Bacterial plasmid addiction systems and their implications for antibiotic drug development

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Abstract
Bacteria frequently carry mobile genetic elements capable of being passed to other bacterial cells. An example of this is the transfer of plasmids (small, circular DNA molecules) that often contain antibiotic resistance genes from one bacterium to another. Plasmids have evolved mechanisms to ensure their survival through generations by employing plasmids segregation and replication machinery and plasmid addiction systems. Plasmid addiction systems utilize a post-segregational killing of cells that have not received a plasmid. In this review, the types of plasmid addiction systems are described as well as their prevalence in antibiotic resistant bacteria. Lastly, the possibility of targeting these plasmid addiction systems for the treatment of antibiotic resistant bacterial infections is explored.
Keywords: plasmid, toxin-antitoxin system, plasmid addiction system, antibiotics, antimicrobial resistance

Introduction

Bacteria often carry mobile genetic elements capable of being passed from bacterium to bacterium. One such element is the plasmid, a small, circular, double-stranded DNA molecule. Plasmids can be transferred to daughter cells upon replication (vertically transferred) or to non-offspring cells (horizontally transferred). Horizontal transfer of genetic material between bacterial cells can occur within the same or different bacterial species. Plasmids often encode genes that provide its host with a survival advantage under specific environmental conditions (ex: metabolic constraints or presence of antimicrobials). On the other hand, maintenance of a plasmid means an added metabolic burden of plasmid replications and gene expression. Thus it is only logical that plasmids may be lost if they do not provide an advantage to the host at the given time (ex: environmental pressures relieved or no longer metabolically advantageous).

Plasmids range in size from less than three kilobases to over a hundred kilobases. Small plasmids can be represented as high as hundreds of copies per cell. Large plasmids on the other hand may be maintained at less than 10 copies per cell. The high copy number of small plasmids usually ensures that plasmids are divided between both daughter cells. However, low-copy number plasmids must use specific mechanisms to ensure that future cell populations retain plasmids. For example, by random segregation a dividing cell with two copies of a plasmid has a 50% chance of both cells acquiring one plasmid and a 50% chance of one cell receiving both plasmids while the other cell receives none. Because bacteria that harbor plasmid DNA often times replicate at slower rates than their isogenic counterparts due to the added energy consumption of plasmid replication, cells bearing plasmids may eventually be outcompeted by cells lacking plasmids under non-selective conditions.

Thus, plasmids remain because they have evolved many mechanisms to ensure their survival. Plasmids large and small encode mechanisms that control plasmid copy number or initiate replication from the plasmid origin of replication. Due to their low copy number, large plasmids must also employ plasmid-partitioning systems. Plasmid-partitioning systems ensure that plasmids are divided amongst daughter cells thus preventing plasmid loss during cell division (1). Plasmids have also evolved to contain plasmid addiction systems, “selfish” DNA elements that ensure that only cells

containing plasmid are viable and those that have lost plasmid are killed. These selfish DNA elements have the sole purpose of replicating itself regardless of cost to the host organism and are especially useful for large plasmids to ensure they are passed on through generations.

Overview of plasmid addiction systems

To prevent plasmid loss from populations, many plasmids encode plasmid addiction systems (PASs) to kill cells that have lost their plasmids through post-segregational killing effects (Fig. 1). PASs found in nature consist of a stable toxin protein and an unstable antitoxin protein. Though the exact mechanism of these toxin-antitoxin systems are diverse, these mechanisms have the following general properties: (1) the toxin and antitoxin are encoded by two adjacent genes and are transcribed as an operon, (2) the antitoxin is encoded upstream of the toxin and is synthesized in excess, (3) the product of one is long-lived and toxic while the product of the second is short-lived and antitoxic, (4) toxin and antitoxin proteins interact, (5) the antitoxin is degraded by bacterial proteases, and (6) the toxin-antitoxin system is self-regulated at the transcriptional level either by the toxin-antitoxin complex or by the antitoxin alone (2).

Types of plasmid addiction systems

There are many distinct mechanisms for toxin-antitoxin pairs, but most fall into three forms: (1) protein-regulated systems, (2) antisense RNA-regulated systems and (3) restriction modification systems.

(1) Protein-regulated plasmid addiction systems

As the name suggests, a toxic protein mediates post-segregational killing of cells that have lost plasmid. In cases where the plasmid is maintained among cells during division, the antitoxin is continually produced, inhibits the toxin through direct interaction, and cells remain viable. However, when the plasmid is lost during replication, the unstable nature of

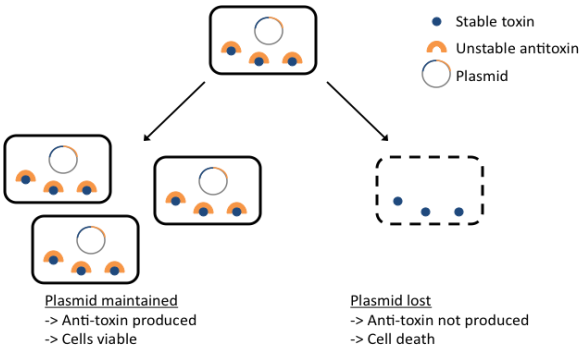


Figure 1: Plasmid addiction system overview. Bacterial cells that carry plasmid addiction systems have two fates upon cell division: (1) Plasmids are maintained post-segregation and cells remain viable due to constant production of the anti-toxin (left) and (2) Plasmids are lost post-segregation resulting in cell death due to degradation of anti-toxin without constant production (right).

the antitoxin leaves the toxin unsequestered, and thus cell death occurs. Toxins in these systems work in many mechanisms.

One of the most well-known toxin-antitoxin systems is the MazE/MazF toxin-antitoxin system. The *mazE* and *mazF* genes were first identified in 1988 (3) but their roles were not elucidated until 1996 (4). MazF is a long-loved toxin while MazE is a short-lived anti-toxin. MazE is constitutively cleaved by the ClpAP protease and without the continual production of MazE, the MazF toxin becomes active. The toxic effect of MazF is due to its ribonuclease activity where it cleaves specific sequences in RNA thus blocking the majority of protein synthesis. MazF has a preference for cleaving ACA motifs in mRNA (5). It is possible that some mRNA sequences encoding cell death proteins do not have ACA motifs making them resistant to MaZF cleavage (6). The degradation of other proteins and the "immunity" of cell death proteins to MazF both synergistically lead to the demise of plasmid-less cells.

(2) Antisense RNA-regulated plasmid addiction systems

Antisense RNA is a single-stranded RNA that is complementary to messenger RNA (mRNA). When antisense RNA base pairs to its cognate mRNA, it inhibits its translation. Like the

antitoxin protein described above, antisense RNA is inherently unstable. Certain plasmid addition systems take advantage of the fact that antisense RNA is unstable compared to the mRNA they inhibit. Antisense RNA-regulated plasmid addition systems consist of a toxin-encoding mRNA and an antisense RNA to the mRNA (7). When improper plasmid segregation occurs, cells without plasmid will eventually lose the antisense RNA. This allows the toxin mRNA to be translated to kill the cells.

One of the most well studied antisense RNA-regulated plasmid addiction systems is the *hok*/*sok* system from the plasmid R1 in *E*. *coli*. The *hok*/*sok* locus consists of three genes: *hok*, *mok*, and *sok*. The *hok* gene encodes a toxic protein and *sok* encodes an unstable antisense RNA that binds to *hok* mRNA. The third gene, *mok*, overlaps with the *hok* gene and is required for translation of *hok* through translational coupling mechanisms (8). Translation of the *mok* transcript is blocked by the *sok* anti-sense RNA (8). The Sok-RNA binds to *hok* mRNA and the resulting RNA duplex is cleaved by RNase III (9). The Hok protein kills the host cell from within by damaging the cell membrane leading to a loss of membrane potential, arrest of respiration, changes in cell morphology, and eventually cell death (10).

(3) Restriction/modification plasmid addiction systems

The third type of plasmid addiction is mediated by restriction/modification-based systems. These systems involve a restriction endonuclease that serves as the toxin and a methyltransferase that serves as the antitoxin. Restriction endonucleases cleave DNA at or near a specific recognition nucleotide sequence called a restriction site. To prevent uncontrolled endonuclease activity, the methyltrasnferase adds a methyl group to a base within a specific nucleotide sequence to prevent the cognate endonuclease from cleaving the DNA. Like the other toxin-antitoxin system described above, the restriction endonuclease is more stable than the methyltransferase. This means that if plasmid is lost, the methyltransferase is degraded and/or diluted as the cell grows leading to the appearance of unmethylated sites in the chromosome. Then, the endonuclease creates double strand breaks in the chromosome eventually causing death of plasmid-free cells.

Restriction-modification systems were first implicated as plasmid addiction systems in 1995 by the study of *Eco*RI (from *E*. *coli*) and *Bsp*6I (from *Bacillus* sp.) (11). These researchers monitored plasmid loss over a hundred generations in cells containing an unstable plasmid (11). In the wild type, 97% of the cells lose plasmid after 100 generations. When genes for a restriction/modification system were introduced in the plasmid, only 25% of the cells lose the plasmid after 100 generations. The researchers showed that the plasmid stability is a direct effect of the endonuclease because when the endonuclease gene was mutated, 98.5% of the cells lost the plasmid after 100 generations. Thus, restriction-modification systems enhanced plasmid segregation stability.

Plasmids carrying both antimicrobial resistance genes and plasmid addiction systems

Many bacteria are resistant to antimicrobials due to genes encoded on plasmids both large and small. Antibiotic resistance genes have transferred from between different bacterial species and have made even the jump from livestock microbes to human pathogens (12). These plasmids have spread widely and easily across bacterial species and across the globe causing public health issues when once treatable infections are no longer treatable. Please see a review from Fernandez-Garcia and coworkers for a more thorough review of plasmid addiction systems in bacterial pathogens (13).

One such example of plasmid-encoded antibiotic resistance microbes are vancomycin-

resistant enterococci (VRE) that are common hospital-acquired pathogens and resistant to many classes of antibiotics. Moritz and Hergenrother probed for the presence of toxin-antitoxin (TA) systems in 75 VRE isolates (14). They found several TA systems in VRE and the MazEF TA system was present in all isolates tested. In more than 90% of strains tested, *mazEF* was encoded on the same plasmid as *vanA* (conferring vancomycin resistance) assuring propagation of a *vanA* gene in subsequent generations.

Extended-spectrum beta-lactamases (ESBLs) were first reported as hospital-acquired pathogens in 1985 (15). Mnif and coworkers analyzed a collection of 125 epidemiologically unrelated ESBL-producing *E. coli* isolated between 1997 and 2002 in Paris, Brest, Amiens and Strasbourg (16). These researchers sought to identify five plasmid toxin-antitoxin systems and three plasmid antisense RNA-regulated systems by PCR. In total, they found 298 plasmid addiction systems in these 125 strains indicating that many isolates encode more than one type of plasmid addiction system.

Methicillin was first used in 1959 to treat penicillin-resistant *Staphylococcus aureus* infections. Strains of bacteria have evolved plasmid-based resistance to methicillin and outbreaks of methicillin-resistant *S. aureus* (MRSA) have become prevalence since the 1980s. A study of 78 methicillin-resistant *Staphylococcus aureus* (MRSA) and 42 *Pseudomonas aeruginosa* identified toxin-antitoxin systems in 100% of strains tested (17). Unlike the toxin-antitoxin systems discussed above, all of these systems were encoded chromosomally. Despite the location of the toxin-antitoxin system, these chromosomally-encoded systems can also be effective targets for drug development.

Another example is the carbapenem resistant *Enterobacteriaceae* (CRE), one of the latest antimicrobial resistant organisms to sweep the globe. Carbapenem is a drug of last resort meaning that they are only used when all other treatment options are exhausted. Thus, resistance to these drugs often results in fatal infections. Carbapenem resistance is encoded on large plasmids, many of which also encode TA systems. TA systems have been found on carbapenemase-encoding plasmids in *K. pneumoniae*, *Acinetobacter baumannii* (18), and *Pseudomonas aeruginosa* (19).

Targeting plasmid addiction systems in treating antimicrobial resistance

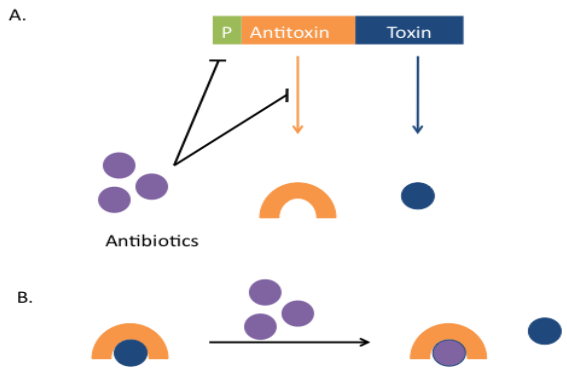
While plasmid addition systems ensure survival of antimicrobial resistance genes among a population, these systems may also become a vulnerable target for drug development. As we have exhausted our antibiotic options, targeting plasmid addiction systems is a viable option for both eliminating plasmids bearing antimicrobial resistance gene and for killing antimicrobial resistant bacteria.

Drugs can target plasmid addiction systems in several ways. For example, a drug can inhibit transcription or translation of the toxin-antitoxin locus. This means that upon drug addition, the anti-toxin is no longer made and the remaining anti-toxin is quickly degraded leading to cell death (Fig. 2A). Alternatively, drugs can disrupt the interaction between the toxin and anti-toxin, also freeing the toxin to kill the cell (Fig. 2B).

Another way small molecules could interfere with plasmid addition systems is to disrupt the usual functioning of the toxin. For example, Wang and Hergenrother developed a high throughput screen to identify small molecules that interfere with MazF/MazE toxin-antitoxin system (20). In this assay, Wang and Hergenrother designed an oligonucleotide with a fluorophore on the 5' end and a quencher on the 3' end. Activity of the MazF ribunuclease

Figure 2:Potential targets sites for plasmid addiction system directed antimicrobials.

(A) Antimicrobials can inhibit transcription of genes encoding the plasmid addiction system or



can inhibit translation of the anti-toxin. (B) Antimicrobials can disrupt interactions between the toxin and the anti-toxin. This frees up the toxin allowing it cause cell death.

would result in an increase in fluorescent signal as the fluorophore is freed from the quencher. Any small molecule that inhibits MazF ribonuclease activity would inhibit this increase in fluorescence. Because of the ubiquity of the MazF/MazE toxin-antioxin system on antibiotic resistance plasmids, assays such as this can identify small molecules that may be used to inhibit plasmid spread and resistance in a myriad of pathogens.

Antibiotics targeting plasmid addiction systems can have an advantage over traditional antibiotics that target bacterial cell walls or translation. Resistance to antibiotics that target plasmid addiction likely means that the toxin has mutated or that the target of the toxin has mutated. Mutations such as these eliminate toxic effects calling into question a further need to keep plasmids in the cell. These mutations mean that over time, the mutated bacteria that are no longer “addicted” to the plasmid will lose plasmid and overtake the bacterial population. During the course of an active infection however, the amount of time needed for bacteria to lose plasmid may be too long to sustain for the host.

Conclusions

Plasmid addiction systems can be thought of as a type of selfish DNA whose sole purpose is to replicate itself regardless if it benefits the host or not. By ensuring that only cells that receive plasmid survive, these systems have allowed plasmids to remain even in the absence of environmental selection. Antibiotics that target these systems have an advantage in that they both treat the infection and rid the cells of plasmids carrying antibiotic resistance genes. While pathogens are becoming more and more resistant to the antimicrobial drugs available today, targeting plasmid addiction systems presents a new way to combat antibiotic resistant bacterial pathogens. By a combination of high throughput screening, medicinal chemistry, and molecular biology, we can better develop antimicrobials that work in this unique fashion.

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References

1. Million-Weaver S, Camps M. 2014. Mechanisms of plasmid segregation: have multicopy plasmids been overlooked? Plasmid 75:27–36. https://doi.org/10.1016/j.plasmid.2014.07.002

2. Engelberg-Kulka H, Glaser G. 1999. Addiction Modules and Programmed Cell Death and Antideath in Bacterial Cultures. Annu Rev Microbiol 53:43–70. https://doi.org/10.1146/annurev.micro.53.1.43

3. Metzger S, Dror IB, Aizenman E, Schreiber G, Toone M, Friesen JD, Cashel M, Glaser G. 1988. The nucleotide sequence and characterization of the relA gene of Escherichia coli. J Biol Chem 263:15699–704.

4. Aizenman E, Engelberg-Kulkat H, Glaser G. 1996. An Escherichia coli chromosomal "addiction module" regulated by 3',5'-bispyrophosphate: A model for programmed bacterial cell death (stringent response/protein degradation/nutritional stress/programmed cell death). Genetics 93:6059–6063.

5. Suzuki M, Zhang J, Liu M, Woychik NA, Inouye M, Inouye M, Xia B, Phadtare S, Ke H,

Acton T, al. et. 2005. Single protein production in living cells facilitated by an mRNA interferase. Mol Cell 18:253–61. https://doi.org/10.1016/j.molcel.2005.03.011

6. Engelberg-Kulka H, Sat B, Reches M, Amitai S, Hazan R. 2004. Bacterial programmed cell death systems as targets for antibiotics. Trends Microbiol 12:66–71. https://doi.org/10.1016/j.tim.2003.12.008

7. Gerdes K, Gultyaev AP, Franch T, Pedersen K, Mikkelsen ND. 1997. ANTISENSE RNA-REGULATED PROGRAMMED CELL DEATH. Annu Rev Genet 31:1–31. https://doi.org/10.1146/annurev.genet.31.1.1

8. Thisted T, Gerdes K. 1992. Mechanism of post-segregational killing by the hok/sok system of plasmid R1. Sok antisense RNA regulates hok gene expression indirectly through the overlapping mok gene. J Mol Biol 223:41–54. https://doi.org/10.1016/0022-2836(92)90714-u

9. Gerdes K, Nielsen A, Thorsted P, Wagner EGH. 1992. Mechanism of killer gene activation. Antisense RNA-dependent RNase III cleavage ensures rapid turn-over of the stable Hok, SrnB and PndA effector messenger RNAs. J Mol Biol 226:637–649. https://doi.org/10.1016/0022-2836(92)90621-P

10. Gerdes K, Bech FW, Jørgensen ST, Løbner-Olesen A, Rasmussen PB, Atlung T, Boe L, Karlstrom O, Molin S, von Meyenburg K. 1986. Mechanism of postsegregational killing by the hok gene product of the parB system of plasmid R1 and its homology with the relF gene product of the E. coli relB operon. EMBO J 5:2023–9.

11. Kulakauskas S, Lubys A, Dusko Ehrlich AS. 1995. DNA Restriction-Modification Systems Mediate Plasmid Maintenance. J Bacteriol 177:3451–3454. https://doi.org/10.1128/jb.177.12.3451-3454.1995

12. Liu Y-Y, Wang Y, Walsh TR, Yi L-X, Zhang R, Spencer J, Doi Y, Tian G, Dong B, Huang X, Yu L-F, Gu D, Ren H, Chen X, Lv L, He D, Zhou H, Liang Z, Liu J-H, Shen J. 2015. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis.

13. Fernández-García L, Blasco L, Lopez M, Bou G, García-Contreras R, Wood T, Tomas M. 2016. Toxin-Antitoxin Systems in Clinical Pathogens. Toxins (Basel) 8:227. https://doi.org/10.3390/toxins8070227

14. Moritz EM, Hergenrother PJ. 2007. Toxin-antitoxin systems are ubiquitous and plasmid-encoded in vancomycin-resistant enterococci. Proc Natl Acad Sci U S A 104:311–6. https://doi.org/10.1073/pnas.0601168104

15. Paterson DL, Bonomo RA. 2005. Extended-spectrum beta-lactamases: a clinical update. Clin Microbiol Rev 18:657–86. https://doi.org/10.1128/CMR.18.4.657-686.2005

16. Mnif B, Vimont S, Boyd A, Bourit E, Picard B, Branger C, Denamur E, Arlet G. Molecular characterization of addiction systems of plasmids encoding extended-spectrum b-lactamases in Escherichia coli.

17. Williams JJ, Halvorsen EM, Dwyer EM, Difazio RM, Hergenrother PJ. Toxin-Antitoxin (TA) Systems are Prevalent and Transcribed in Clinical Isolates of Pseudomonas aeruginosa and Methicillin- Resistant Staphylococcus aureus.

18. Mosqueda N, Gato E, Roca I, Lopez M, de Alegria CR, Fernandez Cuenca F, Martinez-Martinez L, Pachon J, Cisneros JM, Rodriguez-Bano J, Pascual A, Vila J, Bou G, Tomas M, Garnacho J, Pizarraya AG, Marquez Vacaro JA, Cano ME, Farinas MC, Porto AS, Meruendano GE, Vales LB, Ciria JC, Vallejo L, Perez BF, Chao JC V., Ortega BP, Mansilla EC, Irure JJG, del Arco Jimenez A, Cardona CG, Valia JC, Palop NT, Abril V, Rifa J, Jesus M, Garcia M, Pujals J V., Aguirre MN, Vilamala A, Alfaro JAJ, Jaca CR, Casanova PM, Guerreo F, Shaw E, Plasencia V, Mayoral TN, Calavia MJF, de Cruz SG, Mansilla CA, de

Lucas EM, Zorraquino A, Banuls SR, Eseverri EG, Sanchez LL, Gutierrez AF, Guardado AR, Moreno A, Anguera JMG-A, Palmero SL, Maresca MR, Garrote FG, Otero J V., del Pilar Alonso M, Verdu E V., Lopez FR, Sanchez FP, Vizoso EF, Garcia BR, Gurgui M, Pericas R, Pomar V, Astigarraga PMO, Igartua RA, Romero MDM, de Gopegui Bordes ER, Romero MIS, Mata JG, Goyanes MJ, Mateos CM, Quero JH, Lara TE, Bastardie FB, Iftimie S, Bajador IP, Navarro MIG, Gurrea MLC, Antequera CA, Gomez M, Cantudo P, Salas CM, Peragosa JC, Flores AM, Garcia LA, Real SH, Gonzalez PAC, Gonzalez MAP, Fernandez SR, Rojo MP, Tubau F, Alvarez EN, Torres MO, Almaraz SG, Castello MRR, Castillo M, Hortelano E, Sanchez FC, Reyne AG, Gallego JPH, Segura C, Dorado GS, Escudero RY, Hung MED, del Rosario Q C. 2014. Characterization of plasmids carrying the blaOXA-24/40 carbapenemase gene and the genes encoding the AbkA/AbkB proteins of a toxin/antitoxin system. J Antimicrob Chemother 69:2629–2633. https://doi.org/10.1093/jac/dku179

19. Bonnin RA, Poirel L, Nordmann P, Eikmeyer FG, Wibberg D, Puhler A, Schluter A. 2013. Complete sequence of broad-host-range plasmid pNOR-2000 harbouring the metallo- -lactamase gene blaVIM-2 from Pseudomonas aeruginosa. J Antimicrob Chemother 68:1060–1065. https://doi.org/10.1093/jac/dks526

20. Wang NR, Hergenrother PJ. 2007. A continuous fluorometric assay for the assessment of MazF ribonuclease activity. Anal Biochem 371:173–183. https://doi.org/10.1016/j.ab.2007.07.017