PERSPECTIVE

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Bacterial death comes full circle: targeting plasmid replication in drug-resistant bacteria

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Received 7th January 2005, Accepted 19th January 2005
First published as an Advance Article on the web 15th February 2005

It is now common for bacterial infections to resist the preferred antibiotic treatment. In particular, hospital-acquired infections that are refractory to multiple antibiotics and ultimately result in death of the patient are prevalent. Many of the bacteria causing these infections have become resistant to antibiotics through the process of lateral gene transfer, with the newly acquired genes encoding a variety of resistance-mediating proteins. These foreign genes often enter the bacteria on plasmids, which are small, circular, extrachromosomal pieces of DNA. This plasmid-encoded resistance has been observed for virtually all classes of antibiotics and in a wide variety of Gram-positive and Gram-negative organisms; many antibiotics are no longer effective due to such plasmid-encoded resistance. The systematic removal of these resistance-mediating plasmids from the bacteria would re-sensitize bacteria to standard antibiotics. As such, plasmids offer novel targets that have heretofore been unexploited clinically. This Perspective details the role of plasmids in multi-drug resistant bacteria, the mechanisms used by plasmids to control their replication, and the potential for small molecules to disrupt plasmid replication and re-sensitize bacteria to antibiotics. An emphasis is placed on plasmid replication that is mediated by small counter-transcript RNAs, and the "plasmid addiction" systems that employ toxins and antitoxins.

1 Introduction

1.1 The growing problem of bacterial resistance to antibiotics

The 1950s–1970s saw the discovery of multiple classes of antibiotics, and their development into drugs changed a simple bacterial infection from life threatening to trivial. This "golden age" of antibiotics engendered such optimism that it was commonly thought bacterial infections would be rapidly eliminated as a cause of mortality.[1,2] Unfortunately, bacterial resistance to all classes of antibiotics soon appeared.[3] Now, three decades after the end of this era, drug-resistant bacteria are ubiquitous in hospital settings and annually 90 000 people die of such infections each year in the US alone.[4] One quarter of the bacteria that most frequently cause hospital-acquired infections are resistant to the preferred antibiotic treatment,[5] and an alarming 70% of hospital acquired infections are resistant to at least one antibiotic.[6] Methicillin-resistant *Staphylococcus aureus* (MRSA) is the most common drug-resistant bacteria in hospitals, accounting for greater than 30% of all nosocomial infections.[7,8] MRSA can also be community-acquired, causing severe illness and even death.[9] Furthermore, the incidence of extended spectrum β-lactamase (ESBL) production in clinical *Klebsiella* isolates has increased steadily in the past several years, severely curtailing the effectiveness of β-lactam antibiotics.[10] Perhaps most disturbing is the recent estimation that one third of enterococci in intensive care units are resistant to vancomycin, often viewed as the antibiotic of last resort.[11]

The list of drug-resistant pathogens is extensive and growing. These bacterial infections are of particular concern in elderly, infirm, or immuno-compromised patients. Individuals with tuberculosis, AIDS, salmonellosis, gonorrhea, or malaria who contract drug-resistant bacterial infections experience longer hospital stays and have mortality rates more than twice as high as those with antimicrobial-susceptible infections.[5] Thus, resistant bacteria not only complicate medical treatment, but also add billions of dollars to medical costs every year.[12]

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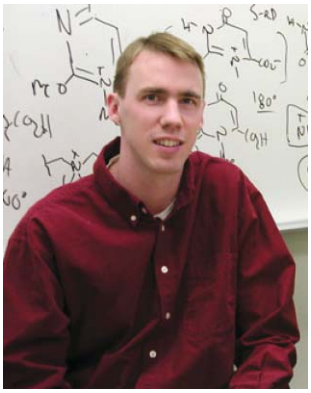

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DOI: 10.1039/b500182j

The problem of bacterial resistance to antibiotics is exacerbated by the downward trend in antibacterial discovery and development. There has been a 56% decrease over the last two decades in the annual number of antibiotics approved by the FDA.[6] In fact, only six antibiotics produced by large pharmaceutical companies are currently in late stage clinical trials, and all of these are derivatives of known antibiotics.[6] Although the reasons for the halting of many antibacterial programs at major pharmaceutical companies are myriad, the acute (not chronic) nature of most bacterial infections and the public expectation for no side effects has made antibacterial research less profitable and more difficult when compared to other disease areas.[6,13]

As bacteria have developed antibiotic resistance to virtually every drug class, it is imperative that new biological targets be found and exploited.[2,14] The majority of therapeutically useful antibiotics target only three operations in the cell: cell-wall biosynthesis (β-lactams, glycopeptides), protein synthesis (aminoglycosides, tetracyclines, macrolides, oxazolidinones), or enzymes involved in bacterial DNA replication (fluoroquinolones).[15,16] Historically, the majority of antibiotics were discovered by screening natural product collections for antibacterial properties and subsequently optimizing lead compounds. In the last decade, however, genomic and proteomic methods have been touted as a means to find new bacterial targets. However, despite progress,[17] a novel compound developed through such discovery methods has yet to come to market.[18]

While most antimicrobial efforts are geared toward finding new ways to kill bacteria, it is not lack of antibiotics *per se*, but rather the increase of resistant bacteria that has made bacterial infections difficult to treat (Table 1). For example *S. aureus*, now the most common cause of hospital-acquired infections, is a bacteria whose resistance rates have risen 15 fold in the last 20 years.[5] While methicillin-susceptible *S. aureus* can be treated with oxacillin at concentrations lower than 2 μg ml^{-1},[27] resistant strains have a minimum inhibitory concentration (MIC) as high as 200 μg ml^{-1}.[8] *E. faecalis*, a cause of enterococcal bacteremia that is lethal in 40% of patients,[28] are susceptible to vancomycin at 0.5 μg mL^{-1} in its nonresistant form,[19] but resistances as high as 128 μg mL^{-1} have been observed in the clinic.[29] Furthermore, *Shigella* species kill 1.1 million people each year due in part to their growing resistance to standard therapies.[30,31] In short, bacterial infections that are susceptible to standard antibiotics are treated more easily, successfully, and at a lower cost than resistant bacteria.

1.2 The role of plasmids in bacterial resistance to antibiotics

Plasmids are small, extrachromosomal pieces of DNA that reside in bacterial cells. Plasmids often encode proteins that allow the bacteria to grow in certain conditions, such as in the presence of an antibiotic.[32] Many plasmids are easily transferable, not only from cell-to-cell of the same bacterial type, but also across genus and species, making them a primary method by which antibiotic resistance genes are disseminated.[32,33] Although bacteria can also be resistant to antibiotics due to mutation of chromosomal genes, this is a relatively rare event, occurring at a rate of 10^{-6} to 10^{-10} per organism.[33] It is believed to be more probable that a nonresistant cell will gain resistance through lateral DNA transfer, which can occur at a rate of one in ten organisms.[33]

Table 1 MIC values (μg mL^{-1}) of resistant and susceptible bacteria

Bacterium	Drug	Susceptible	Resistant
S. aureus	Methicillin	2[8]	4–200[8]
Enterococci spp.	Vancomycin	0.5[19]	>1000[20]
S. pneumoniae	Penicillin	<1[21]	>2[21]
Enterobacteriaceae spp.	Ampicillin	<8[22]	>32[22]
E. coli	Ciprofloxacin	<1[23]	256[24]
P. aeruginosa	Aminoglycosides	4[25]	>1024[26]
Haemophilus spp.	Ampicillin	<2[22]	>4[22]

Bacteria often become resistant to β-lactams,[34] macrolides,[35] tetracyclines,[36] aminoglycosides,[37] quinolones,[24,38,39] and glycopeptides[40] (such as vancomycin) by virtue of plasmid-encoded proteins, and plasmid-mediated resistance is found in both Gram positive[41] and Gram negative[42] bacteria. For example, certain strains of *Yersinia pestis*, the causative agent in the plague, are no longer sensitive to several antibiotics (including ampicillin, chloramphenicol, sulfonamides, and tetracycline) because of plasmid-encoded resistance.[33]

Additionally, vancomycin resistance in *Enterococcus* species is often carried on a plasmid, increasing the chance that resistance will spread to other organisms whose treatment options are already limited.[57] In fact, vancomycin resistance was recently found in MRSA; it is believed that the MRSA acquired resistance to vancomycin through plasmid transfer from vancomycin-resistant enterococci (VRE).[58,59] ESBLs, which mediate resistance to extended spectrum cephalosporins, are typically plasmid encoded and are easily passed among different members of *Enterobacteriaceae* (such as *Shigella* and *Klebsiella* mentioned earlier).[10] Plasmid-mediated resistance to Amp C-type β-lactams has been seen in clinical isolates of *K. pneumoniae*, *E. coli*, *P. mirabilis*, *E. aerogenes*, and *Salmonella*.[60–63] In summary, plasmid-encoded resistance has been observed for a wide variety of bacteria and a large number of antibiotics. Listed in Table 2 are several other examples of plasmid-encoded bacterial resistance to antibiotics.

Although this Perspective focuses on plasmid-mediated antibiotic resistance, it is also worth nothing that plasmids can code for virulence factors that contribute to the ability of bacteria to cause an infection. For example, *Bacillus anthracis*, which causes the life-threatening disease anthrax, is not deadly without the two plasmids PXO1 and PXO2 that contain genes encoding virulence factors.[52,53] *K. pneumoniae*, a cause of pneumonia and a factor in an estimated 8% of nosocomial infections in developed nations, harbors a plasmid that increases its virulence 1000-fold.[52,53]

1.3 Mechanisms of plasmid copy number control

Although plasmids replicate with the assistance of host factors, they have their own replication machinery separate from that of cells they inhabit. In order to ensure that the number of plasmids per cell (copy number) is stable over time, several mechanisms have evolved to regulate plasmid replication and tightly control plasmid copy number.

If the copy number of a plasmid becomes too low, plasmid-free cells (which replicate faster due to their reduced genetic burden) will eventually outnumber the cells with plasmid. Conversely, if there are too many copies of a plasmid in the cell, plasmid replication can deplete resources required for normal cellular function.[64] Although there are multitudes of different plasmids, the delicate balance of plasmid copy number is determined by only a handful of major types of replication/copy number control elements (Fig. 1).

This Perspective details the potential for targeting plasmid copy number control systems to effect the elimination of plasmids from drug-resistant bacteria. Such plasmid-free cells would now be sensitive to standard antibiotics. Two common mechanisms for plasmid replication control are covered in detail: the countertranscript RNA (ctRNA)-based control mechanisms, and the plasmid addiction systems. For each of these examples, the potential for small drug-like organic compounds to bind to specific macromolecules involved in plasmid replication and induce plasmid elimination is discussed.

Table 2 Plasmid-encoded resistance and virulence

Plasmid	Harbored by[a]	Associated disease[a]	Virulence/resistance gene(s)	Plasmid details
pJHCMW1[43,44]	*K. pneumoniae*	Neonatal meningitis & septicemia	Resistance: *aac(6′)-Ib*, *aadA1*, bla_{OXA-9}, and bla_{TEM-1}	Resistance to amikacin, kanamycin, tobramycin, ampicillin; Tn*1331*.
pIP1202[45]	*Y. pestis*	Plague	Resistance: *aph(3′)-I*, *aad*($3''$)(*9*), *tet*(*D*), *sulI*	Resistance to ampicillin, chloramphenicol, kanamycin, streptomycin, spectinomycin, sulfonamides, tetracycline, and minocycline.
pSK41[46,47]	*S. aureus*	Impetigo	Resistance: *smr*, *dfrA*, *aacA-aphD*	Resistance to antiseptics, disinfectants, trimethoprim, aminoglycosides; Tn*4001*.
pACM1[48]	*K. oxytoca*	Neonatal bacteremia	Resistance: SHV-5 extended spectrum β-lactamase (ESBL)	Resistance to β-lactams, trimethoprim, sulfonamides, tetracycline, aminoglycosides, and mercuric chloride.
pBRG1[49,50]	*E. faecium*	Surgical wound infection	Resistance: *vanA*	Resistance to vancomycin.
pLVPK[51]	*K. pneumoniae*	Pneumonia	Virulence: *iucABCDiutA*, *vagCD*, *shiF*, *rmpA2*	Virulence is increased 1000-fold when plasmid is present.
pXO1, pXO2[52–54]	*B. anthracis*	Anthrax	Virulence: *pagA*, *lef* (LF)	Plasmid is essential to pathogenesis.
pSLT[55]	*S. typhimurium*	Gastroenteritis	Virulence: *rck*, *pef*, *spv*	All virulent *S. typhimurium* strains carry this plasmid.
EAF[56]	*E. coli*	Pediatric diarrhoea	Virulence: *bfpA*	Plasmid is essential for adherence and, therefore, virulence.

[a] One example given.

Fig. 1 Plasmid copy number control systems. This Perspective focuses on the control mechanisms highlighted in orange—ctRNA and the addiction systems—as possible targets for antiplasmid agents.†

2 Targeting ctRNA-based replication control to induce plasmid elimination

2.1 Plasmid replication control

Replication control is the primary method of copy number regulation for medium copy number plasmids. The two basic types of replication control, categorized by their control elements, are countertranscript RNA-based systems and iteron-binding systems;[32,64–66] this latter system will not be discussed here in detail. In ctRNA-based systems, the plasmid codes for both an "essential RNA," one that is necessary for plasmid replication, and a countertranscript of the essential RNA (the ctRNA). The ctRNA binds to its complimentary essential RNA, inhibiting (directly or indirectly) replication of the plasmid (Fig. 2). The inhibitory ctRNA is synthesized at a higher rate than that of the essential RNA.[67] Because the ctRNA is transcribed by a constitutive promoter and has a short half-life, its intracellular concentration is proportional to plasmid copy number.[67] ctRNAs can regulate plasmid copy number (either alone or with the help of proteins) by a variety of mechanisms including inhibition of primer maturation, inhibition of translation of the essential Rep protein, transcriptional attenuation, or inhibition of pseudoknot formation.[68] In all cases, the ctRNA is small, untranslated, and highly structured.

The ctRNA often serves as the cellular entity that determines the incompatibility grouping of a plasmid.[68] Two plasmids are said to be incompatible when they fail to co-segregate into daughter cells, leading to elimination of one of the plasmids from the cellular population (Fig. 3A).[66] This natural mechanism for plasmid elimination can be used as a model for designing a small molecule-inducer of plasmid elimination, as overviewed in Fig. 3B. If the plasmid is the sole source of resistance genes, plasmid elimination would cause once-resistant bacteria to become susceptible to standard antibiotics. Because the ctRNA is the incompatibility determinant, using a small molecule to mimic its interaction with the essential RNA should lead to plasmid loss.

2.2 Targeting ctRNA copy number control to inhibit plasmid replication: the IncB system

Plasmids in the IncB incompatibility group contain a ctRNA-based replication control system where RNAI, the ctRNA, acts as the incompatibility determinant (Fig. 4A). As such, IncB plasmids are representative of the larger group of plasmids that use small highly structured RNAs to control copy

† Although low copy number plasmids also use the replication control elements (ctRNA or iteron binding), they are more closely controlled by special systems developed to prevent plasmid loss (*i.e.* addiction and partitioning systems).

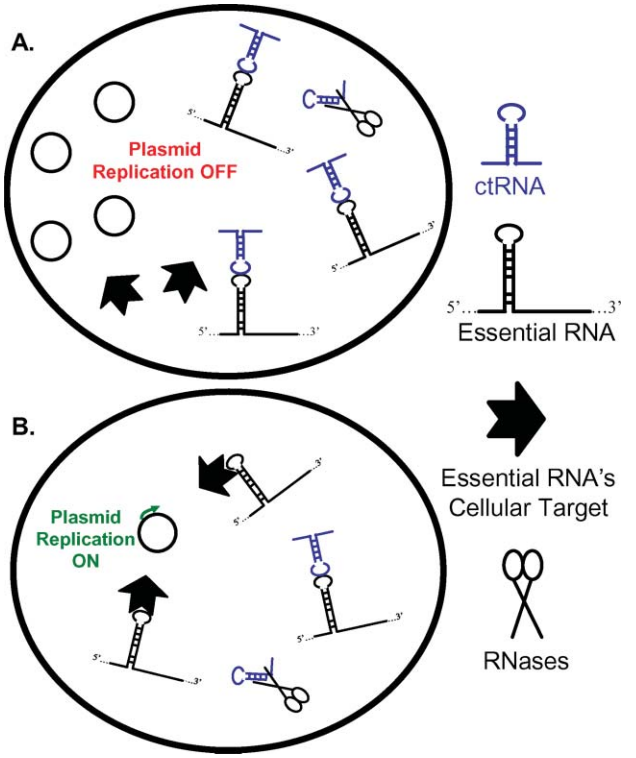

Fig. 2 ctRNA-based replication control. **A**. Cell containing the proper amount of plasmid. ctRNA prevents essential RNA from binding its target and turning on replication. **B**. When plasmid copy number is low the essential RNA turns on plasmid replication.

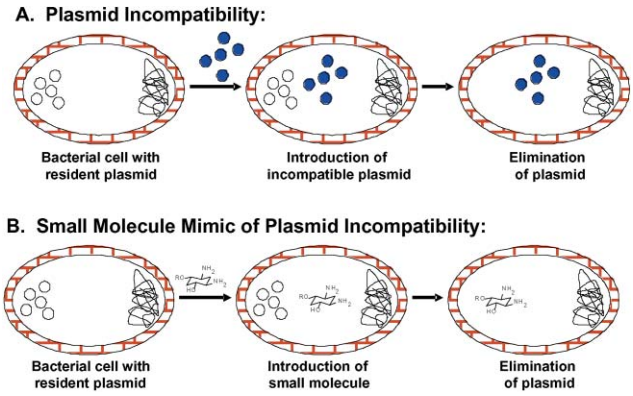

Fig. 3 **A** Plasmid incompatibility. Two plasmids that utilize similar copy number control elements are unable to co-segregate into daughter cells, resulting in elimination of one of the plasmids from the bacterial population. **B**. Mimicking this natural plasmid incompatibility process with a small molecule can cause plasmid elimination and a resensitization of the bacteria to standard antibiotics.

number. In IncB plasmids the RepA protein is essential for plasmid replication.[69,70] Translation of RepA is controlled by a pseudoknot complex between stem-loop I (SLI) and stem-loop III (SLIII) of the RepA mRNA (Fig. 4B);[71] formation of the pseudoknot allows ribosome binding and RepA translation. RNAI acts by binding SLI and preventing formation of the essential pseudoknot and thus shutting down plasmid replication (Fig. 4A).[72]

A recent report details an effort to mimic plasmid incompatibility and effect plasmid elimination with a small molecule. Compounds that bound SLI were sought and apramycin, an aminoglycoside, was found to bind SLI near the important SLI–SLIII regulatory region with a dissociation constant of 93 nM.[73] Further *in vivo* testing demonstrated that apramycin causes dose-dependent plasmid loss; almost complete plasmid elimination was observed at 18 μg mL^{-1} after approximately 250 bacterial generations.[73] Although apramycin was employed

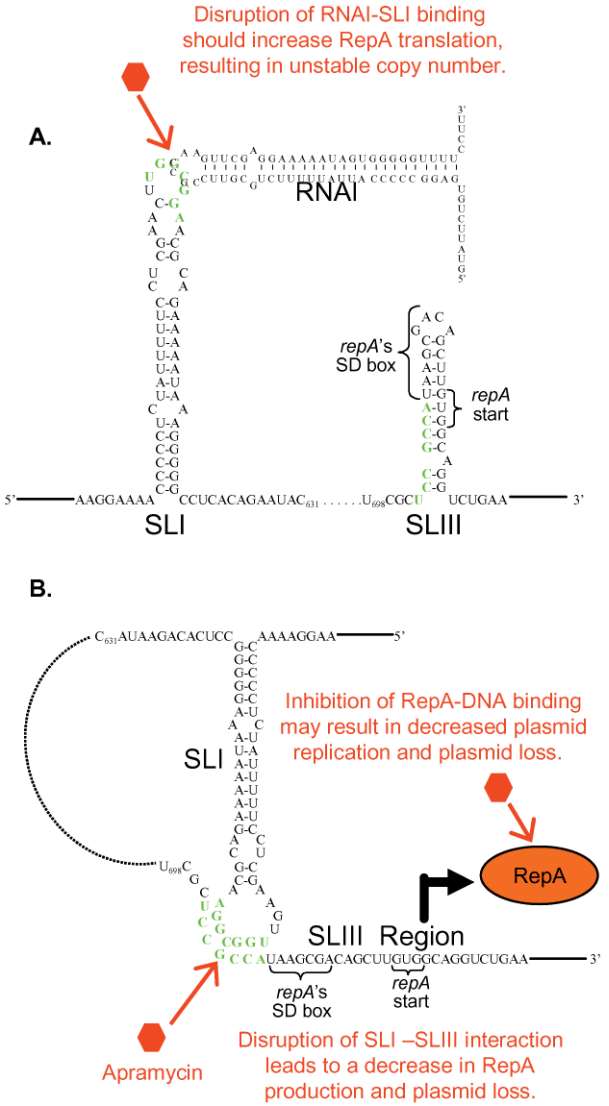

Fig. 4 IncB replication control. **A**. The Shine–Delgarno sequence as well as the start codon of *repA* is sequestered in the stem-loop structure of SLIII, preventing translation. RNAI, the countertranscript to SLI, forms an extended loop–loop kissing complex with SLI, preventing SLI–SLIII binding. **B**. When SLI binds SLIII, translation of RepA can occur. A small molecule (red hexagon) that binds SLI has been used to disrupt this interaction and effect plasmid loss. It is envisioned that a small molecule that disrupts SLI-RNAI binding, or directly interacts with the RepA protein would also lead to plasmid loss.

at subinhibitory concentrations, aminoglycosides are known antibiotics by virtue of their binding the ribosome. Thus, to confirm that binding to SLI (rather than a non-specific stress effect) caused plasmid elimination, a mutant of SLI that does not bind apramycin was identified *in vitro* and tested *in vivo*. No plasmid loss was observed when bacteria containing the mutant plasmid were grown in the presence of apramycin.[73]

While more rapid elimination times and a less toxic class of molecules might ultimately be desired, this study demonstrates that a small molecule can mimic plasmid incompatibility, eliminate plasmid, and re-sensitize bacteria to antibiotics. Moreover, because the majority of plasmids contributing to drug resistance in clinical isolates use a ctRNA as their replication control mechanism and incompatibility determinant,[33] the ctRNA is a potentially general target that could be exploited to combat plasmid-mediated resistance in a variety of bacterial hosts. Furthermore, it appears that many plasmids of this type have a consensus YUNR (Y = pyrimidine, U = uridine, N = any base, R = purine) sequence in the critical RNA stem-loop.[74,75] This sequence, which consists of the first four bases on the 5′ side of the loop sequence, has been found in 45 different

prokaryotic replication control elements and a variety of plasmid incompatibility groups (including IncB), indicating the potential generality of this approach.[74,75]

There are several other cellular entities that could be targeted to disrupt plasmid copy number and cause plasmid elimination from ctRNA-based replication control systems; some of these are highlighted in Fig. 4. As the RepA protein is necessary for plasmid replication, a small molecule that directly inhibits the binding of RepA to DNA could lead to inhibition of plasmid replication. By the same token, if a preprimer is required, disruption of preprimer annealing could lead to plasmid loss. Conversely, compounds that dramatically increase the production of the RepA protein would likely be useful. For example, a molecule that disrupts the SLI–RNAI interaction would cause an unchecked increase in RepA production, possibly resulting in an unstable copy number and eventual loss of plasmid (Fig. 4).

3 Targeting addiction systems to trigger cell death

Plasmid replication is a burden on the host, thus large plasmids are generally present in only a few copies per cell. Plasmids containing several drug resistance genes are generally large and therefore have a low copy number (LCN).[76,77] While LCN plasmids typically control replication through the same mechanisms as medium copy number plasmids (iteron-binding and ctRNA-based replication control systems), they utilize other means to ensure stable plasmid inheritance. Unlike higher copy number plasmids, which are inherited efficiently using random segregation alone,[78] a cell with a copy number of five will give rise to a plasmid-free cell approximately once every 16 divisions if left to random segregation.[78] Therefore, intricate plasmid stability systems have evolved to ensure that bacterial cells retain these large, low copy number plasmids.

One example of this type of stability system is plasmid partitioning. This method, analogous to eukaryotic mitosis, shuttles plasmids into each of the newly formed cells.[78,79] Many plasmids, including salmonella virulence plasmids, IncFII, F, and P1 plasmids, contain some type of partitioning system.[78] Additionally, plasmids that share the same partitioning system are incompatible.[78]

A second mechanism by which LCN plasmids avoid the propagation of plasmid-free daughter cells is through the employment of a plasmid addiction system.[80] Unlike the partitioning system, which facilitates plasmid movement into each of the daughter cells, an addiction system functions as a post-segregational killing mechanism to execute cells that have lost the plasmid.[81] Addiction systems can be thought of as a mechanism of bacterial programmed cell death, comparable to apoptosis in eukaryotic cells.[82] In an addiction system, the plasmid expresses both a toxin and an antitoxin (Fig. 5). The antitoxin is expressed at a higher rate and thus will bind the toxin and prevent cell death, provided that plasmid is present. However, the antitoxin has a much shorter half-life than the toxin. Therefore, when a daughter cell is produced without plasmid, the antitoxin is quickly degraded, leaving the stable toxin to kill the cell. In many cases, both the toxin and antitoxin are proteins.[82] However, there are also types of addiction systems in which the toxin is a protein and the antitoxin is an RNA that functions to prevent the translation of the stable toxin-encoding mRNA.[83] As highlighted below, some examples of plasmids that contain addiction systems include the RNA-based *hok–sok* system from plasmid R1[83] and the protein-based systems *ccd* from plasmid F1 and *pemI–K* from plasmid R100.[81]

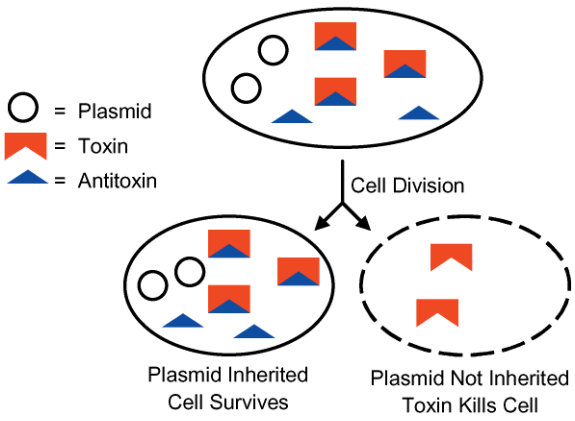

Fig. 5 Toxin–antitoxin system. If a daughter cell is produced without plasmid, the unstable antitoxin is rapidly degraded and the toxin will kill the cell.

3.1 RNA-based addiction systems

In RNA-based addiction systems, the antitoxin is a plasmid-encoded RNA that is constitutively expressed but rapidly degraded. The mRNA of the toxin (also plasmid-encoded) is unusually stable and will therefore be present in daughter cells, even if the plasmid is not inherited. The antitoxin prevents translation of the toxin by binding the mRNA of the toxic protein, leading to RNase cleavage and destruction of toxin mRNA. One well-characterized example of RNA-based addiction is the *hok–sok* system utilized by the R1 plasmid.[83]

R1 confers resistance to several different antibiotics including chloramphenicol, kanamycin, ampicillin, streptomycin, spectinomycin, and sulfonamides.[84] It is a very large plasmid, consisting of approximately 100 kb,[85] and uses several mechanisms to ensure proper copy number. Along with a ctRNA-based replication control system,[68] the *parA* partitioning system,[86] and the kis–kid protein-based addiction system,[80] R1 also employs an RNA-based addiction system to ensure that plasmid-free segregants do not survive. In this system, the Hok (*ho*st *k*illing) protein acts as the toxin by inducing membrane depolarization that releases RNaseI from the periplasm into the cytoplasm, killing the cell from within.[80] Because Hok is not cell permeable, only cells that translate the Hok mRNA and thus produce the toxic Hok protein are killed. *Sok* (*s*uppression *o*f *k*illing), a small antisense RNA transcript of *hok*, acts as the antitoxin by negatively regulating Hok protein expression (Fig. 6).

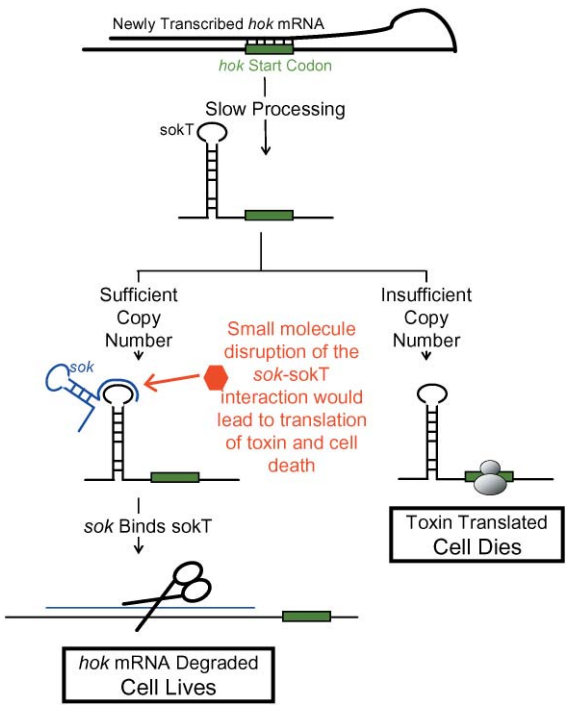

Fig. 6 Hok–Sok addiction system and targets for its inhibition. Disruption of the *sok*–sokT interaction would allow translation of the toxic protein and lead to cell death.

When *hok* and *sok* are transcribed, *sok* transcription occurs at a faster rate than *hok*.[87] As it is transcribed, the *hok* mRNA is folded into a state which cannot be translated; the Shine–Delgarno sequence necessary for ribosome binding is contained in a duplex, as is the start codon. RNases then gradually process the 3′ end of the *hok* mRNA (which has a half-life on the order of hours)[88] until enough is cleaved such that the RNA refolds, revealing the Shine–Delgarno sequence as well as the target for *sok* binding (sokT). Sufficient copy number ensures there will be enough *sok* (which has a half life of less than 30 seconds and is therefore quickly degraded in cells without plasmid)[88] to bind sokT. Once bound, an extended kissing complex is formed between *sok* and sokT. This is followed by RNase III degradation of the Hok mRNA, which prevents translation of the toxic protein.

If a small molecule could be developed to hijack this addiction system by preventing *sok* from binding sokT, then the toxin protein would be translated and kill the cell (Fig. 6).[88] To do this, the molecule could specifically target either sokT or the *sok* RNA itself. Additionally, if a compound bound the Hok mRNA and caused a structural rearrangement such that the binding site for *sok* was not presented, but the Hok Shine–Delgarno sequence and start codon were still exposed, Hok may be translated. Furthermore, because R1 and many other large resistance plasmids use a ctRNA-based replication mechanism, a plasmid elimination strategy (as described for the IncB system) could work to actually eliminate the plasmid, causing the toxin to naturally kill the cell.

3.2 Protein-based addiction systems

Protein-based addiction systems employ proteins as both the toxin and antitoxin.[80,82,89] Several plasmids isolated from patients with drug-resistant infections use a protein-based addiction system to maintain plasmid stability, including pAD1 from *E. faecalis*, and pSM19035 from *S. pyogenes*.[80,81] The intracellular targets of these toxic proteins include DNA gyrase and DnaB, but there are several protein-based addiction systems in which the target of the toxin is unknown.[80]

A well-characterized model for protein-based plasmid addiction is the Pem I–K system of plasmid R100; this plasmid was originally isolated from a patient infected with *Shigella flexneri*.[90,91] In this plasmid system, the 12 kDa PemK (plasmid emergency maintenance killer) protein inhibits the growth of the cell by disrupting DnaB-dependent DNA replication.[92] The 9.3 kDa PemI protein (plasmid emergency maintenance inhibitor) binds PemK, suppressing its function (Fig. 7).[93] Although PemI and PemK are transcribed from the same promoter, PemI is also encoded by a second gene, ensuring a higher concentration of this protein relative to PemK.[93] While PemI is quickly degraded by the Lon protease, PemK is fairly stable; thus if a daughter cell does not contain plasmid, only toxic PemK protein will be present.[94]

Is it possible to turn this intricate toxin–antitoxin against the drug-resistant bacterium? A small molecule that binds either the PemI or PemK proteins in the critical PemI–PemK binding region could disrupt the protein–protein interaction, allowing release of the free PemK toxin and subsequent cell death (Fig. 7). Importantly, there are several examples of other protein-based addiction systems in which the proteins have significant homology to PemI–K, indicative of the potential generality of small molecule toxin–antitoxin disruptors.[80,82,89] Additionally, a small molecule that binds the PemI mRNA may be able to prevent its translation, allowing PemK to persist while the remaining PemI is degraded. Plasmid elimination methods could also be used to force the PemK to inhibit DNA replication.

4 Targeting plasmids as a medicinal strategy

Plasmids offer multiple unique targets for treatment of drug-resistant bacteria. As such, the use of small molecules to target

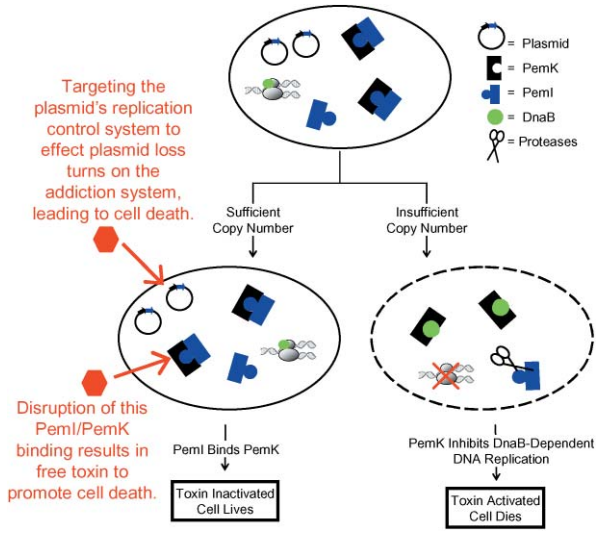

Fig. 7 PemI–K addiction system. If DnaB-dependent DNA replication is inhibited by PemK, the cell dies. A small molecule that disrupted the PemI–PemK interaction could allow PemK to bind DnaB, leading to cell death. Likewise, targeting the plasmid's replication control system (*i.e.* the ctRNA) to effect plasmid loss would turn on the addiction system, killing the cell.

specific copy number control mechanisms and induce plasmid elimination likely has benefits over methods that rely on general cellular stress for plasmid ejection.[95] However, there are many challenges that must be overcome before this strategy can become useful clinically.

For example, whether the target is ctRNA-based replication or an addiction system, studies must be done on clinically relevant bacterial strains in animal models to demonstrate the legitimacy of the approach. One challenge unique to plasmid elimination will be the mode of administration of the antiplasmid agent–antibiotic combination. In one incarnation, the antiplasmid compound could be administered over a period of time sufficient to induce plasmid loss, followed by standard antibiotic treatment. The success of such an approach hinges on the use of a compound that induces a fairly rapid elimination of plasmid, such that the patient does not become gravely ill during the antiplasmid phase of this treatment.

In addition, resistance genes can sometimes be found on transposable elements which can leap from plasmid to chromosome or plasmid to plasmid, rendering elimination of the original resistance plasmid ineffective.[96] Ultimately, however, chromosomally-encoded resistance is less likely to be transferred to another cell and thus the forcing of resistance genes into the chromosome might have some overall benefits. Interestingly, with the plasmid elimination method there is no selective pressure for the bacteria to keep the plasmid in the elimination step of the treatment. Therefore, there is no certainty that transposition to chromosome or mutation will occur.

A final challenge is the heterogeneity that exists among plasmids. If a different small molecule needed to be developed for each plasmid, this would be a less than ideal situation. In this vein, a major challenge will be to define precisely the copy number control mechanisms of plasmids isolated from patients who have a wide variety drug-resistant infections, and to choose those targets possessing the greatest homology.

Although it will undoubtedly take significant effort for antiplasmid agents to become clinically viable, they may one day allow for treatment of currently intractable infections. In addition, there are many known antibiotics that are no longer effective due to the prevalence of resistant strains of bacteria; an antiplasmid approach could rejuvenate these antibiotics, enabling them to be effective once again. Finally, bacteria whose virulence is plasmid encoded could also be vanquished with the help of antiplasmid agents.

5 Conclusion

Plasmid-mediated bacterial resistance to antibiotics is a growing concern in the medical community. Many of these drug-resistant infections are already quite prevalent, like MRSA and bacteria harboring the genes for ESBL. Others, such as VRE or *Bacillus anthracis*, are problems on the immediate horizon that are likely to become more common. Targeting resistance- or virulence-causing plasmids, whether through ctRNA-based replication control or an alternative copy number control system, offers a new weapon in the fight against bacterial infections.

Acknowledgements

This work is supported by the National Institutes of Health (NIHGMS R01-GM68385) and the Office of Naval Research (N00014-02-1-0390). We thank Jason R. Thomas and Dinty J. Musk (University of Illinois) for their input and careful review of this Perspective.

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