Evolutionary Individuality of Bacterial Plasmids

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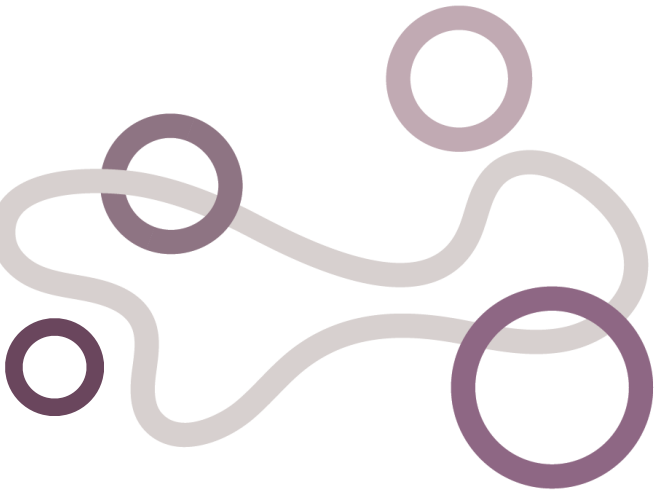

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Abstract

All living forms are organized in a nested hierarchy. Genes and other replicating nucleic acids formed genomes; Eukaryotic cells were formed by the union of free-living eubacteria and proto-eukaryotes. In all cases, previously autonomous entities came together to form new wholes higher in the biological ladder. This dynamic process is a transition in individuality. The objective of this report is to assess the evolutionary individuality of bacterial plasmids, autonomous replicating DNA entities enclosed within bacterial cells. Although plasmids are commonly understood as parts of the bacterial genome, there is a large body of research that considers plasmids are biological individuals in their own right. Here, I will first present key aspects of plasmid biology, exposing the diversity of molecules that the term 'plasmid' encompasses. Then, I'll explain my working concept of evolutionary individuality. After that, I will review and evaluate the distinct views on plasmid individuality and argue that rather than being antagonistic, they are complementary. Finally, I will defend the claim that the current concept of plasmid involves both evolutionary individuals and genetic components of bacterial cells.

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1 The biology of bacterial plasmids

Plasmids are self-replicating DNA entities whose replication is coordinated with the host life cycle but independent from it (Kado, 2014). The term plasmid was coined in 1952 by Joshua Lederberg, to refer to *any extra-chromosomal hereditary determinant* (Lederberg, 1952). The same year, Esther and Joshua Lederberg and Luigi Cavalli discovered the so-called F (for fertility) factor. Of cytoplasmic origin, the F-factor was the first molecule introduced as a plasmid, and it was shown to be responsible for bacterial sexual differentiation via cell-to-cell contact in experiments in *Escherichia coli* (Lederberg et al., 1952). Over the past 70 years, several other plasmids, with a myriad of different functions, have been discovered, characterized and classified.

Because the term plasmid was conceived as an umbrella term, it is not surprising that plasmids resemble other cytoplasmic molecules. In particular, some plasmids resemble bacterial chromosomes, while others resemble bacteriophages[1].

[1] Bacteriophages are a type of virus that infects bacteria.

Figure 1 Intuitions on individuality of molecules similar to plasmids. From left (less individuality) to right (more individuality): a chromosome; a multicopy plasmid, each copy illustrated in a different color and shape; a bacteriophage; a bacterial cell. The chromosome is usually perceived as a molecule with no individuality, being part of a bacterial cell. Bacteriophages and bacteria cells are commonly considered individuals of some sort. [*Original figure.*]

Most plasmids carry genes necessary for their replication and maintenance. In addition, plasmids may carry genes encoding mechanisms for bacterial local adaptation, enabling their bacterial hosts to colonize and compete in natural environments (Levin, 1993; Rankin et al., 2011).

Plasmids are distinguished from chromosomes mainly by the fact that chromosomes carry genes essential for bacterial survival, whereas plasmids do not[2]. However, a large proportion of bacterial genomes harbour a large essential extrachromosomal DNA molecule, known as the bacterial chromid (Harrison et al., 2010). Chromids posses plasmid-type maintenance and replication systems. Because of this, some authors have suggested chromids have a plasmid origin (but see Case Study 1: Chromids) (Harrison et al., 2010). In addition, chromids have a nucleotide composition close to that of the chromosome and encode for essential genes found on the chromosome in other bacterial species. Some authors claim that chromids are on the way to become secondary chromosomes (Fournes et al., 2018; Harrison et al., 2010).

[2]By essential genes, here I refer to genes associated with basic cellular functions such as transcription and replication. Although in an environment supplied with antibiotics a gene encoding for antibiotic resistance would be, by definition, essential, environment-specific essential genes should not be considered essential in the broad sense.

The resemblance between plasmids and chromosomes is relevant to my analysis because chromosomes are seldom, if ever, considered individuals different from the cells they reside in. Chromosomes are so fundamental to the existence of a cell that to picture a living cell without its chromosome is almost inconceivable. On the other hand, among the molecules that resemble plasmids, perhaps the most often perceived as individuals are bacteriophages (Fig. 1).

Phage-plasmids are a particular type of entities somewhere between a plasmid and a bacteriophage (Ravin et al., 1999). Phage-plasmids are bacteriophages which, in addition to stably integrate themselves into the host chromosome (a lifestyle that defines temperate bacteriophages), are found in the host genome as extrachromosomal elements which replicate in line with the cell cycle. In other words, phage-plasmids behave both like temperate bacteriophages and like plasmids. It is worth noting, however, that genomic analysis of phage-plasmids suggest they are not hybrid molecules created from recombination between phages and plasmid, but thought to have arisen independently (Pfeifer et al., 2021).

The great variability of plasmids is not a trivial problem in the genomic nomenclature. However, a large body of plasmid biologists regard the issue as merely semantic and argue that it is not important how DNA molecules are classified if we know how they work (Wegrzyn, 2005). A famous anecdote among plasmid biologists illustrates this point:

> *"A group of the meeting participants wanted to order coffee, and saw 'Greek coffee' in the menu. We had no idea what kind of coffee was that and had to ask a waiter: what is Greek coffee? This was a very strange question to him—I assume he was sure that everybody must know what this kind of coffee is. So, we asked: could you compare Greek coffee with, for example, cappuccino? His answer was: 'Greek coffee is Greek coffee, and cappuccino is different.' It appears that he had an excellent feeling about different kinds of coffee. "* (Wegrzyn, 2005)

For Wegrzyn, most of the people working in the field have a good intuition about what a plasmid is, compared for instance to a chromosome. Hence, plasmid biologists should

stop worrying about how plasmid molecules are classified. Other plasmid biologists claim that the names of these molecules are important, as they often tell us something about the underlying biology (Brockhurst, 2021).

Currently, plasmid classification is based primarily on incompatibility groups. Plasmid incompatibility is the inability of two or more plasmids to be stably[3] maintained in the same host line in the absence of selection (Novick, 1987). In other words, if the introduction of a second plasmid strain destabilizes the vertical transmission of the first, they are said to be incompatible (Novick, 1987). At present, there are 27 known incompatibility groups of plasmids (Shintani et al., 2015). Other plasmid classifications are based on traits such as mobility, capacity to integrate into the chromosome, copy number, function and size.

2 Evolutionary individuality

The problem of biological individuality concerns the distinction of individuals from parts and from groups[4] (Clarke, 2010). Although there are different types of biological individuals, such as metabolic (Dupré and O'Malley, 2009) or immunological individuals (Pradeu, 2010), here I am only interested in evolutionary individuals. Evolutionary individuality understands biological individuals on the basis of natural selection: evolutionary individuals are those objects which participate in the process of evolution by natural selection.

Perhaps the most intuitive biological individuals are higher metazoans. Most people, if not everyone, would probably claim that an octopus or a bee are individuals of some sort. Likely, anyone could easily distinguish one individual octopus from another; perhaps based on physical discontinuity and some integration among the parts that make a functional whole. Octopuses, we might observe, have different behaviors and morphologies;

[3] Throughout this report, I use the word stability to refer to plasmid segregational stability. When daughter cells get at least one copy of a plasmid at cell division (*i.e.* at segregation), the plasmid is said to be segregationally stable (Friehs, 2004).

[4] There are other dimensions to the problem of biological individuality which I will not cover in this report (e.g. identity over time, spatial parts, etc).

octopuses bear adaptations which allow them to compete with each other; they reproduce, form distinct lineages.

Our intuitions about individuality start to fail as we question the individuality of biological entities increasingly dissimilar to ourselves. Is a colony of bees an individual? A troop of baboons? Are genes? *Are plasmids evolutionary individuals?*

To define evolutionary individuals, here I will follow Richard Lewontin's principles of evolution by natural selection (Lewontin, 1970):

> 1. Different individuals in a population have different morphologies, physiologies, and behaviors (***phenotypic variation***).
> 2. Different phenotypes have different rates of survival and reproduction in different environments (***differential fitness***).
> 3. There is a correlation between the parents and offspring in the contribution of each to future generations (***fitness is inheritable***).

The generality of Lewontin's principles implies that any objects – at any level of biological organization – exhibiting differential fitness can be said to be participating in a natural selection process. If such differences in fitness are heritable, then the populations composed of these entities may evolve in response (Damuth and Heisler, 1988; Lewontin, 1970). The challenge is to *"delineate the biological units to which fitness can be properly attributed and which participate in the evolutionary process"* (Clarke, 2013).

3 Evolutionary individuality of bacterial plasmids

Plasmid evolutionary individuality has been approached from two different views throughout the history of plasmid biology: the plasmids as parts view (PAP) and the plasmids as individuals view (PAI). In short, under the PAP plasmids are considered traits of bacterial cells or higher levels of biological organization. In contrast, the PAI considers plasmids are evolutionary individuals with their own fitness interests. To my knowledge, plasmids have not been considered as groups of genes or other genomic elements such as transposons. Although some authors conceive plasmids as mere vehicles of these elements

(Helinski, 1978; Wein et al., 2019).

In the following paragraphs, I will expose in more detail the differences between the PAP and the PAI. Then, I will defend the claim that the current concept of 'plasmid' involves both evolutionary individuals and genetic components of bacterial cells. That is, rather than being antagonistic, the PAP and the PAI are complementary to explain the evolutionary individuality of plasmids. I will illustrate my claim with three case studies, where I will evaluate the individuality of the plasmids involved.

3.1 Plasmids as parts

The PAP understands plasmids as characters, traits or resources of bacterial cells. The PAP can be traced back to the birth of the field of plasmid biology in the late 1940s. At the time, plasmids were first studied and characterized by molecular geneticists interested on bacterial cytoplasmic inheritance. Their discoveries were thus framed under the phenotypic effects that plasmids had on their bacterial hosts. The F-factor mentioned in Section 1, for instance, was proposed to be responsible for *bacterial* sexual differentiation (Lederberg et al., 1952). Likely by the force of habit, most, if not all, of the plasmids discovered since have been described according to the phenotypic consequences they have on their bacterial hosts. Plasmids without a known effect on the bacteria phenotype are considered to be non-functional or 'cryptic' (Novick, 1969).

Although the PAP is not usually explicitly stated, it permeates plasmid biology. The PAP considers plasmids, like chromosomes, are parts of the bacteria genotype, arguing that plasmids do not have phenotypes; *"rather, the plasmid genes are phenotypically expressed by the strain carrying the plasmid"* (Novick et al., 1976). The perception of plasmids as objects with no phenotype is reflected in the language biologists use. In microbiology, it is common to use *resistant bacteria* instead of *resistant plasmids* when talking about the bearers of resistance encoded on a gene physically located on a plasmid. Plasmids, like genes, *confer* resistance to bacteria, they do not *have* it themselves.

However, it is important to note that the problem of evolutionary individuality is not semantic, but empirical. The fact that plasmids are or are not being selected independently

of their bacterial hosts is not contingent on a particular definition of phenotype, but on whether natural selection is able to act on them. The question is then if the existence of a plasmid and its traits can be explained exclusively as a consequence of selection acting at the host level. Here, the PAP finds support on the observation that plasmids often carry genes encoding mechanisms for *bacterial* local adaptation (Rankin et al., 2011).

Most bacterial plasmids are mobile genetic elements. That is, they have the capacity to be transmitted horizontally between bacterial cells without the need of bacterial cell division. Horizontal transmission is often related to the view of plasmids as selfish parasitic genetic elements because it increases the potential for plasmid-host conflict. However, some authors under the PAP considered horizontal transmission to be an adaptation at the bacterial population level, or higher (Nadell et al., 2008). The idea is that plasmids and other mobile genetic elements persists because they accelerate the rate of evolution of bacterial populations (Davies and Davies, 2010; Werren, 2011).

Looking at the regulation of plasmid horizontal transmission, it can be observed that a large share is controlled by genes in the bacterial chromosome (Harrison and Brockhurst, 2012). If we were to take the PAP lenses, we could understand this observation as some sort of functional integration between plasmids and their bacterial hosts. That is, plasmid could be understood as a shareable genetic resource under the control of the bacterial host. Yet, we should be careful with this argument, since the observation could also be explained as a countermeasure undertaken by the host against the parasitic nature of plasmids. To my knowledge, there are no empirical studies tackling this question.

3.2 Plasmids as individuals

The PAI argues that plasmids are evolutionary individuals in their own right. That is, that plasmids have fitness interests different from those of their hosts. The first notions of the PAI can be traced back to the 1950s, where plasmids were described as *'non-lytic infectious agents'*, based on the similarities between plasmids and bacteriophages (Hayes, 1953; Lederberg et al., 1952). However, it was not until much later that the PAI became more prominent. In a seminal paper, William Eberhard adopted multilevel selection theory to argue that multiple levels of selection must be considered to understand plasmid

evolution (Eberhard, 1990). Eberhard regarded both plasmids and chromosomes as reproductive units carrying selfish genes. He then proposed the local adaptation hypothesis to explain why genes for certain functions, such as resistance, tend to consistently occur in plasmids rather than in chromosomes. Eberhard argued that, under some sort of sporadic (that is, fluctuating or rare) selection, for instance when bacterial strains colonize a site containing antibiotics, assuming plasmids have negative effects on host growth, most of those bacteria would not carry a plasmid with a resistance gene. That is, most of those bacterial could potentially receive a plasmid via horizontal transmission. Hence, whenever the plasmid rate of horizontal transmission would be greater than the rate of vertical transmission, the plasmid *genes* would propagate more rapidly than the chromosomal genes.

One of the most interesting features of Eberhard paper is that, by adopting a gene-eyes view of evolution, he understood plasmids neither as individuals nor as parts of bacterial cells: *"Thus, neither prokaryotic species, their chromosomes, nor their plasmids can constitute genetically discrete units or individuals in the sense in which these terms are used in most evolutionary discussions."* (Eberhard, 1990). However, I argue that by recognizing plasmids as reproductive units independent of the chromosome or their bacterial hosts, he recognized plasmids as individuals of some sort.

The capacity of plasmids for great horizontal transmission is one of the most cited features within the PAI. Horizontal transmission gives plasmids control over their transmission, independent of bacterial replication. In this way, plasmids can form unique lineages, which can be selected independently from the lineages formed by their hosts. Horizontal transmission has, in addition, facilitated the study of plasmid population dynamics in analogy to other infectious agents such as bacteriophages. In a large body of research, plasmids are modelled in an epidemiological fashion, where the hosts transit between plasmid-free (susceptible) and plasmid-bearing (infected) states. These studies have largely focused in understanding the persistence conditions of plasmids and the transmission of antibiotic resistance (Bergstrom et al., 2000; Stewart and Levin, 1977; Svara and Rankin, 2011).

Recently, Garoña and Dagan discussed the evolutionary individuality of plasmid defining

plasmid fitness in terms of their stability (Garoña and Dagan, 2021). Stability is determined by the plasmid ability to successfully segregate into daughter cells. Unlike most PAI accounts, Garoña and Dagan do not consider horizontal transmission is an important factor for plasmid individuality. Rather, it is their multiple-copy nature what gives them the qualification. The idea is that, within a bacterial cell, single plasmid molecules compete against mutant variants for increased stability. Under this framework, the DNA sequence of plasmid molecules is their phenotype (phenotypic variation). Different sequences have different rates of replication and effects on multimer resolution[5], leading to differences in their stability (differential fitness).

3.3 No single view can account for the diversity of plasmids

I mentioned before that evolutionary individuality is an empirical problem. If, when assessing the evolution of plasmids, each of the views presented beforehand yields a different prediction, which prediction is correct? I am inclined to believe it depends on the plasmid under scrutiny and its context.

To support my claim, I will use three different case studies. Each of them aligning better with a particular view of plasmid individuality. The first case study is chromids. The relevance of chromids relies on their strong similarity to chromosomes. Thus, I hope to show that the PAP fits their properties. The second case study is the multicopy plasmid pCON, in which the authors use an approach similar to that of Garoña and Dagan, 2021. Last, I will present the case of plasmid R388, which contains a series of genes associated with bacterial local adaptation, I will expose why the PAP is enough to explain its evolution. The key point I want to emphasize is that, even though we might be able to accommodate a particular plasmid either under the PAI or PAP perspectives, none of these views alone can contain the diversity of plasmids.

[5]Multimer resolution refers to the conversion of multimeric plasmids (*i.e.* plasmids made of multiple–formerly individual–molecules) to monomers (*i.e.* plasmids made of a single molecule),thereby increasing the number of plasmid molecules available for distribution at cell division (Austin et al., 1981).

3.4 Case studies

Before continuing, I need to reject the idea that plasmids do not have phenotypes. For if I accepted such idea, my analysis would be done. That is, I would conclude plasmids are not evolutionary individuals. Instead, I'll assume plasmids have phenotypes and evaluate Lewontin's second and third conditions for evolutionary individuality in each of the following case studies.

3.4.1 Case 1: Chromids

One cell-one chromosome is the quintessential model of bacterial cells. However, as mentioned in Section 1, a large portion of bacterial genomes carry an additional large DNA molecule often referred as a secondary chromosome: the bacterial chromid (Harrison et al., 2010). There are three core criteria that define a chromid:

> 1. Chromids have plasmid-type maintenance and replication,
> systems.
> 2. Chromids have a nucleotide composition close to the one
> of the chromosome.
> 3. Chromids carry core genes that are found on the chromosome
> in other species of bacteria.

There are two hypotheses on the origin of chromids. I) *The schisms hypothesis* states that chromids emerged from an ancestral bacterial chromosome split into a primary and a secondary chromosome. The secondary chromosome then fused with a plasmid and became and chromid. II) *The plasmid hypothesis* proposes that essential genes in the chromosome were transferred to a plasmid, transforming it into a now indispensable chromid. None of the hypotheses has been disproved (Fournes et al., 2018). For the purpose of my analysis, however, that is not important. What matters is that both hypotheses propose that chromids are some sort of mixture between a chromosome and plasmid and that, in any case, the consequences for the plasmids involved are the same: plasmids became essential molecules, replicating once and only once per cell life cycle. This eliminates competition between the plasmids' clones at the intracellular level. At the cellular level, it has also been observed that chromid horizontal transmission is greatly impaired due to their large

size. Therefore, chromid reproduction depends entirely on bacterial division. In consequence, chromids do not form lineages that can be selected independently of their hosts. Bacterial chromids and their hosts have thus the same evolutionary fate. Hence, chromids are *not* evolutionary individuals.

3.4.2 Case 2: pCON plasmids

In a study in 2020, Tanita Wein and colleagues investigated the effects of selection for a plasmid-encoded trait on plasmid stability (Wein et al., 2020). Their experimental design is illustrated in Figure 2. In short, they evolved a non-mobile unstable multicopy plasmid, pCON, in selective and non-selective conditions. pCON plasmids have no measurable effects on host growth. Their results showed that, when evolved in non-selective media, pCON stability increased compared to its ancestor. In contrast, when grown in selective media, pCON stability decreased. That is, positive selection for the plasmid-encoded trait promoted the maintenance of unstable plasmids in the population and, assuming fluctuating environments, consequently hinder long-term plasmid persistence.

The study of Wein *et al.* is remarkable because it was designed under a plasmid-centric view of plasmid evolution. Most studies on plasmid evolution understand plasmids in terms of the consequences they have on the fitness of their hosts. That is, plasmids are either parasitic, neutral or beneficial *with respect to the host*. In contrast, for Wein *et al.*, the fitness of the host is not relevant. The conflict emerges because selection for the plasmid-encoded trait diminishes or hinders selection for greater plasmid stability. This observation can only be made by including plasmid intracellular population dynamics into the picture.

I'd argue that the PAI fits pCON plasmids. For this case study, plasmid fitness is defined in terms of plasmid stability[6]. Plasmid stability of pCON plasmids is a product of direct competition among plasmid molecules at the intracellular level (*i.e.* among members of

[6]Here, I am not arguing that plasmid stability *generally* defines plasmid fitness. However, plasmid stability is successful vertical transmission. For non-mobile plasmids, vertical transmission is their only way of reproduction. In addition, pCON plasmids, have no measurable effect on host growth. Hence, stability is fitness in this case.

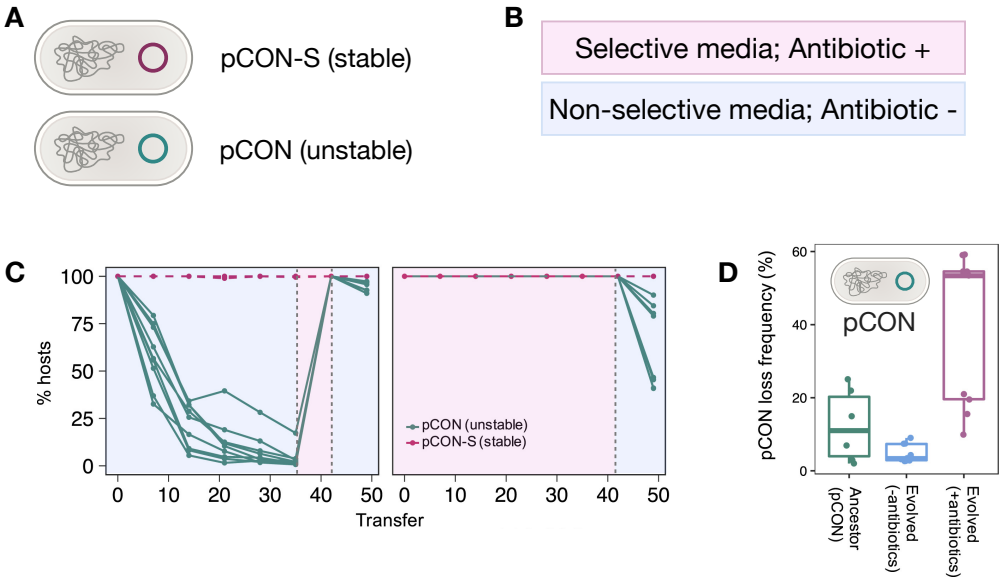

Figure 2 Plasmid stability evolution under selective and non-selective conditions. **A** | The stable pCON-S plasmid and its unstable form pCON. **B** | Culture conditions. **C** | Evolution experiment of plasmid-carrying hosts. Plasmid persistence is shown as the proportion of hosts during the evolution experiment. **D** | Plasmid loss frequency of ancestral pCON plasmids and plasmids that were evolved either with or without antibiotics. [*Modified from Wein et al., 2020*].

the clone), where the plasmid phenotype is its DNA structure. Wein *et al.* found that pCON stability decreased due to multimerization. Plasmid multimerization is the physical fusion between two or more plasmid molecules. Because it reduces the units of inheritable plasmids during cell division, plasmid multimerization often leads to increased plasmid loss.

3.4.3 Case 3: Plasmid R388

Bacteria and their plasmids often encounter stressful environments that impose strong selection pressures on them. Antibiotic resistance, for instance, emerges as a response to the selective pressures imposed by antibiotic molecules. When antibiotics are absent, however, the production of resistance proteins can decrease the fitness of the bacteria. One solution to alleviate this problem is to maintain resistance genes on an cassette. Cassettes are genes or sets of genes found as non-replicative extrachromosomal circular elements that can be expressed by their incorporation into a particular type of plasmid called inte-

gron.

The general structure of an integron is shown (Fig 3). In short, all integrons carry a gene encoding for an integrase. The integrase is an enzyme that can excise and integrate genes into its backbone. The expression of integrases is activated by environmental stressors such as antibiotics. Integrons posses a single promoter controlling the expression of all the genes it carries. The closer a gene is to the promoter, the higher its expression levels. In other words, more proteins are made from the genes closer to the promoter. In this way, the integrase can re-shuffle the order of the cassettes to accommodate its genes to maximize host fitness. It is thought that this mechanism helps bacteria to activate their resistance 'on demand'. That is, to rapidly activate their relevant resistance mechanisms under antibiotic treatment.

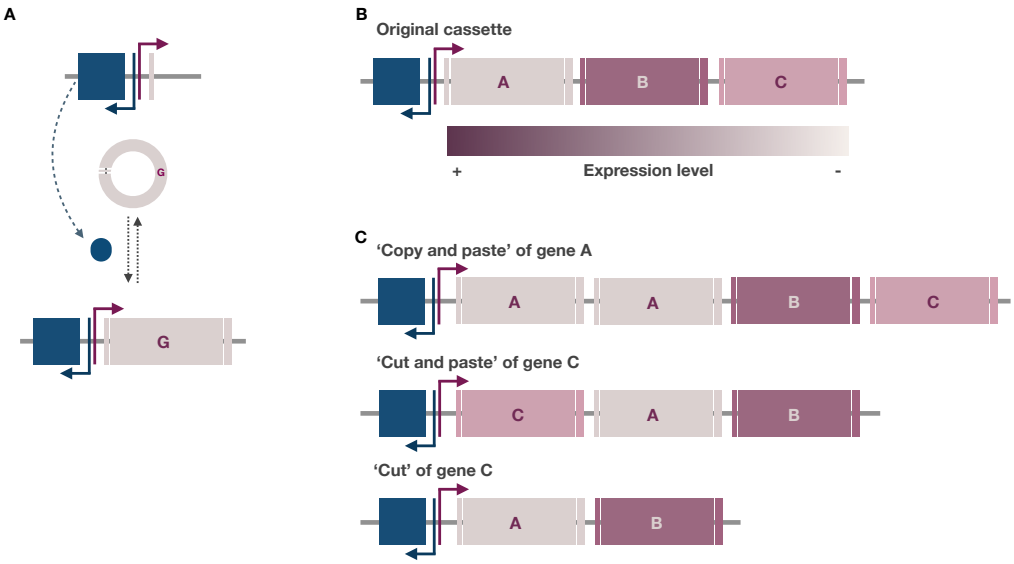

Figure 3 Integron activity. **A** | The general structure of an integron consists in (i) a gene encoding an integrase (blue square), an enzyme (blue circle) that can perform the excision and integration of cassettes (beige circle), and its promoter (blue arrow); (ii) sites of recombination for the integration of cassettes (beige line); (iii) a promoter driving the expression of the cassette (purple arrow). Cassette G can only be expressed once incorporated into the integron. **B** | The original gene cassette composed of genes A, B and C. The closer a gene is to the cassette promoter (purple allow), the higher its expression levels. **C** | Consequences of integrase activity. Genes can get 'copied and pasted', 'cut and pasted', or 'cut'. [*Part A modified from Escudero et al., 2015; parts B and C are original for this report.*]

To test this hypothesis, Souque and her colleagues engineered the bacteria *Pseudomonas aeruginosa* carrying a variant of the non-mobile plasmid R388 (Souque et al., 2021). The plasmid contains an integron and a resistant cassette composed of three genes, here called A, B, and C; each of them encoding resistance mechanisms to a different antibiotic (Fig 3B). Each day, they grow their bacteria on media with increasing concentration on antibiotic. As a media control, they grow their bacteria in (i) antibiotic-free media and in (ii) low concentration of antibiotic. A second variant of the plasmid carrying a non-functional integrase was submitted to the same treatment.

The bacteria surviving at the end of their experiment carried plasmids in which cassettes conferring the relevant resistance to the antibiotic were duplicated, whereas the less beneficial cassettes were eliminated. Thus proving that integrase can accelerate resistance evolution by *"rapidly generating combinatorial variation in cassette composition"* (Souque et al., 2021).

Although different variants arose within populations during the experiment, the authors concluded that these forms were transient since virtually all populations contained a single dominant variant by the end of the evolutionary experiment. Given that plasmids were beneficial for the whole duration of the experiment, plasmid stability is not expected to play a role in the results. Instead, plasmid dynamics can be explained by the fitness benefits they conferred to their hosts.

In short, because R388 plasmids are non-mobile and the genes they carry are being selected for at the cell level, they cannot form lineages which can be selected independently from their hosts. Therefore, R388 plasmids are not evolutionary individuals.

4 Discussion

Plasmids are remarkably diverse. Yet, the way biologists use the term often gives the impression plasmids are an homogeneous well-defined entity. The main thesis of my report is that the current concept of 'plasmid' includes both evolutionary individuals and genetic components of bacterial cells.

The diversity of plasmids, as that of all living entities, came to be through evolution. Natural selection can act on a variety of plasmid traits, from mobility and copy number, to gene content and replication mechanism. Some of these traits are particularly relevant to the question of evolutionary individuality. Importantly, evolutionary individuality is too a property that exists in response to selection.

I mentioned before that plasmids are or are not evolutionary individuals, depending on whether they participate in the selection process. While this is true, I do not wish to give the impression that evolutionary individuality is a discrete property. Quite the opposite, I hold the view that evolutionary individuality is a matter of degree. There are two reasons to support my position: (i) the properties that confer individuality (e.g. the rate of horizontal transmission) are continuous. Some times, selection will push the traits of plasmids towards individuality; others, it will enforce the integration of plasmids and their hosts. (ii) selection acts simultaneously on multiple levels of the biological hierarchy. This means that, the strength of selection acting at the plasmid level, compared to other levels, varies continuously too.

All in all, to the question of evolutionary individuality of bacterial plasmids, there is no single answer. Rather, plasmid individuality depends on traits of the plasmid in question, on its host and the environment. To understand plasmid individuality, it is important to understand the level at which the plasmids are being selected. The level of selection, that is, to which the existence of a plasmid as it is can be attributed.

Let us imagine for a moment that this answer was not satisfactory. That we wished to obtain a unique non-conditional answer to the question of plasmid evolutionary individuality. In such case, one possible solution could be to propose a more fine-grained distinction among plasmid molecules. Then, some of these molecules would be evolutionary individuals and others would not. I hold the view that, since most plasmids are used as molecular tools, without concern for their evolution, any distinction proposed must consider how it serves empirical research. A poor classification of plasmids could, at the very least, lead to the inconvenience of changing the name of a molecule during an experiment. At worst, it could create serious communication problems and inconsistencies.

Up to this point, I have largely ignored levels of selection higher than single bacterial cells. However, it is important to recognize these levels might play an important role in the evolution of plasmids. For example, it has been shown that the amount of mobile DNA in bacteria varies with ecology: the greater the community diversity, the higher the levels of mobile DNA (Newton and Bordenstein, 2011). This suggests that a property of the community (*i.e* diversity) drives the rates of horizontal transmission in mobile elements such as plasmids (Werren, 2011). Of course, it still remains to question whether the observed diversity is a product of selection at the community level.

I mentioned in Section 2 that there are other dimensions to the problem of biological individuality. In particular, I find the problem of identity over time to be of interest to the case of plasmids. The problem of identity over time asks what is the plasmid individual and what makes its identity through time. The question is difficult to answer because plasmids are in constant change: the fuse with each other, they acquire and discard genes. If a plasmid acquires a gene, is it the same plasmid? If two individual plasmid molecules fuse into one, are they still two individuals? Although the aim of my project is not to answer this questions, I recognize their analysis can greatly inform the question of plasmid evolutionary individuality.

I finally wish to address how my account of plasmid evolutionary individuality differs from other accounts. In particular, how it differs from the novel account presented by Garoña and Dagan, 2021. Garoña and Dagan propose what I call 'a plasmid-centric view of plasmid evolution'. Under their view, a plasmid clone is a population and each plasmid molecule is an evolutionary individual. Plasmid fitness is defined in terms of plasmid stability. The bacterial host, they propose, can be considered to be part of the environment. My understanding of plasmid evolution leads me to differ in their last two arguments. First, plasmid stability is only a *component* of plasmid fitness, and horizontal transmission is an important mode of reproduction that should not be ignored. In addition, the bacterial host should not be only considered part of the ecology of plasmids because plasmids and their hosts are in constant co-evolution and their rates of evolution are not distinct enough to separate their time scales (Harrison and Brockhurst, 2012). The work of Garoña and Dagan is relevant because it emphasises a component of plasmid fitness that is often ignored. Nevertheless, I claim that the consideration of selection at multiple

levels is fundamental to understand plasmid evolution–a view that was defended, from a rather different perspective, by Eberhard, 1990.

5 Conclusions

In this work, I evaluated the evolutionary individuality of bacterial plasmids. Focusing on plasmids and their hosts, I concluded that the current concept of 'plasmid' involves both evolutionary individuals and genetic components of bacterial cells. In other words, plasmids are not always themselves the bearers of the fitness they bring about. Some times, it is their hosts who benefit from the existence of plasmids; it is their hosts who engage in competition and form independent lineages of which plasmids are only a part of. Other times, the existence of a plasmid as it is is beneficial only to the plasmid itself. To understand the level at which plasmids are being selected, it is important to consider the traits of the plasmid under examination, to question their relation to the host cell and the role of the environement.

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