

Resistance maintained in digital organisms despite guanine/cytosine-based fitness cost and extended de-selection: implications to microbial antibiotics resistance

Abstract

Antibiotics resistance has caused much complication in the treatment of diseases, where the pathogen is no longer susceptible to specific antibiotics and the use of such antibiotics are no longer effective for treatment. A recent study that utilizes digital organisms suggests that complete elimination of specific antibiotic resistance is unlikely after the disuse of antibiotics, assuming that there are no fitness costs for maintaining resistance once resistance are established. Fitness cost are referred to as reaction to change in environment, where organism improves its' abilities in one area at the expense of the other. Our goal in this study is to use digital organisms to examine the rate of gain and loss of resistance where fitness costs have incurred in maintaining resistance. Our results showed that GC-content based fitness cost during de-selection by removal of antibiotic-induced selective pressure portrayed similar trends in resistance compared to that of no fitness cost, at all stages of initial selection, repeated de-selection and re-introduction of selective pressure. A t-test suggested that prolonged stabilization of resistance after initial loss is not statistically significant for its difference to that of no fitness cost. This suggests that complete elimination of specific antibiotics resistance is unlikely after the disuse of antibiotics despite presence of fitness cost in maintaining antibiotic resistance during the disuse of antibiotics, once a resistant pool of micro-organism has been established.

Keywords: antibiotics resistance, %GC-based fitness cost, digital organism, DOSE

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Clarence FG Castillo,¹ Zhu En Chay,^{2,3}
Maurice HT Ling^{3,4}

¹School of Information Technology, Republic Polytechnic, Singapore

²Department of Accounting, The University of Melbourne, Australia

³Colossus Technologies LLP, Singapore

⁴School of BioSciences, The University of Melbourne, Australia

Correspondence: Maurice HT Ling, School of BioSciences, The University of Melbourne, Victoria, Australia,
Email mauriceling@acm.org

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Introduction

Antibiotic-resistant bacteria area substantial burden on the human population in terms of both morbidity and mortality, and this drives the need for new discovery and use of antibiotics for treatment. Antibiotic resistance can be naturally occurring. An example of that is that Penicillin-resistant *Bacillus licheniformis* pores has were isolated from dried soil from the roots of plants preserved in British Museum since 1689.¹ Lee et al.,² suggests that oral antibiotics and food chemicals, such as preservatives, may not be completely absorbed by the gastrointestinal tract, leading to exposure of sub-lethal concentrations to intestinal flora, which may lead to development of tolerance to oral antibiotics and food chemicals.³⁻⁵ This is supported by the observation of the first vancomycin-resistant bacterium, vancomycin-resistant enterococcus in the mid-1980s, after less than a decade of vancomycin use.⁶ Once traits conferring resistance are acquired, it can be transmitted to sensitive strains using means such as horizontal gene transfer within intestinal micro-organisms.⁷

A number of studies have demonstrated that the maintenance of resistance traits can incur a fitness cost compared to its' antibiotic-sensitive counterparts⁸⁻¹⁰ as resistance mutations may disrupt normal physiological processes. The incurrence of fitness cost had been suggested to be able to revert resistant strains back to sensitivity after extended withdrawal of previously resistant antibiotics¹¹ but with limitations. A few studies suggest that the maintenance of resistance trait in the absence of antibiotics may not incur fitness cost.¹²⁻¹⁴ Blot et al.,¹² and Dutta¹³ have evidence showing that resistant strains may

be fitter than the parental sensitive strains. Experimental examination into the gain-loss-re-gain of resistance traits will require sequencing of individual organisms or bacterium, as each organism may be genetically different from each other during the process. Hence, such experimental studies are difficult and expensive.

Computer-simulated evolution of digital organisms (DOs) has been used in many evolutionary studies¹⁵⁻¹⁷ as it offers the ability to examine each genome non-destructively. Castillo & Ling¹⁸ suggests that complete elimination of resistance is unlikely, even after disuse of antibiotics assuming that no fitness cost is needed to maintain antibiotic resistance, when a resistant pool of micro-organisms have been established.

A number of studies suggest that Guanine-Cytosine (commonly known as GC) content is an important fitness trait. Mann & Chen¹⁹ demonstrated that GC content of bacterial genome correlates to their environmental niche. Light field et al.,²⁰ suggested that translational efficiency can be constraint by GC content. Povolotskaya et al.,²¹ examine the prevalence of stop codons in bacteria and found that TAA is preferred for micro-organisms with low GC content while TGA is preferred for micro-organisms with high GC content. This is supported by Bohlin et al.,²² suggesting that selective pressure towards a varying genomic GC contents in micro-organisms are present. The studies show that GC contents are not random but selective Hershberg & Petrov,²³ suggesting that deviation from an organism-specific GC contents can incur a fitness cost. Castillo & Ling¹⁸ uses a sequence of 5 consecutive zeros, up to a maximum of 10

blocks, to represent antibiotic resistance. In a binary genome, we can consider '0' to represent guanine and cytosine while '1' to represent adenine and thymine. Given that the initial genome of the DOs is a sequence of 500 bases of alternate binaries, the GC content is 50%. Thus, antibiotic resistance can be considered a high GC trait. In this study, we use 50% GC (equal numbers of 1s and 0s in the genome) as the optimal GC content and increasing deviation from this optimal as fitness cost, to examine the gain-loss-re-gain of resistant traits where maintenance of resistant traits will incur a fitness cost.

In this study, we examine the gain-loss-re-gain of resistant traits where maintenance of resistant traits will incur a fitness cost. The fitness cost can be calculated as deviation from expected GC content. The results from this study will be compared against Castillo and Ling.¹⁸ We hypothesize that incurred fitness cost will result in a greater reduction in population resistance in the absence of antibiotics acting as compared to a case whereby no fitness cost is needed to maintain resistance.

Materials and methods

DOSE platform

The details of DOSE (Digital Organism Simulation Environment) have been described.²⁴⁻²⁶ Generally, a population consists of one or more DOs and each DO consists of a genome and a set of status. One or more chromosomes of different lengths make up a genome. Commonly used nucleotides are binary, integer, or alphanumeric. Each chromosome has background mutation rate and an additional mutation rate can be added in the simulation. In addition, defined mutations on a specific base can be made. The genome can be read and executed by Ragaraja interpreter.²⁶ DO statuses contain information regarding the organism (such as identity and deme), as well as vital conditions (such as age), allowing for traceability across generations. One or more populations can reside in the virtual world, consisting of one or more ecological cells arranged in one or more dimensions. During simulation, 6 simulation-dependent operations are executed on each population. Pre-mating population control was first executed to remove unhealthy organisms. Mutation scheme is performed after first step, using point mutations and gene translocation. Thirdly, fitness is measured before and after mating has occurred. Fourthly, post-mating population control is performed to remove unfit organisms. After the processing is done, ad-hoc generational events - catastrophic events are performed. Lastly, organisms are free to migrate to another ecological cell.

Simulation setup

The simulation setup is identical to Castillo and Ling.¹⁸ A population of one hundred Dos is contained within one ecological cell used for simulation. The genome of each DO is one chromosome of 500 binary nucleotides. The ancestral chromosome is 500 bases of alternate binaries; that is, [1010101010]50. 1% or 5 point mutations per generation form the background mutation.

Experiment 1 (Initial gain of resistance)

The simulation experiments are based on Castillo & Ling.¹⁸ 25 replicates of 200 generations per replicate were simulated. Pre-determined nucleotide sequence of 11 zeros is representative of antibiotic-resistance. Fitness score for each DO was defined as the number of consecutive zeros within a block for 10 blocks. Three rules were used. Firstly, a minimum of 2 consecutive zeros was required to be considered a block. For example, '101101011' would result in a fitness score of 0 while '101001011' would result in a fitness score

of 2 as there were at least 2 consecutive zeros. Secondly, each block contributed 10% of the maximum fitness score. This implies that a maximum of 10 blocks were used to calculate fitness score. All DOs will undergo random mutations and replications in each generation, resulting in 200 DOs. Fitness-proportionate selection (FPS) was used to select 100 DOs. The probability of a DO being selected for the next generation is directly proportional to its fitness score. The fitness score of the selected 100 DOs were recorded.

Experiment 2 (Loss of resistance)

Each of the 25 populations at 200th generation (corresponding to final generation of Experiment 1) was revived and simulated for another 5000 generations to mimic the loss of resistant traits, giving the final generation count of 5200. In each generation, a fitness cost of each DO calculated as the percentage deviation between equal numbers of ones and zeros in the entire genome (representative of 50% GC content). The computation of fitness cost is as follows:

$$\text{Fitness cost (\%)} = \frac{\left[\left(x - \frac{x+y}{2} \right) + \left(y - \frac{x+y}{2} \right) \right]}{x+y}$$

where x is the number of ones and y is the number of zeros. For example, if an organism's genome is made up of 200 ones and 300 zeros, its fitness cost will be 20 or 20% deviation from 50% GC content.

$$\left[\text{Fitness cost (\%)} = \left(\frac{(|200 - 250| + |300 - 250|)}{500} \right) \right]$$

The fitness score is defined as the difference between 100 and the fitness cost. In this case, the fitness score will be 80, which will be used for FPS. Fitness score for each of the 100 surviving DOs were recorded.

Similar to Castillo and Ling,¹⁸ we estimated the number of generations needed for the population to revert to pre-selective fitness score, indicated by the average fitness score of un-selected population as control, after the initial decline of fitness score after initial removal of selective pressure. In our previous study (Castillo and Ling),¹⁸ fitness score from generation 2000 to 5200 are used to fit a linear regression model, $\text{Fitness score} = \beta_1(\text{Generation}) + \beta_0$. This model can be used to estimate the number of generations needed for the fitness to decline to pre-selection. 95% confidence interval for the number of generations can be estimated by 95% confidence interval of the gradient of decline (β_1) using Jack-knife re sampling.

Experiment 3 (Re-gain of resistance)

Reviving a population at 5200th generation (corresponding to the last generation of Experiment 2) and continuing the simulation for another 200 generations were used to mimic the re-gain of resistance as a result of re-introduction of antibiotics after extended discontinuation. All processes, including FPS, used in these 200 generations were identical to that of Experiment 1 (initial gain of resistant trait).

Experiment 4 (Repeated loss and re-gain of resistance)

Repeated loss and re-gain of antibiotic resistance was carried out to mimic multiple cycles of re-introduction and disuse of antibiotics. Populations at 5200th generation (corresponding to the last generation of Experiment 3) were revived and simulated for 5000 generations

of resistance loss using the same procedure as Experiment 2 (loss of resistance). This gain and loss of resistant trait was repeated for another 3 cycles, yielding a total of 26000 generations. The average fitness scores for each re-gain of resistance simulation (5201st to 5400th generation as Gain-2, 10401st to 10600th generation as Gain-3, 15601st to 15800th generation as Gain-4, and 20801st to 21000th generation as Gain-5) were analyzed.

Results and discussion

It is postulated that the antibiotic resistant traits may decrease after disuse of the chemical when maintaining resistance traits will incur a fitness cost.⁸⁻¹⁰ However, contradictory results have been reported.²⁸ Dutta¹³ and Jechalke et al.,¹⁴ suggest that maintaining resistance trait in the absence of antibiotics may not incur fitness cost in certain situations. In fact, resistant strains may be fitter than the parental sensitive strains.^{12,13}

We use DOs as a tool to study the gain and loss of resistance traits,

which is both difficult and expensive to examine experimentally. Castillo & Ling¹⁸ suggested that once a reservoir of resistant micro-organism has been established, complete elimination of specific antibiotics resistance is unlikely despite after extended disuse of antibiotics, assuming no fitness cost for maintaining resistance. Our current study will be compared with Castillo & Ling¹⁸ to study if fitness cost is a factor in maintaining the resistant trait.

Our results show that the average population fitness score increases over 200 generations as a result of selectivity (Figure 1A) and is significantly higher than no selective pressure or control ($\text{pairedt-testp-value} < 1 \times 10^{-50}$). This is supported by recent studies showing direct relationship between emergence of antibiotic resistant strains and amount of prescribed antibiotics.²⁹⁻³³ The average population fitness score under selective pressure in this study (generation 1 to 200) is not significantly different ($\text{pairedt-testp-value} > 0.05$) from the corresponding results study by Castillo & Ling,¹⁸ where identical selective pressure and simulation setup are used for comparability of results.

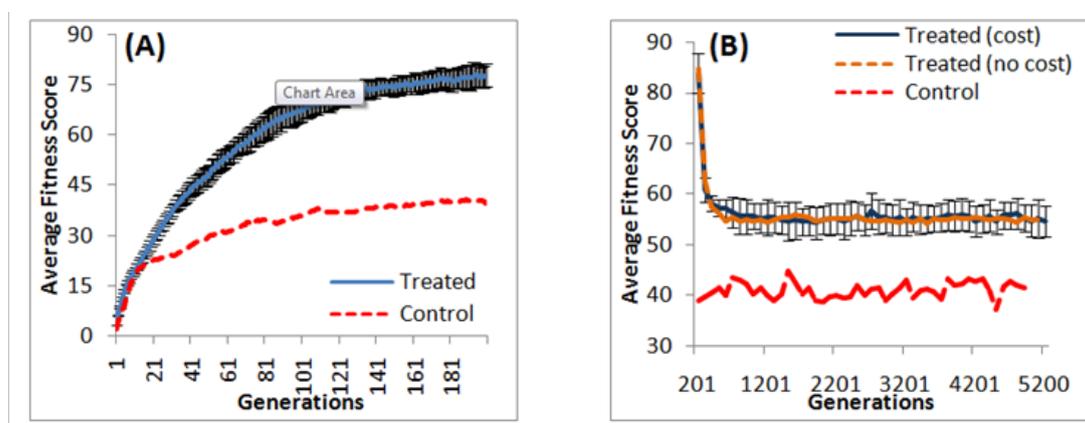


Figure 1 Initial gain and loss of resistance trait. Panel A shows the gain of resistance for the first 200 generations while Panel B shows the loss of resistance for the subsequent 5000 generations (corresponding to generation 201 to 5200). Error bars represent standard errors.

All 25 populations were subjected to 5000 generations of selective pressure withdrawal to mimic the disuse of specific antibiotics after increased antibiotic resistance. However, in this study, we added the deviation from an optimal GC content of 50% as fitness cost. We hypothesize that the fitness cost incurred to maintain resistance will reduce fitness compared to no fitness cost incurred in our previous study (Castillo and Ling).¹⁸ Our results from the first loss of resistant trait (experiment 2) show a rapid decline of fitness immediately after withdrawal of antibiotic-induced selective pressure, followed by a plateau of fitness (Figure 1B). This is consistent with studies showing decline of resistance after discontinuation of antibiotics.^{34,35} Although the trend of fitness is similar with or without fitness cost by comparing results from this study to Castillo and Ling,¹⁸ our results suggest that the incorporation of fitness cost does not significantly reduce average population fitness ($\text{pairedt-testp-value} = 0.013$), and the fitness score is significantly higher than that of control ($\text{pairedt-testp-value} = 4.3 \times 10^{-25}$).

Our previous study (Castillo & Ling)¹⁸ suggested that the average population fitness is not likely to decline to levels before the use of antibiotics based on regression analyses despite extended de-selection (5000 generations) post-selective pressure. We repeat regression analyses on average population fitness and average top fitness from 2000th to 5200th generation (Table 1) from this study. This will estimate the number of generations needed for the average population fitness to decline to the level of pre-antibiotic use, which

can be estimated by the average population fitness of the control. Our results demonstrate that the rate of fitness change over generations is not statistically significant at 99% confidence, with (this study) or without fitness cost (Castillo and Ling).¹⁸ This implies that the average fitness may remain constant, decrease, or even increase during de-selection. A number of studies had reported similar findings. Enne et al.,³⁶ report an increase of 6.2% in the frequency of sulfonamide-resistant *Escherichia coli* in United Kingdom following 98% decline of sulfonamide prescription. Moreover, there is no further reduction in the prevalence of sulfonamide-resistant *E. coli* 5 years post-study.³⁷ Nilsson & Laurell³⁸ report stable proportions of penicillin-resistant *Streptococcus pneumoniae* a decade after intervention and reduced penicillin consumption. However, there are also cases of declined antibiotic prevalence. Okon et al.,³⁹ report decline in penicillin, gentamicin, erythromycin, clindamycin, and methicillin resistance rates in sub-Saharan Africa. Lai et al.,⁴⁰ report decline in cefuroxime, cefotaxime, and cefepime resistance rates in China. These studies suggested that the decline in the occurrence of antibiotic-resistance is not a simple function of stress, but a combination of other factors. Hjalmarsdottir & Kristinsson⁴¹ showed a possibility of redundant resistance traits behaving independently, thus, increasing the unpredictability of antibiotic resistance after antibiotic discontinuation, which is consistent with our results. Although this study only examines GC-content based fitness cost, Hall et al.,⁴² suggested that fitness cost may be a function of conditional environmental

demand on the micro-organism. This means that the role played by fitness costs may not be directly deducible and a possibility of resistant traits being advantageous in certain environmental niches⁴³ as Miskinyte and Gordo⁴⁴ find that 67% of streptomycin-resistant

Escherichia coli survives better than the susceptible bacteria in the intracellular niche of the phagocytic cells. Baker et al.,⁴⁵ find that 6 out of 11 fluoroquinolone-resistant *Salmonella typhi* strains to outgrow susceptible strains on competitive growth assays.

Table 1 Estimated number of generations post-selection that is needed to lose fitness traits. Regression models are generated from gradual loss of fitness after withdrawal of selection pressure (Generation 2000 to 5200) as initial fitness loss (Generation 201 to 1999) may over-estimate the rate of fitness loss.

Study	Regression model	Estimated generations needed to lose fitness
Average Top Fitness	(-95% CI) Fitness = 67.3 – 0.000061 Generation	438,000
	(Mean) Fitness = 67.3 + 0.000021 Generation	Infinity
	(+95% CI) Fitness = 67.3 + 0.000292 Generation	Infinity
	(-95% CI) Fitness = 60.7 – 0.000426 Generation	47,000
	(Mean) Fitness = 60.7 – 0.000269 Generation	74,000
	(+95% CI) Fitness = 60.7 – 0.000100 Generation	201,000
Average Population Fitness	(-95% CI) Fitness = 54.7 – 0.000081 Generation	175,000
	(Mean) Fitness = 54.7 + 0.000072 Generation	Infinity
	(+95% CI) Fitness = 54.7 + 0.000225 Generation	Infinity
	(-95% CI) Fitness = 55.1 – 0.000225 Generation	64,000
	(Mean) Fitness = 55.1 + 0.000043 Generation	Infinity
	(+95% CI) Fitness = 55.1 + 0.000139 Generation	Infinity

Besides the balance of fitness cost incurred and fitness advantage gained from resistance traits, there are studies suggesting that compensatory mutations may occur to circumvent the incurred fitness cost. Albarracín Orió et al.⁴⁶ found that the fitness cost incurred by a mutant form of *penicillin-binding protein 2b (PBP2b)*, giving rise to beta-lactam resistance in *Streptococcus pneumoniae*, is fully compensated by acquisition of mutations in *penicillin-binding protein 1a (PBP1a)* and *penicillin-binding protein 2x (PBP2x)*. Zorzet et al.,⁴⁷ reported that mutations in *accessory gene regulator protein C (AgrC)* gene compensates for the fitness cost from *formyl methyl transferase (fmt)* gene mutation, which resulted in resistance to high levels of actinonin. Brandis & Hughes⁴⁸ report that the most prevalent *bacterial RNA polymerase beta subunit (rpoB)* mutation (S531L), resulting in rifampicin resistance in *Mycobacterium tuberculosis* (the causative agent of tuberculosis), can be compensated by mutations in other bacterial RNA polymerase subunits. These suggested alternative means of bypassing the fitness cost incurred by gain of antibiotic resistant traits. This is a possibility which could explain our results do not differ from Castillo & Ling¹⁸ where fitness cost has minimal contribution to reversion of antibiotic resistance.

It is crucial to consider that the acquisition of an initial resistant gene might be a precursor to subsequent resistance. Castillo & Ling¹⁸ suggested that subsequent rate of gain of resistance are faster compared to the rate of initial gain of resistance, despite being interspaced by 5000 generations of resistance decline. Our results showed that the re-gain of resistance (5201st to 5400th generation) after 5000 generations of de-selection is significant faster and higher as shown in Figure 2, (*paired t-test p-value* = 0.013) than the initial gain of resistance (1st to 200th generation). This is consistent with studies done by Castillo & Ling.¹⁸

Our results also suggested that re-introduction of previous antibiotic resistance is likely to result in more rapid resistant formation. However, this has not been shown experimentally. Rapid resistance

from antibiotic re-introduction might be due the presence of genetic memory of prior events⁴⁹ as suggested by Gajardo and Beardmore⁵⁰ to be a species preservation strategy by retaining resistance. Lee et al.,² suggested that resistance to a specific selective pressure could lead to generic resistance of different selective pressures. A study on single and multi-drug resistant *Mycobacterium smegmatis*. Borrell et al.,⁵¹ reports on cases whereby 6 out of 17 isolates that have resistance to both rifampicin and ofloxacin (first and second line antibiotics respectively) show higher fitness costs compared to single antibiotic resistant strains. A study is done by Saddler et al.,⁵² showed that there are other factors like reproductive numbers and drug efficiency are influential to drug resistance than the cost of resistance fitness.

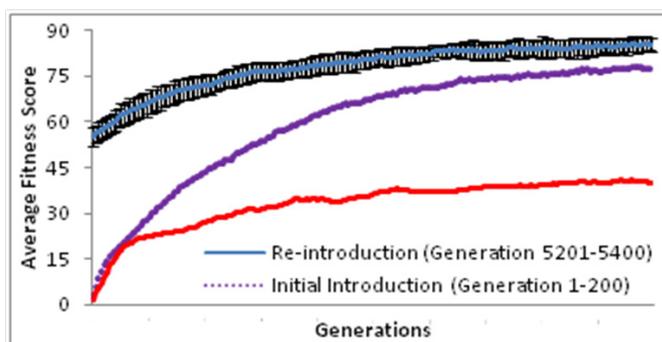


Figure 2 Average population fitness score for 200 generations of re-introduction of selective pressure. Re-introduction of selective pressure was carried out from de-selection experiment (generation 5200 in Experiment 3). This is compared to initial introduction of selection to a native population (Experiment 1) and paired t-test is performed between the generation-matched average fitness of initial introduction (generation 1 to 200) and re-introduction (generation 5201 to 5400) is significantly different (*paired t-test p-value* = 4.9×10^{-56}). Error bars represents standard errors.

Our results from the re-gain of resistance (5201st to 5400th generation) simulation show that the average fitness score without fitness cost incurred (Castillo and Ling [18a]) is significantly higher than the average fitness score with fitness cost incurred (this study). Although this may suggest that fitness cost associated with maintenance of resistance may be acting to revert the population fitness back to susceptibility, our results from subsequent re-gain and loss of selective pressure suggests that such reversion is unlikely as the average

population fitness score is substantially higher than that of control population (Figure 3) (Table 2), ($\text{paired } t\text{-test } p\text{-value} = 4.3 \times 10^{-25}$). Our results suggest that the fitness cost incurred to maintain antibiotics resistance may have an impact in the level of resistance during discontinuation of antibiotic. Such impact is unlikely to revert resistant strains back to susceptibility as the role of fitness cost is limited for such reversion. Other factors other than fitness cost have to be considered for more comprehensive study.⁵³

Table 2 Paired t-test comparisons of average population fitness between de-selections. Paired t-tests were used instead of one-way ANOVA as the former is targeted towards testing the difference of 2 sets of data; hence, a more appropriate test compared to ANOVA. Our results show that there is no statistical difference between the average population fitness (from 25 replicates) from any 2 de-selections, regardless of selection methods. However, the average population fitness from any de-selection is significantly higher than control

Paired t-test Comparisons	p-value
Loss-1 (Generation 201 to 5200):With fitness cost (this study) vs without fitness cost (Castillo and Ling ¹⁸)	0.013
Loss-2 (Generation 5401 to 10400):With fitness cost (this study) vs without fitness cost (Castillo and Ling ¹⁸)	0.053
Loss-3 (Generation 10601 to 15400):With fitness cost (this study) vs without fitness cost (Castillo and Ling ¹⁸)	0.406
Loss-4 (Generation 15801 to 20800):With fitness cost (this study) vs without fitness cost (Castillo and Ling ¹⁸)	0.486
Control vs Loss-2 (Generation 5401 to 10400) with fitness cost	1.3x10-25
Control vs Loss-3 (Generation 10601 to 15400) with fitness cost	1.6x10-25
Control vs Loss-4 (Generation 15801 to 20800) with fitness cost	6.2x10-26

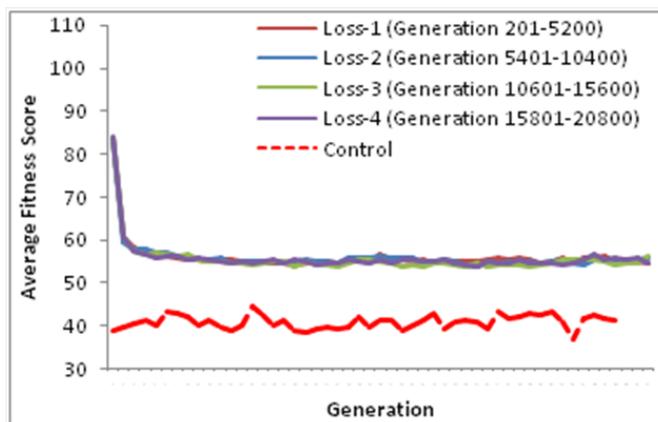


Figure 3 Repeated loss of resistance trait. There is no significant difference (Table 2) between consecutive de-selections between with fitness cost (this study) or without fitness cost¹⁸ but there are significant differences in the fitness scores between de-selections and control population ($\text{paired } t\text{-test } p\text{-value} = 2 \times 10^{-25}$) Table 2.

Conclusion

In this study, we continue from our previous study (Castillo & Ling)¹⁸ to examine the gain-loss-re-gain of resistant traits where fitness cost is incurred to maintain resistant traits, and whether such fitness cost has the potential to revert resistance strains back to susceptibility. Our findings suggest that this is unlikely as the average population fitness in various stages of gain-loss-re-gain of resistant traits with or without fitness cost incurred is not statistically significant. This suggests that fitness cost may have limited role to revert from resistance to susceptibility.

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Conflict of interest

The author declares no conflict of interest.

References

- Pollock MR. Origin and function of penicillinase: A problem in biochemical evolution. *Br Med J.* 1967;4(5571):71–77.
- Lee CH, Oon JS, Lee KC, et al. *Escherichia coli* ATCC 8739 adapts to the presence of sodium chloride, monosodium glutamate, and benzoic acid after extended culture. *ISRN Microbiol.* 2012;2012:965356.
- Furtula V, Farrell EG, Diarrassouba F, et al. Veterinary pharmaceuticals and antibiotic resistance of *Escherichia coli* isolates in poultry litter from commercial farms and controlled feeding trials. *Poult Sci.* 2010;89(1):180–188.
- Goh DJW, How JA, Lim JZR, et al. Gradual and step-wise halophilization enables *Escherichia coli* ATCC 8739 to adapt to 11% NaCl. *Electronic Physician.* 2012;4(3):527–535.
- How JA, Lim JZR, Goh DJW, et al. Adaptation of *Escherichia coli* ATCC 8739 to 11% NaCl. *Dataset Papers in Biology.* 2013;2013:219095.
- Moellering RC. Vancomycin: a 50year reassessment. *Clin Infect Dis.* 2006;42(Suppl 1):S3–S4.
- Broaders E, Gahan CG, Marchesi JR. Mobile genetic elements of the human gastrointestinal tract: Potential for spread of antibiotic resistance genes. *Gut Microbes.* 2013;4(4):271–280.
- Hraiech S, Roch A, Lepidi H, et al. Impaired virulence and fitness of a colistin-resistant clinical isolate of *Acinetobacter baumannii* in a rat model of pneumonia. *Antimicrob Agents Chemother.* 2013;57(10):5120–5121.
- Luangtongkum T, Shen Z, Seng VW, et al. Impaired fitness and transmission of macrolide-resistant *Campylobacter jejuni* in its natural host. *Antimicrob Agents Chemother.* 2012;56(3):1300–1308.
- Vincent BM, Lancaster AK, Scherz-Shouval R, et al. Fitness trade-offs restrict the evolution of resistance to amphotericin B. *PLoS Biol.* 2013;11(10):e1001692.

11. Andersson DI, Hughes D. Antibiotic resistance and its cost: is it possible to reverse resistance? *Nat Rev Microbiol*. 2010;8(4):260–271.
12. Blot M, Meyer J, Arber W. Bleomycin-resistance gene derived from transposon Tn-5 confers selective advantage to *Escherichia coli* K-12. *Proc Natl Acad Sci U S A*. 1991;88(20):9112–9116.
13. Dutta S. Fitness gains hamper efforts to tackle drug resistance. *Elife*. 2013;2:e01809.
14. Jechalke S, Kopmann C, Richter M, et al. Plasmid-mediated fitness advantage of *Acinetobacter baylyi* in sulfadiazine-polluted soil. *FEMS Microbiol Lett*. 2013;348(2):127–132.
15. Adami C. Digital genetics: unravelling the genetic basis of evolution. *Nat Rev Genet*. 2006;7(2):109–118.
16. Grabowski LM, Bryson DM, Dyer FC, et al. A case study of the de novo evolution of a complex odometric behavior in digital organisms. *PLoS One*. 2013;8(4):e60466.
17. Ofria C, Wilke CO. Avida: A software platform for research in computational evolutionary biology. *Artif Life*. 2004;10(2):191–229.
18. Castillo CF, Ling MH. Resistant traits in digital organisms do not revert preselection status despite extended deselection: implications to microbial antibiotics resistance. *Biomed Res Int*. 2014;2014:648389.
19. Mann S, Chen YP. Bacterial genomic G+C composition-eliciting environmental adaptation. *Genomics*. 2010;95(1):7–15.
20. Lightfield J, Fram NR, Ely B. Across bacterial phyla, distantly-related genomes with similar genomic GC content have similar patterns of amino acid usage. *PLoS One*. 2011;6(3):e17677.
21. Povolotskaya IS, Kondrashov FA, Ledda A, et al. Stop codons in bacteria are not selectively equivalent. *Biol Direct*. 2012;7:30.
22. Bohlin J, Brynildsrud O, Vesth T, et al. Amino acid usage is asymmetrically biased in AT- and GC-rich microbial genomes. *PLoS One*. 2013;8(7):e69878.
23. Hershberg R, Petrov DA. Evidence that mutation is universally biased towards AT in bacteria. *PLoS Genet*. 2010;6(9):e1001115.
24. Castillo CFG, Ling MHT. Digital Organism Simulation Environment (DOSE): A Library for Ecologically-Based *In Silico* Experimental Evolution. *Advances in Computer Science: an International Journal*. 2014;3(1):44–50.
25. Joshua ZR Lim, Zhen Qin Aw, Desmond JW Goh, et al. A genetic algorithm framework grounded in biology. *The Python Papers Source Codes*. 2010;2:6.
26. Maurice HT Ling. An artificial life simulation library based on genetic algorithm, 3-character genetic code and biological hierarchy. *The Python Papers*. 2012;7:5.
27. Ling MHT. Ragaraja 1.0: The genome interpreter of Digital Organism Simulation Environment (DOSE). *The Python Papers Source Codes*. 2012;4:2.
28. Johnsen PJ, Townsend JP, Bohn T, et al. Factors affecting the reversal of antimicrobial-drug resistance. *Lancet Infect Dis*. 2009;9(6):357–364.
29. Adam HJ, Hoban DJ, Gin AS, et al. Association between fluoroquinolone usage and a dramatic rise in ciprofloxacin-resistant *Streptococcus pneumoniae* in Canada, 1997-2006. *Int J Antimicrob Agents*. 2009;34(1):82–85.
30. Albrich WC, Monnet DL, Harbarth S. Antibiotic selection pressure and resistance in *Streptococcus pneumoniae* and *Streptococcus pyogenes*. *Emerg Infect Dis*. 2004;10(3):514–517.
31. Greenberg D, Givon-Lavi N, Sharf AZ, et al. The association between antibiotic use in the community and nasopharyngeal carriage of antibiotic-resistant *Streptococcus pneumoniae* in Bedouin children. *Pediatr Infect Dis J*. 2008;27(9):776–782.
32. Hicks LA, Chien YW, Taylor TH, et al. Outpatient antibiotic prescribing and nonsusceptible *Streptococcus pneumoniae* in the United States, 1996-2003. *Clin Infect Dis*. 2011;53(7):631–639.
33. Stevens RW, Wenger J, Bulkow L, et al. *Streptococcus pneumoniae* non-susceptibility and outpatient antimicrobial prescribing rates at the Alaska Native Medical Center. *Int J Circumpolar Health*. 2013;72:22297.
34. Guillemot D, Varon E, Bernede C, et al. Reduction of antibiotic use in the community reduces the rate of colonization with penicillin G-nonsusceptible *Streptococcus pneumoniae*. *Clin Infect Dis*. 2005;41(7):930–938.
35. Haug S, Lakew T, Habtemariam G, et al. The decline of pneumococcal resistance after cessation of mass antibiotic distributions for trachoma. *Clin Infect Dis*. 2010;51(5):571–574.
36. Enne VI, Livermore DM, Stephens P, et al. Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. *Lancet*. 2001;357(9265):1325–1328.
37. Bean DC, Livermore DM, Papa I, et al. Resistance among *Escherichia coli* to sulphonamides and other antimicrobials now little used in man. *J Antimicrob Chemother*. 2005;56:962–964.
38. Nilsson P, Laurell MH. A 10-year follow-up study of penicillin-nonsusceptible *S. pneumoniae* during an intervention programme in Malmö, Sweden. *Scand J Infect Dis*. 2006;38(10):838–844.
39. Okon KO, Shittu AO, Kudi AA, et al. Population dynamics of *Staphylococcus aureus* from Northeastern Nigeria in 2007 and 2012. *Epidemiol Infect*. 2014;142(8):1737–1740.
40. Lai B, Zheng B, Li Y, et al. *In vitro* susceptibility of *Escherichia coli* strains isolated from urine samples obtained in mainland China to fosfomycin trometamol and other antibiotics: a 9-year surveillance study (2004-2012). *BMC Infect Dis*. 2014;14:66.
41. Hjalmsdottir MA, Kristinsson KG. Epidemiology of penicillin-nonsusceptible pneumococci in Iceland 1995-2010. *J Antimicrob Chemother*. 2014;69(4):940–946.
42. Hall AR, Iles JC, MacLean RC. The fitness cost of rifampicin resistance in *Pseudomonas aeruginosa* depends on demand for RNA polymerase. *Genetics*. 2011;187(3):817–822.
43. Burgess DJ. Microbial genetics: Fitness from drug resistance. *Nature Reviews Genetics*. 2014;15:64.
44. Miskinyte M, Gordo I. Increased survival of antibiotic-resistant *Escherichia coli* inside macrophages. *Antimicrob Agents Chemother*. 2013;57(1):189–195.
45. Baker S, Duy PT, Nga TV, et al. Fitness benefits in fluoroquinolone-resistant *Salmonella typhi* in the absence of antimicrobial pressure. *Elife*. 2013:e01229.
46. Albarracín Orió AG, Pinás GE, Cortes PR, et al. Compensatory evolution of pbp mutations restores the fitness cost imposed by beta-lactam resistance in *Streptococcus pneumoniae*. *PLoS Pathog*. 2011;7(2):e1002000.
47. Zorzet A, Andersen JM, Nilsson AI, et al. Compensatory mutations in agrC partly restore fitness *in vitro* to peptide deformylase inhibitor-resistant *Staphylococcus aureus*. *J Antimicrob Chemother*. 2012;67(8):1835–1842.
48. Brandis G, Hughes D. Genetic characterization of compensatory evolution in strains carrying rpoB Ser531Leu, the rifampicin resistance mutation most frequently found in clinical isolates. *J Antimicrob Chemother*. 2013;68(11):2493–2497.
49. Kinloch BB, Dupper GE. Genetic specificity in the white pine-blister rust pathosystem. *Phytopathology*. 2002;92(3):278–280.
50. Gajardo GM, Beardmore JA. The brine shrimp artemia: adapted to critical life conditions. *Front Physiol*. 2012;3:185.

51. Borrell S, Teo Y, Giardina F, et al. Epistasis between antibiotic resistance mutations drives the evolution of extensively drug-resistant tuberculosis. *Evol Med Public Health*. 2013;2013(1):65–74.
52. Saddler CA, Wu Y, Valckenborgh F, et al. Epidemiological control of drug resistance and compensatory mutation under resistance testing and second-line therapy. *Epidemics*. 2013;5(4):164–173.
53. Knight GM, Budd EL, Lindsay JA. Large mobile genetic elements carrying resistance genes that do not confer a fitness burden in healthcare-associated pneumonia-resistant *Staphylococcus aureus*. *Microbiology*. 2013;159(Pt 8):1661–1672.