Stage-Specific Strategies to Limit the Spread of Antibiotic Resistance in Bacteria with a Focus on Conjugation

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Abstract. Horizontal gene transfer (HGT) is the main reason for antibiotic resistance evolution, enabling bacteria to obtain resistance determinants far more rapidly than through point mutations alone. Among HGT mechanisms, conjugation plays a particularly critical role for its efficiency, broad host range, and capacity in disseminating complex resistance plasmids across microbial communities. Despite its clinical significance, conjugation has remained an under-explored target for antimicrobial intervention. This research aims to provide an integrated analysis of bacterial conjugation within the broader context of HGT, emphasizing its evolutionary persistence and mechanistic vulnerabilities. Evidence is synthesized from molecular biology, experimental evolution, and population genetics to uphold the argument for the maintenance of plasmid-mediated resistance through a balance of transfer efficiency, compensatory adaptations, and *post-transfer* stabilization mechanisms. Building on this foundation, a stage-specific framework is proposed for disrupting conjugation by targeting *pre-transfer* cell-cell contact, *peri-transfer* DNA processing, and *post-transfer* plasmid maintenance. Multiple intervention strategies are reviewed and evaluated in this study, including conjugation inhibitors, relaxase-targeting compounds, CRISPR-based barriers, plasmid curing compounds, restriction-modification systems, as well as the plasmid addiction modules. Collectively, these approaches demonstrate that plasmid persistence is an evolvable trait that can be subjected to targeted interference. Overall, this study highlights conjugation-specific interfering strategies as a potential opportunity in slowing down resistance dissemination and preserving the efficacy of existing antibiotics.

Keywords: Bacterial conjugation, horizontal gene transfer, plasmid transmission inhibition, antibiotic resistance evolution, conjugation-targeted therapeutics.

1. Introduction

Antibiotic resistance originates from a natural process in which bacteria evolve to resist antibiotics, either by point mutations in the genes or by acquiring resistant genes from other bacteria [1]. Antibiotic resistance remains one of the most critical challenges in the medical field today, acting as a powerful evolutionary pressure on microbial populations [1]. When exposed to antibiotics, bacteria often experience an intense selection process, whereby only the variants carrying resistance genes survive and proliferate [1]. Their rapid replication, high mutation rates, and extensive gene flow all contribute to the phenomenon of adaptive responses in pathogenic bacteria emerging far faster than clinical drug development can keep pace with [1]. From an evolutionary perspective, as proposed by Richard Dawkins, genes, including the resistance determinants, behave as "replicators" that are seeking transmission; while bacterial plasmids, phages, and chromosomes serve as the "vehicles" that enable the persistence of the "replicators" [1-3]. Thus, when exposed to the pressure of antibiotics, any genetic element that is capable of enhancing its own ability to propagate will be strongly favoured, driving the rapid spread of resistance genes within its communities [1, 3].

HGT plays a central role in this mechanism [4, 5]. While genetic transmission is restricted to the parent-to-offspring pathway in vertical inheritance, HGT allows bacteria to acquire genetic material laterally from unrelated donor organisms [4, 5]. Conjugation, transduction, and transformation are the three principal mechanisms of HGT in bacteria [5, 6]. Conjugation is the direct transfer of plasmids via pili through direct contact [2, 5-7]. Transduction uses bacteriophages to inject genetic material into a bacterium, whereas transformation involves the uptake of free DNA in the environment by transformable bacterial cells [8, 9]. Together, these processes allow a bacterium to

bypass the slow accumulation of point mutations in its genes, and instead allow the import of entire resistance operons, virulence modules or metabolic cassettes in a single event, thus resulting in the rapid rise of multi-drug-resistant strains of bacteria [1, 5]. This capacity in mobilizing large, pre-assembled genetic systems make HGT much more impactful than evolutions resulting from point mutations in generating multi-drug-resistant lineages within clinically relevant timeframes [1].

Among the three forms of HGT, conjugation, being the most efficient and experimentally tractable process, naturally became the dominant driver of clinically significant antibiotic resistance dissemination [5, 6, 10]. Conjugative plasmids often possess a broad range of hosts, enabling them to cross phylogenetic boundaries and colonize new microbial niches [2, 4]. Many of these plasmids also carry accessory genes that reduce or mitigate their fitness costs, thereby increasing the evolutionary stability of the bacterial population [2, 3, 11]. Conjugation excels at spreading antibiotic resistance genes because bacteria can easily adapt to new environments, transfer genes efficiently via elements such as transposons (which are genes that jump move between bacterial plasmids and chromosomes) and other mobile genetic elements to move resistance genes between different microbes, making conjugation a prime method for the widespread expansion of antibiotic resistance [11, 12]. This suggests that conjugation would represent both a central evolutionary strategy for plasmids and the most practical target for interventions seeking to reduce antibiotic resistance from the root cause [3-6].

Despite decades of work on antibiotic stewardship and drug discovery, relatively little research has focused on interventions that directly target the evolutionary machinery that enables the rise of antibiotic-resistant bacteria, particularly the conjugative transfer of plasmids, which remains largely unaddressed in both clinical and environmental settings [4, 5]. Current studies overwhelmingly describe the spread of resistance genes, but few investigated how conjugation persists under selective pressures, how plasmids maintain a long-term evolutionary stability, or which stages of the transfer cycle are the most susceptible to disruption [3-6]. Thus, this paper aims to provide a consolidated assessment of conjugation within the broader context of HGT, outlining the evolutionary pressures that give rise to its persistence and to examine some strategies that target specific stages of the conjugation process [7, 9, 13]. By breaking down the conjugation process into the *pre-transfer*, *peri-transfer*, and *post-transfer* phases, this study should highlight multiple opportunities for scientists and physicians to interfere with plasmid transmission at multiple checkpoints [7, 9, 13, 14]. A sound understanding of these mechanisms is essential in designing effective interventions that are capable of slowing down or preventing the spread of antibiotic resistance within a microbial population to a certain degree, therefore preserving the effectiveness of existing antibiotics while protecting healthcare systems from the significant consequences of antibiotic resistance [3-6].

2. A Comparative Overview of HGT Mechanisms

In addition to the gradual accumulation of point mutations, HGT provides the bacteria with a sudden leap in their adaptation to new environmental stresses such as exposure to antibiotics. Unlike vertical inheritance, whereby progenies inherit genetic material directly from their parents, HGT allows bacteria to exchange genetic material laterally among a population, sometimes even across species or generations, accelerating evolutions and facilitating the rapid emergence of multi-resistant phenotypes [5, 8]. There are three principal mechanisms by which HGT occurs (Fig. 1), namely conjugation, transduction and transformation. Each one of them has its own biological process, ecological preferences, and epidemiological consequences.

Conjugation is believed to be the most efficient and experimentally tractable form of HGT with the current level of technology (though not in Streptococcus pneumoniae, with transformation being the most efficient; or in some bacteriophages in high population density, where transduction is the more dominant driver). Donor cells containing conjugative plasmids assemble a pilus, which acts as a mating bridge, and attaches to a recipient cell, enabling the direct transfer of plasmid. The plasmid typically encodes all necessary machineries (*tra* genes, origin of transfer, mobilization functions),

allowing autonomous propagation without relying on external vectors or sequence homology [8]. Because plasmids replicate independently and carry ready-to-use genes (e.g., antibiotic resistance genes), recipient cells acquire those traits instantly. This allows survival without the need for integration, making plasmids key drivers of rapid bacterial adaptation and the spread of the resistance genes [11]. Hence, conjugation enables both gene acquisition and functional take-over as a recipient cell becomes a donor cell, propagating the resistance gene even further. Its high efficiency, capacity to transfer in a broad range of hosts, as well as its success in both laboratory and natural conditions, make conjugation a dominant method of resistance dissemination [5].

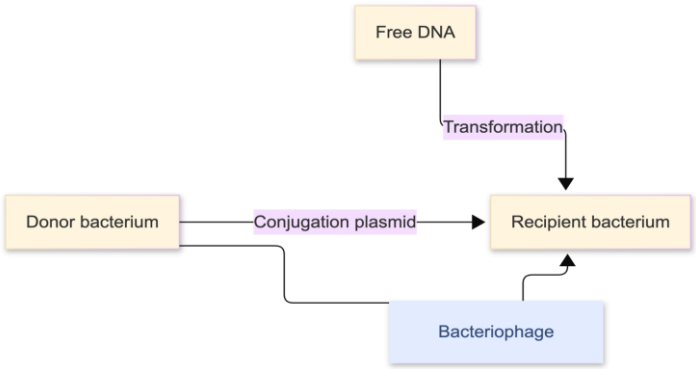

Fig. 1 Major mechanisms of HGT contributing to the dissemination of antibiotic resistance genes [5, 6]. This figure provides a simplified overview of the 3 principal mechanisms of HGT in bacteria: conjugation, transduction, and transformation. Conjugation involves the direct cell-cell transfer of plasmids via a conjugative pilus; transduction is mediated by a bacteriophage that packages and transfers bacterial DNA during infection cycles; transformation relies on the uptake of free DNA by competent cells.

Transduction, in contrast, relies on bacteriophages. During a phage's lytic cycle, fragments of bacterial DNA which may potentially include resistance genes, can be packaged into new phage particles [8]. When these phages infect other bacteria, the DNA can be injected and sometimes integrated, thereby transferring the genes without direct cell contact such as that observed in conjugation [5, 8]. Although transduction is equally clinically important as the other two forms of HGT in the understanding of antibiotic resistance, its utility, however, is constrained by the multi-faceted requirements such as phage-host compatibility, DNA packaging limits, and its dependency on environmental conditions favouring phage activity [5, 8]. Hence, transduction tends to be sporadic and unpredictable than conjugation, thus making it a weaker but still notable contributor to resistance dissemination by HGT.

Transformation, the third method of HGT in bacteria, is the acquisition of free DNA from the environment. Free DNA are the DNA released into the environment from other bacteria that have died and lysed. The DNA then gets picked up by naturally competent bacteria. Competent bacteria express surface machinery that binds, internalizes, and integrates free DNA they picked up, often via homologous recombination [5, 9]. While transformation allows the acquisition of genes from lysed donors and potentially from a larger environmental reservoir, its efficiency is limited by several factors, including but not limited to the availability of extracellular DNA, the need for competence induction, degradation of nucleases, and the need for sequence homology to ensure stable integration [8, 9]. Thus, although transformation largely contributes to genetic diversity and the occasional uptake of resistance genes, its role in the rapid and widespread dissemination of resistance genes, particularly of those carried by plasmids, remains comparatively limited when compared with conjugation.

When viewed side by side, these mechanisms often reveal a hierarchy of relevance for the spread of antibiotic resistance within a population. Conjugation stands out among the three mechanisms for its capacity for cross-species transfer, high efficiency of plasmid transfer, self-replication of plasmids and its epidemiological relevance. Transduction and transformation, while biologically significant, face ecological or mechanistic constraints that make them less efficient for large-scale dissemination

in clinical or environmental contexts. This comparison justifies the primary focus on conjugation when developing strategies contributing to counteract HGT-mediated resistance.

3. Conjugation as an Evolutionary Strategy

To understand why conjugation reliably propagates resistance across bacterial populations even without selective pressure from antibiotics, one should view plasmids as “selfish genetic elements”, a term used by evolutionary biologists such as Richard Dawkins himself. In this framework, the “selfishness” of genes refers to their tendency to maximize their own replication and survival rates and that is viewing the organisms as mere "survival machines" built by genes to propagate them. Thus, from a selfish-gene perspective, conjugation is beneficial for resistance genes dissemination because it lets them jump between bacteria, spreading rapidly beyond simple inheritance, directly accelerating their own replication chances by giving bacteria resistance, making those bacteria (and the genes within) more likely to survive antibiotics and copy themselves into new "survival machines", ensuring the gene's “selfish” propagation across many hosts, not just one lineage [3, 4, 8].

However, carriage of plasmid in bacterial cells often comes with costs. Hosts would often suffer fitness penalties such as slowed growth, increased metabolic burdens as well as regulatory disruption in exchange for the plasmid’s presence. Under purely vertical inheritance, such costs should select against plasmid maintenance when selective pressure from antibiotics is absent [2, 11]. Early studies have found that plasmid-bearing bacteria were outcompeted by plasmid-free ones in an antibiotic-free environment, suggesting a barrier to long-term plasmid stability [1]. Yet, plasmids persist widely in natural populations, largely due to compensatory evolution [2]. Experimental evolution studies demonstrated that after relatively few generations (i.e., fewer than 600), host bacteria would often evolve chromosomal mutations that mitigate plasmid-imposed fitness costs. These mutations often target host regulatory networks or helicase-plasmid interactions, transforming plasmid carriage from costly to neutral or even beneficial [2, 15]. Such compensatory mutations can mitigate costs, though the specific effect will depend on host-plasmid compatibility.

Furthermore, recent empirical work on clinically relevant resistance plasmids, such as those carrying *mcr-1*, has shown that with an increased conjugation rate alone via a single mutation enhancing transfer genes can rescue previously unstable plasmids from extinction in a bacterial population [16]. This reveals another axis of plasmid strategy: not only minimizing the cost but also maximizing transmissibility. A plasmid that spreads rapidly before being lost gains an evolutionary advantage that is independent of host fitness.

Moreover, ecological and community-level dynamics further reinforce the effectiveness of conjugation. In heterogeneous microbial communities, such as gut microbiota, wastewater and soil, plasmid persistence arises from community structures, species diversity and the varied ecological interactions even when antibiotic selection is absent. In such contexts, high conjugation rates and ecological noise allow plasmids to hide, spread and re-emerge, thereby making them more persistent to threats such as antibiotics.

Collectively, these evidence present conjugation as a deeply evolved strategy, by which “selfish” genetic elements ensure survival and propagation. For researchers aiming to intercept antibiotic resistance dissemination, this implies that interventions must take evolutionary logic into consideration. Conjugation, with its efficient transfer, compensatory adaptation, and a community-level persistence, ought to be the logical target for intervention.

4. Stage-specific Interference Strategies Targeting Conjugation

Since conjugation is a multi-step process involving donor recognition, DNA processing, and plasmid establishment, interfering with any of these processes can significantly reduce the level of resistance dissemination in the bacterial population. Accordingly, interference strategies can be categorized into *pre-transfer*, *peri-transfer*, and *post-transfer* interventions.

4.1. Pre-transfer Interference

Conjugative pili formation represents the earliest irreversible commitment to horizontal plasmid transfer and therefore constitutes a critical upstream control point in the dissemination of resistance genes [6, 7]. Unlike downstream strategies that focus on events after plasmid entry, interference at this pre-entry stage is able to prevent both DNA transfer and the establishment of donor-recipient networks that amplify resistance at the population level [12]. Experimental and theoretical work has shown that, prior to transfer, conjugation is limited by its dependence on costly and socially regulated structures, such as pili, whose expression is favoured only under specific ecological and population density conditions [9, 12]. This upstream position is particularly evolutionarily advantageous, as it imposes a relative weak selective pressure on resistance determinants themselves, thereby reducing the probability of counter-adaptation compared to interventions targeting at plasmid replication or expression directly [7, 11]. Hence, *pre-transfer* approaches should be paying more attention in monitoring and controlling the ecological conditions required for antibiotic resistance genes to circulate [6].

4.1.1. Inhibition of conjugation by targeting at pilus formation

Conjugative transfer heavily relies on the successful assembly and operation of the type IV secretion system (T4SS) and its associated pili, placing a significant energy demand on the donor cell [7, 12]. Ripoll-Rozada et al. (2016), and later Cabezón et al. (2017), systematically reviewed molecules that are capable of interfering with conjugation (e.g. unsaturated fatty acids, 2-hexaecynoic acid, and 2-alkynoic fatty acids) and emphasized that the fatty acids mentioned in their work, primarily function by targeting essential proteins involved in the conjugation process, especially the T4SS system, by blocking the ATPase activity of proteins like TrwD (a crucial hexameric traffic ATPase in bacterial conjugation) [7]. A similar experimental approach demonstrated by Jia et al. (2022) achieved the same effect by collapsing the proton motive force (PMF) using melatonin [14]. Note that both methods of interference (Cabezón et al. and Jia et al.) reduce the rate of plasmid transfer without having to inhibit bacterial growth. This means that the use of melatonin and conjugation inhibitors only target the "vehicle", which only disarms the bacteria (such as preventing them from forming protective biofilms) and make them more vulnerable to the immune system or the existing antibiotics. It is also worth noting that an interference method not affecting bacterial growth puts less evolutionary pressure on the bacteria to mutate, preserving the effectiveness of the antibiotics used [3, 7, 11, 14].

In short, these two methods of interference mentioned above prevent the efficiency of pilus extension as well as DNA transfer among bacteria without affecting host viability. It is crucial to emphasize this because it reveals that conjugation is physiologically fragile, and this fragility represents a bottleneck whereby plasmids dependent on high transfer rates are largely vulnerable to subtle energy disturbances. This suggests that *pre-transfer* inhibition can make the most of this systemic weakness that is inherent to conjugation, instead of features that are only plasmid-specific.

Innovatively, these findings imply that future conjugation inhibitors and methods of inhibition could be screened for their capacity to selectively destabilize membrane energetics or of secretion systems' efficiency under more realistic environmental conditions, such as those found in human gut or hospital wastewater systems.

4.1.2. Inhibition of conjugation via bacteriophages

An alternative approach involves the use of bacteriophages that recognize and bind to conjugative pili as receptors. While conjugation is often discussed as a cell-autonomous process, evidence provided indicates that conjugative activities are frequently coordinated at a population level through quorum sensing (QS) networks. QS network is a pathway for bacterial communication through the use of chemical signals known as autoinducers [9]. Jiang et al. (2019) demonstrated that QS regulates a wide array of collective bacterial behaviours, including the formation of biofilms, virulence, as well as HGT [9]. In several conjugative systems, plasmid transfer genes are upregulated only when the

density of donor cells reaches a certain threshold, ensuring that transfer only takes place under the most favourable conditions for plasmid persistence.

Overall, QS creates optimal conditions for bacterial plasmid persistence by ensuring that plasmid dissemination only occurs when recipient availability is relatively high [9]. Thus, it is reasonable to conclude that QS ensures the energetically expensive and socially dependent behaviours in a population of bacteria are only expressed when the chances of pay-offs are high [9, 11].

In elaboration for the reason why QS interference (QSI) can be one of the conjugative inhibition strategy, while referring to Fig. 2, the logic is meant to be self-explanatory: plasmid persistence depends on efficient conjugative transfer, but efficient conjugative transfer is energetically costly and probabilistic [9]. However, QS optimizes and hence makes conjugative transfer more efficient by linking gene expression to population density, which stabilizes plasmid persistence across fluctuating environments [9]. Thus, interfering with QS reduces conjugative transfer by preventing the population density from reaching the activation threshold and hence the dissemination of resistance genes can be controlled [9].

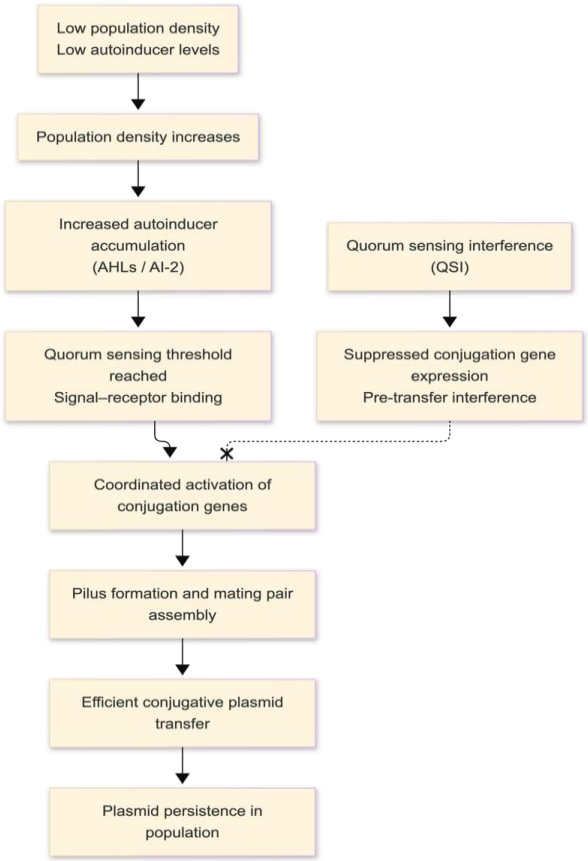

Fig. 2 Quorum sensing-mediated regulation of conjugative plasmid persistence and its disruption in the *pre-transfer* stage [9]. This figure illustrates how QS coordinates conjugative plasmid transfer within a bacterial population in a density-dependent manner. At low population densities, autoinducer concentrations remain below the activation threshold, limiting the expression of energetically costly conjugation machinery. As cell density increases, autoinducers bind to their cognate receptors, triggering a coordinated transcriptional response that activates genes involved in conjugation. The activation of these genes promotes pilus assembly, mating pair formation, and efficient plasmid transfer, thereby enhancing the persistence of plasmid in the population. The figure also shows QSI as a *pre-transfer* interference strategy to disrupt this process by suppressing the accumulation of signals, hence reducing the dissemination of resistance genes in the population.

4.1.3. Inhibition of conjugation by genetic disruption

Genetic mutations affecting pilus biogenesis or stability or both can also prevent conjugative transfer from taking place. Conjugative transfer, as mentioned multiple times before, is highly

dependent on the assembly and function of the conjugative pilus, and more importantly, this specialised surface appendage is encoded by plasmid-borne transfer (*tra*) genes [6]. By introducing mutations to the genes coding for the pili, this *pre-transfer* interference strategy is able to prevent the transfer of plasmid before DNA processing or transport can occur. From an evolutionary perspective, this approach directly interferes with the core mechanism that allows resistance genes to behave as successful "selfish replicators" [3].

In well-characterised conjugative systems such as the *F* plasmid in *Escherichia coli* (*E. coli*), formation of pilus requires the coordinated expression of multiple tra genes. The *tra* genes are needed to encode for pilin, the assembly of pilus and its stabilization, as well as connecting the formation of conjugative pilus to DNA processing at the origin of transfer (oriT) [6]. A disruption to any of these essential components of this system will produce a conjugation-deficient phenotype despite the fact that the plasmid will remain otherwise intact [6]. Cabezón et al. (2017) highlights those mutations affecting the processing of pilin, extrusion of pilus, or the anchorage of pilus to the bacterial cell envelope, consistently result in dramatic reductions in conjugation frequency, which often occurs at several orders of magnitude [7].

As an example, targeted deletions or point mutations in *traA* will lead to truncated or misfolded pilin proteins that fail to form a functional pilus, thus making donor cells unable to establish mating pairs despite preserving the plasmid [6]. Similarly, mutations in genes encoding for components of the T4SS prevent the stable cell-cell junction formation and effectively block transmission of plasmids at *pre-transfer* stage [6, 7]. This implies that, beyond loss-of-function mutations occurring naturally, engineered genetic interventions can be utilised to deliberately suppress conjugation. One of the approaches involves introducing dominant-negative alleles of pilus-associated genes into bacterial populations. Products from these defective genes can interfere with normal pilus assembly even in the presence of functional wild-type (WT) genes, resulting in a reduction in conjugation efficiency in the entire population of bacteria [7]. Strategies as such are particularly appealing because they take advantage of the cooperative nature of pilus biogenesis, where partial disruption can collapse the entire structure of a pilus [7].

Another proposed strategy involves chromosomal integration of regulatory elements that repress expression of tra genes [7]. As discussed by Cabezón et al. (2017), conjugation is majorly regulated at the transcriptional level to minimise fitness costs under unfavourable conditions. To artificially reinforce this repression, e.g., by constitutive expression of negative regulators or antisense RNAs (asRNAs) targeting transcripts, could lock plasmids into a permanently non-conjugative state without eliminating them completely [7].

Although these approaches remain largely experimental, they, nonetheless, align with population-level models proposed by Stewart and Levin (1977). Thus, with evolutionary theories in mind, genetic disruption of pilus formation is effective as it makes use of plasmid's transmission advantage rather than imposing direct lethality on the host. Additionally, Millán and MacLean (2019) also emphasize that plasmids persist only when the balance between transmission benefits and fitness costs favours maintenance [11]. Hence, by inhibiting conjugation, this equilibrium is shifted against the plasmid by a large extent, exposing the metabolic burden of plasmid carriage without providing the compensatory dissemination [11].

However, this strategy is not entirely evolution-proof. Hall et al. (2021) have also demonstrated that plasmids and hosts can rapidly acquire compensatory mutations which alleviate fitness costs or restore functionality through alternative genetic pathways [2]. In the context of pilus formation disruption, natural selection may favour plasmid variants with modified transfer systems, adjusted regulatory thresholds, or increased reliance on other HGT mechanisms. This reflects Dawkins' broader argument of "selfish" genetic elements selecting for persistence when host benefit and its persistence comes with a dilemma, and this means that the selection pressure will exploit any available pathway to maintain the replication and transmission of genetic elements and plasmids [4].

Furthermore, strategies using genetic disruptions may face practical challenges in complex microbial communities [7]. In natural environments such as wastewater systems, HGT occurs across

a diverse range of species with heterogeneous conjugation systems [6, 7]. A mutation or regulatory construct that is effective against one plasmid family may have little or no impact on unrelated plasmids with distinct pilus architectures or transfer machineries [6, 7].

Despite these limitations, genetic disruption of pilus formation as one of the *pre-transfer* interference mechanisms remains relevant, for it operates upstream of DNA mobilization, and it does not rely on antibiotic pressure. When integrated with complementary approaches such as QSI or *post-transfer* fitness-cost usage, genetic disruption targeting specifically at pilus can significantly reduce the baseline rate of conjugation within a population of bacteria. More importantly, by lowering the frequency of transmission of resistance instead of selecting for resistance, this strategy aligns with the evolutionary principles that favour long-term suppression of plasmid-mediated antibiotic resistance.

4.2. Peri-transfer Interference

Once cell-cell contact has been established, conjugation proceeds through a strictly controlled DNA processing and transport phase, which is generally referred to as the *peri-transfer* stage in this paper. Interfering at this stage will directly block plasmid mobilization regardless of mating pairs forming successfully in the *pre-transfer* stage.

The *peri-transfer* stage exposes an essential molecular bottleneck, where plasmids must transiently abandon host control to be processed and transferred by specialized enzymes. The evolutionary constraint which limits the free bypass of plasmids without losing transmissibility acts in favour of the *peri-transfer* strategies, targeting at DNA processing and/or transfer machineries.

4.2.1. Relaxase inhibitors

The relaxase enzyme initiates plasmid transfer by introducing a site-specific nick at the oriT, the relaxase enzyme then guides the single-stranded DNA (ssDNA) through the conjugative pore [17]. After cleavage, the relaxase remains covalently bonded to the 5’ end of the ssDNA via a conserved tyrosine residue, forming a nucleoprotein complex that guides the DNA through the T4SS into the recipient cell [17]. It is worth noting that relaxases are able to act as highly specific targets for intervention because they are plasmid-encoded enzymes that are mechanistically and structurally distinct from host chromosomal replication as well as repair proteins, such as DNA gyrase or DNA polymerase III [17]. Lujan et al. (2007) took advantage of this distinction by screening small-molecules inhibitors targeting the relaxase *traI* of *IncF* plasmids in *E. coli* [17]. Their study showed that inhibition of relaxase-mediated nicking at oriT largely reduces conjugation frequencies by up to two orders of magnitude while exerting no effect on bacterial growth rates and their viability, thus providing evidence for the feasibility of targeted treatments that inhibit bacterial conjugation [17]. However, because relaxase inhibition disrupts the plasmid life cycle, it directly weakens the evolutionary strategy of plasmids as “replicators” [3, 17].

In accordance with Stewart and Levin (1977), inhibition of relaxase activity should suppress HGT without immediately eliminating the costs induced on hosts from bearing a plasmid, thereby shifting the cost-benefit balance against plasmid maintenance [12]. This effect directly weakens plasmids as “selfish replicators” in the Dawkins-ian sense [3]. While compensatory mutations can mitigate plasmid fitness costs over time, such adaptations cannot restore transmission if the molecular machinery required for DNA processing at oriT is disabled. Hence, relaxase inhibition exerts an evolutionary pressure on the reproductive capacity of the plasmid itself, reducing its effective population size and long-term persistence [3, 4].

Together, these findings place relaxase inhibition as a highly specific, evolutionarily informed strategy for disrupting conjugation at a critical mechanistic bottleneck. By targeting enzymes that are specific to plasmids for HGT, relaxase inhibitors offer means to suppress the dissemination of antibiotic resistance genes while minimizing selective pressure for host-level resistance [17].

4.2.2. CRISPR-based barriers to DNA transfer

CRISPR-Cas systems function as adaptive immune mechanisms that protect bacteria against invading mobile genetic elements, including bacteriophages and conjugative plasmids, by recognising specific sequences and then cleaving the foreign DNA to neutralise threat [13, 18]. In conjugation, CRISPR interference operates at the late- *peri-transfer* stage, where incoming plasmids ssDNA or double-stranded DNA (dsDNA) is recognised and degraded before stable establishment can take place [13].

Experimental and genomic studies have shown that many bacterial species encode CRISPR spacers that specifically match conserved regions of conjugative plasmids, including tra genes and replication initiators (repA), indicating historical selection pressure against plasmid acquisition [18]. As an example, type I-E CRISPR-Cas systems in *E. coli* have been shown to efficiently target the *IncF* and *IncI* plasmids when spacer-protospacer complementarity is present, leading to an almost complete abolition of plasmid acquisition without influencing the viability of host cells [18]. This specificity effectively demonstrates that CRISPR is able to block horizontal gene establishment after DNA transfer has occurred while reducing the efficiency of conjugation in a population of bacteria.

More recent advances have extended this principle into therapeutic design [19]. Engineered CRISPR-Cas-based antimicrobials that are delivered using phagemids or conjugative delivery vectors have been shown to selectively eliminate plasmids that carries resistance determinants such as *blaNDM*, *mcr-1*, and *tetM*, while sparing bacteria free of plasmids in a population [13]. Tao et al. (2022) highlighted that CRISPR constructs targeting at resistance genes on plasmids can lead to, in some cases, cell death due to unresolved double-strand breaks, thus amplifying their antimicrobial effect [18].

However, CRISPR-based barriers impose strong evolutionary pressures that may select for escape mutations, including protospacer mutations, anti-CRISPR proteins, or loss of plasmid in targeted regions [13]. Moreover, CRISPR efficacy is highly dependent on the environment and context. For instance, biofilm environments and polymicrobial communities may reduce efficiency in DNA delivery and promote HGT pathways that bypass CRISPR surveillance [18]. Clinically, CRISPR interventions have successfully demonstrated the strength of a stage-based framework by targeting at plasmids after entry but before fixation.

Nonetheless, there are always limitations. Just as with combination antiretroviral therapies, timing, delivery, and adherence are all crucial. Failure at any one of these stages may result in resistance to persist or rebound [13]. As a conclusion, while CRISPR-Cas systems are powerful late-*peri-transfer* barriers, they are only the most effective when integrated into a broader, multi-stage strategy that acknowledge conjugation as necessary but insufficient on its own to explain resistance persistence.

4.3. Post-transfer Interference

Following successful transfer, plasmid persistence is majorly controlled by a balance between fitness costs, compensatory evolution, and host-plasmid conflict. Interference strategies in the *post-transfer* stage utilizes this evolutionary effect by amplifying the inherent costs of plasmid carriage or disrupting the plasmid maintenance systems, thereby imposing a long-term selective disadvantage on a population of bacteria. Nevertheless, it is also important to note that interference at the *post-transfer* stage does not necessarily result in the complete elimination or inhibition of resistance gene dissemination in a bacterial population.

4.3.1. Plasmid curing compounds

Once a conjugative plasmid has successfully entered a recipient cell, its long-term persistence will depend on stable replication, segregation, and expression of maintenance systems. Plasmid curing strategies aim to selectively eliminate plasmids from bacterial populations without necessarily killing the host cell, thereby reversing antibiotic resistance phenotypes [4]. Unlike the *pre-* and *peri-transfer* interventions, plasmid curing compounds directly target the *post-transfer* maintenance stage, where plasmids must behave as reliable intracellular replicons.

Buckner et al. (2018) reviewed multiple classes of plasmid curing agents, including intercalating agents such as acridine orange, replication inhibitors as well as compounds that destabilize plasmid partitioning systems [4]. By way of illustration, acridine dyes have been shown to preferentially interfere with plasmid DNA replication in *E. coli*, leading to progressive plasmid loss across generations while allowing chromosomal replication to continue [4]. More modern approaches focused on disrupting plasmid-encoded replication initiators which are essential for low-copy-number (LCN) plasmids to segregate reliably during cell division [4].

Essentially, plasmid curing makes use of the fact that plasmids are genetically autonomous yet physiologically dependent. Because plasmid replication is semi-independent from the host chromosome, selective interference can push plasmids below the threshold required for stable inheritance, especially in the absence of antibiotic pressure [12]. However, addiction systems such as toxin-antitoxin (TA) modules (e.g., hok/sok, ccdAB) can counteract plasmid curing attempts by killing plasmid-free segregants, creating a major evolutionary barrier to this strategy [10].

From a clinical point of view, plasmid curing compounds showed the strength of a stage-specific treatment framework by targeting resistance after transfer has already occurred. Yet their effectiveness is limited in real-world settings, where constant antibiotic exposure maintains selection for plasmid retention and mixed microbial communities facilitate rapid acquisition of plasmids [4]. As a result, plasmid curing would be the most effective when combined with strategies that reduce both incoming transfer and selective pressure.

4.3.2. Restriction-modification systems

Restriction-modification (R-M) systems represent an innate bacterial defence mechanism that discriminates between self and non-self DNA through sequence-specific restriction endonucleases paired with cognate DNA methyltransferases [15]. In conjugation, R-M systems act at the early post-entry stage, degrading incoming plasmid DNA before it can be fully established or expressed [15].

Kulakauskas et al. (1995) demonstrated that plasmid maintenance in *Lactococcus lactis* (*L. lactis*) was strongly influenced by the host R-M systems, with unmethylated incoming plasmid DNA being efficiently cleaved upon entry [15]. Their experiments showed that plasmids lacking appropriate methylation patterns suffered dramatic reductions in establishment efficiency, whereas pre-methylated plasmids could evade restriction and persist [15]. This highlights how intracellular molecular recognition shapes the success of HGT after physical transfer has already taken place.

Evolutionarily, R-M systems exert selective pressures on plasmids to either acquire compatible methylation motifs or evolve mechanisms to evade restriction, effectively functioning as a *post-transfer* compatibility filter [12]. Yet, plasmids are not passive targets. Many plasmids encode for anti-restriction proteins or evolve sequence architectures that minimize restriction sites, allowing the plasmids to bypass host defences [4]. Repeated exposure to the entry of foreign DNA to bacterial cells in a population can overwhelm R-M systems in dense microbial communities or biofilms. Thus, while R-M systems present themselves as powerful natural *post-transfer* interference strategies, they have also illustrated how evolutionary arms races can rapidly erode single-point interventions [3].

4.3.3. Exploiting fitness costs and evolutionary trade-offs

Many conjugative plasmids persist because they encode plasmid addiction systems that actively eliminate plasmid-free segregants after cell division, which ensure the maintenance of plasmids even when plasmid carriage imposes a fitness burden on the host [10, 11].

A typical example is the hok/sok system found in LCN plasmids of *E. coli*, where the stable Hok toxin and unstable Sok asRNA create a conditional lethal system [10]. As long as the plasmid is kept, Sok suppressed the expression of Hok; however, if the plasmid is lost, rapid degradation of Sok allows the translation of Hok, resulting in death of the host cell [10]. Similar post-segregational killing systems include ccdAB (DNA gyrase inhibition) and parDE, all of which act after conjugative transfer, but during inheritance and expression [10].

Addiction systems represent a critical weak spot for conjugation in the *post-transfer* interventions. Tsang (2017) emphasized that these systems are mechanistically distinct from essential host processes,

making them suitable targets for therapeutic disruption [10]. In exemplification, inhibiting toxin activity and stabilizing antitoxin molecules, or interfering with TA gene expressions, would convert plasmid maintenance from an involuntary state to a selectable trait, thereby allowing plasmid-free cells to survive and replicate [10]. Such interference aims to directly impair the plasmid's ability to function as a stable "replicator" [3].

Cranenburgh et al. (2001) engineered *E. coli* strains with plasmid maintenance that relied on repressor titration rather than antibiotic selection, demonstrating that plasmids can be maintained or lost depending on the stability of regulatory circuits rather than external selection pressure [20]. Their work highlighted how plasmid persistence is conditional and how altering *post-transfer* regulatory dynamics can shift populations toward plasmid loss.

The R-M systems intersect with addiction mechanisms by influencing plasmid stability after entry, illustrating that *post-transfer* survival is not guaranteed but dependent upon successful navigation of host molecular control systems [15]. Further, Buckner et al. (2018) argued that plasmid curing strategies are most effective when they target maintenance systems instead of transfer itself [4]. Taken together, disrupting addiction systems can remove the selective penalty associated with plasmid loss, allowing natural segregation errors to eliminate resistance plasmids over time [4]. This is especially clinically relevant where antibiotic pressure fluctuates and changes, which create windows during which destabilized plasmids can be removed from populations.

5. Future Directions and Discussion

While each interference strategy discussed promised isolation, the most effective long-term solutions are likely to involve a combination of approaches that target at multiple stages of conjugation simultaneously [4, 7]. Additionally, future research must prioritize testing these strategies in ecologically realistic settings, such as communities and biofilms consisted of multiple species, where conjugation dynamics differ substantially from laboratory monocultures [3, 6].

Essentially, interventions should be evaluated for both their immediate effectiveness and evolutionary consequences. Strategies that minimize selective pressure for resistance genes while disrupting plasmid transmission closely align with the long-term goal of slowing antibiotic resistance from its origin [4]. By viewing conjugation as an evolutionary process in addition to a mechanistic one, future therapies may shift from attempting to eliminate resistant bacteria to targeting the genetic systems that allow resistance to spread.

6. Conclusion

Antibiotic resistance is fundamentally caused by evolution, driven by both selections acting on individual bacterial genomes and the rapid dissemination of resistance determinants through HGT. Among the three major HGT mechanisms, conjugation acts a central role due to its efficiency, broad host range, and capacity in transferring complex, multi-gene resistance architectures across phylogenetic boundaries. This paper argued that effective mitigation of antibiotic resistance, therefore, requires interventions that directly target the conjugative lifecycle of plasmids rather than relying solely on traditional bactericidal or bacteriostatic approaches. In essence, the main contribution of this paper lies in the proposal of interference strategies targeting at the conjugation lifecycle.

Through integrating molecular, evolutionary, and population-level perspectives, this study framed conjugation as a multi-stage process consisting of *pre-transfer*, *peri-transfer*, and *post-transfer* phases, each controlled by different molecular mechanisms. Evidence from both experimental and theoretical studies have exemplified that plasmid persistence is not entirely inevitable, though contingent upon successful navigation of each of these three stages. Thus, all strategies reviewed in this study are capable of reducing resistance gene dissemination without necessarily imposing lethal selection on bacterial hosts.

Furthermore, this stage-specific framework also reveals why conjugative plasmids have remained evolutionarily stable despite imposing fitness costs on bacterial host cells: through overviews and explanations on compensatory evolution, addiction systems, and regulatory integration enabling plasmids to function autonomous “replicators” within bacterial populations, it indicates that these adaptations generate vulnerabilities that can be strategically targeted.

Nevertheless, focusing on conjugation alone is insufficient. Resistance genes can still propagate through transformation, transduction, and clonal expansion, particularly in structured environments such as biofilms or polymicrobial communities. Therefore, effective long-term control systems or methods will require coordinated strategies that integrate interventions focused on conjugation with ecological considerations and management, surveillance, as well as the prudent prescription & use of antibiotics.

Taken together, this work highlights conjugation as both a mechanism of resistance gene dissemination and a tractable evolutionary process whose disruption may slow the propagation of antibiotic resistance from the root, while also providing a conceptual and operational framework for the treatment and prevention of antibiotic resistance in the future medical field.

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