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Title : Resistance is a numbers game

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Standfirst

Plasmids are well known for spreading antibiotic resistance genes between bacterial strains. Recent experiments show that they can also act as catalysts for evolutionary innovation, promoting rapid evolution of novel antibiotic resistance.

Main text

Plasmids — non-essential loops of DNA — are well known for their ability to fuel bacterial adaptation by moving genes between bacterial lineages, a process known as horizontal gene transfer (HGT). This is particularly important in the case of antibiotic resistance, where acquisition of plasmid-borne resistance genes can instantly render a strain impervious to antibiotic treatment. However, in a recent article in *Nature Ecology and Evolution*, San Millan and colleagues show that plasmids — specifically small, multi-copy plasmids — can facilitate bacterial adaptation through an alternative and complementary mechanism[1]. Many plasmids, particularly those that are small (< 10 kb), do not carry sophisticated partitioning systems, instead ensuring transmission to daughter cells by existing in multiple copies within a cell. A consequence of this strategy is that any resistance genes they carry will also be multi-copy, leading to both amplification of the encoded phenotype through greater gene expression, and plasticity through random fluctuations in plasmid, and therefore gene, copy number. Most intriguingly however, is the prediction that multi-copy plasmids act as catalysts for evolutionary innovation, due to the combined effect of gene duplication and freedom from linkage between alleles, accelerating the appearance, and exaggerating the impact, of novel mutations.

To test this prediction, San Millan *et al*. established experimentally evolving *Escherichia coli* populations to contrast the adaptive potential of genes carried on plasmids compared with the same gene on the chromosome. The authors placed the ampicillin resistance gene bla_{TEM-1} on either the chromosome or on a multi-copy (~19 replicons/cell) plasmid and challenged bacterial populations to survive in the presence of increasing concentrations of ceftazidime, a beta-lactam antibiotic to which bla_{TEM-1} provides only minimal resistance. While populations with chromosomal bla_{TEM-1} were able to evolve some resistance to the antibiotic, all forty-eight independently-evolving populations went extinct when concentrations reached between 5 and 16 x their ancestral MIC, well below clinically relevant concentrations. On the other hand, ~15% of plasmid-containing populations were able to evolve extreme levels of ceftazidime resistance, surviving in concentrations >4000 x their ancestral MIC; a concentration that easily surpasses those used in clinical applications. DNA sequencing revealed that bla_{TEM-1} evolution, associated with high levels of resistance, only occurred when the resistance gene was present on the plasmid, indicating a role for plasmids in enhancing bla_{TEM-1} evolution (common mutations occurred in both the chromosomal bla_{TEM-1} and plasmid-borne bla_{TEM-1} treatments, but these occurred outside of the bla_{TEM-1} gene).

What factors drive this adaptive potential of multi-copy plasmids? Multi-copy plasmids increase the evolvability of the genes they carry in several important ways (fig 1). Firstly, by increasing mutation supply: having multiple copies of bla_{TEM-1} increases the probability of beneficial mutations occurring in the first place. This has been previously explored in the 'amplification-mutagenesis hypothesis' where increasing plasmid copy number can increase the probability of mutations in plasmid-borne genes, making mutation rates at those loci appear elevated[2]. The authors found that mutations in the bla_{TEM-1} gene were detectable in these populations at very early time points, consistent with this hypothesis. Secondly, multi-copy plasmids rapidly generate variation in allele dosage. Random partitioning of plasmids at cell division leads to variation in the number of mutant alleles between daughter cells, allowing selection to act efficiently. Thus a single beneficial mutation occurring on one plasmid can quickly become fixed across all plasmid copies within the cell, enhancing the beneficial effect of the novel mutation. It is worth noting that this would not be the case were gene copies to be connected by linkage, for instance carried in duplicate on the chromosome (figure 1), and therefore represents a benefit unique to multi-copy extrachromosomal elements such as plasmids. This is important: reconstruction of the evolved bla_{TEM-1} mutations in the ancestral background doubled ceftazidime resistance, but when present on the ~19 copies of the plasmid, the mutation increased resistance 128-fold. Finally, maximum levels of ceftazidime resistance were reached through additional mutations which increased plasmid copy number. However, copy number increase was not an effective resistance strategy by itself: despite wildtype bla_{TEM-1} gene conferring weak resistance to ceftazidime, higher copy number lead to only small gains in resistance, which were accompanied by a substantial fitness cost associated with the increased plasmid burden. Therefore the authors convincingly demonstrate that the evolution of ceftazidime resistance observed in their study was due primarily to the innovative potential of multi-copy plasmids, rather than to copy number alone.

The carriage of accessory genes by plasmids is an evolutionary puzzle[3]. Plasmids tend to levy a considerable cost on their bacterial hosts and are often unstable, driving loss from populations. Even when beneficial, plasmid accessory genes can be 'captured' by the host chromosome, making the

plasmid dispensable. Experimental studies of the evolution of plasmids and bacteria are beginning to tease apart the mechanisms by which plasmids might persist through, for example, compensatory evolution or high conjugation rate[4-6]. But as San Millan and colleagues have shown, extrachromosomal elements also have unique features that predispose them to act as engines of evolutionary change. Providing a flexible and efficient platform for hosting multiple copies of a gene could expand the functional range of a plasmid, and consequently the conditions under which plasmid carriage is favoured. This opens up the question of what conditions favour the maintenance of this 'evolvability'. Along with other drivers of rapid phenotypic change, such as contingency loci and increased mutation rate, it is possible that multicopy plasmids form part of a toolkit employed by bacteria to deal with rapidly changing environmental challenges[7]. Importantly, these results may also explain the rapid evolution and dissemination of antibiotic resistance in bacterial populations, as ColE1 plasmids of the type used in this study are widespread and often carry relevant phenotypic traits such as antibiotic resistance[8], and $bla_{\text{TEM-1}}$ is highly adaptable with >170 known variants and many different antibiotic resistance phenotypes[9]. Plasmids can appear deceptively simple, consisting sometimes of just a few kilobases and carrying only a handful of genes. Yet, as this study shows, the evolutionary opportunities they provide can be formidable.

(900-1200 words)

Figure legend

Figure 1. Comparing the adaptive potential of a resistance gene carried on the chromosome, a multicopy plasmid and, hypothetically, in multiple copies on the chromosome. (1) The impact of acquiring a gene conferring weak antibiotic resistance is amplified when multiple copies of a gene are present. (2) Mutations (z) in the gene can increase the level of resistance — the probability of such beneficial mutations occurring on one copy increases with copy number. In the case of blaTEM1 these mutations have relatively small effects in single copies of the gene therefore we can assume that, even where the bacteria carries multiple copies of the original gene, carrying a single mutated gene leads to similarly small increases in resistance. (3) Random partitioning of plasmids into daughter cells leads to the rapid accumulation of variability in the frequency of the mutant allele, allowing selection on genotypes with more copies of the mutant allele and therefore greater resistance. In the hypothetical case, where duplicate genes are carried on the chromosome, linkage between gene copies means that daughter cells receive the same quota of mutant alleles, and variation could only be generated

by slower multi-step processes such as gene conversion or transposition. (4) Finally the authors found that increased resistance could be fine-tuned by mutations which increased plasmid copy number, amplifying the number of resistance genes yet further. (350 words max)

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