	4.4745	< 0.0001
Ampicillin * Strain	19.4151	2	9.7075	58.0653	< 0.0001
Time * Ampicillin * Strain	15.0745	3	5.0248	30.0560	< 0.0001
Error	14.7121	88	0.1672		
Total	298.9434	132

Figure 2.3: Growth curves of *E. coli* DH5 α grown separately in both Luria-Bertani liquid solid media in three different concentrations of ampicillin (0, 16, and 128 mg/l and corresponding colours dark red, orange, and light green respectively). Diamond symbols correspond to the antibiotic resistant strain and triangle symbols correspond to the antibiotic sensitive strain.

(b) Frequency results

The overall effect of the frequency of the sensitive bacteria (relative to the resistant bacteria) on the selection coefficient, s , shows a statistically significant effect (Table 2.7). The relationship between the frequency of the sensitives and s is negative (Figure 2.4) which is evidence of negative frequency dependent selection. Further evidence of negative frequency dependent selection is found in the negative parameter

estimate of the Frequency variable (Table B2.1). No difference is found in s if ampicillin is present or absent as seen in the Time and Frequency interaction term in Table 2.7 and in the similarity of the fitted lines in Figure 2.4.

Table 2.7: Full factorial ANOVA results table for the full frequency dependent analysis in the shaken liquid environment. The model includes the effect of Time (three time points during the experiment, categorical variable), Ampicillin (three ampicillin concentrations, categorical variable), and Frequency (relative frequency of the sensitives, covariate) on the selection coefficient of the sensitives, s (dependent variable). Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	<i>P</i>
Time	1.0365	2	0.5182	0.5697	0.5668
Ampicillin	0.2932	2	0.1466	0.1612	0.8513
Frequency	12.0600	1	12.0600	13.2568	0.0004
Time * Ampicillin	3.9946	4	0.9987	1.0978	0.3594
Time * Frequency	0.4264	2	0.2132	0.2344	0.7913
Ampicillin * Frequency	0.0545	2	0.0272	0.0299	0.9705
Time * Ampicillin * Frequency	3.6448	4	0.9112	1.0016	0.4083
Error	155.5618	171	0.9097		
Total	175.0649	188

Figure 2.4: The effect of the frequency of the sensitives on the selection coefficient of the sensitives (s) over the entirety of the experiment at different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the liquid environment. For each trend line, the R^2 values are in the following order (from top to bottom): 0, 16, 128 mg/l ampicillin concentration and are exclusively for the fitted lines (not the overall model). The p -values for each regression line are 0.0426, 0.0534, and 0.0859 for the 0, 16, and 128 mg/l lines respectively.

To better understand the effect of frequency dependent selection on the sensitives I examined the change in the selection coefficient (s) at each sampling point (at hour 24,

48, and 72). Hour 0 was excluded since as seen in Equation 2.1, the equation will be comparing the same data at the same time point ($t=0$). Comparing the same time points will result in s having a value of zero for all values since there is no change when comparing values at the same time point. As a final point, with all the values of s being zero, it would also violate the assumption that pertains to the normal distribution of data for an ANOVA.

As is seen in Figure 2.5, there is a general negative trend at all time points as the frequency of the sensitives increase when ampicillin is both present and absent. What is important to note here is what each time point represents. First, Time=24 represents the end of the exponential growth phase where both strains grow at an exponential rate. Time=48 represents the phase of the experiment when both the sensitive and resistant populations are in a stable equilibrium with each other. Finally, Time=72 represents an environment where the changes to the environment made by both populations are beginning to have an effect on said populations. The main change for Time=72 can include a decreased level of β -lactam antibiotics due to increasing levels of β -lactamase in the environment produced by the resistants. It is important to note that at least in the mid to late portions of the experiment, as seen in Figure 2.5, when Time=48 and Time=72, there is a negative slope of s when ampicillin is absent.

Figure 2.5: The effect of the frequency of the sensitives on the selection coefficient of the sensitives (s) at three sampling points during the experiment at three different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the liquid environment. All R^2 values correspond with the fitted ampicillin concentration lines. The p -values for each regression line for Time=24 are 0.9762, 0.4594, and 0.0755 for the 0, 16, and 128 mg/l lines respectively. For Time=48, the p -values (in the same order as in the previous sentence) are 0.0393, 0.2554, and 0.7136. For Time=72, the p -values (in the same order as in the Time=24 sentence) are 0.1362, 0.1678, and 0.2586.

(c) *Density results*

The analysis of the effect of population density on s (Figure 2.6) shows that there is no significant main effect on s but there is a significant main effect of Time (Table 2.8). Time has an increasingly positive effect on s when examining the parameter estimates from the analysis presented in Table 2.8 (Table B2.2). The interactions of ampicillin concentration and population density also show a very small positive influence on s as the ampicillin concentration increases (Table B2.2). The absence of three-way interactions in Table 2.8 is due to the decrease in the degrees of freedom when such an interaction is examined. When the three-way interaction is included the resulting analysis is invalid since the subsequent lack of the degrees of freedom invalidates the results by not providing all the data.

Table 2.8: Full factorial ANOVA results table for the full density dependent analysis in the shaken liquid environment. The model includes the effect of Time (three time points during the experiment, categorical variable), Ampicillin (three ampicillin concentrations, categorical variable), and Density (population density of the sensitives, covariate) on the selection coefficient of the sensitives, s (dependent variable). Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	P
Time	3.0737	2	1.5368	5.5752	0.0058
Ampicillin	1.1879	2	0.5939	2.1546	0.1239
Density	0.1585	1	0.1585	0.5748	0.4510
Time * Ampicillin	1.1738	4	0.2935	1.0646	0.3810
Time * Density	1.0876	2	0.5438	1.9728	0.1471
Ampicillin * Density	2.5617	2	1.2809	4.6467	0.0129
Error	18.4689	67	0.2757		
Total	29.0016	80

Figure 2.6: The effect of the population density of the sensitives on s over the entirety of the experiment at different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the liquid environment. For each trend line, the R^2 values are in the following order (from top to bottom): 0, 16, 128 mg/l ampicillin concentration. The p -values for each regression line are 0.0017, 0.9295, and 0.1173 for the 0, 16, and 128 mg/l lines respectively.

In examining the change at each sampling point (Figure 2.7) we can see that when ampicillin is present, s increases as population density increases for the first two time

points (Time=24 and Time=48). Subsequently, s decreases when ampicillin is present at Time=72. When ampicillin is absent, s shows a negative trend for all sampling points.

Figure 2.7: The effect of the final population density of the sensitives on the selection coefficient of the sensitives (s) at three sampling points during the experiment at three different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the liquid environment. The p -values for each regression line for Time=24 are 0.0996, 0.2493, and 0.0415 for the 0, 16, and 128 mg/l lines respectively. For Time=48, the p -values (in the same order as in the previous sentence) are 0.0873, 0.7575, and 0.1579. For Time=72, the p -values (in the same order as in the Time=24 sentence) are 0.1639, 0.1342, and 0.1982.

of a reduced carrying capacity. A possible reason for the lower carrying capacity of the resistant bacteria can likely be attributed to the number of copies of pBR322 present in the bacterial genome (Burton and Kaguni, 1997). Consequently, multiple copies of pBR322 will lead to a larger bacterial genome and subsequently more energy is used to maintain the bacteria. Generally speaking, the larger genome leads to larger individual bacteria. Larger individuals will thereby have a lower carrying capacity since they take up less physical space than smaller ones and consume more.

(b) *Frequency dependence*

The presence of negative frequency dependence in the shaken liquid environment was confirmed in this study (Figure 2.4, Table 2.7). The resulting effect of the frequency of the sensitives on s is shown to be negative (Table B2.1) and is supporting evidence of negative frequency dependent selection and hence the frequency dependence hypothesis. An observation to note is the negative slope of the trend line of s when ampicillin is absent (Figure 2.4). A negative slope of s is not expected (but could be due to the increasing competition between sensitive individuals) as there is no ampicillin present in the environment to hamper the growth of the sensitives. Here, the expectation is that the growth of the sensitives will at least reach equilibrium with the resistants which would result in a slope of zero. A slope of zero would be a result of no changes in the relative ratios to each of could be a result of the sensitives' actual relative frequency to the resistants.

The situations where the sensitives are rare lead to an increase in their numbers relative to the resistants in order to reach equilibrium, as seen in Figure 2.2 and Appendix C. The reverse could be true where when at high relative frequencies the sensitives decrease in numbers to achieve the same equilibrium with the resistants. Both of the examples presented show that frequency dependent selection is indeed occurring. Also, despite the starting frequency of the sensitives and the levels of ampicillin present in the media, the sensitives all come to approximately the same carrying capacity (as seen in Figure C.1). A likely reason for the sensitives reaching the same carrying capacity could be that small numbers of resistants can influence the levels of ampicillin in the environment. The influence of the ampicillin could lead to the sensitives taking advantage and rising to levels consistent with those where ampicillin is absent. Increased levels of sensitives can be due to a higher than expected degradation of ampicillin by β -lactamase or an increased production (and subsequent leakage) of β -lactamase by the resistants. It is unknown which of these speculations could be correct but there is evidence that at least one is correct as seen in the consistent carrying capacities in each experimental condition for the sensitive populations. The evidence that the sensitive bacteria are susceptible to the effects of ampicillin (Figure 2.3) is confirmation that the resistants may be compensating to a greater extent that was expected. It was shown that when the resistants are present in any numbers, the sensitives do very well, as evidenced by the raw data (Figure C.1).

The effect of the presence and absence of ampicillin over the course of the experiment is shown to produce similar trend lines at each sampling point (Figure 2.5).

For instance, there is a negative or neutral (no change) trend for all sampling points. The effect of the relative frequency of the sensitives, as is shown by the results, is negative on the selection coefficient, s , despite the stage at which the sampling takes place. Each sampling point used in this experiment is important since each one covers interesting points within the bacteria growth curve seen in Figure 2.1 and Figure 2.2. The first sampling point, at hour 24, can be used to determine the effect of the bacterial populations just entering the stationary phase and its potential effect on s . The sampling point at hour 48 can be used to determine the effects of the resistants and sensitives when the populations have had stable populations for an extended period of time. It can be argued that there may be some environmental changes occurring due to the metabolic effects of both bacterial populations but the resistant bacteria in particular. Such changes can lead to decreased levels of ampicillin due to increasing levels of β -lactamase already within the environment and possibly increased levels of waste products produced by both populations of bacteria. The ultimate environmental change could be examined using the last sampling point, hour 72 since the environment has gone through the most changes by this point.

There is no direct evidence of cooperation that influences frequency dependence. Cooperative population interactions could be linked to the frequency of the sensitives in the entire population. When the frequencies of the sensitives are low, the sensitives take advantage of the increasing β -lactamase levels due to leakage from the relatively large numbers of resistants. Higher frequencies of the sensitives lead to a decreased selection

coefficient as a consequence of the decreasing frequencies of the resistants (as seen in the negative coefficient of the variable Frequency in Table B2.1).

The experimental results are compared with the results presented by the model presented in Appendix A (Figure A.1) and the comparison suggests that the initial ratios of the sensitives to the resistants influence how the populations will behave as the experiment progresses (this is in fact, the very definition of frequency dependent selection). The behaviour of the sensitive bacteria is independent of the starting conditions and seems to imply that the projection of both the resistant and sensitive populations is highly dependent on the initial numbers of both populations.

(c) *Density dependence*

When examining the overarching effect of population density on s in the shaken liquid environment, density was shown not to be a significant main effect (Table 2.8). A possible reason for the lack of significance of the main effect of density may be due to an underlying interaction between the sensitives and resistants. Potentially, there may have been something that has not been accounted for, such as a possible increase of the ability of β -lactamase to degrade ampicillin found in the environment. Degrading β -lactamase at a higher rate could be independent of the amount of ampicillin present. Therefore, if the degradation is equal to the negative effect of the antibiotics (to the sensitives), then no effect will be seen. The possibility that no net effect of the degradation of β -lactamase and the effect of the antibiotics can be seen as shown in the raw data presented in Figure

C.2 where the sensitive population densities are generally higher than the resistant populations.

The statistical interactions of the effects of ampicillin within the environment and the population density of the bacteria are important to note here. For instance, there is evidence that the presence of antibiotics (at both ampicillin concentrations) causes s to increase as the density of the bacterial populations increase (Figure 2.6) in a statistically significant manner (as seen in the significance of the ampicillin concentration-population density interaction term in Table 2.8). These results are expected as at lower population densities the resistants produce a relatively small amount of β -lactamase available to breakdown the ampicillin within the environment. Subsequently the amount of ampicillin that is present within the environment affects the sensitives since a low β -lactamase level will not degrade the ampicillin quickly. Conversely, at higher population densities, the sensitives are expected to have a higher relative fitness since higher densities of the resistants will be producing more β -lactamase. In the absence of antibiotics, the reverse behaviour is true. Lower population densities will have few competitors for resources which would lead to a higher relative fitness.

As the density experimental results suggest, it can be said that there is a significant density dependence effect on the relative fitness of the sensitive bacteria. This density dependent effect could involve cooperative behaviour within the liquid environment when ampicillin is present. As a consequence of this, the null hypothesis of the density dependence hypothesis ($NH3$) can be rejected, and the alternative hypothesis, where density dependence is present, can be accepted. In the absence of ampicillin, the

opposite behaviour occurs. The opposite result is expected since lower numbers of the bacteria will lead to relatively higher levels of resources since there are fewer individuals competing for those resources. As a result, when population densities increase, so does competition for those resources and subsequently a lower relative fitness for the competitors.

Examining the effect of the population density on s over the three different sampling points shows an interesting pattern (Figure 2.7). When examining the effect of the presence or absence of antibiotics and its effect on s we can see that at Time=24 and Time=48, the patterns are very similar to Figure 2.6. When ampicillin is absent, there is a decrease in s as the density of the sensitives increase. The decrease in s could be attributed to increasing competition between individuals as the population increases. There is an opposite effect in the presence of antibiotics where the ampicillin will affect the sensitives more readily due to the low concentrations of β -lactamase within the environment. What is interesting here is the slope of s at Time=72. All the slopes here are negative despite the presence or absence of ampicillin (Figure 2.7). The most likely explanation for the negative slopes is due to the global degeneration of the ampicillin by the β -lactamase within the environment.

Conclusions

To conclude, this chapter presents strong evidence that there is a cost of maintaining the plasmid pBR322 and this cost is expressed as a reduced carrying capacity (Figure 2.1 and Figure 2.2). As presented in the introduction of this chapter, the null

hypothesis of Cost of Resistance Hypothesis can be rejected. The acceptance of the alternative hypothesis is supported by the evidence presented in this chapter to show that a cost of resistance is present. There is also evidence to support the existence of weak negative frequency selection between s and the frequency of the sensitives (despite the presence or absence of ampicillin) (Table 2.8) which supports the Frequency Dependence Hypothesis. As for the density dependence hypothesis, there is evidence that density has a direct effect on s when taking the presence of ampicillin into account.

Chapter 3

The Role of Frequency and Density Dependence in a Solid Environment and a Comparison between the two Environments

Introduction

As a continuation from the previous chapter, we will now examine the role of the frequency and density dependent effects on the relative fitness of the sensitives in a solid environment. As the shaken liquid environment is uniform in its distribution of resources, the solid environment is initially uniform in its resource distribution. The uniform distribution of resources will change gradually due to the metabolic processes that the bacteria undergo. Specifically, since the bacteria are not mobile on solid media, any utilisation of resources can alter the local environment. An alteration of the local environment can result in the accumulation of waste metabolites or the permanent removal and subsequent consumption of resources by the bacteria. Eventually, local effects would tend to take precedence over global effects. In other words, the media directly surrounding a colony will be affected to a greater degree when compared to the media beyond the colony's influence. Local effects (both frequency and density dependent effects) may be directly influenced by the spatial structure of the population itself due to the structure of the environment (Ellis *et al.*, 2007).

For a broader context, the effect of both environmental structure and density dependent selection can be due to any number of reasons that includes the transmission of pathogens (whether bacterial or viral) between host species that require different modes

of transmission (Andre and Gandon, 2006). Another possible reason for the effect of environmental structure and density dependence could be the migration from one area of the host to another such as a localised infection (a skin wound, for example) to a global infection where the infection can spread to the blood. The behaviour of such infections is important to understand as it could effect treatment of various types of disease that can change infection sites. Infections changing sites can possibly alter the evolutionary pressures that would come with such a change.

Nevertheless, the current literature does not examine the effect of spatial structure on bacterial populations in detail with some exceptions (Perfeito *et al.*, 2006; Ellis *et al.*, 2007; Perfeito *et al.*, 2008). Perfeito and colleagues (for both studies) studied the effects that spatial structure had on mutation rates. Ellis *et al.* (2007) examined the effects of proximity and the structure of media on elucidating the effect of spatial structure and is an important step in understanding how bacteria can react to changes in their local environments. Ellis *et al.* provides some insight that the structure of the environment can indeed have an influence on how populations behave.

In this chapter, I will examine the effect of both frequency and density dependence in a structured environment. More importantly, an examination of the difference of these frequency and density effects in both of the spatial structures (liquid and solid) will be undertaken. These examinations will test some of the hypotheses introduced in Chapter 1 concerning frequency and density dependence. These examinations are important in the context of this chapter in order to develop an

understanding of how the two different selections are important in a solid environment.

For a reminder, the first hypothesis is:

- **Frequency Dependence Hypothesis:**

- *NH2: There is no frequency dependent selection*
- *AH2: The sensitives have a relatively higher fitness than the resistants at low frequencies due to antibiotic breakdown by the β -lactamase leaked by the resistants and decreases as the frequencies of the sensitives increase*

Here the expectation is the same as in the liquid environment; there is a negative relationship between the frequency of the sensitives and the selection coefficient of the sensitives, s . The negative relationship is expected since lower relative frequencies of the sensitives will have greater access to any β -lactamase leaked from the relatively large numbers of resistants present along with a higher relative fitness. Conversely, higher relative frequencies of the sensitives are expected to have less access to β -lactamase due to the relatively small number of resistants producing the enzyme and consequently a lower relative fitness. The second hypothesis examining density hypothesis is:

- **Density Dependence Hypothesis:**

- *NH3: There is no density dependence*
- *AH3: The sensitives will have a lower relative fitness compared to the resistants when at a low total population density due to the lack of accessibility of the β -lactamase and will increase as the total population densities increase*

Again, the expectation here is the same as the liquid environment presented in Chapter 2. At low densities, the sensitives are expected to have a lower relative fitness

due to the decreased levels of β -lactamase produced by the low densities of the resistants breaking down the existing levels of ampicillin. As population densities increase, it is expected that β -lactamase levels increase (due to the increasing numbers of resistants) and consequently, the sensitives will have an increasing relative fitness due to the increased rate of ampicillin degradation.

The main portion of this chapter will involve the examination of the differences between the shaken liquid and solid experiments in terms of both frequency and density dependence. Examining both the frequency and density is important as it will add to the understanding of what the role of spatial structure in population interactions are. To examine these types of interactions the following hypothesis will be tested:

- **Environmental Structure Hypothesis:**

- *NH4: There is no difference in the nature of frequency dependence and density dependent selection between media*
- *AH4: Sensitives will have a higher relative fitness in the liquid environment when compared to a solid environment for both frequency and density dependent selection due to increased accessibility of β -lactamase in the liquid environment*

In the Environmental Structure Hypothesis, the sensitives in the liquid environment are expected to have a higher relative fitness than the sensitives in the solid environment due to the increased accessibility of β -lactamase in the liquid environment. The resulting increased accessibility of β -lactamase is due the continuous mixing of the liquid environment. Due to the constant agitation of the environment, the β -lactamase

leaked from the resistants will be uniformly distributed within the environment. Even distribution of β -lactamase through the environment is predicted to breakdown ampicillin on a global scale and therefore allows the sensitives to take advantage of the decreasingly hostile conditions.

Methods and Materials

The methodology in this chapter is very similar to the methodology presented in Chapter 2. The differences between the two methodologies are listed as follows:

1. The basic setup of the experiment differs by using 1 ml of solid agar media in each well of a 24 well plate instead of 2 ml of liquid media. Each well contained 1 ml of M9 minimal solid media supplemented with 20% glucose (MM + Glucose) and incubated at 37°C over the course of the experiment. At hours 0, 4, 8, 12, 16, 24, 36, 48, 60, and 72, one millilitre of the resulting solid culture was spun down in 2 ml of M9 minimal salt solution for 45 seconds using a mini-vortex (VWR VM-3000). The resulting spinning solution was then frozen down with a 50% glycerol solution to a 60:40 (culture: glycerol) ratio in a -80°C freezer (Thermo 900 Series Forma -86C ULT) for later use.
2. To create the initial starting frequencies, the volumes of the sensitive-resistant mixtures were used to sum up to one millilitre. These frequencies were set up as sensitive to resistant ratios and include 10:90, 50:50, and 90:10 as measured by volume. The initial frequencies were chosen to ensure a range of starting frequencies as initial population numbers were highly variable. 20 μ l of each

resulting culture were added to each of the experimental conditions and are summarised in Table 3.1. It should be noted that these frequencies are different from those in the liquid experiments presented in Chapter 1. The favourable spread of data points in this media, as seen in Figure 3.1 is the reason for this discrepancy. To be precise, the spread of the frequencies of the sensitives was large enough to exclude the use of all the frequencies used in Chapter 2.

3. The starting densities are also summarised in Table 3.1.

		Volume of Inoculate (μ l)			
		2	10	20	
Initial Sensitive:Resistant Ratio (Frequency Dependent Conditions) by Volume (μ l)	10:90	x	x	✓	0
		x	x	✓	16
		x	x	✓	128
	50:50	✓	✓	✓	0
		✓	✓	✓	16
		✓	✓	✓	128
	90:10	x	x	✓	0
		x	x	✓	16
		x	x	✓	128

Table 3.1: Experimental setup of the **solid** portion of this experiment examining frequency dependence and density dependence. x signifies an unexamined experimental condition and ✓ signifies an examined experimental condition. Each test was performed in triplicate.

Results

(a) Frequency results

The statistical results of the effect of frequency of the sensitives on s (each in 20 μ l inoculates) in the solid media provides evidence of frequency dependence as a main effect (Table 3.2). The overall effect of frequency on s in the solid environment on the sensitive populations is negative (parameter estimate for Frequency variable, Table B2.3).

Table 3.2: Full factorial ANOVA results table for the full frequency dependent analysis in the solid media environment. The model includes the effect of Time (three time points during the experiment, categorical variable), Ampicillin (three ampicillin concentrations, categorical variable), and Frequency (relative frequency of the sensitives, covariate) on the selection coefficient of the sensitives, s (dependent variable). Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	P
Time	3.5653	2	1.7827	2.1121	0.1295
Ampicillin	1.6522	2	0.8261	0.9788	0.3814
Frequency	4.9219	1	4.9219	5.8315	0.0187
Time * Ampicillin	1.0075	4	0.2519	0.2984	0.8779
Time * Frequency	2.8638	2	1.4319	1.6965	0.1916
Ampicillin * Frequency	2.2569	2	1.1285	1.3370	0.2700
Time * Ampicillin * Frequency	0.7265	4	0.1816	0.2152	0.9291
Error	53.1726	63	0.8440		
Total	84.4839	80			

The negative effect of the frequency of the sensitives on s when ampicillin is present is shown in Figure 3.1 with the negative slope of s as the sensitive frequency increases. Figure 3.1 also shows that the absence of antibiotics results in a positive slope for s and the presence of antibiotics exhibit a negative slope. However, it should be noted that there is a wide distribution of data which leads to the non-significance of the interaction between the ampicillin concentration and the relative frequency of the

sensitives (Table 3.2). The wide distribution of data would also explain the low R^2 values for each of the fitted lines shown in Figure 3.1.

Figure 3.1: The effect of the frequency of the sensitives on s over the entirety of the experiment at different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the solid environment. For each trend line, the R^2 values are in the following order (from top to bottom): 0, 16, 128 mg/l ampicillin concentration were calculated for the regression lines. The p -values for each regression line are 0.5440, 0.4432, and 0.3581 for the 0, 16, and 128 mg/l lines respectively.

To determine any possible effect of the frequency of the sensitives on s the data was divided by sampling time (Figure 3.2). As in Chapter 1, the sampling point at Time=0 is not included since the subsequent values of s will be zero. There is a general negative trend of s despite the presence or absence of antibiotics at Time=24 and Time=72. However, a positive slope is found at Time=48 when ampicillin is absent.

Figure 3.2: The effect of the frequency of the sensitives on the selection coefficient of the sensitives (s) at three sampling points during the experiment at three different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the solid environment. The R^2 values correspond to the 0, 16, and 128 mg/l ampicillin trend lines from top to bottom respectively. The p -values for each regression

line for Time=24 are 0.6066, 0.4434, and 0.1593 for the 0, 16, and 128 mg/l lines respectively. For Time=48, the p -values (in the same order as in the previous sentence) are 0.6823, 0.7240, and 0.9265. For Time=72, the p -values (in the same order as in the Time=24 sentence) are 0.5284, 0.0585, and 0.0938.

(b) *Density results*

The role of population density on s within the solid environment is shown to be significant when interacting with the effect of sampling time (Time) (Table 3.3). When examining the effect of time on s , there is evidence that time has a general positive effect on s as the value of Time increases (as determined by the parameter estimates in Table B2.4). Time's positive main effect on s is somewhat different in the interactions between time and density. The results show there is a negative effect of time and density when Time=24 and a positive effect when Time=48 but the magnitude of the effect of the Time*Density interaction is negligible. There is no three-way interaction in Table 3.3 since including it will decrease the degrees of freedom to such a point as to make the analysis invalid.

Table 3.3: Full factorial ANOVA results table for the full density dependent analysis in the solid environment. The model includes the effect of Time (three time points during the experiment, categorical variable), Ampicillin (three ampicillin concentrations, categorical variable), and Density (population density of the sensitives, covariate) on the selection coefficient of the sensitives, s (dependent variable). Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	P
Time	6.5498	2	3.2749	5.9505	0.0042
Ampicillin	0.3664	2	0.1832	0.3329	0.7181
Density	0.6588	1	0.6588	1.1971	0.2778
Time * Ampicillin	3.4631	4	0.8658	1.5731	0.1916

Time * Density	10.3417	2	5.1708	9.3955	0.0003
Ampicillin * Density	0.2301	2	0.1151	0.2091	0.8119
Error	36.8737	67	0.5504		
Total	67.6605	80			

The graphical examination of the effect of population density on s (Figure 3.3) shows that there is a positive relationship between s and population density. That is, as the density of the entire bacterial populations increase, so does s . All the data from each sampling time is used for this graph (with the exception of the data at Time=0) from the initial 50:50 (sensitive to resistant) ratio to obtain this information. Generally, for each ampicillin concentration, earlier sampling points correspond to lower population densities since as time progresses, populations increase in numbers and consequently increase in densities when the volume of habitat is constant. The relationship between population density and time does not change despite the presence (or absence) of ampicillin. In fact, the slopes are not significantly different from each other as seen in the non-significance of the ampicillin and density interaction term (Table 3.3).

Figure 3.3: The effect of the population density of the sensitives on s over the entirety of the experiment at different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the solid environment. For each trend line, the R^2 values are in the following order (from top to bottom): 0, 16, 128 mg/l ampicillin concentration. The p -values for each regression line are 0.3334, 0.0002, and 0.0297 for the 0, 16, and 128 mg/l lines respectively.

Breaking down the effect of the effect of population density on s to different sampling points, we can see that with the exception of Time=48 a general negative trend exists (Figure 3.4). When ampicillin is absent there is generally a negative slope with the

exception of the positive slope at Time=48. It can be implied by the results that as population density increases in the presence of antibiotics the sensitives have an increasing value of s . However, as it is shown in Figure 3.4 in the absence of antibiotics, and as time progresses, the direction of the slope of s varies greatly in magnitude and direction.

Figure 3.4: The effect of the population density of the sensitives on the selection coefficient of the sensitives (s) at three sampling points during the experiment at three different ampicillin concentrations (blue = 0 mg/l, green = 16 mg/l, red = 128 mg/l) with trend lines in the solid environment. The R^2 values correspond to the fitted lines only and represent (from top to bottom): 0, 16, and 128 mg/l ampicillin. The p -values for each regression line for Time=24 are 0.0141, 0.1987, and 0.2870 for the 0, 16, and 128 mg/l lines respectively. For Time=48, the p -values (in the same order as in the previous sentence) are 0.0303, 0.0150, and 0.1104. For Time=72, the p -values (in the same order as in the Time=24 sentence) are 0.2833, 0.8283, and 0.3413.

(c) *Comparison between Solid and Liquid Environments*

The comparison between the two environments when examining the effect of the relative frequency of the sensitives on s shows that there is no significant effect of environment on s (Table 3.4). The effect that the environmental structure has on s can be seen on a scatter plot of the data (Figure 3.5) where the two trend lines representing the change in s in the different environments have similar slopes. The main effect of the frequency of the sensitives is the only variable that provides evidence of any effect on s in the analysis presented here. The frequency of the sensitives is also shown to have a negative effect on s as shown in the negative parameter estimates (Table B3.1). This negative parameter estimate indicates negative frequency dependence.

Table 3.4: Factorial ANOVA results examining the effect of the independent variables, including the frequency of the sensitive bacteria (Frequency), sampling points (Time), ampicillin concentrations (Ampicillin), and the environmental structure (Environment), on the dependent selection coefficient variable (*s*). Time, Ampicillin, and Environment are all categorical variable and Frequency is a continuous variable. Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	<i>P</i>
Time	4.5872	2	2.2936	2.5712	0.0786
Ampicillin	1.5188	2	0.7594	0.8513	0.4282
Environment	0.1585	1	0.1585	0.1777	0.6737
Frequency	12.2286	1	12.2286	13.7088	0.0003
Time * Ampicillin	3.1347	4	0.7837	0.8785	0.4774
Time * Environment	1.4232	2	0.7116	0.7977	0.4516
Time * Frequency	2.4783	2	1.2391	1.3891	0.2513
Ampicillin * Environment	1.0995	2	0.5498	0.6163	0.5408
Ampicillin * Frequency	1.8392	2	0.9196	1.0309	0.3583
Environment * Frequency	0.2584	1	0.2584	0.2897	0.5909
Time * Ampicillin * Environment	0.2516	4	0.0629	0.0705	0.9909
Time * Ampicillin * Frequency	2.1320	4	0.5330	0.5975	0.6648
Time * Environment * Frequency	2.2672	2	1.1336	1.2708	0.2825
Ampicillin * Environment * Frequency	1.8126	2	0.9063	1.0160	0.3636
Time * Ampicillin * Environment * Frequency	0.3135	4	0.0784	0.0879	0.9862
Error	208.7345	234	0.8920		
Total	259.6800	269

Figure 3.5: Comparison of the effect of the relative frequency of the sensitives (to the resistants) on the selection coefficient of the sensitives (s) in the two different environments (Liquid environment – purple, Solid environment – Orange). The p -values for each regression line are 0.0013 and 0.6086 for the liquid and solid environment regression lines respectively.

There is a similarity in most cases in the directionality (the sign of the gradient) of the linear slope for s against starting frequencies when examining the effect of ampicillin concentration between the two different environments (Figure 3.6). The direction of the s slope in most cases is negative as the frequency of the sensitive populations increase in

the overall population of the bacteria. There is one exception: the solid media line in the 0 mg/l ampicillin condition. However, despite this difference, the two spatial structures are not statistically significant from each other when controlling for environmental structure (Table 3.5). Frequency has a significant effect on s (Table 3.5) for all ampicillin concentrations as seen in the negative parameters value and provides evidence of negative frequency dependence (Table B3.2). As seen in the same appendix, the analysis also shows that the liquid environment has a negative effect on s relative to the solid environment.

Table 3.5: Factorial ANOVA analysis examining the effect of Environment (liquid or solid environment, categorical variable), Time (time, categorical variable), and Frequency (frequency of the sensitives, covariate) on the selection coefficient s (dependent variable) in three different ampicillin concentrations. Items in **bold** are statistically significant ($\alpha=0.05$).

Ampicillin Concentration (mg/l)	Source	Type III Sum of Squares	df	Mean Square	F	P
0	Time	2.7535	2	1.3768	1.8495	0.1641
	Environment	0.0317	1	0.0317	0.0425	0.8371
	Frequency	1.2091	1	1.2091	1.6243	0.2063
	Time * Environment	0.3814	2	0.1907	0.2562	0.7746
	Time * Frequency	0.2991	2	0.1495	0.2009	0.8184
	Environment * Frequency	0.6710	1	0.6710	0.9014	0.3453
	Time * Environment * Frequency	1.5893	2	0.7946	1.0675	0.3488
	Error	58.0617	78	0.7444		
Total		73.5194	89			
16	Time	3.1341	2	1.5671	1.4131	0.2496
	Environment	1.2245	1	1.2245	1.1041	0.2966
	Frequency	6.2109	1	6.2109	5.6006	0.0204
	Time * Environment	0.9409	2	0.4705	0.4242	0.6558
	Time * Frequency	1.3468	2	0.6734	0.6072	0.5474
	Environment * Frequency	0.2749	1	0.2749	0.2479	0.6200
	Time * Environment * Frequency	0.3621	2	0.1811	0.1633	0.8496
	Error	86.4997	78	1.1090		
Total		102.5469	89			
128	Time	2.8926	2	1.4463	1.7579	0.1792
	Environment	0.0386	1	0.0386	0.0469	0.8291
	Frequency	5.6035	1	5.6035	6.8108	0.0109
	Time * Environment	0.3256	2	0.1628	0.1979	0.8209
	Time * Frequency	2.6962	2	1.3481	1.6385	0.2009
	Environment * Frequency	0.9379	1	0.9379	1.1400	0.2889

Figure 3.6: The effect of the frequency of sensitive *E.coli* on the selection coefficient s in the two different environments (liquid and solid) examined in this study. The examinations are split according to the concentrations of ampicillin present in the environment. R^2 values are for the fitted lines only. The p -values for each regression line are 0.0426 and 0.5440, 0.0534 and 0.0859, and 0.4432 and 0.3581 each for the liquid and solid environment regression lines in the 0 mg/l, 16 mg/l, and 128 mg/l ampicillin conditions respectively.

Examining the effect of population density on s for the population as a whole is significant when controlling for sampling time (Time), the Time and total population

density interaction (Time*Density), and the Time, Density and environmental structure interaction (Time*Density *Environment) (Table 3.6). These results provide evidence that density does indeed have an effect on s but only with the interaction of other variables. Figure 3.7 shows the comparison of each environment by examining the trend of s as population density increases. The slopes of s in each environment prove not to be significant when fitting a GLM (Environment and Density interaction, Table 3.6) despite the apparent difference of the best fit lines (Figure 3.7). The difference in best fit lines is likely due to the distribution and overlapping of the data. When examining the parameter estimates of Table 3.6, the estimates for Time show a general increasing trend as the value of Time increases (Table B3.3). The parameter estimates also show that interaction of Time and Density has a very small influence on s . The liquid environment also shows a negative influence on s relative to the solid environment (Table B3.3).

Table 3.6: Factorial ANOVA analysis examining the effect of Environment (liquid or solid environment, categorical variable), Time (time, categorical variable), and Density (population density of the sensitives, covariate) on the selection coefficient s (dependent variable) in three different ampicillin concentrations. Items in **bold** are statistically significant ($\alpha=0.05$).

Source	Type III Sum of Squares	df	Mean Square	F	P
Time	7.2302	2	3.6151	8.6285	0.0003
Ampicillin	0.2886	2	0.1443	0.3445	0.7092
Environment	1.2743	1	1.2743	3.0416	0.0835
Density	0.5615	1	0.5615	1.3403	0.2491
Time * Ampicillin	3.6247	4	0.9062	2.1628	0.0767
Time * Environment	1.7955	2	0.8978	2.1428	0.1215
Time * Density	6.8305	2	3.4153	8.1516	0.0005
Ampicillin * Environment	0.6864	2	0.3432	0.8191	0.4431
Ampicillin * Density	0.3842	2	0.1921	0.4585	0.6332
Environment * Density	0.0035	1	0.0035	0.0084	0.9272
Time * Ampicillin * Environment	1.0736	3	0.3579	0.8542	0.4668
Time * Ampicillin * Density	0.8766	4	0.2192	0.5231	0.7189
Time * Environment * Density	3.9457	2	1.9729	4.7088	0.0106
Ampicillin * Environment * Density	0.7979	2	0.3989	0.9522	0.3886
Error	54.4660	130	0.4190		
Total	108.0112	161

Figure 3.7: Comparison of the effect of the population density of the sensitives on the selection coefficient of the sensitives (s) in the two different environments (Liquid environment – purple, Solid environment – Orange). R^2 values are for the fitted lines only. The p -values for each regression line are 0.1764 and <0.0001 each for the liquid and solid environment respectively.

The effect of the different ampicillin concentrations used in this study show that the effect of population density on s is important but only if interacting with another variable (Table 3.7). The interaction with Time for both the 0 mg/l and 128 mg/l conditions and the interaction with Environment for the 16 mg/l condition are significant.

The effect of ampicillin on the different environments can be seen in the increased slope present in the solid environment data when compared to the liquid data (Figure 3.8). The observation is in line with the parameter estimates calculated from the ANOVA used to formulate Table 3.7, where the liquid environments have a negative effect on s when compared to the solid environments (Table B3.4). The estimates involving the interaction terms (the Time*Density term for the 0 mg/l and 128 mg/l ampicillin conditions and the Environment*Density term for the 16 mg/l ampicillin condition) have also shown significance for each ampicillin concentration, and subsequently each has a very small effect on s .

Table 3.7: Factorial ANOVA analysis examining the effect of Environment (liquid or solid environment, categorical variable), Time (time, categorical variable), and Density (population density of the sensitives, covariate) on the selection coefficient s (dependent variable) in three different ampicillin concentrations. For the purpose of clarity, only the main effect of Frequency is included within this table since it is the only significant effect present in the analysis. Items in **bold** are statistically significant ($\alpha=0.05$).

Ampicillin Concentration (mg/l)	Source	Type III Sum of Squares	df	Mean Square	F	P
0	Time	3.4563	2	1.7282	2.9293	0.0639
	Environment	0.2915	1	0.2915	0.4941	0.4858
	Density	0.8024	1	0.8024	1.3601	0.2498
	Time * Environment	1.3007	2	0.6503	1.1023	0.3411
	Time * Density	3.8324	2	1.9162	3.2480	0.0483
	Environment * Density	1.0058	1	1.0058	1.7048	0.1984
	Error	25.9582	44	0.5900		
	Total	46.3057	53			
16	Time	0.2455	1	0.2455	0.6093	0.4392
	Environment	0.3065	1	0.3065	0.7606	0.3879
	Density	0.3430	1	0.3430	0.8514	0.3612
	Time * Environment	0.5484	2	0.2742	0.6805	0.5116
	Time * Density	1.4003	2	0.7001	1.7376	0.1878
	Environment * Density	2.3182	1	2.3182	5.7532	0.0208
	Error	17.7292	44	0.4029		
	Total	33.6856	53			
128	Time	4.4929	2	2.2465	6.7130	0.0029
	Environment	0.9792	1	0.9792	2.9261	0.0942
	Density	0.5126	1	0.5126	1.5317	0.2224
	Time * Environment	0.5527	2	0.2763	0.8258	0.4446
	Time * Density	3.1275	2	1.5638	4.6730	0.0144
	Environment * Density	0.0521	1	0.0521	0.1557	0.6951
	Error	14.7242	44	0.3346		
	Total	27.9456	53

Figure 3.8: The effect of the population density of sensitive *E.coli* on the selection coefficient s in the two different environments (liquid and solid) examined in this study. The examinations are split according to the concentrations of ampicillin present in the environment. R^2 values are for the fitted lines only. The p -values for each regression line are 0.0017 and 0.3334, 0.9295 and 0.0002, and 0.0951 and 0.0297 each for the liquid and solid environment regression lines in the 0 mg/l, 16 mg/l, and 128 mg/l ampicillin conditions respectively.

Discussion

(a) *Frequency results*

The effect on s by the frequency of the sensitives was shown to be both significant and negative (Table 3.2 and Table B2.3) and suggests that there is negative frequency dependence. The results provide evidence that frequency dependence is present in the solid environment and therefore, the null of the frequency dependence hypothesis within the solid environment can be rejected and the alternative accepted. Further evidence of frequency dependence can be seen in Figure C.1 where, when sensitives are rare, they achieve population densities lower than that of the resistants. When increasing in frequency, the sensitive bacteria start to swell in numbers to similar endpoints as the resistants (in the 50:50 sensitive to resistant conditions) and eventually surpass them in the 90:10 conditions. As the results suggest, the ratio of the sensitives at the onset of the experiment influences the endpoint. These results are not surprising as this was also seen in the model examining the frequency dependent effects in Appendix A where the relative frequencies at the beginning of the experiments influences the endpoint of the simulations, even in the absence of antibiotics (Figure A.1).

The experimental findings show that in terms of cooperation, the resistants could be facilitating the increased survivorship of the sensitives at lower sensitive frequencies and this facilitation decreases as the frequencies of the sensitives increase. However, any cooperation, if present, is likely very weak and could be weighted towards the sensitives. These frequency findings were expected as the relative large frequencies of resistants would produce proportionately large amounts of β -lactamase and subsequently have a

positive effect on s . Alternatively, large frequencies of the sensitives lead to a lower s as smaller proportions of the resistants cannot leak enough β -lactamase to effectively breakdown enough ampicillin to have an effect on s . It is interesting to note that the negative frequency selection of s seems to be contrary of the raw data as seen in Figure C.1. The increase in the frequency of the sensitives, as stated above, seems to contradict the negative frequency dependence as seen in the negative parameter estimates (Table B2.3). The increase in sensitive frequencies could lead to the possibility that could result in a decrease in the selection coefficient of sensitive bacteria.

(b) *Density results*

The effect of density on s in a solid environment was shown to be significant when interacting with Time (sampling time) (Table 3.3). The density effect is in a generally positive direction (as seen in the parameter estimates in Table B2.4) that infers positive density dependence. Positive density dependence is not surprising since it was predicted as the density of the bacteria increase over time the sensitives would have an increasingly higher fitness due to the increased levels of β -lactamase present in the environment leaked from the increasing number of resistants when in the presence of ampicillin. The sampling time (Time) appears to have an important effect on the analysis of s , as a main effect (Table 3.3) which suggests that there is a temporal component to s and is in line with the explanation for the Density and Time interaction term previously discussed. There may also be a degradation or build up of metabolites that could alter

how the bacteria grow by changing the environment in which they grow and can either improve or diminish their relative fitness.

With the evidence presented here, the null of the density dependence hypothesis can be rejected and the alternate accepted. As a result, it is shown here that there is a density dependent effect and the effect has a positive influence on s (when interacting with Time) (Table B2.4).

(c) Comparison between solid and liquid environments

Differences between the two different spatial structures did not arise as expected which is an interesting result. The results of this study show that there are similarities in the trends of s in both environments when examining the frequency of the sensitives (Figure 3.5). When examining the difference in the two environments during the frequency dependence stage of this experiment there was no significant effect on s by the spatial structure of the environment when everything else is controlled for (Environment variable, Table 3.4). This observation provides evidence that spatial structure has no bearing on s , which is surprising at least in terms of frequency dependence. Further evidence for the lack of statistical significance between the environments can be found in the raw growth curves of the frequency data (Figure C.1) where there is a great deal of similarity between the curves.

What would be expected between the environments is that the sensitives in the solid (structured) environment, at higher frequencies, would have a lower chance of survival since there would be less access to β -lactamase. A lower chance of survival at

high sensitive frequencies is due to the local effects that dominate the solid environment. Local effects dominate the solid environment since the bacteria are not mobile and any β -lactamase leaked out of the resistant cell can only diffuse outwards and therefore is only beneficial to sensitives located close to resistant colonies. Higher concentrations of β -lactamase will lead to a faster breakdown of any ampicillin present and would result in a more favourable environment for the sensitives. As a result, the sensitives will have a higher relative fitness when located within the zone of influence of the resistants and the number of colonies will be higher. The increased number of colonies is thought to occur since one colony of the resistants can theoretically influence multiple colonies of the sensitives. By contrast, in the shaken liquid environment, any β -lactamase leaked by the resistant bacteria is evenly distributed into the media. The resulting even distribution of the β -lactamase throughout the liquid environment could constitute a global effect. The β -lactamase would then breakdown any ampicillin it would come into contact with, and lower the ampicillin present throughout the media. Hence, with the local effects of the solid environment and the global effects of the liquid environment in mind the bacteria in the liquid environment would be expected to have a higher s than in the solid environment. Nevertheless, this is not the case as shown in the parameter estimates of all the tests examining differences in the effect of environmental structure on s (Table B3.1-B3.4). In all the analyses, it is shown that the liquid environment has a negative effect on the relative fitness of the sensitives when compared to the solid environment. The experimental results can lead to the conclusion that the global availability of β -lactamase does not have the same effect on the sensitives as the

local β -lactamase does in the solid environment. The local effect of the enzyme can possibly be explained by the higher concentrations of β -lactamase in the areas around the resistant colonies by virtue of the diffusion in the solid environmental structure.

What is similar with both environments is that the frequency of the sensitives has a negative effect on s when examining the liquid environment (Table B2.1), the solid environment (Table B2.3) and the two environments together (Table B3.1). Further support is also shown in Figure 3.6 where all the slopes of s in the presence of ampicillin are negative. All the evidence has shown here points to the fact that in the presence of antibiotics, negative frequency dependence is present. When antibiotics are absent, there are conflicting results between the two environments. The liquid environmental results show that there is a negative relationship between the frequency of the sensitives and s (Figure 2.4 2.4) where the opposite is true for the solid environment (Figure 3.1). A possible reason for the relationship between the sensitive frequency and s is likely a result of the distribution of the resources in both environments, such as the even distribution of resources in the shaken liquid environment or the fixed distribution in the solid environment. In the case of this study, the length of the experiment did not allow the resources within the solid environment to be consumed. Hence there could be a higher availability of resources in the solid environment. The increased availability of resources would allow the sensitives to achieve higher population sizes when not in the presence of antibiotics. As can be seen in Figure C.1, the behaviour of the sensitives is unexpected when they are relatively common where after twenty-four hours there is a noticeable increase in the numbers of the sensitives while the resistants increase at a smaller rate.

The increase in the numbers of the sensitives is further evidence that the initial number of the resistants and the sensitives play an important role in their numbers at the endpoint of the experiment.

As a whole, the frequencies of the sensitives do play an important role in the maintenance of diversity. Both the sensitive and resistant populations grow in the liquid environment until the same carrying capacity is reached despite the amount of environmental ampicillin and the starting frequency of the sensitives (Figure C.1). The implication of the higher sensitive count at least for the duration of this experiment is that antibiotic sensitive populations of bacteria are in greater numbers than previously thought. Sensitive bacteria in greater numbers could lead to a further understanding of how this type of system functions by showing that there are alternate behavioural patterns than what the theory presented in the previous chapters suggest. Alternate behavioural patterns suggest that the resistant bacteria could be producing more β -lactamase than previously thought or that β -lactamase can break down ampicillin at a higher rate than expected. As a result with fewer resistant bacteria present in the system, it may be possible to manipulate the system in such a way to target the resistant individuals only. One such system manipulation could include the creation of novel bacteriophages that specifically target resistant bacteria (Coates and Hu, 2007; Rey *et al.*, 2009). With the resistant bacteria removed from the population the remaining sensitive populations will, barring any spontaneous mutation events conferring resistance to the sensitives, die off at a similar rate to the sensitives exposed to ampicillin in Figure 2.3.

When broken down into the different ampicillin concentrations, it is shown that for frequency dependence to have any effect, the presence of ampicillin is needed (Table 3.5). Ampicillin concentrations at 16 mg/l and 128 mg/l show that the frequencies of the sensitives have a significant impact on s . The results are evidence that the pressure exerted on the sensitives and the resistants by the presence of ampicillin is needed to maintain frequency dependence (although ampicillin itself does not have a significant effect on s). The pressure exerted by the presence of ampicillin, as expected, would result in the sensitives (at low frequencies) taking advantage of the β -lactamase being leaked from the relatively high frequencies of the resistants. As a result of the β -lactamase leakage from the resistants, the increases the frequencies of the sensitives would decrease the available amount of β -lactamase and therefore would result in a lower s .

Despite the experimental results showing that the two environments did not differ statistically significantly from each other was surprising. Fortunately, there is evidence in the literature to show that this result is not a novel finding. Perfeito *et al.* (2008) examined adaptation on populations of *E. coli* in two different spatial structures, as in this study, both liquid and solid media were used. The authors observed no significant difference in the cost of adaptation due to environmental structural differences. Using a different experimental system, Greig and Travisano (2008) examined the ability of rare types to invade populations of common types. Greig and Travisano did this by examining the effect of toxin production in structured and unstructured environments in varying density and frequency starting levels of the yeast *Saccharomyces cerevisiae*. The

results obtained by Greig and Travisano (2008) indicate that *S. cerevisiae* has an advantage in spatially structured (solid) environments which contain high population densities. Conversely, at low density and low frequency conditions, the rare toxin producers cannot invade the yeast populations of common, sensitive individuals. Ultimately, Greig and Travisano conclude that this occurs due to the interaction between the toxin producers and the sensitives depend on both the densities and the frequencies of both competitors. At least where the frequency of the sensitives is concerned, the frequency experimental results do fall in line with the results from the model developed in Appendix A where the initial frequencies influence the relative fitness of the sensitives (Figure A.1).

The difference in the change of s as the bacteria population density increases is shown to be statistically significant between the two environments when examining density but only when interacting with another variable (Table 3.6). The interaction with the sampling time (Time) (Table 3.6 and Table 3.7) and the environmental structure of the media (Environment) do produce a highly significant effect (Table 3.7). Population density alone, as indicated by the interaction terms does not influence s but it does in conjunction with time and ampicillin concentration. A likely scenario for the interaction between density, time and ampicillin concentration may be that as time progresses in the experiment the ampicillin present affects the sensitives. The affect of ampicillin on the sensitives could therefore subsequently have a negative affect on lower densities and positively effect higher densities. At lower population densities, the availability of β -lactamase to the sensitives is reduced (due to the small numbers of resistants producing β -

lactamase) and increases as the population density increases due to the increasing numbers of resistants. When including the environmental structure into the interaction, we can see that a difference between the solid and liquid environment manifests itself. The environmental difference involves the sensitives having a higher s in the solid environment when compared to the liquids for the same reasons as stated above. The higher values of s in the solid environment are apparent in both the statistical analysis (Table 3.6) and in Figure 3.7 and Figure 3.8 where the trend lines are distinct.

As discussed above no difference was found between the environments in the frequency experiments (and Table 3.5). Still, there is a small difference in the density experiments (Table 3.7). One conclusion that can be drawn from this finding is that frequency dependent selection is not influenced by the spatial structure of the environment when examining both selections on a global scale. The lack of influence of spatial structure on frequency dependent selection implies that cooperation between the sensitives and the resistants do not play a significant role in increasing the fitness of the sensitives between the two environments when examining frequency dependence. However, the examination of density dependence yielded evidence that shows a significant effect on s when interacting with the environment on only one occasion. The only significant affect of density dependence occurred under the 16 mg/l ampicillin condition where the Environment*Density interaction was statistically significant but cannot justify rejecting the null hypothesis of the Density Dependence Hypothesis. Further evidence to support the lack of difference between the two environments can be found in the raw growth curves of the density data (Figure C.2). Figure C.2 shows that

there is a great deal of similarity in the growth trajectories between the two environments which could confound the results by providing similar data. However, there are some differences worth noting. To illustrate these differences, the sensitives achieve relatively high population numbers in the low to mid population density conditions and tend to decrease in the higher density condition (especially in the solid environment). The high numbers of the sensitives is somewhat surprising as the population numbers do not seem to be affected by the amount of ampicillin present in the system. Another example of high sensitive population sizes occurs in the frequency dependent conditions (Figure C.1) where the sensitives attain high population numbers despite the amount of ampicillin present. The rise and fall of the sensitives correspond to the subsequent rise and fall of the resistants in most cases in both environments. The evidence shows that the numbers of sensitives are dependent on the number of resistants which in turn implies that the levels of β -lactamase present within the environment influences the numbers of sensitives. Further evidence to support that the number of sensitives are dependent on the number of resistants can be found in the significant Time*Density interaction (Table 3.6). The significance of the interaction is noteworthy since it could mean that density could have a small effect on s during the early stages of the experiments since there is an initially small inoculum (as seen in the negative parameter value in the [Time=24]*Density interaction in Table B3.3). With a small inoculate there can be potentially more resources available to the bacterium since competition will likely be low. During the later stages of the experiments, once the resources change in accessibility, strong selective effects may take shape.

As stated above, frequency and density dependence are important factors for the determination of the relative fitness of the sensitives. Unfortunately, there is no strong evidence that the Environmental Structure Hypothesis is valid. That is, the environmental structure of the environment did not have a large detectable effect on s . Therefore, the null of the Environmental Structure Hypothesis cannot be rejected and at least for the purposes of this study, it can be stated that there is no difference between the two environmental structures at least when detecting for frequency and density dependence. An effect of environmental structure on s may exist when population density changes but this effect is not large enough to be able to reject the null of the Environmental Structure Hypothesis.

Conclusions

When examining the effects of both frequency and density dependence in a solid environment, a number of key observations were noted and discussed. It was shown that there is significance of the frequency of the sensitives on s . The frequency of the sensitive bacteria alone did have an effect on s over the entire course of the experiment (Table 3.2). In fact Figure 3.1 shows that there is significant directionality for s as the frequency of the sensitives increase. When ampicillin is present, there is negative frequency dependence and when ampicillin is absent, there is positive frequency dependence. There is enough statistical evidence present so that the null of the frequency dependence hypothesis can be rejected.

The examination of the density dependence hypothesis over the course of the experiment shows that density does play a significant role in selection (Table 3.3) when interacting with Time. The effect of density over the entirety of the data shows a strong positive density dependency, independent of the amount of ampicillin present in the environment. There is also a strong statistical effect on s when density interacts with both time and ampicillin concentration. The statistical results provide evidence to support the density dependence hypothesis. Therefore, the null of density dependence hypothesis can be rejected and the alternative hypothesis, that there is indeed an effect of density, accepted.

The role of spatial structure in this study has been shown not to be a significant one. There is little evidence that a difference exists between the solid and liquid environment under both the frequency and density dependent conditions. Consequently, the null of the Environmental Structure Hypothesis cannot be rejected. Therefore, at least in the terms of this study, the effect of the two environments on s is not statistically significant using either frequency or density dependence as a detection method.

Chapter 4

General Conclusions

The role of frequency and density dependence in a population composed of multiple populations can be complicated when examining how differing environmental structures affects them. The main purpose of this study was to look at how the effect of frequency dependent selection and density dependent selection affected antibiotic sensitive *E. coli* in the presence of antibiotic resistant *E. coli* in two environmental structures (shaken liquid and solid media). To recap, the antibiotic resistant bacteria passively diffuse β -lactamase from the periplasmic space into the external environment where it subsequently breaks down β -lactam antibiotics and tetracycline. To measure the changes in relative fitness, a selection coefficient for the sensitive bacteria was calculated. This study is also to carry on the works of Perfeito *et al.* (2008) who looked at the effect of spatial structure on adaption in *E. coli* and also Greig and Travisano (2008) who looked at the ability of rare types to invade populations of common types.

A series of hypotheses were proposed and tested to look at the role of both frequency and density dependence and are presented here again:

- **Cost of Resistance Hypothesis:**
 - *NH1: There is no cost to resistance*
 - *AH1: The resistants pay an energetic/metabolic cost to producing β -lactamase*
- **Frequency Dependence Hypothesis:**
 - *NH2: There is no frequency dependent selection*

- *AH2: The sensitives have a relatively higher fitness than the resistants at low frequencies due to antibiotic breakdown by the β -lactamase leaked by the resistants and decreases as the frequencies of the sensitives increase*

- **Density Dependence Hypothesis:**

- *NH3: There is no density dependence*
- *AH3: The sensitives will have a lower relative fitness compared to the resistants when at a low total population density due to the lack of accessibility of the β -lactamase and will increase as the total population densities increase*

- **Environmental Structure Hypothesis:**

- *NH4: There is no difference in the nature of frequency dependence and density dependent selection between media*
- *AH4: Sensitives will have a higher relative fitness in the liquid environment when compared to a solid environment for both frequency and density dependent selection due to increased accessibility of β -lactamase in the liquid environment*

First, in order to proceed on these hypotheses, it must be shown that there is a cost associated with maintaining resistance. To recap, the presence of a cost of maintaining resistance is important as a cost will be measureable between the resistant and sensitive bacterial populations. Any lack of a cost of resistance will result in both populations behaving in such a similar manner as to make the populations indistinguishable from each

other. Therefore, the cost of resistance was examined in the Cost of Resistance Hypothesis to determine if the resistants do indeed pay a cost to maintain resistance. It was shown in Chapter 2 that there is indeed a cost associated with resistance and this cost is manifested in the form of a reduced carrying capacity as seen in Figure 2.1 and Figure 2.2. The cost itself is not surprising as there are numerous examples of resistance cost present in the literature (Andersson, 2006; Courvalin, 2008; Pranting *et al.*, 2008; Sandegren *et al.*, 2008; Fernandez-Reyes *et al.*, 2009; Ward *et al.*, 2009). Since we now know that there is a cost associated with resistance the investigation of frequency and density dependence can continue.

Both frequency and density dependence in a homogeneous liquid media are what was examined next. Starting with the frequency dependence hypothesis, it was shown that there is evidence that the frequency of the sensitives does have an effect on s , whereas when looking at the role of density on s (density dependence hypothesis) an effect was found when sampling time was taken into account (or when sampling time interacted with s). The significance of the frequency dependence hypothesis shows that there may be some population interactions influencing how the frequency of the sensitives and the resistants influence each other. When the sensitives are low in frequency, the sensitives have a higher selection coefficient than when compared to the condition where the sensitives are common. The change in the selection coefficient is significant since the greater the frequency of the sensitives leads to fewer resistants present and therefore less β -lactamase being produced to breakdown the ampicillin in the environment. Further evidence for the density dependent effect is also shown using the

model presented in Appendix A where the same negative frequency dependence is present (Equation A.1). Therefore, the null of the frequency dependence hypothesis can be rejected and the alternative, that the sensitives have a higher fitness when rare than when common can be accepted.

What is interesting with the density result is that there was a significant interaction between sampling time (Time) and population density (Density). The significant interaction between Time and Density provides evidence that population density does play a significant role in the behaviour of s and therefore is evidence that the null of the density dependence hypothesis can be rejected. Therefore, there is an important relationship between the population density of the bacteria and s .

The experimental results show that there was no difference of the environment on s when looking at the effect of frequency or density on s . The lack of an effect on s for both frequency and density dependence implies that the sensitive-resistant interactions are similar in the frequency and density experiments as main effects. However, despite the similarity of the sensitive-resistant interactions to the frequency and density main effects, environmental structure was found not to have an effect on s . The frequency results were surprising but are not unprecedented as shown in a study by Greig and Travisano (2008). Density dependent effects on the sensitive bacteria were not surprising and involved interactions with other factors. Also, there does seem that the solid media has a greater effect on s when compared to the liquid media.

The effect of both frequency and density dependent on s in each environment was also examined in this study. Even though there are some positive results for both density

and frequency dependence in each environment, some future work would still shed some light on these two selective forces. Future work involving the study of more structured environments could prove fruitful. Such environments might include a checker board agar plate where there are alternating areas of differing antibiotic concentrations. The checkerboard pattern could determine whether varying levels of antibiotics within the environment could affect the sensitives due to the release of β -lactamase from the resistants from areas with differing levels of antibiotics. In particular, would a sensitive colony have a higher relative fitness when located in an area of high antibiotic concentration located next to an area of low concentration when there also happens to reside a resistant colony leaking β -lactamase? The examination of the changes of the population over time could also prove useful to investigate the role of long-term exposure to a constant level of antibiotics. A long term experiment could be achieved through the use of a chemostat where a constant input of resources and antibiotics along with a constant removal of waste products and antibiotics. Therefore, a consistent environment will allow for testing any long term effects of frequency or density dependence that is beyond the scope of this study.

Appendix A - Modelling Population Interactions in a Liquid Environment

A1. Background and Derivations

Mathematical models are a very powerful tool used in both theoretical and experimental studies examining the details of antibiotic resistance. Mathematical models can be used for various purposes, such as examining the pharmacokinetics of antibiotics (Maglio *et al.*, 2005), transmission/population (in terms of host populations) dynamics (Austin *et al.*, 1997; Bonhoeffer *et al.*, 1997; Stewart *et al.*, 1998; Lipsitch *et al.*, 2000; Bergstrom *et al.*, 2004; Cooper *et al.*, 2004; Boni and Feldman, 2005; Hotchkiss *et al.*, 2005; Reluga, 2005; Webb *et al.*, 2005; Wang and Lipsitch, 2006), bacterial population dynamics (Stewart and Levin, 1973; Jin *et al.*, 2005), agricultural antibiotic use affecting antibiotic resistant bacteria in humans (Smith *et al.*, 2002), basic bacterial growth (Monod, 1949), and antibacterial reactions with bacteria (Andre and Godelle, 2005).

The modeling of antibiotic resistance itself and the cost of resistance have been studied under multiple paradigms (Peck, 2001; O'Neill *et al.*, 2006; Trawick and Schilling, 2006). The model presented in this paper will look at the interactions between resistant individuals and susceptible individuals in increasing concentrations of ampicillin. Resistant individuals produce β -lactamase to break down β -lactam antibiotics (such as ampicillin and tetracycline) as it enters the cell (Dugatkin *et al.*, 2005a; Dugatkin *et al.*, 2005b). Some of the produced β -lactamase is diffused through the cellular membrane from the bacteria into the extra cellular space where it proceeds to break down

antibiotics (Feizabadi *et al.*, 2006). The breakdown of the antibiotics will allow the sensitive individuals to take advantage of the decreasing amount of antibiotics to increase in numbers.

To examine the interactions between the resistant and sensitive populations, a logistic based model has been developed, studied, and compared to the experimental data to determine its validity. The numerical integration will be performed as a frequency-dependent and density-dependent experiment. To characterize the microbial dynamics in this model, let's begin by assuming a homogeneous system in which the growth rate of resistants (R) and sensitives (S). The continuous change in the concentrations of ampicillin (A) and β -lactamase (L) over time is also important to examine. The resulting rate equations are:

Equation A.1: Rate equation for the resistant strain

$$\frac{dR}{dt} = r_R R \left(1 - \frac{R + \alpha_{RS} S}{K_R} \right)$$

Equation A.2: Rate equation for the sensitive strain

$$\frac{dS}{dt} = r_S S \left(1 - \frac{S + \alpha_{SR} R}{K_S} \right) (1 - Az)$$

Equation A.3: Rate equation for the β -lactamase produced by the resistants

$$\frac{dL}{dt} = pR - qL$$

Equation A.4: Rate equation for the ampicillin present in the system

$$\frac{dA}{dt} = -xAL$$

Where r_R and r_S , K_R and K_S are the growth rates and carrying capacities of R and S respectively, α_{RS} and α_{SR} are the interaction terms of R on S and vice versa respectively, z reflects the sensitivity of susceptible growth rate to ampicillin, p and q are the rate of production of lactamase by resistants and the degradation rate of lactamase respectively, and x is the rate of binding of lactamase with ampicillin. It is assumed that β -lactamase production is costly, such that the overall carrying capacity of the resistant form is lower than the overall growth rate of the sensitives in the absence of ampicillin, which in this model is shown by $K_S > K_R$ and $\alpha_{RS} > \alpha_{SR}$. Subsequently, this is also modeled by the prohibitive term $(1-Az)$ in Equation A.3 when A is present.

To examine the model, a number of analyses were performed. First, the determination of the equilibria of the system was calculated for all of the variables involved. By setting the rate of change for R (Equation A.1) and S (Equation A.2) to zero, one can see the equilibrium states for Equation A.1 are:

$$\hat{R} = 0, \quad r_R = 0,$$

and

Equation A.5: Equilibrium state equation for the resistants

$$\hat{R} = K_R - \alpha_{RS} \hat{S}$$

the states for Equation 3 are:

$$\hat{S} = 0, r_S = 0, \hat{A} = \frac{1}{z}, z = \frac{1}{\hat{A}}$$

and

Equation A.6: Equilibrium state equation for the sensitives

$$\hat{S} = K_S - \alpha_{SR} \hat{R}$$

the states for Equation A.3 are:

$$\hat{L} = \frac{p\hat{R}}{q}$$

and

$$p = 0 \text{ or } \hat{R} = 0$$

and the states for

Equation A.4 are:

$$\hat{A} = 0, \hat{L} = 0 \text{ or } x = 0$$

It is intuitive that there are equilibria present when S and R are set to zero since, no growth can happen when there are no individuals present (in the case of S and R). In the other case, where both R and S are equal, their carrying capacity minus the interaction term is also intuitive. The second case is true since for a logistic equation, K is an equilibrium point by default. The equilibrium states for r_R , r_S , p , x and z are all special cases of the parameters and are not relevant since they are not biologically interesting or are unrealistic. For instance, if r_R or r_S are zero then no growth would occur, if p is zero no β -lactamase would be produced, x being zero is not valid since β -lactamase is known to breakdown β -lactam antibiotics (Babic *et al.*, 2006; Harbottle *et al.*, 2006), and if z was zero then S would also be resistant and the interaction would be moot.

Looking at the equilibrium state for L shows that it is dependent on the value of R and this makes sense, as R is the sole producer of β -lactamase. When examining the equilibrium states for A , several equilibria were found. However, only two of the possible three equilibria are relevant. The state where $A=0$ is intuitive and relevant since there is no addition of ampicillin to the system after time zero. A value of zero for L could also be relevant due to the absence of β -lactamase and the resulting lack of degradation of ampicillin due to this enzyme.

A2. Discussion of Model Results and Tie-in with Study Results

To determine if this model can help explain the results of this experiment, the comparison of its results to experimental data are warranted. Figure A.1 shows the affect of increasing sensitive frequencies in three different ampicillin levels. What is important here is the evidence that frequency dependence is present in this model. For example, as the frequency of the sensitives increase, the value of s decreases, which is evidence of negative frequency dependence as expected. It should be important to note that s decreases as the frequencies of the sensitives increase in all concentrations of ampicillin. The results do indeed match with the experimental data (in direction, not magnitude of the change in s) where there is also negative frequency dependent selection (Figure 2.4 and Figure 2.5; Table B2.1 and Table B2.3) and consequently provides support for the alternative frequency dependency hypothesis of the frequency dependence hypothesis.

The examination of the density data derived from this model shows an increase in s as population density increases (Figure A.2). Again, this is the expected result and corresponds with the alternate hypothesis in the density dependence hypothesis. For example, without antibiotics, there is no real change in the positive slope of s as time progresses. How the model runs when antibiotics are present is interesting in this model. When ampicillin is present, low population densities have a markedly lower s than in conditions with higher population densities. The affect of ampicillin on s is drastic and only occurs when population densities are very small. As the simulations near the final time point, population densities also have s stabilising slightly above zero. With s stabilising slightly above zero, the implication is that the population density reaches the

same endpoint when population densities are moderate to high. Moreover, when densities are low the endpoint is not reached in the duration that is used in this model. The results from the model do indeed match the experimental conditions in the directionality of the slope when ampicillin is present and absent (Figure 2.6 and Figure 2.7). The trajectories of s over varying densities show a positive slope that indicates positive density dependence and falls in line with the experimental results. Positive density dependence is evidence that the model and the experimental results have a strong similarity to the model results.

One important factor that the result of this model demonstrates is that there is a slight frequency dependence effect already built into the system. The inherent frequency dependence of the model was unintentional but it can be seen in Figure A.1a and becomes more prevalent as time increases. The inherent frequency dependence is due to the logistic component within both Equation A.1 and Equation A.2. The logistic equation has a built in sigmoidal curves that contain two horizontal asymptotes that simulate a carrying capacity (on high values of the dependent variable) and an initial population size (on low values of the dependent variable). The inherent carrying capacity two populations will cause the populations to behave as if under frequency dependent conditions. Once each the resistants (R) and the sensitives (S) reach their respective carrying capacities, the inherent frequency dependence can influence the model results. The value of this behaviour (in terms of carrying capacity) is constant and will still allow for the examination of both the frequency and density dependent conditions.

Also inherent in the in-built frequency dependence in the model is the initial starting numbers of both R and S. Even if there were no interactions between the two populations (where α_{SR} and α_{RS} both equal zero) the starting values of R and S can provide an inherent frequency dependence. To be specific, high values of either R or S will influence the model by influencing the rates of change of R or S by altering the ratio of the specific population to its respective carrying capacity. For instance, R will influence K_R and S will influence K_S . The resulting influence between K_S and K_R would lead to values that could slightly change the behaviour of R and S in the absence of antibiotics. In the absence of antibiotics, the behaviour of R and S is evident in Figure A.1a where there is a gradient of decreasing s values as the frequency of the sensitives increase at the end of the simulation run (at Time=72 hours). Decreasing s values show that at low frequencies, the S to K_S ratio has a greater impact than the R to K_R ratio and vice versa at higher frequencies. Although the affect of a strain to its respective carrying capacity (S to K_S or R to K_R) is small, it must be kept in mind when interpreting the frequency results obtained from this model. The density dependent simulations are not influenced by this in-built frequency dependence since there are no variations in the frequency of the sensitives (the S to R ratio is maintained at 50:50).

One last point, as seen in Figure A.1(c), all the s values are below zero. The s values below zero indicated that the sensitives could go extinct. However, the s values do increase as time progresses in the simulation. An increase in s suggests that given enough time s would rise to above zero and the sensitives would have a positive relative fitness. Consequently, if s does not increase above zero, then it is likely that the sensitives will eventually go extinct.

Figure A.1: Simulation results of the effects of the frequency of the sensitives and time on the selection coefficient s in three different ampicillin concentrations: a) 0 mg/l; b) 16 mg/l; and c) 128 mg/l. Model equations are found in Appendix A.

Figure A.2: Simulation results of the effects of population density and time on the selection coefficient s in three different ampicillin concentrations: a) 0 mg/l; b) 16 mg/l; and c) 128 mg/l. Model equations are found in Appendix A.

Appendix B - Tables from Statistical Analyses

B1. Cost of Resistance Parameter Estimates

Table B1. 1: Abridged parameter estimates from the full model ANOVA presented in Table 2.2. Included are the parameter estimates of only the strain (resistant or sensitive bacterial population) parameter.

Parameter	95% Confidence Interval					
	B	Std. Error	t	P	Lower Bound	Upper Bound
[Strain=Resistant]	-0.252	0.131	-1.918	0.057	-0.511	0.008
[Strain=Sensitive]	0					

B2. Frequency Dependence and Density Dependence Parameter Estimates

Liquid Analysis

Table B2.1: Abridged parameter estimates from the full model factorial ANOVA presented in Table 2.7. Included are the parameter estimates of only the significant effects in the full frequency analysis in the liquid environment.

Parameter	95% Confidence Interval					
	B	Std. Error	t	P	Lower Bound	Upper Bound
Frequency	-0.7656	0.7792	-0.9826	0.3272	-2.3036	0.7724

Table B2.2: Abridged parameter estimates from the full model factorial ANOVA presented in Table 2.8. Included are the parameter estimates of only the significant effects in the full density analysis in the liquid environment.

Parameter	B	Std. Error	t	P	95% Confidence Interval	
					Lower Bound	Upper Bound
[Time=24]	-0.7123	0.3027	-2.3530	0.0216	-1.3166	-0.1081
[Time=48]	-0.4625	0.3021	-1.5309	0.1305	-1.0654	0.1405
[Time=72]	0					
[Ampicillin=0] *						
Density	-2.2808x10 ⁻⁰⁸	7.6726x10 ⁻⁰⁹	-2.9727	0.0041	-3.8123x10 ⁻⁰⁸	-7.4936x10 ⁻⁰⁹
[Ampicillin=16] *						
Density	-7.2012x10 ⁻⁰⁹	8.1577x10 ⁻⁰⁹	-0.8827	0.3805	-2.3484x10 ⁻⁰⁸	9.0817x10 ⁻⁰⁹
[Ampicillin=128] *						
Density	0					

Solid Analysis

Table B2.3: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.2. Included are the parameter estimates of only the significant effects in the full frequency analysis in the solid environment.

Parameter	B	Std. Error	t	P	95% Confidence Interval	
					Lower Bound	Upper Bound
Frequency	-2.1477	1.5907	-1.3502	0.1818	-5.3264	1.0310

Table B2.4: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.3. Included are the parameter estimates of only the significant effects in the full density analysis in the solid environment.

Parameter	95% Confidence Interval					
	B	Std. Error	t	P	Lower Bound	Upper Bound
[Time=24]	-1.0905	0.6167	-1.7683	0.0816	-2.3215	0.1404
[Time=48]	-1.4932	0.6342	-2.3546	0.0215	-2.7590	-0.2274
[Time=72]	0					
[Time=24] * Density	-2.9122x10 ⁻⁰⁸	2.6318x10 ⁻⁰⁸	-1.1066	0.2724	-8.1653x10 ⁻⁰⁸	2.3408x10 ⁻⁰⁸
[Time=48] * Density	5.0426x10 ⁻⁰⁸	1.6362x10 ⁻⁰⁸	3.0819	0.0030	1.7768x10 ⁻⁰⁸	8.3085x10 ⁻⁰⁸
[Time=72] * Density	0					

B3. Environmental Structural Parameter Estimates

Frequency Analysis

Table B3.1: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.4. Included are the parameter estimates of only the significant effects in the full frequency analysis in both environments.

Parameter	95% Confidence Interval					
	B	Std. Error	t	P	Lower Bound	Upper Bound
[Environment=Liquid]	-0.3964	1.0972	-0.3613	0.7182	-2.5580	1.7652
[Environment=Solid]	0					
Frequency	-2.1477	1.6353	-1.3133	0.1904	-5.3695	1.0741

Table B3.2: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.5. Included are the parameter estimates of only the significant effects in the full frequency analysis in both environments separated by ampicillin concentration.

Ampicillin Concentration (mg/l)	Parameter	95% Confidence Interval					
		B	Std. Error	t	P	Lower Bound	Upper Bound
0	[Environment=Liquid]	-0.3526	0.8453	-0.4172	0.6777	-2.0355	1.3303
	[Environment=Solid]	0					
	Frequency	-0.6166	1.1775	-0.5237	0.6020	-2.9608	1.7275
16	[Environment=Liquid]	-1.3691	1.1434	-1.1974	0.2348	-3.6454	0.9072
	[Environment=Solid]	0					
	Frequency	-2.5053	1.6696	-1.5005	0.1375	-5.8292	0.8187
128	[Environment=Liquid]	-0.3964	1.0537	-0.3762	0.7078	-2.4941	1.7014
	[Environment=Solid]	0					
	Frequency	-2.1477	1.5705	-1.3675	0.1754	-5.2744	0.9789

Density Analysis

Table B3.3: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.6. Included are the parameter estimates of only the significant effects in the full frequency analysis in both environments separated by ampicillin concentration. Not included within this table is the Time, Ampicillin, Environment, and Density interaction term as all parameter values are very small (but positive).

Parameter	B	Std. Error	t	P	95% Confidence Interval	
					Lower Bound	Upper Bound
[Time=24]	-1.2271	0.5876	-2.0882	0.0387	-2.3896	-0.0645
[Time=48]	-1.4662	0.6028	-2.4324	0.0164	-2.6587	-0.2737
[Time=72]	0					
[Environment=Liquid]	-1.2341	0.5745	-2.1483	0.0335	-2.3706	-0.0976
[Environment=Solid]	0					
[Time=24] * Density	-1.5236x10 ⁻⁰⁸	2.9151x10 ⁻⁰⁸	-0.5227	0.6021	-7.2907x10 ⁻⁰⁸	4.2435x10 ⁻⁰⁸
[Time=48] * Density	4.9561x10 ⁻⁰⁸	1.6103x10 ⁻⁰⁸	3.0778	0.0025	1.7704x10 ⁻⁰⁸	8.1418x10 ⁻⁰⁸
[Time=72] * Density	0					

Table B3.4: Abridged parameter estimates from the full model factorial ANOVA presented in Table 3.7. Included are the parameter estimates of only the significant effects in the full density analysis in both environments separated by ampicillin concentration and with less than three interaction terms.

Ampicillin Concentration (mg/l)	Parameter	95% Confidence Interval					
		B	Std. Error	t	P	Lower Bound	Upper Bound
0	[Environment=Liquid]	-0.2129	0.5097	-0.4177	0.6782	-1.2402	0.8144
	[Environment=Solid]	0					
	[Time=24] * Density	-5.170x10 ⁻⁰⁹	1.629x10 ⁻⁰⁸	-0.3174	0.7524	-3.800x10 ⁻⁰⁸	2.766x10 ⁻⁰⁸
	[Time=48] * Density	2.767x10 ⁻⁰⁸	1.523x10 ⁻⁰⁸	1.8167	0.0761	-3.027x10 ⁻⁰⁹	5.838x10 ⁻⁰⁸
	[Time=72] * Density	0					
16	[Environment=Liquid]	-0.0563	0.5830	-0.0966	0.9235	-1.2314	1.1187
	[Environment=Solid]	0					
	[Environment=Liquid] * Density	-3.372x10 ⁻⁰⁸	1.406x10 ⁻⁰⁸	-2.3986	0.0208	-6.205x10 ⁻⁰⁸	-5.387x10 ⁻⁰⁹
	[Environment=Solid] * Density	0					
128	[Time=24]	-1.6138	0.4553	-3.5448	0.0009	-2.5313	-0.6963
	[Time=48]	-1.1220	0.5009	-2.2401	0.0302	-2.1314	-0.1126
	[Time=72]	0					
	[Environment=Liquid]	-0.7971	0.4359	-1.8289	0.0742	-1.6756	0.0813
	[Environment=Solid]	0					
	[Time=24] * Density	3.526x10 ⁻⁰⁸	1.546x10 ⁻⁰⁸	2.2807	0.0275	4.102x10 ⁻⁰⁹	6.641x10 ⁻⁰⁸
	[Time=48] * Density	3.875x10 ⁻⁰⁸	1.300x10 ⁻⁰⁸	2.9807	0.0047	1.255x10 ⁻⁰⁸	6.495x10 ⁻⁰⁸
	[Time=72] * Density	0					

Appendix C- Raw Data Graphs

Figure C.1: Raw data of the growth data in both environments for the frequency experiments for both the resistants and sensitives. Included are a representative sample of the volume ratios used (sensitive to resistant) in the experiments and the three different ampicillin concentrations used (0 mg/l, 16 mg/l, and 128 mg/l).

Figure C.2: Raw data of the growth data in both environments for the density experiments for both the resistant and sensitive strains. Included are the inoculate volumes (μl) used in the experiments and the three different ampicillin concentrations used (0 mg/l, 16 mg/l, 128 mg/l).

Appendix D - Glossary

Antibiotic Cycling: Process in which antibiotics from two or more classes are given to a patient one after the other once resistance is acquired

Antibiotic Mixing: Process in which two or more classes of antibiotics are administered simultaneously to a patient

Antibiotic Resistance: condition in which a microbe has an inherent resistance to a single, a class of, or multiple classes of antibiotic drugs

Bactericidal Antibiotic: an antibiotic that kills the microbe outright

Bacteriostatic Antibiotic: an antibiotic that does not kill the microbe outright but prevents the cell from dividing

Commensal: A bacterial species found in a host what is part of the normal fauna within the host in a non-pathogenic form

Compensatory mutation: A rare mutation that decreases the cost of another beneficial mutation

Conjugation: process in which plasmids are transferred from one microbe to another and are often facilitated by surface pili

Gene cassettes: a modular genetic sequence that codes for one or more genes

Horizontal gene transfer: the uptake of genetic material in a microbe from the environment or another unrelated organism

In vitro: outside a living organism, such in a test tube

In vivo: in a living organism

Integrans: DNA elements with a specific structure consisting of two conserved segments flanking a central structure in which genes, often referred to as gene cassettes can be inserted and are the main mechanism of resistance transfer in Gram negative bacteria

MIC: Minimum Inhibitory Concentration – the minimum concentration of a antimicrobial substance which when given to a microbial culture growth is inhibited

Monte Carlo Simulation: a class of stochastic computer algorithms used for simulating the behaviour of various physical and mathematical

MRSA: Methicillin Resistant *Staphylococcus aureus*

Nosocomial: acquisition of a new disease, condition or infection within a healthcare setting

Pharmacokinetics: the study of the metabolism and action of drugs with particular emphasis on the time required for absorption, duration of action, distribution in the body and method excretion

Plasmid: extra-chromosomal segments of DNA that replicate independently of the chromosome and can be donated to other organisms

Super-Integrans: similar to integrans with the exception of a greater ability to insert gene cassettes into the genome

Transposons: gene sequences that can move from one location to another within the microbe's DNA (both chromosomal and plasmid DNA are included)

Vertical gene transfer: process in which genes are passed to offspring

VRE: Vancomycin-Resistant Enterococci

References

- Andersson, D. I. and Levin, B. R. 1999 The biological cost of antibiotic resistance. *Current Opinion in Microbiology*, **2**, 489-493.
- Andersson, D. I. 2006 The biological cost of mutational antibiotic resistance: any practical conclusions? *Current Opinion in Microbiology*, **9**, 461-465.
- Andre, J. B. and Godelle, B. 2005 Multicellular organization in bacteria as a target for drug therapy. *Ecology Letters*, **8**, 800-810.
- Andre, J. B. and Gandon, S. 2006 Vaccination, within-host dynamics, and virulence evolution. *Evolution*, **60**, 13-23.
- Austin, D. J., Kakehashi, M. and Anderson, R. M. 1997 The transmission dynamics of antibiotic-resistant bacteria: the relationship between resistance in commensal organisms and antibiotic consumption. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **264**, 1629-1638.
- Babic, M., Hujer, A. M. and Bonomo, R. A. 2006 What's new in antibiotic resistance? Focus on beta-lactamases. *Drug Resistance Updates*, **9**, 142-156.
- Bergstrom, C. T., Lo, M. and Lipsitch, M. 2004 Ecological theory suggests that antimicrobial cycling will not reduce antimicrobial resistance in hospitals. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 13285-13290.
- Besier, S., Ludwig, A., Brade, V. and Wichelhaus, T. A. 2005 Compensatory adaptation to the loss of biological fitness associated with acquisition of fusidic acid resistance in *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*, **49**, 1426-1431.

- Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. and Falkow, S. 1977 Construction and Characterization of New Cloning Vehicles .2. Multipurpose Cloning System. *Gene*, **2**, 95-113.
- Bonhoeffer, S., Lipsitch, M. and Levin, B. R. 1997 Evaluating treatment protocols to prevent antibiotic resistance. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 12106-12111.
- Boni, M. F. and Feldman, M. W. 2005 Evolution of antibiotic resistance by human and bacterial niche construction. *Evolution*, **59**, 477-491.
- Bryan, A., Shapir, N. and Sadowsky, M. J. 2004 Frequency and distribution of tetracycline resistance genes in genetically diverse, nonselected, and nonclinical *Escherichia coli* strains, isolated from diverse human and animal sources. *Applied and Environmental Microbiology*, **70**, 2503-2507.
- Burton, Z. F. and Kaguni, J. M. 1997 *Experiments in molecular biology : biochemical applications*, Academic Press, San Diego.
- Coates, A. R. M. and Hu, Y. 2007 Novel approaches to developing new antibiotics for bacterial infections. *British Journal of Pharmacology*, **152**, 1147-1154.
- Cooper, B. S., Medley, G. F., Stone, S. P., Kibbler, C. C., Cookson, B. D., Roberts, J. A., Duckworth, G., Lai, R. and Ebrahim, S. 2004 Methicillin-resistant *Staphylococcus aureus* in hospitals and the community: Stealth dynamics and control catastrophes. *Proceedings of the National Academy of Sciences of the United States of America*, **101**, 10223-10228.
- Courvalin, P. 2008 Predictable and unpredictable evolution of antibiotic resistance. *Journal of Internal Medicine*, **264**, 4-16.

- Dugatkin, L. A., Perlin, M. and Atlas, R. 2005a Antibiotic resistance and the evolution of group-beneficial traits. II: A metapopulation model. *Journal of Theoretical Biology*, **236**, 392-396.
- Dugatkin, L. A., Perlin, M., Lucas, J. S. and Atlas, R. 2005b Group-beneficial traits, frequency-dependent selection and genotypic diversity: an antibiotic resistance paradigm. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **272**, 79-83.
- Ellis, R. J., Lilley, A. K., Lacey, S. J., Murrell, D. and Godfray, H. C. J. 2007 Frequency-dependent advantages of plasmid carriage by *Pseudomonas* in homogeneous and spatially structured environments. *Isme Journal*, **1**, 92-95.
- Feizabadi, M. M., Etemadi, G., Yadegarinia, D., Rahmati, M., Shabanpoor, S. and Bokaei, S. 2006 Antibiotic-resistance patterns and frequency of extended-spectrum beta-lactamase-producing isolates of *Klebsiella pneumoniae* in Tehran. *Medical Science Monitor*, **12**, BR362-BR365.
- Fernandez-Reyes, M., Rodriguez-Falcon, M., Chiva, C., Pachon, J., Andreu, D. and Rivas, L. 2009 The cost of resistance to colistin in *Acinetobacter baumannii*: a proteomic perspective. *Proteomics*, **9**, 1632-1645.
- Gordo, I. and Campos, P. R. A. 2006 Adaptive evolution in a spatially structured asexual population. *Genetica*, **127**, 217-229.
- Greig, D. and Travisano, M. 2008 Density-dependent effects on allelopathic interactions in yeast. *Evolution*, **62**, 521-527.
- Habets, M., Rozen, D. E., Hoekstra, R. F. and de Visser, J. 2006 The effect of population structure on the adaptive radiation of microbial populations evolving in spatially structured environments. *Ecology Letters*, **9**, 1041-1048.

- Handel, A., Regoes, R. R. and Antia, R. 2006 The role of compensatory mutations in the emergence of drug resistance. *Plos Computational Biology*, **2**, 1262-1270.
- Harbottle, H., Thakur, S., Zhao, S. and White, D. G. 2006 Genetics of antimicrobial resistance. *Animal Biotechnology*, **17**, 111-124.
- Horwitz, A. H., Heffernan, L., Cass, L., Miyada, C. G. and Wilcox, G. 1980 CONSTRUCTION OF PBR322-ARA HYBRID PLASMIDS BY INVIVO RECOMBINATION. *Molecular & General Genetics*, **179**, 615-625.
- Hotchkiss, J. R., Strike, D. G., Simonson, D. A., Broccard, A. F. and Crooke, P. S. 2005 An agent-based and spatially explicit model of pathogen dissemination in the intensive care unit. *Critical Care Medicine*, **33**, 168-176.
- Jin, Y. D., Xu, J. P., Zhang, W. H., Luo, J. L. and Xu, Q. W. 2005 Simulation of biological waves in single-species bacillus system governed by birth and death-diffusion dynamical equation. *Mathematics and Computers in Simulation*, **68**, 317-327.
- Kaymak, B. and Haas, C. N. 2008 Effect of initial microbial density on inactivation of *Escherichia coli* by monochloramine. *Journal of Environmental Engineering and Science*, **7**, 237-245.
- Kim, C., Cha, J. Y., Yan, H. G., Vakulenko, S. B. and Mobashery, S. 2006 Hydrolysis of ATP by aminoglycoside 3'-phosphotransferases - An unexpected cost to bacteria for harboring an antibiotic resistance enzyme. *Journal of Biological Chemistry*, **281**, 6964-6969.
- Kugelberg, E., Lofmark, S., Wretling, B. and Andersson, D. I. 2005 Reduction of the fitness burden of quinolone resistance in *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy*, **55**, 22-30.

- Lenski, R. E., Rose, M. R., Simpson, S. C. and Tadler, S. C. 1991 Long term experimental evolution in *Escherichia coli* .1. Adaptation and divergence during 2,000 generations. *American Naturalist*, **138**, 1315-1341.
- Levin, B. R., Perrot, V. and Walker, N. 2000 Compensatory mutations, antibiotic resistance and the population genetics of adaptive evolution in bacteria. *Genetics*, **154**, 985-997.
- Levin, B. R. and Bull, J. J. 2004 Population and evolutionary dynamics of phage therapy. *Nature Reviews Microbiology*, **2**, 166-173.
- Lipsitch, M., Bergstrom, C. T. and Levin, B. R. 2000 The epidemiology of antibiotic resistance in hospitals: Paradoxes and prescriptions. *Proceedings of the National Academy of Sciences of the United States of America*, **97**, 1938-1943.
- Maglio, D., Kuti, J. L. and Nicolau, D. P. 2005 Simulation of antibiotic pharmacodynamic exposure for the empiric treatment of nosocomial bloodstream infections: A report from the OPTAMA program. *Clinical Therapeutics*, **27**, 1032-1042.
- McDermott, P. F., Walker, R. D. and White, D. G. 2003 Antimicrobials: Modes of action and mechanisms of resistance. *International Journal of Toxicology*, **22**, 135-143.
- Mindlin, S. Z., Petrova, M. A., Bass, I. A. and Gorlenko, Z. M. 2006 Origin, evolution, and migration of drug resistance genes. *Russian Journal of Genetics*, **42**, 1257-1271.
- Monod, J. 1949 The growth of bacterial cultures. *Annual Review of Microbiology*, **3**, 371-394.
- Nilsson, A. I., Zorzet, A., Kanth, A., Dahlstrom, S., Berg, O. G. and Andersson, D. I. 2006 Reducing the fitness cost of antibiotic resistance by amplification of initiator tRNA genes. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 6976-6981.
- Normark, B. H. and Normark, S. 2002 Evolution and spread of antibiotic resistance. *Journal of Internal Medicine*, **252**, 91-106.

- O'Neill, A. J., Huovinen, T., Fishwick, C. W. G. and Chopra, I. 2006 Molecular genetic and structural modeling studies of *Staphylococcus aureus* RNA polymerase and the fitness of rifampin resistance genotypes in relation to clinical prevalence. *Antimicrobial Agents and Chemotherapy*, **50**, 298-309.
- Peck, S. L. 2001 Antibiotic and insecticide resistance modeling - is it time to start talking? *Trends in Microbiology*, **9**, 286-292.
- Perfeito, L., Gordo, I. and Campos, P. R. A. 2006 The effect of spatial structure in adaptive evolution. *European Physical Journal B*, **51**, 301-306.
- Perfeito, L., Pereira, M. I., Campos, P. R. A. and Gordo, I. 2008 The effect of spatial structure on adaptation in *Escherichia coli*. *Biology Letters*, **4**, 57-59.
- Perlman, D., William, B., Pastan, J. and Pastan, I. H. 1979 Use of antibiotics in cell culture media. *Methods in Enzymology* 110-116. Academic Press,
- Pranting, M., Negrea, A., Rhen, M. and Andersson, D. I. 2008 Mechanism and fitness costs of PR-39 resistance in *Salmonella enterica* serovar *Typhimurium* LT2. *Antimicrobial Agents and Chemotherapy*, **52**, 2734-2741.
- Reluga, T. C. 2005 Simple models of antibiotic cycling. *Mathematical Medicine and Biology-a Journal of the Ima*, **22**, 187-208.
- Ross-Gillespie, A., Gardner, A., West, S. A. and Griffin, A. S. 2007 Frequency dependence and cooperation: theory and a test with bacteria. *American Naturalist*, **170**, 331-342.
- Sandegren, L., Lindqvist, A., Kahlmeter, G. and Andersson, D. I. 2008 Nitrofurantoin resistance mechanism and fitness cost in *Escherichia coli*. *Journal of Antimicrobial Chemotherapy*, **62**, 495-503.

- Sanders, C. C., Sanders, W. E., Goering, R. V. and McCloskey, R. V. 1987 Leakage of Beta-lactamase - a 2nd Mechanism for antibiotic potentiation by amdinocillin. *Antimicrobial Agents and Chemotherapy*, **31**, 1164-1168.
- Smith, D. L., Harris, A. D., Johnson, J. A., Silbergeld, E. K. and Morris, J. G. 2002 Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 6434-6439.
- SPSS Inc. 2006 SPSS for Windows, 15.0.0 SPSS Inc. Chicago
- Stewart, F. M. and Levin, B. R. 1973 Partitioning of resources and outcome of interspecific competition - model and some general considerations. *American Naturalist*, **107**, 171-198.
- Stewart, F. M., Antia, R., Levin, B. R., Lipsitch, M. and Mittler, J. E. 1998 The population genetics of antibiotic resistance II: Analytic theory for sustained populations of bacteria in a community of hosts. *Theoretical Population Biology*, **53**, 152-165.
- Stewart, P. S. and Costerton, J. W. 2001 Antibiotic resistance of bacteria in biofilms. *Lancet*, **358**, 135-138.
- Trawick, J. D. and Schilling, C. H. 2006 Use of constraint-based modeling for the prediction and validation of antimicrobial targets. *Biochemical Pharmacology*, **71**, 1026-1035.
- Velicer, G. J. 2003 Social strife in the microbial world. *Trends in Microbiology*, **11**, 330-337.
- Vulic, M. and Kolter, R. 2001 Evolutionary cheating in *Escherichia coli* stationary phase cultures. *Genetics*, **158**, 519-526.
- Walsh, C. 2000 Molecular mechanisms that confer antibacterial drug resistance. *Nature*, **406**, 775-781.

- Wang, Y. C. and Lipsitch, M. 2006 Upgrading antibiotic use within a class: Tradeoff between resistance and treatment success. *Proceedings of the National Academy of Sciences of the United States of America*, **103**, 9655-9660.
- Ward, H., Perron, G. G. and Maclean, R. C. 2009 The cost of multiple drug resistance in *Pseudomonas aeruginosa*. *Journal of Evolutionary Biology*, **22**, 997-1003.
- Webb, G. F., D'Agata, E. M. C., Magal, P. and Ruan, S. 2005 A model of anti biotic-resistant bacterial epidemics in hospitals. *Proceedings of the National Academy of Sciences of the United States of America*, **102**, 13343-13348.
- Wiener, P. 2000 Antibiotic production in a spatially structured environment. *Ecology Letters*, **3**, 122-130.
- Wijngaarden, P. J., van den Bosch, F., Jeger, M. J. and Hoekstra, R. F. 2005 Adaptation to the cost of resistance: a model of compensation, recombination, and selection in a haploid organism. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **272**, 85-89.
- Willms, A. R., Roughan, P. D. and Heinemann, J. A. 2006 Static recipient cells as reservoirs of antibiotic resistance during antibiotic therapy. *Theoretical Population Biology*, **70**, 436-451.
- Zehnder, A. J. B. and Wuhrmann, K. 1977 Physiology of a Methanobacterium Strain AZ. *Archives of Microbiology*, **111**, 199-205.