Does gene mobility promote the persistence of a bacterial social trait?

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MSc by Research

University of York

Biology

June 2015

ABSTRACT

The mer operon confers bacteria with resistance to mercury toxicity whilst simultaneously allowing them to bioremediate their local environment. This 'collective' effect of mercury resistance provides the potential for social conflict wherein mercury sensitive cells may act as 'cheats' benefiting from the detoxification performed by resistant cells whilst bearing none of the cost; it is challenging therefore to explain how mercury resistance is maintained. One potential solution for the maintenance of social traits is for their location on mobile genetic elements allowing infectious transfer of the trait and enforcement of cooperation on cheat cells. This study explores the dynamics of mobile versus non-mobile mercury- resistance in populations of *Pseudomonas fluorescens SBW25.* When the mer operon is located on a costly plasmid the short-term dynamics show that mercury resistance is a Snowdrift Game. Intermediate concentrations of mercury select for the stable coexistence of plasmid-carrying and plasmid-free cells due to negative frequency dependent selection. In the longer term, environmental mercury selects for resistance to be encoded on the bacterial chromosome allowing cells to eliminate the costly plasmid. However, in the absence of mercury, resistance is maintained at a significantly greater proportion of the population when located on a mobile plasmid, as conjugative transfer promotes the spread of mercury resistance. These findings suggest that gene mobility plays an important role in the dynamics of microbial social traits. In the short term, location on a costly plasmid enables the persistence of 'cheats', however, in the longer term conjugative transfer can overcome these costs by enforcing cooperation on the entire population.

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ACKNOWLEDGEMENTS

I would like to thank Mike Brockhurst for his wonderful supervision throughout this project, Ellie Harrison and James Hall for all their help with experimental design and analysis- it is very much appreciated, and Dave Guymer for his brilliant knowledge of molecular biology. I would also like to thank my TAP panel Peter Young and Calvin Dytham for the opportunity to discuss my work and their helpful advice throughout.

AUTHORS DECLARATION

I, Cagla Stevenson declare that all the material contained within this thesis is a result of my own work and has been written solely by myself. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as references.

INTRODUCTION

Bacteria exhibit a variety of social traits: behaviours which are not only beneficial to the individual cell that performs them but also benefit neighbouring cells in their vicinity (Crespi, 2001; West et al., 2006). These social traits encompass a whole host of microbial behaviours. Microbes can secrete products which promote growth, for example, in glucose limiting environments, cells of the budding yeast *Saccharomyces cerevisiae* secrete the enzyme invertase which allows hydrolysis of sucrose into readily available glucose and fructose (Greig and Travisano, 2004). Other secreted products can bind and scavenge nutrients: P*seudomonas aeruginosa* relies on secreted products called siderophores which allow the capture of iron (West and Buckling, 2003). Furthermore microbes use secretions to communicate to one another: secreted molecules known as autoinducers allow cells to detect the density of their neighbours by a process known as quorum sensing (Diggle et al., 2007; Williams et al., 2007). As well as extracellular products, microbes can also share the benefits of intracellular processes including the detoxification of harmful substances like antibiotics, for example ampicillin-sensitive *E.coli* are able to benefit from the detoxification carried out by ampicillin-resistant cells and thus survive in toxic environments not ordinarily available to them (Yurtsev et al., 2013).

Cooperative behaviours are often costly to the individual cell performing them. The production of extracellular secreted products can result in a metabolic cost on the cell. This is apparent in autoinducer production (Brown and Johnstone, 2001) and also in siderophore production where producers exhibit a fitness disadvantage relative to non-producers (Griffin et al., 2004). Because of this fitness cost the evolution of microbial social behaviours presents an evolutionary conundrum: social traits are vulnerable to

'cheating' where cells which are able to receive the benefits of cooperation whilst bearing none of the individual cost should invade populations of cooperators (West et al., 2006, 2007). 'Cheats' have been shown to spontaneously arise within bacterial populations, both in the laboratory (Harrison et al., 2007) and in clinical settings (Köhler et al., 2009) and these dynamics may have important consequences for microbial communities. On the one hand, the presence of cooperators can allow microbial communities to survive in stressful environments including high doses of antibiotic ordinarily lethal to individual cells (Vega and Gore, 2014). On the other hand, populations with high levels of non-producing 'cheats' have been shown to be subject to population collapse (Rainey and Rainey, 2003). Understanding these population dynamics can help us manipulate bacterial communities to our advantage, and may even provide a useful tool for medical interventions (Diggle, 2010; Foster, 2005).

Previous work has suggested that environmental mercury detoxification may be a microbial social trait (Ellis et al., 2007). Populations of mercury-sensitive *Pseudomonas fluorescens* were found to co-exist alongside mercury-resistant cells at a range of mercury concentrations demonstrating that the benefits of resistance were available to both mercury-sensitive and mercury-resistant cells (Ellis et al., 2007). Mercury resistance is prevalent among bacterial populations the most common mechanism of which is the mer operon, which encodes a number of genes involved in the detection (MerR/MerD), import (MerP/MerT) and eventual reduction (MerA) of ionic mercury (Hg^{2+}) into elemental mercury (Hg^{0}), a gaseous form which dissipates from the local environment reducing the concentration of mercuric ions (Barkay et al., 2003). The detoxification of mercury provided by resistant cells may well have collective benefits beyond the individual cell because the detoxification they provide allows the local environment to be de-

contaminated from mercury pollution, providing all neighbouring cells a benefit. Similar collective benefits have been found elsewhere: *Escherichia coli* which were originally sensitive to ampicillin were able to co-exist alongside resistant cells (Yurtsev et al., 2013). This was because resistant cells produced β-lactamase enzymes which degraded ampicillin, thus lowering the concentration of antibiotic and allowing non-producing sensitive cells to persist in the environment (Yurtsev et al., 2013).

Because mercury resistance is energetically costly to the cells performing it (Boyd and Barkay, 2012), it is likely that, as with other putatively social microbial traits, it will be vulnerable to invasion by cheats which receive the collective benefits of mercury detoxification whilst bearing none of the individual costs (West et al., 2006, 2007). Game theory provides a framework to understand how the social environment affects the evolution of social traits (Maynard Smith, 1982). Game theoretical models incorporate the idea that the payoff to an individual playing a social strategy depends upon the strategies adopted by other players. In game theory models, two players simultaneously decide whether to cooperate or cheat, yielding a 2-by-2 matrix of payoffs from all possible interactions. The structure of the payoff matrix can then be used to determine the evolutionarily stable social strategy. Two classical game theory scenarios have been explored in the context of microbial cooperation: the Prisoner's Dilemma and the Snowdrift Game (Table 1).

Table 1. Theoretical payoff matrix for a | Prisoner's Dilemma and b | the Snowdrift Game. The benefits b exceed the costs c of co-operation.

Prisoners Dilemma	Cooperator	Cheat
Payoff to Cooperator	b - c	-c
Payoff to Cheat	b	0

Snowdrift Game	Cooperator	Cheat
Payoff to Cooperator	b - c / 2	b - c
Payoff to Cheat	b	0

In Prisoner's Dilemma, cooperators perform costly (c) cooperative acts the benefits of which (b) accrue to their opponent, whereas cheats do not cooperate thereby avoiding the costs of cooperation but potentially benefiting from the cooperation of their opponents. The pay-off matrix of this game is given in Table 1a and yields a pay-off structure of $T > R > P > S$: under this scenario cheating is the only evolutionarily stable strategy in the simple single-play version of the game. Fitness payoffs consistent with this have been demonstrated in biological systems (Turner and Chao, 1999): during infection, defective viral genotypes which were unable to produce components of the capsid had the highest fitness payoff when co-infecting with wild-type viruses that produced all proteins needed for replication. By contrast, the lowest fitness pay-off was for wild-type viruses co-infecting with the defective virus.

Despite predictions of the Prisoner's Dilemma it is often observed that in microbial systems cheats and cooperators can stably coexist (Greig and Travisano, 2004; Hauert and Doebeli, 2004). This implies that another model might be better suited to explain these scenarios. In one particular model, the Snowdrift Game, cheats still prosper at the expense of cooperators but, in contrast to the Prisoner's Dilemma, cooperators gain some individual benefit from their cooperative acts and can share the costs of cooperation with cooperative opponents; the resulting pay-off matrix is given in Table 1b and yields a pay-off structure of $T > P > R > S$. Now, cheating is no longer the evolutionarily stable strategy, rather, the optimal strategy is the opposite of your opponent's; hence both cheating and cooperation can be stably maintained in the population (Hauert and Doebeli, 2004). This stable co-existence between cooperators and cheats has been seen in microbial systems (Gore et al., 2009): cooperative yeast

cells which hydrolyse sucrose through the production of costly invertase were shown to co-exist with cheating yeast cells that do not produce invertase.

As well as the microbial social environment, other factors have been implicated in the maintenance and spread of costly social traits. Alongside their chromosome, bacterial cells often harbour plasmids, selfish elements which replicate independently of the host chromosome and are thus able to spread through a bacterial host population by conjugative 'infectious' transfer. As well as encoding traits required for their own replication, plasmids often carry 'accessory' genes: traits which are potentially useful to their bacterial host under particular conditions. Because these accessory genes often encode proteins secreted outside the cell, they are likely to affect neighbouring cells (Nogueira et al., 2009), and therefore it has been hypothesised that plasmids may play a role in the dynamics of microbial social traits (McNally et al., 2014; Rankin et al., 2011). Furthermore, gene transfer has been shown to promote the maintenance of social traits within theoretical models of bacterial populations (Smith, 2001): if cooperative traits were encoded on plasmids, any 'cheats' which arose through loss of the plasmid would subsequently be re-infected through conjugative plasmid transfer enforcing the cooperative trait on all members of the population. This has been experimentally demonstrated in spatially structured populations of *E.coli* where genes encoding the production of a cooperative public good were able to invade a bacterial population when encoded on a conjugative plasmid as opposed to when on a non-conjugative plasmid (Dimitriu et al., 2014). The mer operon is often found on mobile genetic elements such as transposons and plasmids and is widely distributed among environmental taxa indicating a prevalent role for horizontal gene transfer in its evolution and spread (Boyd and Barkay, 2012).

Previously, a number of mercury resistant pQBR plasmids were isolated from soil in Wytham, Oxfordshire (Lilley and Bailey, 1997; Lilley et al., 1996). These plasmids encode the transposon Tn5042 which harbours the mer operon allowing cells which bear the plasmid to detoxify their local environment. This study investigates the social dynamics surrounding mercury detoxification and the environmental conditions which promote its maintenance using populations of *Pseudomonas fluorescens SBW25* (Rainey and Bailey, 1996) and the mercury resistance plasmid pQBR57 (Hall et al., 2015). This study determines the game theoretical payoff matrices of plasmid-encoded mercury resistance across a range of mercury concentrations, how the fitness of mercury resistance varies with its frequency, and whether private benefits exist to carrying a mercury resistance plasmid. Finally, this study looks at whether horizontal gene transfer plays a prevalent role in the spread of mercury resistance and how this might vary with different mercury environments.

METHODS

Strains and culture conditions

To investigate the short term dynamics of mercury detoxification I used *P. fluorescens SBW25* (Rainey and Bailey, 1996) and its naturally co-occurring plasmid pQBR57 which encodes a *mer* operon (Lilley and Bailey, 1997). SBW25 was previously differentially marked with gentamicin resistance (Gm^R) and streptomycin resistance + lacZ cassettes (Sm^RlacZ) using the Tn7 delivery system (Koch et al., 2001) in order to distinguish between strains in mixed cultures when plated onto King's Medium B (KB) agar (King et al., 1954) (10 g glycerol, 20g proteose peptone no. 3, 1.5g K2HPO4•3H2O, 1.5g MgSO4•7H2O, per litre) supplemented with X-gal (50 μg/ml). Fitness assays were conducted by competing mercury resistant plasmid-bearers and mercury sensitive plasmid-free controls against a differentially marked mercury-sensitive control strain SBW25-ΩSm^RlacZ.

To investigate the effect of mobility on *mer* dynamics I took a previously constructed strain SBW25-ΩGm^Rmer, which carries the mercury resistance transposon Tn5042 on the bacterial chromosome, and from this from this constructed a *mer* knockout to give a mercury-sensitive control strain SBW25-ΩGm^R-Δmer. I then conjugated pQBR57 into this strain to construct SBW25-ΩGm^R-Δmer-pQBR57 which carries the mercury resistance on the plasmid. Both chromosome-encoded and plasmid-encoded strains were competed against the mercury-sensitive reference strain SBW25-ΩSm^RlacZ. In addition the

mercury-sensitive control strain SBW25-ΩGm^R-Δmer was also competed against the reference to determine whether there was any effect of marker. There was no significant effect of transfer (X^2 = 1.59, p = 0.21) or mercury treatment (X^2 = 1.34, p = 0.5). on the proportion of Gm^R marked cells indicating no major effect of marker.

All the above experiments were conducted in 6ml KB broth in 30ml microcosms shaking at 180rpm and incubated at 28°C. For all of the, sterile water to the same volume was added to the microcosm instead of $HgCl^2$ solution.

Construction of mercury sensitive control strain

Mercury sensitive control strain SBW25-ΩGm^R-Δmer was constructed from SBW25-ΩGm^{R}mer by deleting the mer encoding transposon Tn5042 using site directed mutagenesis (Heckman and Pease, 2007; Ho et al., 1989). Initially upstream primers A l (for: 5'-TATAGGTACCACTAGTCCTCGATACTGCCCAAAACC- 3') & B l (rev: 5'-ATCCTATTGCACGTAAGCGGACGGTTACACCGTTTGCGACTTTTG-3') and downstream C l (for: 5'-CAAACGGTGTAACCGTCCGCTTACGTGCAATAGGATCCG 3') & D l (rev: 5'-TATATCTAGAACTAGTTAGACGCGAAAGGAATGACTC 3') were used to amplify regions flanking the transposon. B and C include a short stretch of the transposon sequence in order to put the resultant ORF out of frame and maintain the non-functionality created by insertion of the transposon. The flanking primers A and D were then used to generate a final product. The final product was inserted into the cloning vector pUC19 and transformed into E.coli strain DH5α with PCR and sequencing used to confirm successful, error-free ligation. The recombinant vector was purified using

QIAprep Miniprep Kit (Qiagen) and excised using SpeI restriction enzyme for ligation into the suicide vector pUIC13 (Rainey, 1999) in the E.coli host strain DH5α λpir. The recombinant vector was purified, as above, and transformed by electroporation into *Pseudomonas fluorescens SBW25*-ΩMer. The flanking regions allow homologous recombination of the suicide plasmid into the host chromosome and successful recombination was obtained by selection for tetracycline resistance and screening for lacZ. Cultures were then sub-cultured without selection in order for de-integration of the plasmid. PCR was performed to confirm the presence of the desired mutation using primers targeting outside of the transposon flanking regions; upstream E l (for: 5'-ATGAAGTCAATCCTCGATACTGC - 3') and downstream F l (rev: CAAGGGAAAAGTACTCGTAGACG - 3'). Due to the size of Tn5042 (~4000 bp), a product would only be expected if Tn5042 had been successfully deleted.

Table 2. Table of strains.

STRAIN	DESCRIPTION	REFERENCE
SBW25-ΩSm^{R}lacZ	lacZ marked reference	(Hall et al., 2015)
SBW25-ΩGm^{R}	mercury-sensitive	(Hall et al., 2015)
SBW25-ΩGm^{R}.pQBR57	plasmid-encoded resistant	(Hall et al., 2015)
SBW25-ΩSm^{R}lacZ.pQBR57	plasmid-encoded resistant	(Hall et al., 2015)
SBW25-ΩGm^{R}Mer	chromosome-encoded resistant	Unpublished data
SBW25-ΩGm^{R}-ΔMer	mercury-sensitive control	Constructed here
SBW25-ΩGm^{R}-ΔMer.pQBR57	plasmid-encoded mer	Constructed here

Fitness assays

The fitness pay-offs and frequency-dependence of mercury detoxification were estimated using competitive fitness assays. Independent overnight cultures of test and reference strain were mixed at a given ratio and 60 μl (~10^7 cells ml^{-1}) used to inoculate treatment microcosms. Samples of populations were diluted and spread onto KB agar + X-gal at the beginning and end of each competition in order to determine viable numbers. Fitness was estimated from these counts as the ratio of Malthusian parameters $W = \ln(test_{end} / test_{start}) / \ln(reference_{end} / reference_{start})$ or selection rates $r = (\ln(test_{end} - test_{start})) - \ln(reference_{end} - reference_{start})) / time$ ((Lenski et al., 1991).

Game theoretical payoffs were estimated using relative fitness (W) by conducting pairwise 24-hour mixed and mono-culture competitions with mercury-sensitive and mercury-resistant strains. All competitions were set up from a 1:1 starting ratio at one of three mercury treatments (0, 20 or 40 μM $HgCl^2$). Six replicates were founded for each competition per mercury treatment; 3 replicates used the Gm^R as the reference marker and 3 used the Sm^RlacZ as the reference marker to control for any marker effect. W was used here because I was interested only in populations with positive growth rates, therefore if populations were unable to grow their fitness was set to 0.

Frequency dependence of mercury resistance was estimated using selection rates (r) by conducting competitions from starting frequencies of 1:100, 1:10, 1:1, 10:1 and 100:1 mercury-sensitive: mercury-resistant cells. Using r allowed estimation of fitness despite

population decline during the competition. Populations were exposed to three mercury treatments as stated above. Four replicates were founded per treatment, half of the replicates used the Gm^R as the reference marker while half used the Sm^RlacZ as the reference marker to control for the effect of marker genotype.

Selection experiment

The effect of gene mobility on mercury-resistance was estimated by conducting pairwise competitions between test Gm^R (plasmid- and chromosome- encoded resistant and control mercury-sensitive) strains against the reference Sm^RlacZ strain over 28 days. Independent overnight cultures of test and reference strain were mixed at a 1:1 ratio and 60 μl (~10^7 cells ml^{-1}) used to inoculate treatment microcosms. Mercury treatments comprised of low (KB + 0 μM $HgCl^2$), medium (KB + 20 μM $HgCl^2$) or high mercury (KB + 40 μM $HgCl^2$) environments. Six replicate populations were founded per treatment. Populations were propagated by serial transfer of 1% of culture to fresh media every 48 hours for a total of 14 transfers. Samples of populations were diluted and spread onto KB agar + X-gal at the end of transfer 1, 2, 4, 8, 12 and 14 in order to determine viable numbers. Plasmid prevalence was estimated by replica plating agar plates onto KB agar + 100 μM $HgCl^2$ + 50 μg/ml X-gal in order to determine the proportion of each population bearing the plasmid.

Quantifying differences between chromosome-encoded and plasmid-encoded mer

MICs for the plasmid- and chromosome- encoded mercury resistant strains as well as the two mercury sensitive control and reference strains were determined by inoculating ~10^4 cells into 96-well plates containing KB media + mercury (range: 0 - 80 μM $HgCl^2$). The MICs were calculated as the lowest mercury concentration that yielded no appreciable growth (OD_{600} <2) after 24 and 48 hours incubation at 28°C.

The expression of merA for plasmid-encoded and chromosome-encoded strains across 3 mercury environments (0, 20 & 40 μM $HgCl^2$) using quantitative PCR. Clones of plasmid- and chromosome- encoded mercury resistant strains as well as the two mercury sensitive control and reference strains were grown in treatment microcosms for 48 hours and then transferred to fresh treatment microcosms for 6 hours prior to RNA extraction. Treatments consisted of KB + 0 μM $HgCl^2$ for all populations, and KB + 20 and 40 μM $HgCl^2$ for resistant populations. RNA was extracted using the RNeasy Mini Kit (Qiagen). Extracted RNA was then transformed into cDNA using the High Capacity RNA-to-cDNA Kit (Life Technologies, USA). Gene expression was quantified by qPCR using an ABI StepOnePlus Real-Time PCR system (Life Technologies, USA). MerA gene expression was compared to gene expression of a housekeeping gene rpoD. Gene expression was quantified by qPCR of merA primers (for: 5'-TGACCACACTGCGAATCCA-3') and (rev: 5'- GGAGATTCCCTCCAGAGCATCT-3'), efficiency: 80% and rpoD (for: 5'-GCAGCTCTGTGTCCGTGATG-3') and (rev: 5' TCTACTTCGTTGCCAGGGAATT-3'), efficiency: 80% .Efficiency of reactions was calculated from the standard curve generated by performing qPCR with 3, 5-fold dilutions of template (range: ~20ng/ml - 20pg/ml).

qPCRs were performed using Applied Biosystems SYBR Green PCR Master Mix (Life technologies) (10 μl into each 20 μl reaction). Initial denaturation for 3s at 95°C, followed by 40 cycles of denaturation for 3s at 95°C, annealing and extension for 30s at 60°C. After the amplification was complete, melt curve analysis was performed by cooling the reaction to 60°C and then heating slowly to 95°C.

Statistical analyses

To analyse the relative fitness of cooperators and cheats one-sample t-tests were used to compare the relative fitness of each strain with 1. To analyse the effect of starting frequency on strain fitness linear regression models were used for 0 and 20μM $HgCl^2$ treatments. For 40 μM $HgCl^2$ treatment, a regression model with a quadratic term was used as this significantly improved the fit of the model to the data (ANOVA $F_{1,17} = 26.3$, $p < 0.001$). To test for an individual benefit of carrying mercury resistance an anova was used to compare relative fitness across mercury treatments (0, 20, 40 and 80 μM $HgCl^2$ treatments). To analyse the effect of mobility on the proportion of resistant cells a generalised linear mixed model was constructed with population and transfer as random effects to account for the repeated sampling of a population over time. Including a three way interaction in the model significantly improved the fit of the model to the data (ANOVA $X^2 = 51.93$, $p < 0.001$). To analyse the control data, a generalised linear mixed model was constructed with population and transfer as random effects as before. To analyse the plasmid dynamics across mercury treatments an anova was used to compare the endpoint proportion of transconjugant resistant cells across mercury (0, 20 and 40 μM $HgCl^2$ treatments). All analyses were conducted in R statistical package version 3.1.3 (R Foundation for Statistical Computing). Packages used were ‘multcomp’

to conduct post-hoc Tukey comparisons and ‘lme4’ to construct the generalised linear mixed model.

RESULTS

Mercury resistance is a context-dependent Snowdrift Game

To determine whether the fitness payoffs of mercury-resistance were consistent with a Snowdrift Game, the relative fitness of plasmid-carrying mercury-resistant and plasmid-free mercury-sensitive cells were calculated by carrying out mixed- and mono- culture competitions across three mercury treatments (0, 20, 40 μM $HgCl^2$), given in Table 3. Only at 20 μM $HgCl^2$ was the payoff structure consistent with a Snowdrift Game. The highest payoff accrued to mercury-sensitive cells competing against mercury-resistant cells: sensitive cheats were able to benefit from the mercury detoxification whilst bearing none of the cost. The second highest fitness payoff accrued to mercury-resistant cells competing against other mercury-resistant cells: the costs and benefits of detoxification were shared between the cells yielding equal fitness payoffs to both competitors. The third highest payoff accrued to mercury-resistant cells competing against mercury-sensitive cells: despite the costs of mercury bioremediation, resistant cells jointly shared the resulting benefits with sensitive cells. The lowest payoff occurred when mercury-sensitive cells were competed against other mercury-sensitive cells: in the absence of mercury detoxification sensitive cells were unable to grow. At 0 μM $HgCl^2$ the mercury resistance plasmid was costly, and the plasmid-free mercury-sensitive state was the evolutionary stable strategy. Finally, at 40 μM $HgCl^2$ mercury-resistant cells were able to outcompete mercury-sensitive cells suggesting that in highly toxic environments the individual benefits of mercury bioremediation outweigh the costs and resistance is the evolutionary stable state.

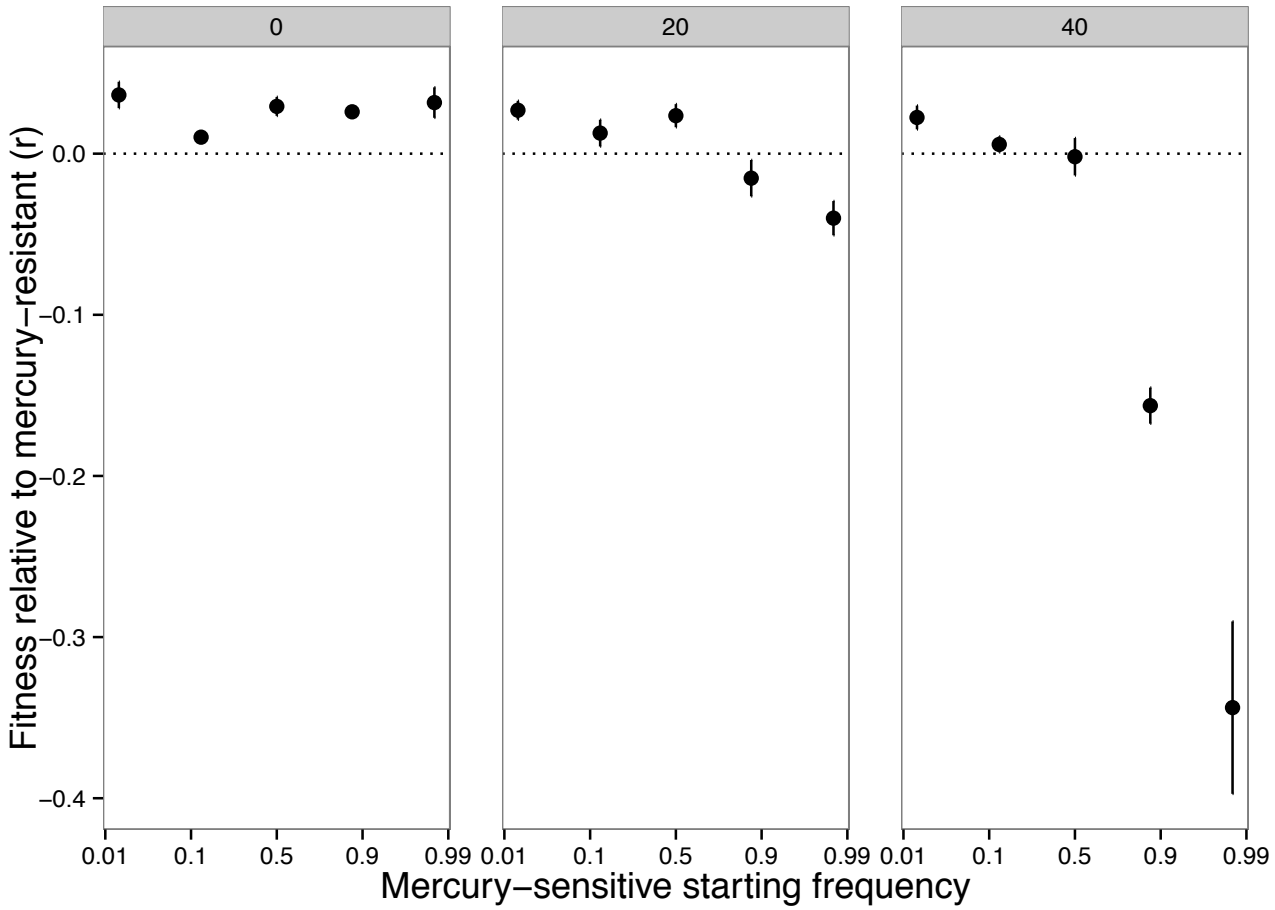
Table 3. Observed payoff matrix for mixed and mono- culture competitions between mercury-resistant and mercury-sensitive cells across three mercury environments. A value of 1 indicates equal competitive fitness. Asterisks indicate significant deviation from 1 ($p < 0.05$). Values of 0 indicate populations unable to persist in the test environment.

	0		20		40	
	Resistant	**Sensitive**	**Resistant**	**Sensitive**	**Resistant**	**Sensitive**
Payoff to Resistant	1.03	0.85 *	1.01	0.82 *	1.00	1.41 *
Payoff to Sensitive	1.26 *	0.96	1.26 *	0	0.79 *	0

Mercury resistance is subject to negative frequency dependent selection in mercury contaminated environments

Snowdrift Game theory predicts that plasmid-bearing mercury-resistant and plasmid-free mercury-sensitive cells should stably coexist (i.e. both should be able to invade from rare). To test this, mixed- culture competition experiments were performed between mercury-resistant and mercury-sensitive cells across a range of starting frequencies (1:100, 1:10, 1:1, 10:1 and 100:1 mercury-sensitive : mercury-resistant cells) in 3 mercury environments (0, 20, 40 μM $HgCl^2$). The effect of starting frequency on the fitness of mercury-sensitive cells depended on the concentration of mercury (FREQUENCY X MERCURY $F_{8,51} = 67.94$, $P < 0.001$; Figure 1). At 0 μM $HgCl^2$, there was no effect of starting frequency on fitness, with mercury-sensitive cells outcompeting mercury-resistant at all starting frequencies (simple effect of FREQUENCY $F_{1,18} = 0.0580$, $p = 0.812$). Whereas at 20 μM $HgCl^2$, consistent with the Snowdrift Game, mercury-sensitive fitness declined linearly with starting frequency (simple effect of FREQUENCY $F_{1,18} = 25.94$, $p < 0.001$) indicating the potential for stable coexistence of cooperators and cheats through negative frequency dependent selection. At 40 μM $HgCl^2$, a quadratic effect of starting frequency was observed wherein fitness of mercury-sensitive cells decreased dramatically with increasing starting frequency (simple effect of FREQUENCY X FREQUENCY $F_{2,17} = 75.57$, $p < 0.001$).

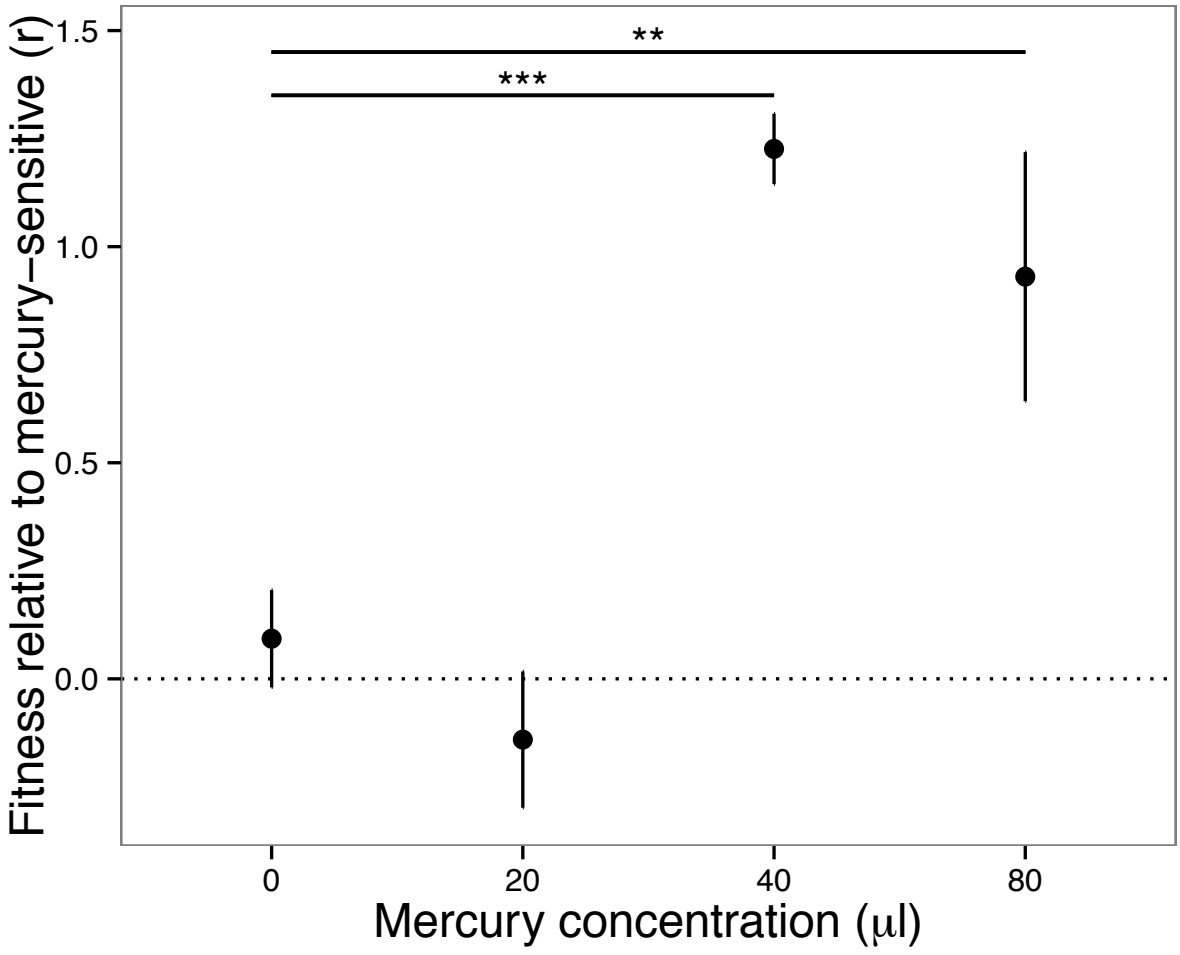
Figure 1. Mercury-sensitive fitness shows negative frequency dependence in the presence of mercury. Mercury-sensitive and mercury-resistant cells were grown in mixed culture competitions at varying starting frequencies across three mercury environments. Mercury-sensitive competitive fitness was calculated as (r) and plotted as means ± SE. A value of 0 indicates equal competitive fitness. Starting frequency was plotted as ln (proportion mercury-sensitive/ proportion mercury-resistant).



Individual benefits of carrying the mercury resistance plasmid

To test whether, consistent with a Snowdrift Game, resistant cells have access to an individual benefit from carrying the mercury resistance plasmid, populations were initiated with a 50:50 ratio mercury-sensitive: mercury-resistant cells and grown to exponential phase before adding $HgCl^2$ at a range of concentrations (0, 20, 40 and 80 μM) Figure 2. Population declines of mercury-sensitive and mercury-resistant fractions were calculated over the hour following the addition of $HgCl^2$. Mercury-resistant cells showed significantly higher survival than mercury-sensitive cells in the presence of mercury (MERCURY $F_{3,8}$ = 13.48, P = 0.00171). Post-hoc Tukey HSD comparisons revealed that this effect was driven by the high $HgCl^2$ treatments which were significantly different from the 0 μM $HgCl^2$ controls (p = 0.00874 and p = 0.0422 for the 40 and 80 μM $HgCl^2$ respectively). Interestingly, mercury-resistant cells survived no better than mercury-sensitive cells in mixed culture upon addition of 20 μM $HgCl^2$ (p = 0.79). These data suggest that at high mercury concentrations mercury-resistant cells benefit from individual level resistance against mercuric ions. However, ~2 hours after addition of $HgCl^2$, surviving mercury-sensitive cells were able to grow at rates comparable to mercury-resistant cells, indicating that the individual benefit of mercury resistance is relatively short lived.

Figure 2. Mercury-resistant cells have greater individual fitness at high concentrations of mercury. Mercury-sensitive and mercury-resistant cells were grown in mixed culture competitions at a 1:1 ratio in mercury environments. Mercury-resistant competitive fitness was calculated as (r) and plotted as means ± SE. A value of 0 indicates equal competitive fitness. Starting frequency was plotted as ln (proportion mercury-resistant/ proportion mercury-sensitive).



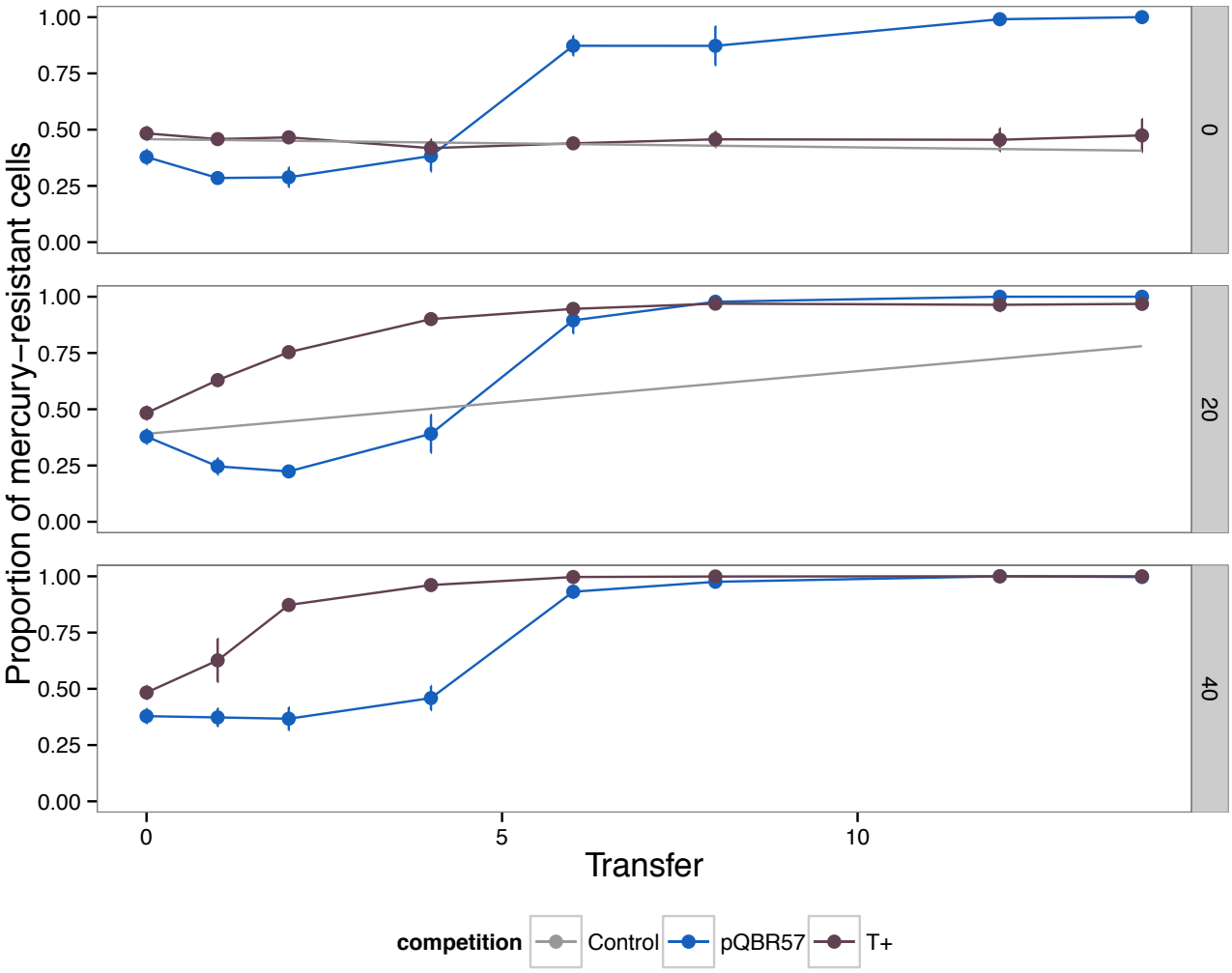
Horizontal gene transfer maintains resistance in the absence of selection

One potential solution for the maintenance of social behaviours is through horizontal gene transfer of the trait on a mobile genetic element. To test whether mobility can play a role in the maintenance of mercury resistance within a bacterial population, the proportion of chromosome- and plasmid-encoded resistant cells was compared over time across 3 mercury environments (0, 20, 40 μM $HgCl^2$).

The interaction between mobility and mercury had a significant effect on the proportion of resistant cells in the population over time (MOBILITY X MERCURY X TRANSFER: X^2 = 51.93, $p < 0.001$). Whereas plasmid-encoded mercury resistance could invade under all conditions, chromosomal mercury-resistance only increased in frequency over time in the presence of mercury (Post-hoc Tukey comparing the proportion of chromosome-encoded resistant cells between 0 and 20 μM $HgCl^2$ treatments $z = 7.17$ $p < 0.001$ and 0 and 40 μM $HgCl^2$ treatment $z = 10.76$, $p < 0.001$). This confirms that conjugation can play an important role in the spread of mercury resistance. When resistance was mobile, there was a significantly greater proportion of resistance cells in the population in the absence of mercury than when the trait was non-mobile (Post-hoc Tukey comparing the proportion of resistant cells between chromosome and plasmid encoded populations at 0 μM $HgCl^2$ $z = -11.22$, $p < 0.001$) indicating that conjugation allowed the resistance trait to invade even in the absence of positive selection. In the presence of high mercury (40 μM $HgCl^2$) resistance invaded faster when encoded on the chromosome as opposed to on the plasmid (Post-hoc Tukey comparing proportion of resistant cells between plasmid- and chromosome- encoded resistance in 40 μM $HgCl^2$ $z = 6.47$ $p < 0.001$). This suggests that

when under positive mercury selection the additional cost of plasmid-carriage slows down the spread of the resistance trait.

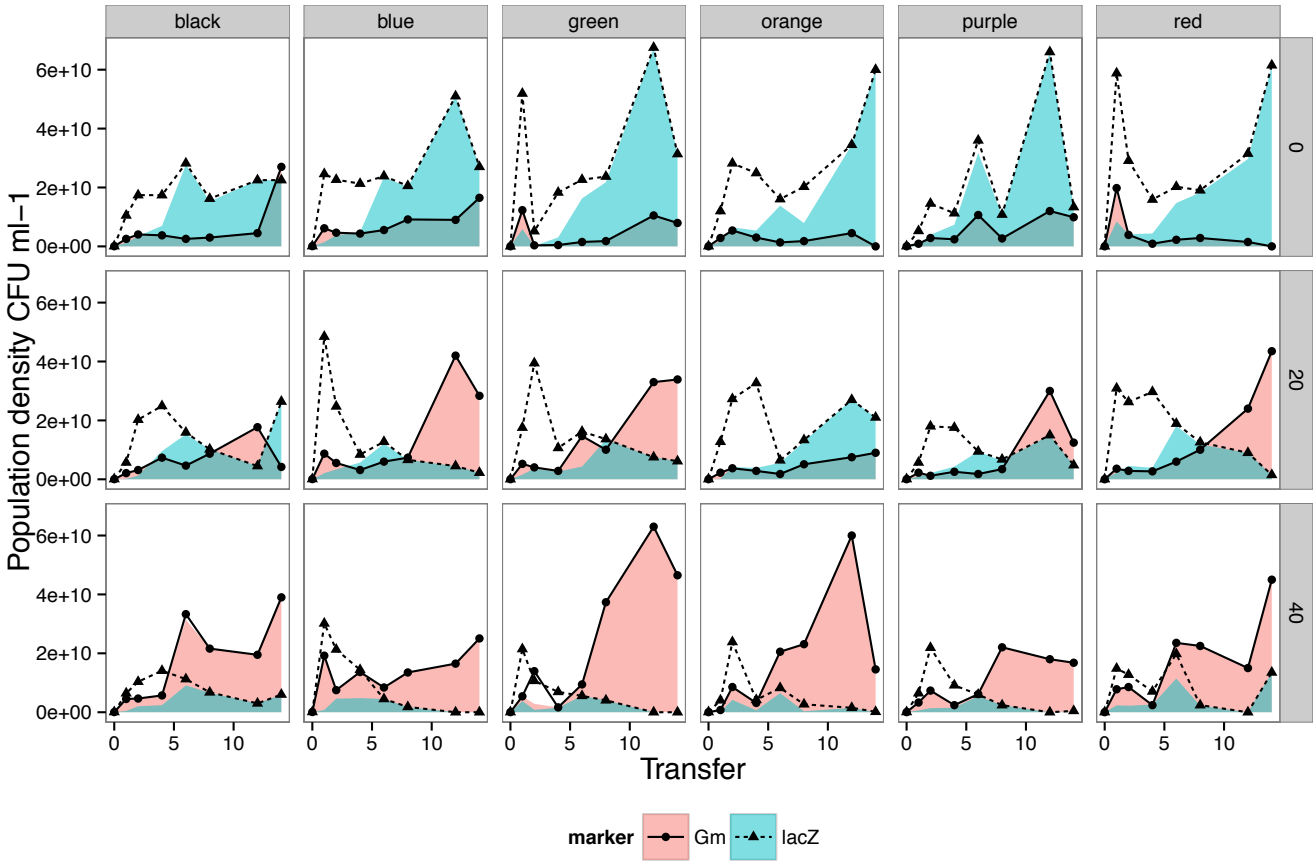
Figure 3. Mobility allows the mercury-resistant trait to sweep through populations in the absence of positive selection. Mercury-resistant containing mer either on the plasmid or chromosome were grown in mixed culture competitions at a 1:1 ratio with mercury-sensitive cells in 3 mercury environments. Proportion of resistant cells plotted as mean ± SE. For the control mercury-sensitive control strain was grown in mixed-culture competition with mercury-sensitive lacZ marked reference strain and proportion of control plotted as a regression line.



Selection and conjugation play a role in the maintenance of plasmid-encoded mercury-resistance

To investigate the plasmid dynamics across mercury treatments the spread of the plasmid through donor and recipient backgrounds was tracked across 3 mercury environments (0, 20 and 40μM $HgCl^2$). To determine the specific effect of mercury on host background the endpoint proportion of transconjugants (i.e. recipient cells who had gained the plasmid) was compared across the 3 mercury environments. Mercury significantly decreased the proportion of plasmid-bearing cells that were transconjugants (simple effect of MERCURY: $F_{2,15} = 11.38$, $p < 0.001$) highlighting in 0uM $HgCl^2$, conjugation plays a more important role in the spread of the plasmid whereas in 40uM $HgCl^2$, the proportion of original donors increases under positive selection.

Figure 4. Mercury-resistance is maintained by both selection and conjugation. Mercury-resistant containing mer either on the plasmid or chromosome were grown in mixed culture competitions at a 1:1 ratio with mercury-sensitive cells in 3 mercury environments. For each replicate population the total population density of each marker: lacZ and Gm for recipient and donor respectively were plotted by the black lines. The presence of plasmid in each marker background is depicted by the shaded region.



Mer shows similar activity when encoded on the chromosome and on a plasmid

To determine whether any phenotypic difference existed between mer encoded on the plasmid or the chromosome MICs were calculated across the plasmid- and chromosome-encoded resistant strains as well as the two mercury sensitive control and reference strains. There was no significant difference between the MICs of the two resistant strains ($t_{3.2} = -0.866$, $p = 0.4465$) indicating no obvious difference between the level of resistance conferred by plasmid- or chromosome- encoded mer.

MerA expression was determined by qPCR across the plasmid- and chromosome-encoded mercury resistant strains as well as the two mercury sensitive control and reference strains in 3 mercury environments (0, 20 and 40 μM $HgCl^2$). There was no significant difference in merA expression across the two resistant strains: ($F_{1,14} = 1.838$, $p = 0.197$) indicating plasmid- and chromosome- encoded mer display a similar level of activity. MerA expression did not differ with mercury treatment: ($F_{2,14} = 3$, $p = 0.08$). This is perhaps due to the quality of RNA extracted from my populations: many of my samples had high 230/260 ratios indicative of salt contamination and it is possible that this contamination interfered with the accuracy of the qPCR reaction.

DISCUSSION

This study demonstrates that mercury-resistance offers collective social benefits beyond the individual cell: at intermediate mercury concentrations both resistant and sensitive cells were able to benefit from mercury-resistant cells being present in the population. Furthermore, I demonstrate that when resistance is encoded on the conjugative plasmid pQBR57, cells that harbour this plasmid are subject to a fitness cost. Despite this cost, conjugative transfer of the plasmid enables the resistance to persist in populations even in the absence of positive mercury selection.

This data confirms that mercury-resistance is a microbial social trait, and subject to the population dynamics predicted by game theory. Mercury resistance provided by a plasmid is a context dependent Snowdrift Game: at intermediate concentrations of mercury (20 μM $HgCl^2$), the evolutionary stable state is a mixed population of mercury-resistant and mercury-sensitive cells. This arises because, in game theoretical terms, the best strategy is to do the opposite of your opponent. Consistent with this I observed that in the short term mercury resistance was under negative frequency dependent selection in this environment, suggesting the capacity for stable coexistence of mercury-resistant and mercury-sensitive cells. Frequency-dependence has also been demonstrated for other microbial social traits, both with experimental laboratory populations (Ross-Gillespie et al., 2007) and with natural isolates (Vos et al., 2001) highlighting that Snowdrift dynamics may apply in other microbial systems.

Assessing microbial social traits within their environmental context is crucial to understanding what drives their evolution (Ghoul et al., 2014; Zhang and Rainey, 2013). In this study selection for mercury resistance was affected by both physiochemical and social environment. Altering the physiochemical environment i.e. mercury concentration, changed the balance of costs and benefits associated with mercury resistance. At 0 μM $HgCl^2$, mercury resistance was a purely costly trait, whereas at 40 μM $HgCl^2$ the individual benefits of mercury resistance outweighed the costs. The social effects, described above dominated the system at intermediate levels of $HgCl^2$. The effect of environment on the selection for a cooperative trait has been demonstrated previously with regard to ampicillin resistance in E.coli (Yurtsev et al., 2013). Here, resistant cells degrade the ampicillin through the action of extracellular enzymes, allowing ampicillin-sensitive cells to persist. When the environment was altered by increasing the concentration of antibiotic, it was found that a greater fraction of resistant cells was stably maintained in the population (Yurtsev et al., 2013).

To understand what drives the longer term dynamics of plasmid-encoded mercury resistance plasmid-bearers were compared against populations where the trait was encoded on the chromosome. In the absence of positive mercury selection, plasmid-borne mercury-resistance was maintained at a significantly greater proportion compared to chromosome-encoded resistance. This arises because the plasmid is able to spread by conjugative transfer through the population: horizontal transfer of the plasmid is frequent enough to overcome the costs associated with plasmid-carriage. When resistance was encoded on the chromosome in the absence of mercury it remained at ~50% throughout the experiment: only under positive mercury selection was the trait able to increase in frequency. However, when mercury was present in the environment, the

spread of the trait did occur at a faster rate than when on the chromosome, presumably because resistant individuals did not carry the additional fitness burden associated with the plasmid.

Alongside previous theoretical work, these results demonstrate that being mobile can promote the maintenance of a microbial social trait (Rankin et al., 2011; Smith, 2001). When encoded on a mobile element, social traits are able to spread through the population enforcing the cooperative detoxifying behaviour on resident mercury-sensitive cells. Under, these circumstances, even in the absence of positive selection, conjugation can act as a strong force to maintain the social trait. This is contrary to previous studies which have suggested that conjugation is not a strong enough force to maintain plasmids within populations (Levin, 1993). It remains to be seen whether similar plasmid dynamics would occur in a more naturalistic environment like soil. Unlike the well-mixed environment of this experiment, soil is heterogenous and therefore the spread of the plasmid may be potentially impeded by the spatial structure within a soil population due to the more limited opportunity for conjugation. Furthermore, the fitness cost that was seen with plasmid carriage here is likely to be exaggerated in soil where the conditions are nutrient limited and more stressful for the individual cell.

This study has only considered one plasmid in isolation however the dynamics are likely to differ in natural communities where other resident plasmids may be present within the population. It has been theoretically demonstrated that were a less costly plasmid to arise through mutation without the social trait, it would outcompete the resistant plasmid and prevent its spread (Dimitriu et al., 2014; Rankin et al., 2011).

Given that when chromosome encoded resistance allows cells to bypass the fitness cost of the plasmid, under consistent positive selection traits should integrate onto the host chromosome allowing loss of the costly plasmid backbone (Bergstrom et al., 2000). Plasmid-borne accessory genes are often carried on smaller mobile elements embedded within the plasmid, such as transposons, allowing them to mobilise within and between plasmids as well as integrate onto the host chromosome. Given this, it is perhaps surprising that mercury-resistance is so commonly found on mobile elements (Boyd and Barkay, 2012). One possibility is through co-selection where plasmid-borne traits can increase in frequency by selection on other plasmid-borne genes (Baker-Austin et al., 2006). Even very low environmental concentrations of heavy metals and antibiotics have been shown to sufficiently select for clinical multi-drug resistant plasmids (Gullberg et al., 2014).

In summary, my findings suggest that transitions from social to non-social selection may frequently occur for microbial social traits as a result of environmental context dependence and, along with other recent studies, highlight the importance of quantifying the fitness of such traits across a range of environments. Furthermore, I find that mobility and positive selection both play important roles in the maintenance of social traits within bacterial populations.

CONCLUSION

This work demonstrates that plasmid-borne bacterial mercury detoxification is a social trait with affects beyond the individual cell. I have highlighted that in the short term the trait is under snowdrift dynamics: despite the trait being located on a costly plasmid, negative frequency dependent selection help to maintain it within the population. In the longer term, conjugation plays a much larger role in the maintenance of the trait allowing its spread through the population despite the additional plasmid-associated costs. When encoded on the chromosome, cells avoid the cost of the plasmid, allowing rapid selection for the trait under positive mercury selection. These results demonstrate that both environmental conditions and gene transfer play important roles in the evolution of bacterial social traits.

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