Bacteriophage selection against a plasmid-encoded sex apparatus leads to the loss of antibiotic-resistance plasmids

Matti Jalasvuori1,*, Ville-Petri Friman2, Anne Nieminen1, Jaana K. H. Bamford1 and Angus Buckling2,3

1Department of Biological and Environmental Science and Nanoscience Center, University of Jyväskylä, PO Box 35, 40014 Jyväskylä, Finland
2Department of Zoology, University of Oxford, The Tinbergen Building, South Parks Road, Oxford OX1 3PS, UK
3Biosciences, University of Exeter, Penryn TR10 9EZ, UK
*Author for correspondence (matti.jalasvuori@ju.fi).

Antibiotic-resistance genes are often carried by conjugative plasmids, which spread within and between bacterial species. It has long been recognized that some viruses of bacteria (bacteriophage; phage) have evolved to infect and kill plasmid-harboring cells. This raises a question: can phages cause the loss of plasmid-associated antibiotic resistance by selecting for plasmid-free bacteria, or can bacteria or plasmids evolve resistance to phages in other ways? Here, we show that multiple antibiotic-resistance genes containing plasmids are stably maintained in both Escherichia coli and Salmonella enterica in the absence of phages, while plasmid-dependent phage PRD1 causes a dramatic reduction in the frequency of antibiotic-resistant bacteria. The loss of antibiotic resistance in cells initially harbouring RP4 plasmid was shown to result from evolution of phage resistance where bacterial cells expelled their plasmid (and hence the suitable receptor for phages). Phages also selected for a low frequency of plasmid-containing, phage-resistant bacteria, presumably as a result of modification of the plasmid-encoded receptor. However, these double-resistant mutants had a growth cost compared with phage-resistant but antibiotic-susceptible mutants and were unable to conjugate. These results suggest that bacteriophages could play a significant role in restricting the spread of plasmid-encoded antibiotic resistance.

Keywords: antibiotic resistance; conjugative plasmids; bacteriophages

1. INTRODUCTION

The lateral spread of antibiotic-resistance genes is often dependent on mobile genetic elements [1]. For example, antibiotic-resistance-conferring conjugative plasmids can spread rapidly both within and between species of bacteria by expressing proteins (mating-pair complexes) that physically connect cells in close proximity [1]. Antibiotic-resistance plasmids (AB-plasmids) can exist at high frequencies in bacterial populations, even in the absence of antibiotic selection [2]. This is because plasmids often impose little cost to bacterial fitness [2], and where there are costs, they can be readily compensated by mutations in the bacteria, plasmid or both [2,3]. Moreover, some plasmids encode long-lasting toxins that kill the cell if the plasmid is lost, while other plasmids are so efficient at promoting bacterial conjugation that their loss is compensated by their spread [4]. Interventions that specifically target the spread of plasmids may help in the fight against antibiotic resistance and thus prolong the effective lifespan of conventional antibiotics.

Bacteriophages (viruses that infect bacteria) that specifically bind to the mating-pair complex encoded by conjugative, drug-resistance-conferring plasmids have the potential to limit the spread of AB-conferring plasmids [5]. Despite identification of these ‘male-specific phages’ more than four decades ago [6], their potential role in limiting the spread of antibiotic resistance has yet, to our knowledge, to be experimentally addressed. Here, we investigate this possibility by cultivating AB-plasmid-carrying Escherichia coli and Salmonella enterica in the presence or absence of plasmid-dependent bacteriophage PRD1 in the absence of antibiotics for 10 days. One of the plasmids used was the well-studied IncP-type RP4 [7], which has sophisticated toxin–antitoxin mechanisms that help maintain the plasmid within dividing bacterial cells. The other plasmid used was the less-studied conjugative IncN-type plasmid RN3 [8].

2. MATERIAL AND METHODS

(a) Bacteria, plasmids and bacteriophages

The bacterial strains, plasmids and bacteriophages used in the study are listed in table 1. Bacteria were cultured in Luria-Bertani (LB) medium [13] at 37°C. When antibiotic selection was required, kanamycin (final concentration 25 µg ml⁻¹), chloramphenicol (final concentration 25 µg ml⁻¹) and tetracycline (final concentration 20 µg ml⁻¹) were used. Liquid cultures were steadily shaken at 220 revolutions per minute. LB agar (1%) was used for plating.

(b) Serial culture experiments

The experiments were started by transferring 5 µl of overnight-grown bacterial culture to tubes containing 5 ml of LB medium. The bacteria were grown for approximately 24 h and 5 µl of the culture were transferred to fresh medium (5 ml) every day for 10 days. The effect of bacteriophage selection was studied by adding 5 µl of PRD1 stock (approx. 10¹² plaque-forming units per millilitre) to the bacterial cultures and measuring the percentage of antibiotic-resistant bacteria after 1, 3 and 10 days. Bacteriophages were added either only on the first day or every day, whereas no phages were added to control treatments. The percentage of antibiotic-resistant cells was measured by plating the bacteria on an LB plate and on an LB-antibiotic plate and counting the number of colony-forming bacteria. Five replicates were used in all but one studied combination of bacteria, plasmids and bacteriophages; in the case of RP4 plasmid and the daily addition of phage, four replicates were used.

(c) Polymerase chain reaction assay

The presence of the conjugative plasmid RP4 was tested by polymerase chain reaction (PCR) from several antibiotic-susceptible cells expelled their plasmid and on an LB-antibiotic plate and counting the number of colony-forming bacteria. Five replicates were used in all but one studied combination of bacteria, plasmids and bacteriophages; in the case of RP4 plasmid and the daily addition of phage, four replicates were used.

for _aphA_ and GCATCCTTCTTGCGGCTGGC and TGCAC GACGAGGACCTACAGCG for _traC_.

The absence of PCR product was taken as indicating a lack of plasmid DNA in the sample. For positive controls, we used antibiotic-selected bacteria known to harbour RP4. Note that the genome sequence is not available for RN3, and generic IncN primers failed to work. Thus, we were unable to test for RN3 using PCR.

(d) **Bacterial growth measurements**

At the end of the serial culture experiment, we selected the treatment where the *E. coli* had been seeded daily with PRD1 phage and isolated two double-resistant and two phage-resistant but antibiotic-susceptible clones per replicate population. Also 10 ancestral clones were isolated. The maximal densities of all clones were measured as maximum observed optical density by using a wide-band filter (420–580 nm) on bacterial culture growing on Luria Broth medium with shaking at 37 °C in the absence of PRD1 phage for 24 h.

(e) **Conjugation experiments**

The kanamycin- and phage-resistant bacterial clones were mixed together with chloramphenicol-resistant recipient bacterial cells (50–50) and incubated at 37 °C for 24 h without shaking. The bacterial mixture (about 10⁷ cells) was spread on plates containing both antibiotics (kanamycin and chloramphenicol) and the number of colonies counted. The clone was scored as conjugation-defective if no colonies appeared on the plate.

(f) **Statistical analysis**

The log-transformed cell density and arcsine-transformed colony frequency data (to meet general linear model assumptions) were analysed with repeated measures ANOVA. Two-way ANOVA was used to analyse differences in bacterial growth. Statistical analyses were performed with SPSS-software (v. 17.0, SPSS Inc., Chicago, IL, USA).

3. RESULTS

Practically all bacteria (approx. 100%) retained the antibiotic resistance in the absence of phages (figure 1). However, the frequency of antibiotic-resistant bacteria decreased rapidly in the presence of the bacteriophage PRD1 to an average of 5 per cent by day 10 (*F*₁,₁₀ = 1575.3, *p* < 0.001, figure 1), regardless of the phage addition frequency (single versus daily phage addition, *F*₁,₂₂ = 0.001, *p* = 0.977, figure 1). Phage selection reduced the antibiotic-resistant phenotype in RN3-plasmid-carrying *E. coli* (*F*₁,₂₂ = 28.8, *p* < 0.001, figure 1c) and *S. enterica* species most efficiently (*F*₁,₂₂ = 5.2, *p* = 0.033, figure 1b). Note that phages were driven to extinction by the end of the experiment in all cases under the single phage addition treatment.

We confirmed the link between the loss of RP4 plasmid, antibiotic sensitivity and phage resistance from randomly isolated evolved antibiotic-susceptible cells (18 *E. coli* and 14 *S. enterica* cells); all were free of plasmids (as determined by the absence of three plasmid-specific genes, see §2) and were resistant to phages. The evolved bacterial isolates that had retained the resistance were shown to contain RP4 plasmid-specific genes. The genomic sequence is not available for RN3-plasmid and thus the presence of RN3-plasmid in evolved bacterial cells was not investigated beyond the antibiotic-resistant phenotype in this study. Furthermore, phage selection did not significantly alter the mean bacterial population densities, which could otherwise have affected the system dynamics (phage addition frequency, *F*₂,₃₅ = 2.12, *p* = 0.135; phage addition frequency × bacterial strain, *F*₂,₃₅ = 0.13, *p* = 0.88 and phage addition frequency × plasmid type, *F*₁,₃₅ = 0.4, *p* = 0.66; electronic supplementary material, figures S4–S6, respectively).

We next investigated more closely the small frequency of bacteria (approx. 5%) that retained antibiotic resistance and found that all had evolved phage resistance. Given the relative ease with which phage resistance could evolve in cells that retained the plasmid, we investigated whether fitness costs associated with such double-resistant mutants had limited their spread. First, the double-resistant bacteria reached lower maximal densities than phage-resistant but antibiotic-susceptible bacteria (*F*₂,₆₃ = 213.8, *p* < 0.001; Bonferroni adjusted pair wise comparison, *p* = 0.009, figure 2), presumably as a result of plasmid carriage per se, given the low maximal densities of the plasmid-carrying ancestral strain. Second, previous work suggests that some PRD1-resistant mutant plasmids are incapable of conjugating [14]. We therefore isolated 12 double-resistant mutants from four independent replicates and measured their conjugation ability. All our isolates were conjugation-defective, suggesting that phage resistance was associated with the disturbance of the functionality of the bacterial sex apparatus.

4. DISCUSSION

Our results demonstrate that plasmid-targeting bacteriophage PRD1 can dramatically reduce the frequency of antibiotic resistance in bacterial populations. In the case of bacterial cells initially harbouring plasmid RP4, the phage was shown to favour bacteria that had discarded their plasmids. The rate of frequency reduction of the antibiotic-resistant phenotype did not depend on phage addition frequency for the plasmids RP4 and RN3, suggesting that the phage-resistant phenotypes were selected very early in the experiment. Indeed, phage-resistant phenotypes appear to have dominated cultures by 24 h, based on growth dynamics in both the presence and absence of phages (electronic supplementary material, figures S1–S3). This is consistent with the observation that phage went extinct by the

---

Table 1. Bacterial strains, plasmids and bacteriophages used in the study.

<table>
<thead>
<tr>
<th>strain</th>
<th>bacteria</th>
<th>phenotype/genotype</th>
<th>reference number</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMS174</td>
<td><em>E. coli</em> K-12</td>
<td>F’ <em>recA</em> <em>hisD</em></td>
<td>[9]</td>
</tr>
<tr>
<td>JE2571</td>
<td><em>E. coli</em> K-12</td>
<td><em>leu</em> <em>thr</em> <em>thi</em> <em>lacY</em> <em>thy</em> <em>pil</em></td>
<td>[10]</td>
</tr>
<tr>
<td>DS88</td>
<td><em>S. enterica</em> serovar Typhimurium</td>
<td><em>SL5676</em>(pLM2)</td>
<td>[11]</td>
</tr>
<tr>
<td>plasmids</td>
<td>drug-resistance marker used in the study</td>
<td>incompatibility group</td>
<td></td>
</tr>
<tr>
<td>RP4 (pLM2)</td>
<td>kanamycin</td>
<td>IncP</td>
<td>[7,12]</td>
</tr>
<tr>
<td>RN3</td>
<td>tetracycline</td>
<td>IncN</td>
<td>[8]</td>
</tr>
<tr>
<td>bacteriophage</td>
<td>characteristics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRD1</td>
<td>conjugative plasmid-dependent virus (IncP, IncN and IncW)</td>
<td></td>
<td>[5]</td>
</tr>
</tbody>
</table>
end of the experiment if phages were not continuously introduced to the cultures. Double-resistant mutants (that retained the RP4 plasmid and the antibiotic resistance) did evolve, but as expected, they showed growth costs relative to RP4 plasmid-free bacteria and were unable to conjugate, explaining their relatively low frequency at the end of the experiment.

There are, however, a number of important caveats to these promising preliminary results. First, selection was carried out in the absence of antibiotics: simultaneous antibiotic and phage selection would inevitably select for double-resistant mutants. Second, it is unclear whether conjugation ability would have been regained and the cost of resistance compensated had the populations evolved for longer. Third, given the variety of different types of plasmids, it is possible that some plasmids can avoid phage infection by simply repressing the genes encoding phage-receptors. Fourth, heterogeneity in the bacterial population (or community) with respect to expression of conjugative machinery has been shown theoretically to limit the impact of phages on plasmid loss [15] and, empirically, potentially to enhance plasmid transmission by the presence of ‘super-conjugators’ [16]. Fifth, as with all test-tube studies, the relevance to natural environments is unclear [17].

Previous studies have reported other methods that can reduce the frequency of antibiotic-resistant plasmids. First, bacteria have been cured to some extent of their (non-conjugative) plasmids with simple molecular compounds that mimic plasmid maintenance molecules [18]. Second, infection with non-lysing filamentous phages can result in antibiotic sensitivity of Pseudomonas aeruginosa carrying a plasmid that normally confers gentamicin resistance, although the mechanism is unclear [19]. The phage approach suggested in this study is not mutually exclusive with the molecular compounds and possibly not with the filamentous phages, and thus the methods could potentially be combined in future applications.

Many conjugative plasmids can move between species [20], and hence conjugative plasmid-dependent phages are likely to play an important role in the evolution and ecology of many bacterial species within microbial communities. For example, horizontal gene transfer between species is now believed to play a crucial role in the evolution of bacteria [4], and phages could limit this. Moreover, coevolutionary interactions between plasmids, phages and a particular species of bacteria could be played out simultaneously across the community. Future work will explore the consequences of specific plasmid–phage interactions for the microbial community as a whole.

We thank S. Brown for constructive comments on the manuscript. The study was supported by the Finnish Centre of Excellence Programme of the Academy of Finland (2006–2011) grant 1213467 (J.K.H.B) and Marie Curie Intra European Fellowship within the 7th European Community Framework Programme (V.F.). A.B. acknowledges support from the European Research Council.

Figure 1. Changes in the frequency of antibiotic-resistant cells. (a) Escherichia coli carrying the RP4 plasmid, (b) S. enterica carrying the RP4 plasmid, and (c) E. coli carrying the RN3 plasmid. Black bars denote absence of PRD1. Grey and white bars denote experiments where PRD1 bacteriophage was added only once or daily to bacterial cultures, respectively. Bars show ± 1 s.e.m.

Figure 2. Changes in the maximal bacterial densities of E. coli harbouring plasmid RP4. The black bar denotes ancestral genotypes, light grey bar the phage-resistant but antibiotic-susceptible genotypes, and the dark grey bar both antibiotic- and phage-resistant genotypes. Bacterial growth was measured in the absence of PRD1 phage. Bars show ± 1 s.e.m.


