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Exposing Plasmids as the Achilles' Heel of Drug-Resistant Bacteria

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Abstract

Many multi-drug resistant bacterial pathogens harbor large plasmids that encode proteins conferring resistance to antibiotics. While the acquisition of these plasmids often enables bacteria to survive in the presence of antibiotics, it is possible that plasmids also represent a vulnerability that can be exploited in tailored antibacterial therapy. This review highlights three recently described strategies designed to specifically combat bacteria harboring such plasmids: Inhibition of plasmid conjugation, inhibition of plasmid replication, and exploitation of plasmid-encoded toxin-antitoxin systems.

Introduction

Bacterial resistance to antibiotics is a worldwide health crisis [1]. Resistance to multiple antibiotics has been reported in nearly all pathogenic bacteria, with vancomycin-resistant enterococci (VRE), methicillin-resistant *Staphylococcus aureus* (MRSA), multi-drug resistant (MDR) *Pseudomonas aeruginosa*, extensively-drug resistant (XDR) *Mycobacterium tuberculosis*, MDR *Acinetobacter baumannii*, β -lactam-resistant Enterobacteriaceae, and penicillin-resistant *Streptococcus pneumoniae* (PRSP) being particularly notorious [1-3]. Resistance typically occurs as a result of chromosomal mutation or acquisition of a mobile genetic element, such as a plasmid, that harbors resistance-mediating genes. The looming threat of a “post-antibiotic” era where untreatable bacterial infections are common is exacerbated by the shift of research programs in the pharmaceutical industry away from the development of novel antibacterials [4,5]. New strategies to combat drug-resistant bacteria are necessary to keep pace with ever-evolving bacterial resistance.

Plasmids as mobile genetic elements that mediate drug resistance

Lateral transfer of mobile genetic elements between diverse bacteria leads to a rapid dissemination of genes encoding resistance to antibiotics. These mobile genetic elements include plasmids, which are extrachromosomal DNA that transfer horizontally within and across bacterial genera and species by conjugation. Plasmids can also serve as vehicles for transposons and integrons; thus, through plasmid conjugation bacteria are exposed to a wide array of genes from the mobile gene pool. Plasmid-encoded resistance to multiple antibiotics, including β -lactams, aminoglycosides, tetracyclines, macrolides, and glycopeptides is prevalent in a plethora of pathogenic bacteria including VRE and MRSA [6,7]. In fact, recent

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analyses of >100 VRE isolates from humans, animals, and food show that *vanA*, the gene cluster encoding vancomycin resistance, resides in the *Tn1546* transposon carried on plasmids [8,9]. In addition, plasmid-encoded virulence and antibiotic resistance contribute to the pathogenicity of biowarfare agents such as *Bacillus anthracis* and *Yersinia pestis* [10-12]. Most frightening is the recently observed transfer of plasmids from VRE to MRSA, resulting in the virtually untreatable vancomycin resistant *S. aureus* (VRSA) [13,14].

However, the very nature of their importance to the antibiotic resistant phenotype may expose plasmids as the Achilles' heel of drug-resistant bacteria. Indeed, creative strategies have recently been devised to prevent the transfer of plasmids between bacteria, to inhibit plasmid replication and hence induce the elimination of plasmids from bacteria, and to exploit plasmid maintenance systems to directly and selectively induce death in drug-resistant bacteria (Figure 1). [15-24]. Although compounds based on these approaches have not yet progressed to clinical trials, the well-documented prevalence of plasmids within the most problematic drug-resistant bacteria makes the targeting of plasmid-encoded elements an intriguing antibacterial option. This Current Opinion focuses on these recent efforts to exploit plasmids in antibacterial therapy.

Inhibition of plasmid conjugation

To prevent the transfer and dissemination of resistance-mediating plasmids, the inhibition of plasmid conjugation has been postulated as a prophylactic strategy [15,25]. Using a cell-based assay involving the transfer of a plasmid containing the *lux* gene (encoding luciferase) from a donor strain to a recipient strain (Figure 2A), chemical libraries and bacterial/fungal extracts were screened for inhibitors of plasmid conjugation [15,16]. Through these screens dehydrocrepenynic acid (DHCA) and linoleic acid were identified as conjugation inhibitors (Figure 2B). The compounds depicted in Figure 2B were found to only inhibit the transfer of plasmids with similar DNA replication and transfer machinery and did not inhibit the proteins involved in the mating bridge (mating pair formation). Secondary assays ruled out general inhibitory effects of these unsaturated fatty acids, suggesting that these compounds may act through a conjugation-specific mechanism [15].

A subsequent study on conjugation inhibition also used the same fluorometric, cell-based assay to identify intrabodies that specifically inhibit conjugation [16]. Intrabodies are intracellularly-expressed antibodies that have been used to inactivate proteins in yeast [26,27], plants [28, 29], mammals [30-32], and bacteria [33-35]. The relaxase enzyme, which catalyzes the cleaving and religating of plasmid DNA, is an essential component of plasmid conjugation systems (Figure 3A). Recognizing the critical importance of relaxases to plasmid conjugation, Garcillan-Barcia and co-workers expressed intrabodies in the recipient cell to inactivate the TrwC relaxase enzyme encoded by plasmid R388 in a proof-of-concept study [16]. Mice were immunized with the TrwC relaxase domain (the N-terminal 293 amino acids (N293)), and single chain Fv antibody clone libraries were created from splenocytes. Screening of the intrabody libraries for their binding to TrwC-N293 and for their inhibition of conjugation using the aforementioned fluorescence-based assay yielded two conjugation inhibitors, scFv-P4.E7 and scFv-P1.F2. Whereas scFv-P4.E7 recognizes a region of TrwC not known to be involved in catalysis, scFv-P1.F2 binds to the conserved motif 1 of the MOB_F relaxase family, which is a mobile loop containing the catalytic tyrosine-26 [36,37]. TrwC relaxase function depends on two catalytic tyrosines: Y18 carries out the initial cleavage event at *oriT* and Y26 is thought to catalyze a transesterification, which recircularizes the T-DNA, the DNA that is transferred, in the recipient cell [38]. The observed 20-fold conjugation inhibition of scFv-P1.F2 matches the reduction in activity observed by the TrwC-Y26F mutant [38], suggesting that the binding of scFv-P1.F2 to the mobile Y26-containing loop may prevent the transesterification and recircularization of T-DNA in the recipient cell. Another intriguing result is that mutant TrwC-

Y18F but not wild-type TrwC could partially rescue the reduced conjugation of TrwC-Y26F, suggesting different roles for each tyrosine and possible different conformations of TrwC during conjugative DNA processing. Using a target-based approach to study conjugation, these results confirm previous evidence that TrwC is active in the recipient cell and suggests relaxase inhibition is a viable strategy for preventing plasmid conjugation. However, because these intrabodies do not actually induce cell death, this type of prophylactic strategy would only be useful for preventing the dissemination of genes that mediate antibiotic resistance.

Although the use of relaxase-targeting intrabodies validated the notion that interference with relaxase function could inhibit plasmid conjugation, the therapeutic application of intrabodies will be difficult due to the biological stability, cell permeability, and pharmacokinetic problems faced by any macromolecular drug. In a recent study by Lujan and co-workers, however, a series of *small molecule* relaxase inhibitors were identified and shown to prevent plasmid conjugation [17]. Through X-ray crystallographic analysis of the F plasmid TraI-N300 relaxase domain, it was hypothesized that simple bisphosphonates could interact with the active site Mg^{2+} ion and two catalytic tyrosine residues to inhibit relaxase. Thus, an enzymatic assay was developed that measured the relaxase-catalyzed cleavage of a fluorescently-labeled ssDNA containing the F plasmid *oriT* sequence. This assay showed that nanomolar concentrations of imidobisphosphate (PNP) (Figure 3B) inhibited relaxase-catalyzed cleavage of *oriT* ssDNA. The crystal structure of PNP-bound relaxase revealed a phosphate of PNP within 3.7 Å of the Mg^{2+} metal center. Of twelve bisphosphonates tested in the kinetic assay, six compounds (shown in Figure 3B) were found to potently inhibit relaxase.

A conventional conjugation assay showed that PNP inhibited transfer of the F plasmid between two *E. coli* cells with an EC_{50} of 10 μM . Surprisingly, PNP was found to selectively kill F^+ *E. coli* expressing the TraI relaxase but had no effect on strains containing TraI relaxase but no F plasmid, F plasmid but no TraI relaxase, or F plasmid in which all four relaxase active site tyrosines were mutated to phenylalanines. These data suggests that PNP inhibits conjugation and produces a bactericidal effect dependent on the presence of active relaxase and F plasmid. The exact mechanism behind this relaxase-dependent antibacterial activity of bisphosphonates is unknown. All six bisphosphonates in Figure 3B inhibited conjugation and displayed F plasmid specific killing in the nanomolar-to-low-micromolar range, making them significantly more selective over cells lacking the F plasmid. Two of these potent bisphosphonates, Clodronate and Etidronate, are clinically approved for the treatment of bone disease. These compounds are promising candidates for use in combination with current antibiotics to prevent dissemination of plasmid-encoded antibiotic resistance in the gastrointestinal tract, and may have potential as single entity antibacterial agents against bacteria harboring plasmid-encoded relaxases. Before either of the relaxase-targeting strategies described above can be broadly utilized, there will need to be a demonstration that homologous relaxases are present and active in clinically significant bacterial pathogens.

Inhibition of plasmid replication by mimicking plasmid incompatibility

Another novel approach to combat bacteria harboring plasmid-encoded resistance genes is the use of small molecules to inhibit plasmid replication and hence eliminate the plasmid from the bacterial population. Plasmid incompatibility is a natural phenomenon for plasmid elimination; two plasmids of the same incompatibility group will not stably cosegregate to a daughter cell. Studies by DeNap *et al.* [18] and Thomas *et al.* [19] exploit this natural mechanism in the identification of small molecule mimics of plasmid incompatibility, “antiplasmid” compounds that eliminate plasmids from the bacterial population and re-sensitize the bacteria to antibiotics.

Plasmid incompatibility is determined by the plasmid replication machinery, which has been extensively studied in the incompatibility group B (IncB) plasmids [39-46]. IncB plasmid

replication is tightly controlled by the levels of the phosphodiesterase RepA [47,48], the translation of which is controlled by a small, untranslated RNA, called RNA I (Figure 4A). RepA mRNA forms an intramolecular pseudoknot between stem-loop I (SLI) and stem-loop III (SLIII), which allows RepA translation and hence plasmid replication [43]. RepA translation is shut down when the countertranscript RNA I binds SLI [39,44-46]. In an effort to mimic this process with a small molecule, antiplasmid compounds that bound to SLI were sought. A variety of aminoglycosides were tested for their ability to bind SLI RNA, and apramycin (Figure 4B) was found to bind with a K_d of 93 nM. Through mutagenesis studies it was determined that bases A22 and A23 on SLI were essential for apramycin binding, as this binding event was completely abolished in the SLI-A22G/A23G double mutant. Plasmid stability assays showed that the IncB plasmid was almost completely eliminated after 250 generations, and a general correlation between SLI binding affinity and plasmid loss was observed [19]. In contrast, when the SLI-A22G/A23G mutations were created on the same IncB plasmid (abolishing the apramycin binding site), this plasmid was not eliminated by apramycin. These studies demonstrate that plasmids can be eliminated from bacterial cells in a mechanistically distinct fashion, that is, through the identification of compounds that bind tightly to RNAs essential to plasmid replication control. For this approach to find clinical utility, the homology of the RNAs that mediate plasmid replication control in pathogenic bacteria will need to be investigated. The little information that is available does indicate that some plasmids do indeed have homologous regions in these key countertranscript RNAs [49, 50]. Furthermore, identification of compounds that cause more rapid plasmid loss will improve this strategy and increase its potential in antibacterial therapy.

Toxin-antitoxin systems

Proteic toxin-antitoxin systems, found on both bacterial plasmids and chromosomes, produce a stable toxic protein and a labile antitoxin protein. The possibility of exploiting toxin-antitoxin (TA) systems as a novel antibacterial strategy with a compound that activates the latent toxin through one of two pathways (Figure 5), has been proposed [6,20-22,51]; although the end result (toxin-induced cell death) is the same, the two strategies depicted in Figure 5 differ mechanistically. In pathway 1, a compound acts at either the transcriptional or translational level to prevent the synthesis of new antitoxin. Thus, when the highly labile pre-existing antitoxin molecules are degraded, the stable toxin is freed to kill the cell. The second mechanism for the exploitation of TA systems as antibacterial targets involves the direct disruption of the toxin-antitoxin protein-protein interaction, freeing the toxin to induce cell death (pathway 2 in Figure 5) [6,20,21,51]. When considering TA systems as an antibacterial target, it is helpful to make a distinction between TA systems found on chromosomes and those found on plasmids, although compounds acting through either mechanism should be effective against plasmid- and chromosomally-encoded TA systems alike.

Chromosomally-encoded toxin-antitoxin systems

Although the genes for toxin-antitoxin proteins have been found on bacterial and archaeal chromosomes, the function of chromosomally-encoded TA systems remains elusive. Data from several studies indicate that these systems function to halt bacterial growth during times of stress (Figure 6A). For example, the *mazEF* TA system has been described as a suicide module that causes programmed cell death (PCD) in response to extreme amino acid starvation. In this scenario *relA* synthesizes the stringent response molecule guanosine 3',5'-bispyrophosphate (ppGpp), inhibiting *mazEF* transcription, activating MazF, and ultimately leading to cell death [52-54]. Furthermore, addition of antibacterials that inhibit transcription (rifampicin), translation (chloramphenicol and spectinomycin) or that cause thymine starvation (trimethoprim and sulfonamide) cause *mazEF*-dependent cell death [23,55,56]. Based on these studies it has been proposed that a new class of antibacterials could be developed that would

stress the cells such that the toxin protein(s) are activated, causing cell death [22,23]. The recent discovery of a short peptide that appears to induce bacterial cell death in a MazF-dependent fashion in *E. coli* bolsters the argument that chromosomally-encoded TA systems are a tractable antibacterial target [24,57].

However, other studies offer conflicting evidence, including a recent report in which the genes for several chromosomally-encoded TA systems were systematically knocked out in *E. coli*, and the resulting bacterial strain had no obvious change in phenotype in response to the cellular stresses that were tested [58]. Given this contradictory evidence, a variety of potential functions for chromosomally-encoded TA systems have been postulated, including the possibility that they have no function [59]. TA systems have also been reported as modulators of the persister cell phenotype, in which cells neither grow nor die in the presence of bactericidal antibiotics, resulting in multi-drug tolerance (MDT) [60-62]. HipA, of the TA operon *hipBA*, was the first validated persister-MDT protein; knocking out *hipA* significantly reduces the occurrence of persister cells [61]. However, knocking out other TA systems shown to be involved in producing the persister cells in *E. coli* resulted in no phenotype, thus suggesting that persister genes are redundant [62].

Although several genomic studies have revealed the presence of TA genes on the chromosomes of a variety of different bacteria [63-65] and their absence in obligate host-associated organisms, definitive evidence showing that chromosomally-encoded TA genes are functional in clinical isolates of pathogenic bacteria will be required before disruption of chromosomally-encoded TA systems can be considered a viable antibacterial strategy. Furthermore, understanding the role of chromosomally-encoded TA systems in the formation of persister cells will help to further evaluate TA systems as an antibacterial target.

Plasmid-encoded toxin-antitoxin systems

The role of plasmid-encoded TA systems is to function as post-segregational killers (PSKs) (Figure 6B) [66-68]. Proteic TA systems are utilized by plasmids to ensure that only those daughter cells that inherit the plasmid survive after cell division. When both proteins are present, the antitoxin binds to the toxin, preventing its toxic activity. However, if during cell division a plasmid-free daughter cell arises, the labile antitoxin is quickly degraded (and not replenished), freeing the toxin to induce cell death. Because of this indelible link between plasmid maintenance and bacterial life, TA systems have been termed 'plasmid addiction systems' [69].

Before the search for toxin activators could commence, it was necessary to know if the genes for TA systems were present on plasmids isolated from major drug-resistant bacterial pathogens, if a certain TA systems was more prevalent than others (making it a more attractive antibacterial target), and if these plasmid-encoded TA genes were functional in the drug-resistant bacteria. A recent epidemiological survey of VRE isolates provided answers to these questions [21]. In this survey, plasmids were purified from 75 different VRE clinical isolates, and then probed by PCR for the presence of TA genes. Surprisingly, the genes for TA systems were found to be ubiquitous on plasmids from VRE and physically linked to the *vanA* gene cluster; 75 out of 75 VRE isolates contained plasmids harboring genes for TA systems. Certain TA systems were indeed more prevalent than others, with *mazEF* (75 out of 75), *axe-txe* (56 out of 75), *relBE* (35 out of 75), and ω - ϵ - ζ (33 out of 75) being the most common. RT-PCR showed that the *mazEF* transcripts are produced in the VRE isolates. Furthermore, plasmid pS345RF, which contains *mazEF* as the only detectable TA system, was shown to be highly stable in the absence of selection. Finally, the cloning of *mazEF* and its native promoter into the unstable enterococcal vector pAM401 was shown to impart a significant increase in plasmid stability, thus suggesting that TA systems are functional in VRE [21].

Targeting toxin-antitoxin systems

The discovery that certain TA systems are ubiquitous in clinical isolates of difficult-to-treat drug-resistant pathogens suggests the exciting possibility that disruption of TA systems could be a viable target for tailored antibacterial therapy. The next challenge is to develop high-throughput screens and use them to identify compounds that induce toxin-dependent death. In this vein, a continuous fluorometric assay that follows the ribonuclease activity of MazF was recently developed [70]. This assay employs a short oligonucleotide containing the MazF cleavage sequence 5'-labeled with 6-carboxyfluorescein (6-FAM) and 3'-labeled with a black-hole quencher (BHQ1). Cleavage of the oligonucleotide releases the fluorophore from the quencher, resulting in a large increase in fluorescence emission of 6-FAM. This *in vitro* assay could be used to screen compounds for their ability to induce activation of MazF activity from the MazE-MazF complex. Cell-based assays to identify compounds that selectively restrict growth of toxin-antitoxin producing bacteria can also be envisioned.

It is possible that targeting plasmid-encoded TA systems could have advantages over traditional antibiotics. One could envision resistance to such toxin-activating compounds arising through an inactivating mutation in the toxin protein, or through mutation of the target of the toxic protein. However, both situations are problematic from the bacteria's perspective. If the toxin protein is mutated and inactivated, a compound that released the toxin would indeed no longer be an effective antibacterial. On the other hand, this mutation would also eradicate the plasmid stabilization system, and hence the plasmid (containing the drug-resistance genes) would be eliminated from the bacterial population, re-sensitizing the bacteria to conventional antibiotics. Mutation of the target would be equally complicated as a resistance mechanism. It is difficult to foresee how toxic RNase activity (such as in MazF) could be abolished through target mutation. For the toxins that inhibit DNA gyrase (such as CcdB), mutants of this enzyme could indeed arise that are resistant to the toxin proteins. However, once again this sort of mutation would eliminate the natural function of the TA systems, that of plasmid stabilization; gyrase mutants would be resistant to the post-segregational killing effect, and plasmid-free daughter cells (that are sensitive to the effect of antibiotics) would likely arise quickly.

Analysis, Summary, and Future Directions

The very fact that plasmids are responsible for large swaths of drug-resistance in bacteria makes them attractive antibacterial targets. It would seem that prophylactic strategies, for example those that are designed to stop the spread of drug-resistance genes through inhibition of plasmid conjugation, are less attractive and less practical than those that directly induce bacteria cell death. However, as shown by the creative work of Lujan and co-workers [17], the prevention of plasmid conjugation through the inhibition of relaxase can indeed directly induce cell death, a surprising and welcome discovery. While strategies that rely on compounds to induce plasmid elimination may have some utility, the heterogeneity of the plasmid replication elements and the number of generations required for elimination will need to be defined in clinical isolates before this approach can be implemented. The direct induction of cell death through the disruption of toxin-antitoxin systems appears to hold considerable promise, given the ubiquity of certain TA systems on plasmids isolated from VRE and the strong toxicity of the various toxic proteins. Although the lack of plasmids in XDR *M. tuberculosis* presents a limitation for the proposed plasmid conjugation and replication inhibition antibacterial strategies, toxin-antitoxin systems have been shown to reside on the *M. tuberculosis* chromosome [63,71].

As these approaches toward utilizing mobile genetic elements against bacteria are further explored and exploited, a major effort will need to be made to move studies past proof-of-concept work in *E. coli* with model plasmids and into demonstrations in actual clinical isolates. Given the wide array of naturally occurring plasmids, target heterogeneity will be a major question for any strategy seeking to exploit a plasmid-encoded trait; an ideal target would be

one that is fully conserved throughout a variety of difficult-to-treat bacteria. Because plasmids often harbor the genes for resistance-mediating enzymes, standard mechanisms of resistance may not be as applicable to compounds that target plasmid-encoded elements. Just as dipping Achilles into the river Styx gave him overall strength but left his heel vulnerable, so too does the very resistance conferred on bacteria by a plasmid make them susceptible to plasmid-targeting strategies.

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- of special interest
 - of outstanding interest
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- REF 6. Moritz and Hergenrother: The Prevalence of Plasmids and Other Mobile Genetic Elements in Clinically-Important Drug-Resistant Bacteria. This paper presents a comprehensive review of the mobile genetic elements that contribute to the dissemination of genes conferring antibiotic resistance that are associated with a number of the most problematic bacterial pathogens.
- REF 12. Fernandez-Lopez *et al.*: Unsaturated fatty acids are inhibitors of bacterial conjugation. This study reports the development of a fluorometric conjugation assay that detects plasmid transfer via

the production and activity of luciferase. This assay was used in a high-throughput screen and identified dehydrocrepenynic acid (DHCA) and linoleic acid as inhibitors of plasmid R388 conjugation.

- REF 13. Garcillan-Barcia *et al.*: Conjugative transfer can be inhibited by blocking relaxase activity within recipient cells with intrabodies. This study utilized the recently developed technology of bacterial cytoplasmic antibody expression to create a library of intrabodies that were found to bind the TrwC relaxase domain encoded by plasmid R388. Expression of the intrabodies in the recipient cell inhibited conjugation, further confirming the transfer of a functional relaxase in conjugal DNA transfer.
- REF 14. Lujan *et al.*: Disrupting antibiotic resistance propagation by inhibiting the conjugative DNA relaxase. This report highlights the ability of several bisphosphonates to bind the F plasmid TraI relaxase domain inhibit conjugation, and selectively kill bacteria that harbor the F plasmid and functional TraI. Two of these bisphosphonates are clinically approved, making them interesting candidates for novel antibiotic therapy.
- REF 15. DeNap *et al.*: Combating drug-resistant bacteria: small molecule mimics of plasmid incompatibility as antiplasmid compounds. This study combined with [16] report the binding of apramycin to the *repA* SLI RNA of an IncB plasmid, shutting down *repA* translation, inhibiting plasmid replication, and thus eliminating plasmids from the populations. In this way, apramycin act as small molecule mimic of plasmid incompatibility.
- REF 18. Mortiz and Hergenrother: Toxin-antitoxin systems are ubiquitous and plasmid-encoded in vancomycin-resistant enterococci. This paper reports a comprehensive survey of the prevalence of toxin-antitoxin (TA) genes on plasmids within individual clinical isolates of VRE. Analysis of 75 isolates revealed that TA genes are ubiquitous on plasmids in VRE, and that certain individual TA systems are highly prevalent.
- REF 61. Pandey and Gerdes: Toxin-antitoxin loci are highly abundant in free-living but lost from host-associated prokaryotes. This exhaustive database search of 126 sequenced prokaryotic genomes identified 671 toxin-antitoxin (TA) loci representing the 7 major TA system families in free-living prokaryotes; whereas in contrast, obligate intracellular prokaryotes were found to lack the genes for TA systems. The observed TA systems were commonly clustered with mobile genetic elements.

Fig. 1.

Three approaches to exploit plasmids in antibacterial therapy. 1. Plasmids are transferred between bacteria through conjugation. Inhibition of the relaxase enzyme (blue oval) has been proposed as an antibacterial strategy, and several relaxase inhibitors have been identified. 2. Plasmids replicate to maintain themselves in the bacterial population. Strategies have been devised and small molecules have been identified that inhibit plasmid replication, thus resulting in the elimination of the plasmid from the bacterial population and re-sensitizing the bacteria to antibiotics. 3) Large plasmids rely on elaborate mechanisms to ensure their faithful segregation to daughter cells after cell division. One of the most common plasmid maintenance systems is the toxin-antitoxin (TA) post-segregational killing mechanism. In this mechanism, if a plasmid-free daughter cell arises, the labile antitoxin is degraded and the toxin induces cell death. TA genes are ubiquitous in clinical isolates of certain drug-resistant bacteria, and it has been postulated that compounds that disrupt the toxin-antitoxin interaction could free the toxin to kill the bacterial cell.

Fig. 2.

The identification of inhibitors of plasmid conjugation. (A) To screen for inhibitors of plasmid conjugation, a donor cell harboring an F plasmid derivative with the *lux* gene under control of the *lac* promoter is utilized. This cell also harbors a second plasmid, a non-mobilizable plasmid containing *lacI*, encoding the lac repressor protein LacI; LacI binds *lacO*, repressing the expression of *lux*. Transfer of the F plasmid derivative to the recipient cell results in luciferase production and light emission. (B) This screen was used to identify two conjugation inhibitors: DHCA and linoleic acid.

A

B

Fig. 3.

Targeting relaxase as a strategy to inhibit plasmid conjugation. (A) Overview of the relaxase mechanism. The relaxase domain (shown as the blue circle) domain of TrwC binds the *oriT* (1) of the T-strand. Tyrosine-19 (shown as an orange circle) of relaxase catalyzes a cleavage at the *nic* site and forms a covalent phosphotyrosyl intermediate with the 5' terminus of *oriT* (2). Rolling circle replication using the uncleaved strand as a template displaces the T-strand (3) and Y26 cleaves at the T-strand *nic* site (4). The T-strand is re-ligated and transferred to the recipient cell. Figure adapted from Llosa *et al.* [72] and Llosa and de la Cruz [73]. (B) Certain bisphosphonates will inhibit relaxase and prevent plasmid conjugation. The

bisphosphonates shown are inhibitors of F plasmid TraI relaxase; these compounds prevent plasmid conjugation and induce plasmid- and relaxase-dependent cell death.

A

B

Fig 4.

“Antiplasmid” antibiotics induce plasmid elimination from the bacterial cell population, resensitizing bacteria to antibiotics. A) Plasmid replication control by RepA. In IncB systems, an intramolecular pseudoknot forms between SLI and SLIII of the RepA mRNA, allowing for ribosome binding and Rep protein translation (left). Rep protein translation and thus plasmid replication is inhibited by the reversible binding of RNA I (middle) or a small molecule (right, orange hexagon) to SLI. Figure adapted from Thomas *et al.* [19]. B) The compound apramycin was discovered to bind to SLI *in vitro*, and induce the elimination of an IncB plasmid from bacterial cells in culture.

Fig. 5.

Activation of the toxin of a toxin-antitoxin system, and subsequent cell death. At least two mechanisms for toxin activation are possible. In pathway **1** (left), the small molecule acts to directly or indirectly to inhibit transcription and/or translation of the TA locus, preventing the replenishment of the labile antitoxin, which is quickly degraded. This frees the toxin to kill the cell. This half of figure adapted from Engelberg-Kulka *et al.* [22,23,74] In pathway **2** (right), the small molecule directly disrupts the toxin-antitoxin protein-protein interaction, freeing the toxin to kill the cell.

Fig. 6.

The genes for toxin-antitoxin systems have different functions, depending on their location. A) Chromosomally-encoded TA systems are possibly used by the cell to respond to stress. Stress causes toxin activation and reversible inhibition of cell growth; if the “point of no return” is reached, cell death occurs. B) Plasmid-encoded TA systems function as a “post-segregational killing” system, resulting in the elimination of plasmid-free bacterial cells.