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Bacterial recombination promotes the evolution of multi-drug-resistance in functionally diverse populations

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Bacterial recombination is believed to be a major factor explaining the prevalence of multi-drug-resistance (MDR) among pathogenic bacteria. Despite extensive evidence for exchange of resistance genes from retrospective sequence analyses, experimental evidence for the evolutionary benefits of bacterial recombination is scarce. We compared the evolution of MDR between populations of *Acinetobacter baylyi* in which we manipulated both the recombination rate and the initial diversity of strains with resistance to single drugs. In populations lacking recombination, the initial presence of multiple strains resistant to different antibiotics inhibits the evolution of MDR. However, in populations with recombination, the inhibitory effect of standing diversity is alleviated and MDR evolves rapidly. Moreover, only the presence of DNA harbouring resistance genes promotes the evolution of resistance, ruling out other proposed benefits for recombination. Together, these results provide direct evidence for the fitness benefits of bacterial recombination and show that this occurs by mitigation of functional interference between genotypes resistant to single antibiotics. Although analogous to previously described mechanisms of clonal interference among alternative beneficial mutations, our results actually highlight a different mechanism by which interactions among co-occurring strains determine the benefits of recombination for bacterial evolution.

Keywords: horizontal gene transfer; antimicrobial resistance; experimental evolution; community ecology; cycling therapy; *Pseudomonas aeruginosa*

1. INTRODUCTION

The recent and rapid rise in bacteria resistant to multiple antibiotics has aggravated healthcare costs and mortality around the world, making it a leading public health issue [1]. Described as one of the most important human-induced evolutionary changes [2,3], the evolution of antibiotic resistance presents a serious challenge for evolutionary biologists and microbiologists [4,5]. Multi-drug-resistance (MDR) can arise via two genetic mechanisms: either by de novo mutation (e.g. point mutations, insertions, deletions or duplications) or by bacterial recombination, which is defined as the transfer of DNA from one bacterium to another by means other than vertical transmission. However, the relative importance of mutation and recombination in the evolution of MDR, and in bacterial adaptation generally, remains unclear. Genomic analyses of clinical isolates show that many known resistance genes probably spread via horizontal gene transfer between different species [6–8], yet many clinical pathogens also evolve MDR de novo through novel genetic changes [9,10]. Evolution experiments offer a useful approach to uncover the factors determining the evolution of resistance, but most experiments have studied clonal populations without any contribution of recombination [4].

As most bacteria live in communities of dozens to thousands of microbial species [11,12], the ubiquity and the diversity of other microbes present in the environment are likely to be major factors influencing bacterial evolution and the effect of recombination. On one hand, the breadth of genetic material being released into the extracellular milieu can provide a large reservoir of new genes. Although the quality of DNA being released into the environment is a matter of debate [13,14], resistance genes can be disseminated in soil and other environments rich in nucleic acids [15,16]. On the other hand, standing variation present among different species of bacteria might result in competition among functional classes, such that strains that are pre-adapted to new conditions will limit the opportunity for the evolution of novel traits in less well pre-adapted strains [17]. This functional interference should be especially important in soil and gut microbial communities where there are a high diversity and number of existing bacteria types, ecological functions and resistance determinants [18–21]. Clearly, microbial diversity is a key component in understanding the benefits of recombination, yet its general effect on bacterial evolution has been largely overlooked in experimental studies.

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Here, we investigate the effect of recombination via natural transformation on the evolution of MDR in functionally diverse populations of bacteria. We use transformation as a model for the evolutionary benefits of bacterial recombination because, unlike the alternative mechanisms of conjugation or transduction, natural transformation is encoded entirely by the bacterial chromosome [22]. Teasing apart the benefits for the bacterium is, therefore, simpler than in multi-partner systems in which recombination depends on coevolutionary interactions with selfish genetic elements such as plasmids and bacteriophages [23]. Furthermore, transformation has played a role in the rapid evolution of MDR in human pathogens such as *Acinetobacter baumannii* [24] and can therefore be considered as a model to study bacterial recombination and adaptation.

Although rates of recombination vary dramatically among bacterial species [25], a key benefit of transformation is believed to be the bringing together of beneficial genes present in the extracellular milieu into a single lineage [26]. For example, the pathogenic bacterium *A. baumanii* is believed to have recently acquired more than 45 resistance genes from other genera such as *Escherichia*, *Pseudomonas* and *Salmonella* through this mechanism [24]. However, some authors have argued that the uptake of DNA by bacterial cells might serve primarily for nutrition or DNA repair and have little or no adaptive significance to bacterial evolution [13,27]. Under this hypothesis, it is argued that recombinational repair of DNA damage is the main short-term benefit of transformation and that exchange of beneficial genes is a chance by-product that contributes infrequently to adaptation [27].

Which of these adaptive benefits of transformation explains its maintenance within populations remains unclear. Recent experiments show that both conjugation and transformation can promote adaptation in clonal populations of bacteria such as *Escherichia coli* [28] and *Helicobacter pylori* [29]. In both studies, it was argued that recombination reduces competition between multiple beneficial mutations that arise within separate lineages, a phenomenon known as clonal interference [30]. Recombination mitigates the effect of clonal interference by combining alternative beneficial mutations within a single lineage. However, the loss of transformability in populations of *Acinetobacter baylyi* grown on rich medium shows that benefits of recombination are context-dependent [31]. Furthermore, these studies considered evolution by de novo mutation arising during the experiment. Given the ubiquity of resistant strains in the environment, MDR in real situations is likely to evolve in bacterial communities already harbouring an array of resistance genes for one or more of the multiple antibiotics used for treatment. How does recombination affect the evolution of MDR in the face of standing diversity in resistance genes?

Here, we investigate the effect of transformation on the evolution of MDR in functionally diverse populations of *A. baylyi*. This bacterium has high competency for natural transformation [32] and occasionally causes antibiotic-resistant infections in humans [33]. No plasmids or viruses are present in our experimental strains and therefore the only mechanism of recombination is transformation. Through genetic manipulation, we inhibited the bacterium's recombinogenic activity without impeding its capability to take up extracellular DNA, making *A. baylyi* a powerful model system to study the evolutionary benefits of recombination (rather than just DNA uptake). Measuring the evolution of MDR, we show that diversity tends to inhibit adaptation in the absence of recombination. However, when recombination is allowed, the exchange of resistance genes between the bacterial strains leads to rapid evolution of MDR within a few generations. We also show that only the presence of DNA harbouring resistance genes in the extracellular environment promotes evolution, which excludes the effect of other possible benefits of recombination in our experiments. Together, these results show that alleviation of functional interference between single drug-resistant strains might explain the high frequency of recombination among microbial populations evolving MDR.

2. MATERIAL AND METHODS

(a) *Bacterial strains*

In our experiments, we used *A. baylyi* strain ADP1, a soil bacterium that is naturally competent for homologous and non-homologous recombination [32]. Recombination into the recipient chromosome is achieved via a RecA-dependent mechanism and usually requires minimal levels of homology between donor and recipient DNA sequences [34]. We constructed a non-recombinogenic mutant by knocking out the *recA* gene and deleting 4 bp in the *salR* gene of the naturally transformable *A. baylyi* ADP1. The *salR* deletion prevents growth on the medium with salicylate as sole carbon source and was used as a selective marker that has no detectable phenotypic effect in our culture conditions (for a complete description of the construction procedure and a list of strains and plasmids used, see the electronic supplementary material, table S1). In the text, we refer to strain ADP1 as Rec(+) and the non-recombinogenic ADPWH_Δ*salR_recA* mutant as Rec(−), short for recombination plus and recombination minus, respectively. Because the deletion of *recA* only affects recombination ability, Rec(−) can still take up plasmids by transformation (data not shown).

To verify the recombination rate of our constructed strains, we compared the transformation capacity of Rec(−) to that of an ADPWH_*lux*_Δ*salR* mutant that had the selective *salR* deletion marker, but without the knockout of *recA* [35]. Transformation of both mutants was attempted by mixing cells with PCR products of intact *salR* fragments using ADP1 colony as DNA template (PCR primers are listed in electronic supplementary material, table S2). Transformation of intact *salR* DNA into the ADP1 mutants would rescue the 4 bp deleted *salR* and restore the cells' capacity to metabolize salicylate, which we assayed by growing cells on minimal medium with salicylate as sole carbon source.

(b) *Choice of antibiotics and selection of antibiotic-resistant mutants*

Ten clones resistant to either gentamicin or rifampicin were isolated for both Rec(+) and Rec(−) strains using the modified fluctuation test described in Ward *et al.* [36]. In brief, a culture of each ancestral strain was diluted to 10^{-6} into Miller's Lysogeny Broth (LB, per litre: 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl). From this dilution, 200 µl were inoculated into each well on a 96-well microtitre plate. After incubating the plate for 24 h at 30°C, we plated 4 µl of a 100× dilution of each culture onto an LB agar plate containing either rifampicin or gentamicin. Both antibiotics were used as recommended by the manufacturer (Sigma Aldrich, St-Louis,

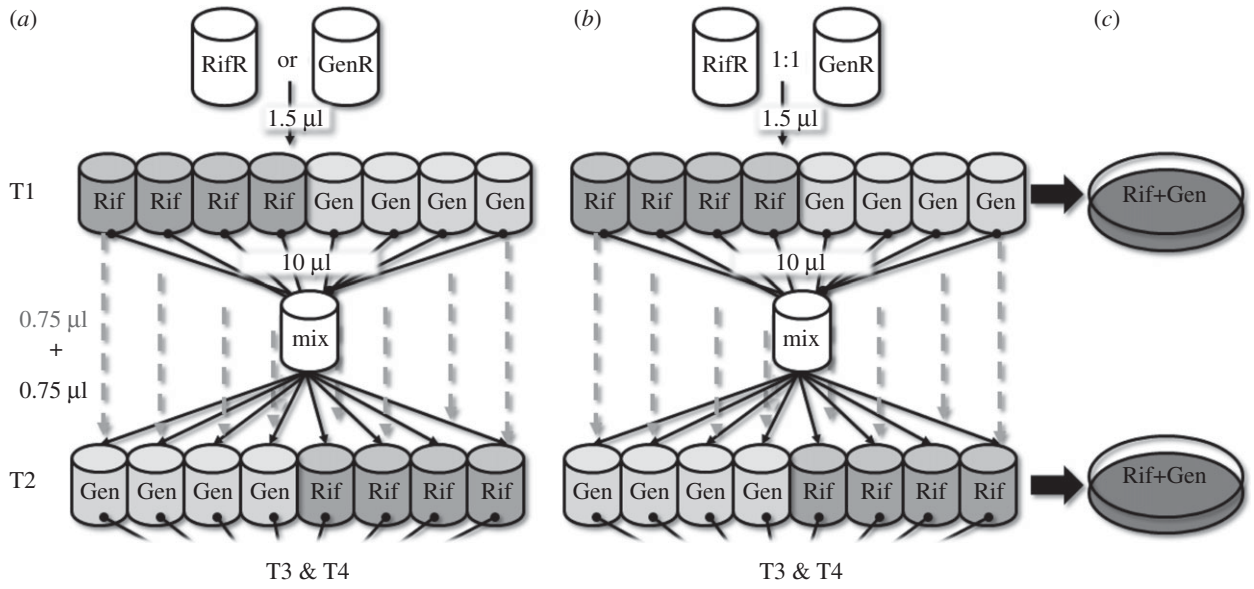

Figure 1. Experimental design investigating the evolution of multi-drug-resistance (MDR) in diverse microbial populations under the effects of recombination. Two antibiotics, rifampicin (Rif) and gentamicin (GenR) were cycled at each transfer, and we compared MDR evolution between: (*a*) monocultures of the bacterium *A. baylyi* resistant to either rifampicin RifR or GenR; and (*b*) polycultures of RifR and GenR mixed (1 : 1) together. The effect of recombination was tested by conducting the experiment in a naturally recombinogenic strain, Rec(+), and a strain lacking recombination, Rec(−). For each treatment and each strain combination, eleven independent replicate metapopulations were maintained for four dilutions. (*c*) The presence of MDR in each microcosm was screened after every two transfers by pipetting 4 µl of 1000× dilution onto an LB agar supplemented with GenR and RifR.

MO, USA) and the minimal inhibitory concentration (MIC), which is the concentration that inhibits at least 90 per cent of the growth of the bacteria under normal conditions, was found to be 0.75 µg ml^{-1} for both antibiotics. Colonies indicate resistant mutants and were picked from the plate. Confidence intervals were estimated using the binomial distribution.

From the library of antibiotic-resistant mutants, we selected four as starting genotypes for our experiments: one Rec(+) mutant resistant to gentamicin, one Rec(+) mutant resistant to rifampicin, one Rec(−) mutant resistant to gentamicin and one Rec(−) mutant resistant to rifampicin (see electronic supplementary material, table S1). Because resistance mutations often incur fitness costs on the host and can therefore affect the mutation supply rate of a population, we selected mutants that have similar growth parameters. To do so, we minimized the difference between the carrying capacity, *K*, and the maximal growth rate, *r*, between all four resistant mutants. *K* and *r* were measured from optical density (OD_{600}) using an automated spectrophotometer (BioTek, Winoosky, VT, USA). None of the mutants could grow on the alternative antibiotic without further mutation. All strains and mutants were stored at −80°C in 25% $^{m}/_{v}$ glycerol solution.

(c) *Selection experiment in diverse populations*

We maintained cultures in an environment that favours the evolution of MDR by cycling the antibiotic gentamicin and rifampicin between each transfer, following the study of Perron *et al.* [37]. The design mirrors cycling treatments known to promote the evolution of MDR in clinical infections under certain conditions [38,39]. Bacteria were grown in static microcosms containing LB at 30°C and were transferred (1%) into fresh media every 24 h. Also every 24 h the antibiotic added to the growth medium in each microcosm was switched from one antibiotic to the other. Because the order in which antibiotics are cycled can affect resistance evolution [37], we initiated half of our microcosms in gentamicin and the other half in rifampicin.

Microcosms were then grouped into metapopulations containing eight microcosms linked by migration at each transfer. At any one time, half of the microcosms within a metapopulation were treated with gentamicin and the other half with rifampicin. Each transfer was done to allow migration between all microcosms within a metapopulation. First, we transferred 0.75 µl of saturated cultures directly from the old microcosm to the fresh microcosms. Second, we pooled together 0.75 µl of saturated culture from all the microcosms within a metapopulation and from this mixture, we transferred 0.75 µl to all the fresh microcosms the metapopulation. Therefore, every 24 h, a total of 1.5 ml (approx. 10^4 cells) were transferred into every fresh microcosm, with approximately 56.25 per cent coming from one local microcosm and approximately 6.25 per cent coming from each other microcosm of a metapopulation (figure 1). This transfer protocol enabled individual genotypes to track their optimal local environment across the metapopulation and therefore allowed genotypes resistant to single antibiotics to survive and replicate enough to generate new mutations at appreciable frequency, yet still experience selection favouring resistance to both drugs [37].

To investigate the effects of recombination and functional diversity on the evolution of MDR in this system, we then set up replicate metapopulations with each factorial combination of the following treatments. First, we initiated some metapopulations with just a single antibiotic-resistant genotype present (monocultures with either RifR or GenR in turn) and some with a mixture of both antibiotic-resistant genotypes present (polycultures with RifR and GenR). For polycultures, we mixed a 1 : 1 ratio (±5%) of cells resistant to rifampicin (RifR) or gentamicin (GenR) in 5 ml of LB. We then transferred 1.5 µl (approx. 10^4 cells) of this GenR : RifR mix into microcosms containing 150 µl of

fresh LB supplemented with one of the antibiotic treatments described above. The initial total population size and growth conditions were the same in both monocultures and polycultures. Second, we conducted each treatment for Rec(+) and Rec(−) genotypes in turn. For each treatment and strain combination, 11 independent replicate metapopulations were maintained for four transfers (approx. 40 generations).

At every second transfer, the presence of MDR was screened in each microcosm by pipetting 4 µl of 1000× dilution (thus minimizing the chance of sampling cells with a frequency of 10^{-6} or less) onto an LB agar supplemented with gentamicin and rifampicin, so that only cells resistant to both antibiotics could grow. MDR was scored as present in a microcosm if colonies appeared on the plates.

(d) *Selection experiment with extracellular DNA*

To identify the mechanism enhancing antibiotic-resistant evolution in the Rec(+) strain in our experiments, we set up a series of selection experiments exposing Rec(+) and Rec(−) strains to different types of extracellular material. Starting with strains lacking resistance to rifampicin, we compared the evolution of resistance with rifampicin between populations of Rec(+) and Rec(−) grown with the following supplements to their growth media in turn: (i) saline; (ii) *A. baylyi* wild-type DNA; and (iii) *A. baylyi* DNA harbouring resistance to rifampicin. If the uptake of extracellular DNA promoted evolution of resistance irrespective of the type of DNA, we would expect Rec(+) populations grown with any type of extracellular DNA to evolve resistance more rapidly than in the absence of extracellular DNA. Alternatively, if the uptake of resistance genes was the dominant mechanism, we would expect Rec(+) populations grown with extracellular resistant DNA to evolve resistance more rapidly than Rec(+) populations grown with wild-type DNA. Twenty microcosms were established for each combination of treatments for a total of 60 lines. To check the effect of severity of antibiotic pressure on the outcome, we repeated the experiments for a constant environment (rifampicin provided at every dilution) and a fluctuating environment (rifampicin only provided at every alternate dilution).

Each line was initiated by inoculating 1.5 µl of stationary phase culture (approx. 10^4 cells) into 150 µl LB plus rifampicin and transferred daily (1%) into fresh medium for five days. Growth medium was supplemented daily with 1 µl of the appropriate extracellular material treatment (approx. 100 ng of genetic material when DNA was provided). Genomic DNA was extracted using a Qiagen DNeasy Blood & Tissue Kit (Qiagen, Crawley, UK), following manufacturer's instructions for extraction from Gram-negative bacteria, and was purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK). DNA weight was estimated from comparison with a molecular ladder and gel electrophoresis. Growth of lines was monitored daily as optical density (OD_{600}). Scores were corrected to that of a blank.

(e) *Statistical analyses*

To compare the evolution of MDR between monocultures and polycultures of Rec(+) and Rec(−), we compared the proportion of metapopulations in which MDR evolved using pairwise χ^2-tests. Because of the low sample sizes of the comparisons, the significance values were simulated using 100 000 replications. For simplicity, we combined the data for individual genotypes into a single monoculture category.

To compare the evolution of antibiotic resistance during the selection experiment with extracellular DNA, we modelled the temporal dynamics of bacterial growth using a hierarchical linear mixed model with optical density as the response variable (lme function of the nlme package of the R v. 2.11.1 software; http://www.r-project.org). While time was considered as a random variable, extracellular DNA treatment (three levels) was fixed. We accounted for the nonlinear growth by computing the quadratic term for time. Replicates were taken to be random effects and were nested within treatments. We simplified the full model by sequential backward selection using *F*-tests. A variance function (varIdent of nlme library) that permits different variances for each level treatment was used to model heteroscedasticity. We used the corAR1 function to model the autocorrelation structure in the time series. Model parameters and confidence intervals were estimated with restricted maximum-likelihood methods [40]. All analyses and model assumptions were performed and verified using R v. 2.11.1 software.

3. RESULTS

(a) *Recombination and mutation rates of strain Rec(−)*

The salR assay confirmed that our Rec(−) strain had lost the ability to recombine. Transformation rate was below the detection limit (10^{-9}) for Rec(−), but was 6.4×10^{-4} for ADPWH_*lux*_Δ*salR*, the strain constructed with the same deletion in *salR* (a selective marker) as Rec(−) but without the *recA* knockout. Because of its role in DNA repair, one possible consequence of deleting *recA* is to increase the mutation rate [41], which might influence evolutionary rates in later experiments. The rate of mutations conferring antibiotic resistance in the fluctuation test to generate resistant mutants was marginally higher in strain Rec(−) (4.02×10^{-8} resistant-mutations per cell division, 95% CI: 3.62 to 4.48×10^{-8}) than in the transformable wild-type strain Rec(+) (1.94×10^{-8} resistant-mutations per cell division, 95% CI: $1.47–2.43 \times 10^{-8}$). This difference is insufficient, however, to explain differences in evolutionary dynamics between the two strains described below (see §4).

(b) *The effects of functional diversity and recombination on evolution of multi-drug-resistance*

In the absence of recombination, MDR evolved less frequently in polycultures than in monocultures (figure 2). While MDR evolved and spread in every gentamicin-resistant and rifampicin-resistant monoculture (grey bars, figure 2*a*), MDR evolved in only five out of 11 polycultures (grey bars, figure 2*b*; comparison at day 4: $\chi^2 = 10.97$; d.f. = 1, $p = 0.0024$; figure 2). The evolution of MDR was therefore inhibited by initial diversity in single-antibiotic resistance in bacterial populations.

In marked contrast, recombination promoted the evolution of MDR in polycultures (figure 3). Notwithstanding the opportunity for each single drug-resistant genotype to track its optimal environment across the metapopulations, MDR evolved in all metapopulations in which Rec(+) single drug-resistant mutants were mixed together (comparison between polycultures at day 4: $\chi^2 = 8.25$; d.f. = 1, $p < 0.01$; black bars, figure 2*b*). Interestingly, MDR evolved more rapidly in polycultures with recombination present

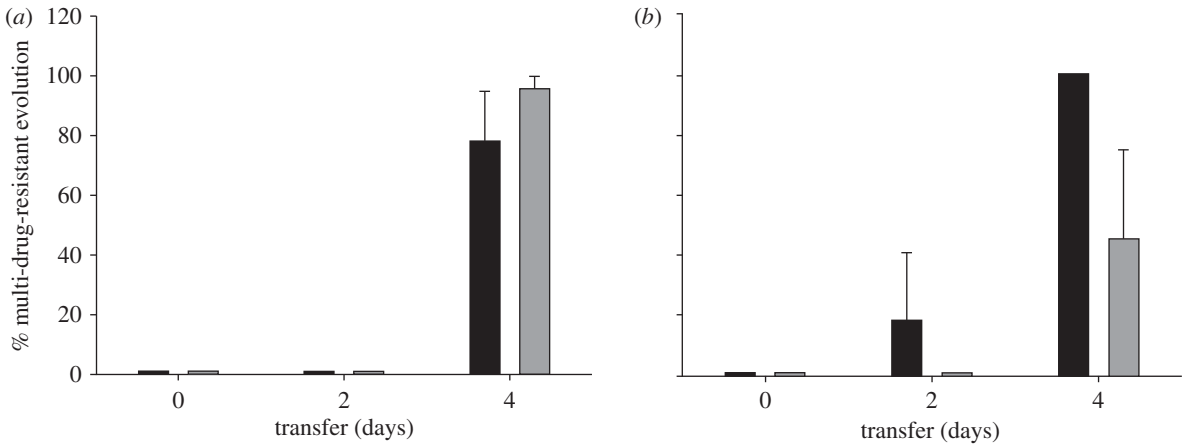

Figure 2. The effect of recombination on the evolution of MDR in (*a*) monocultures and (*b*) polycultures of antibiotic-resistant strains of *A. baylyi*. Monocultures were started with just one genotype, initially resistant to a single antibiotic (either GenR or RifR). Polycultures were started with a 1 : 1 mixture of genotypes resistant to GenR and genotypes resistant to RifR. (*a*,*b*) Proportion of independent metapopulations in which MDR evolved is compared between the highly recombinogenic Rec(+), black bars, and Rec(−), a construct lacking any detectable recombination, grey bars. Binomial 95% confidence intervals are shown.

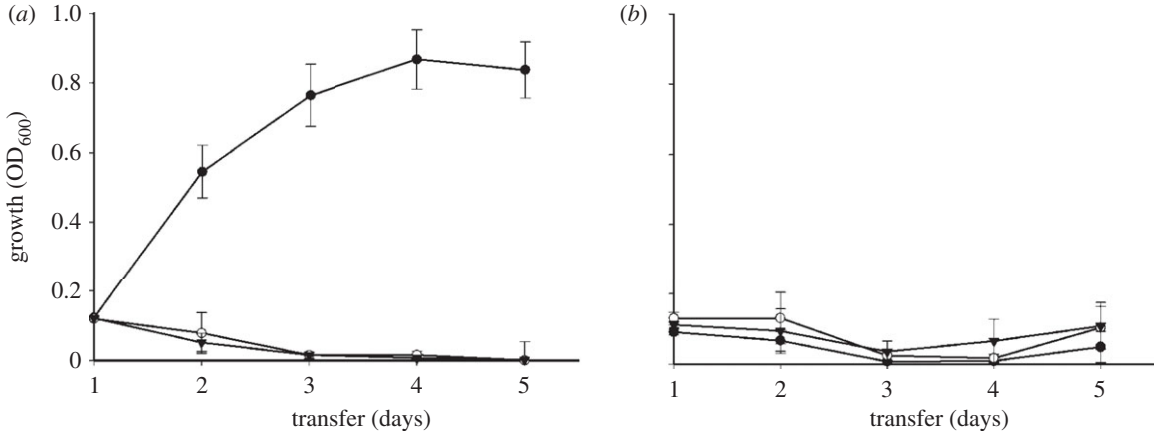

Figure 3. Evolution of resistance to treatment of rifampicin in experimental populations of *A. baylyi* with natural recombination, Rec(+) in (*a*) and no recombination, Rec(−) in (*b*). Microcosms were supplemented either with: sterilized water (filled triangles), wild-type susceptible DNA (open circles) and *A. baylyi* DNA harbouring rifampicin-resistant mutations (filled circles). Resistance was measured as growth represented by average density (OD_{600}) in the presence of rifampicin, and error bars depict standard error of the means.

than in any other treatment (comparison of Rec(+) polycultures versus Rec(−) polycultures and all monocultures by day 2: $\chi^2 = 9.66$; d.f. = 1, $p < 0.01$; figure 2*a*,*b*), suggesting that evolution through recombination of existing diversity was more important than through novel mutations. The occurrence of recombination had no effect on evolution in monocultures: most experimental metapopulations evolved MDR under cycling therapy by day 4 both in Rec(+) and Rec(−) ($\chi^2 = 3.09$; d.f. = 1, $p = 0.19$; figure 2*a*). Therefore, recombination alleviated the inhibitory effect of initial diversity of single antibiotic-resistant mutants, and promoted the evolution of MDR.

(c) *Recombination and extracellular material*

Only the addition of extracellular DNA harbouring resistance to rifampicin promoted a rapid and large increase in growth of Rec(+) populations (constant regime: treatment × strain × time: $F_{2,378} = 33.033$; $p < 0.0001$; figure 3*a*; closed circles). Although the fluctuating regime resulted in an overall larger population density than in the constant environment (environment: $F_{1,240} = 125.1$; d.f. = 1, 240; $p < 0.0001$), again only the addition of resistant DNA promoted resistance evolution in Rec(+) populations (treatment × strain × time: $F_{2,186} = 6.603$; $p = 0.0017$ and electronic supplementary material, figure S1*a*,*b*, open circles). The addition of wild-type *A. baylyi* DNA or sterilized water did not promote evolution of rifampicin resistance in this experiment. These results indicates that the uptake of resistance genes is the main mechanism leading to enhanced evolution of resistance in Rec(+).

Several observations allowed us to reject alternative explanations for the fitness benefit of HGT observed in the above experiments. First, in the absence of extracellular DNA, no population could evolve resistance to rifampicin in the constant environment (figure 3*a*,*c*, closed triangles) or in the fluctuating regime (electronic supplementary material, figure S1*a*,*b*, closed triangles). Combined with the fact that recombination did not increase rates of adaptation of monocultures in the previous experiment, this result indicates that the increase in mutation rate afforded by the bacterial exchange system reported above is not

sufficient to promote adaptation under these conditions. Second, the addition of wild-type DNA did not promote the evolution of resistance in Rec(+) (figure 3*a* and electronic supplementary material, figure S1*a*, open circles) or Rec(−) populations (figure 3*b* and electronic supplementary material, figure S1*b*; open circles). This indicates that our results cannot be explained either by any benefits for recombining alternative resistance mutations arising within the populations during the experiment or by the potential nutritional benefit of extracellular DNA, which both Rec(+) and Rec(−) would derive.

4. DISCUSSION

Our results show that bacterial recombination can promote the evolution of antibiotic resistance beyond levels obtained purely by mutation. The transfer of resistance genes is well known from retrospective sequence analyses [42], but we know of no previous study that has compared the evolution of resistance in recombining and non-recombining populations. In response to severe antibiotic stress, transformation allowed bacteria to adapt to the presence of single and multiple antibiotics in just a few generations when resistance genes were present in the environment. Furthermore, we can reject alternative hypotheses for the benefit of transformation based on nutrition and recombinational repair [13,27].

The diversity of resistance genes present at the start of the experiment was a key determinant of whether MDR evolved and of the differences between recombining and non-recombining strains. In non-recombining strains, the initial presence of two strains resistant to different single antibiotics in the polycultures inhibited the evolution of MDR compared with monocultures started with just one genotype present. We have observed the same inhibitory effect in antibiotic-resistant polycultures of *Pseudomonas aeruginosa*, an opportunistic pathogenic bacterium of human with a naturally low recombination rate (electronic supplementary material, figure S2). Our findings are, therefore, likely to be relevant to many other bacterial systems. In recombining strains, however, MDR evolved faster in the polycultures than in the monoculture, i.e. the inhibitory effect of diversity observed in non-recombining strains was reversed. The difference between monoculture and polyculture treatments shows that the benefits of recombination derived from bringing together resistance genes present at the start of the experiment, rather than by combining new mutations arising de novo within the populations (which would occur at the same rate in monocultures and polycultures).

One explanation consistent with some of our findings is that the chance of an MDR genotype originating is higher in recombining populations, because the effective recombination rate is faster than the mutation rate. This effect is to be expected only in polycultures, because only then can recombination enhance the evolution of MDR above mutation, and it is therefore supported by the observation that MDR evolved faster in Rec(+) polycultures than any other treatment. However, it cannot explain why diversity inhibited the evolution of MDR in Rec(−): MDR genotypes arose frequently enough in monocultures to yield MDR, so why not in polycultures?

A better explanation for our findings is that the fitness of an MDR genotype relative to single drug-resistant genotypes (and hence the chance of it spreading to detectable frequency) depends on whether the MDR genotype originated by mutation or by recombination. An MDR genotype will only spread in polycultures if its average fitness in the cycling environment is greater than the average fitness of the single drug-resistant genotypes. In non-recombining populations, this will depend on whether a de novo mutation providing (for example) rifampicin-resistance to a GenR genotype has sufficient benefit to be able to compete with the existing RifR genotypes when growing in microcosms treated with rifampicin. In monocultures, irrespective of whether recombining or non-recombining, a new MDR genotype should always spread (as long as the new RifR mutation does not have an outweighing cost to growth in gentamicin treatments) because there are no competing RifR genotypes initially. The inhibitory effect we observe in non-recombining populations suggests therefore that most de novo mutations leading to MDR genotypes had insufficient benefit to outcompete the single drug-resistant genotypes in polyculture populations.

In contrast, the spread of a recombinant MDR genotype in recombining populations will depend on whether there are genetic interactions between RifR and GenR genes following recombination. If there are no genetic interactions, the fitness of the MDR genotype should always exceed that of either single drug-resistant genotype in the cycling environment, since it gains the fitness benefits of both single drug-resistant genotypes. Only strong negative epistasis between RifR and GenR would reduce the average fitness of the recombinant MDR genotype below that of the single drug-resistant genotypes and prevent the spread of the MDR genotype. Although the comparison is complicated because we can only sample 'winning' genotypes, higher average fitness of MDR genotypes in recombining populations than those in non-recombining populations supports this explanation (electronic supplementary material, figure S3).

Our study highlights what is, to our knowledge, a new mechanism explaining the benefits of recombination in populations of bacteria. In the absence of recombination, standing diversity inhibits the evolution of novel traits such as MDR that rely on bringing together different traits already present in the population. Superficially, our findings are similar to those of studies showing that bacterial recombination reduces clonal interference between competing beneficial mutations [28]. However, the mechanism and the context are different. Theories of clonal interference rely on recombination increasing the rate at which genotypes with multiple beneficial mutations arise in the population. In our case, however, MDR genotypes arose readily even in Rec(−) monoculture, suggesting that the chance of MDR genotypes originating was not lower in the absence of recombination. Instead, the fitness of MDR genotypes, and whether they spread or not, depended on whether MDR genotypes arose by mutation or by recombination. Our proposed mechanism, therefore, has more in common with theories of the evolution of generalists versus specialists [43]: whether the 'generalist' MDR genotype evolves depends on its fitness relative to the 'specialist' single drug-resistant genotypes. However, the trade-off between specialism and generalism in our explanation derives from whether new mutations needed to generate the generalist in a non-recombining population can match the fitness effect of genotypes already present in the population.

This mechanism requires that genes present in the population have greater fitness effects than de novo mutation. In our experiments, the initial single drug-resistant genotypes were chosen to have desirable growth properties and it is therefore perhaps unsurprising that they should tend to have greater fitness effects than new mutations arising spontaneously in the experimental cultures. If we chose initial mutants with lower fitness effects, we might not have observed the positive effect of recombination on the evolution of MDR. However, we believe this condition is likely to be the characteristic of real situations. In most bacterial populations, except monocultures grown in the laboratory, genetic diversity among co-occurring populations is likely to exceed genetic variation originating by new mutations. Furthermore, the fitness effects of existing genes should tend to exceed de novo mutations, because they have already been tried and tested in the environment. Any trait that enables a strain to evade this inhibitory effect is therefore likely to increase in frequency. This could explain the prevalence of recombination (or recombinogenic elements) in clinically relevant bacteria today: mainly bacteria with recombinogenic capability have been capable of evolving MDR after the introduction of commercial antibiotics [44]. Given a longer period of time, the rare mutant needed for MDR to spread might also arise in non-recombining metapopulations. However, if both recombining and non-recombining bacteria were present, by that time the recombining populations would have already evolved MDR and probably driven the single drug-resistant non-recombining genotypes extinct. The recent discovery of hyper-recombinogenic phenotypes in MDR clinical isolates of bacteria such as *Pneumococcus* species further supports this hypothesis [6].

One possible confounding difference between diversity treatments that could have affected our experiments is population size. Monocultures initially had lower effective population sizes than polycultures because until cells evolved MDR they could only grow in half of the microcosms (those with the antibiotic they were already resistant to), whereas populations in polyculture metapopulations could grow in all microcosms (i.e. rifR in half of them and GenR in the other half). Increased population size might have increased the chance of MDR mutations arising in polycultures than in monocultures. Although such an effect might have occurred, it cannot explain our findings. The same population size effect would be expected in both Rec(+) and Rec(−) strains and, if anything, it would increase the rate of origin of MDR in polycultures compared with monocultures, which contradicts results for the non-recombining strain.

Another possible confounding factor in our experiment is that although we selected resistant bacteria with identical growth parameters, it is likely that Rec(+) and Rec(−) genotypes harbour different resistance mutations. Depending on the nature of epistatic interactions between mutations [36], it is possible that the particular resistance mutations in Rec(+) and Rec(−) might constrain or enhance subsequent evolution of MDR differently from one another. Constructing Rec(+) and Rec(−) strains of each resistant genotype was not practical in this study. However, in our experiments, every resistant mutant could rapidly evolve MDR when grown in monocultures (figure 2*a*), meaning that the observed effects were due to the manipulation of recombination and its effects in polycultures, rather than possible epistatic effects.

In conclusion, our experiments highlight the need for a better understanding of the ecology of antibiotic resistance and bacteria in general. The overwhelming diversity of microbes combined with the prevalence of recombination in natural populations of bacteria is likely to confound the development of novel therapeutic strategies for years to come. Not only is the use of novel antimicrobial molecules likely to foster evolution of de novo resistance mechanisms [45], but it can also favour the emergence of pathogens harbouring yet unidentified combinations of resistance genes [20,21,46]. For this reason, increasing pharmaceutical efforts as the sole strategy to fight antibiotic resistance [47] is bound to offer a short-lived remedy to the actual antibiotic-resistant crisis.

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