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New Methods of Pathogenic Bacteria Elimination

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A b s t r a c t

The growing bacterial resistance to antibiotics calls for the elaboration of new pathogens elimination strategies. Some of these methods are based on the conjugative transfer of recombinant plasmids able to eliminate pathogenic recipients by plasmid run-away replication or by killing activity of plasmid-encoded bacteriocins. Using live bacteria as donors of plasmid vectors carrying killing determinants requires meeting many safety restrictions in order to eliminate potential biohazard.

K e y w o r d s: bio-therapeutic, recombinant plasmids, conjugation

Introduction

Enormous amounts of anti-microbial chemical agents, mainly antibiotics, are produced and used to treat human and animal infections and to promote the growth of animals and plants (Levy, 1998). This overusage, sometimes misusage of antibiotics is correlated with the growing emergence of bacterial resistance to multiple drugs whose dissemination is greatly facilitated by the association of resistance encoding determinants with the mobile genetic elements, *e.g*. integrons, transposons and plasmids (Mazel and Davies, 1999).

The growing bacterial resistance to antibiotics causes that they are no longer effective and many people die from bacterial infections that previously were easily treated with antibiotics. It should be mentioned here that some anti-microbial agents themselves promote the evolution of resistance. Tetracycline, for example, stimulates the transmission of transposons coding for tetracycline resistance (Miller and Sulavik, 1996). Moreover anti-microbial agents that often cause DNA damage or decrease in translation fidelity directly enhance the frequency of mutations and thus can potentially influence bacterial susceptibility to antibiotics (Heinemann, 1999). Another problem hampering safe antibiotic usage in therapy is that they are frequently broad spectrum, indiscriminately killing “good” and pathogenic bacteria.

Many approaches have been taken to find better antimicrobial agents and this task is executed by modification and improvement of traditional antibiotics and development of fundamentally new chemical antibiotics by modern genomic-identified, target-based discovery (Walsh, 2003). This includes, for example, antagonists of bacterial communication (such as quorum sensing) to turn off the expression of virulence factors. A better understanding of bacterial molecular biology will eventually allow us to discover new efficient “smart” antibiotics which will not cause resistance spread (Heinemann, 2001). These drugs probably might have a more limiting therapeutic spectra and will be more expensive and therefore many big pharmaceutic companies have greatly curtailed their antibacterial research (Projan, 2003).

The alarming data presented above indicate a need for alternative anti-microbial agents, mainly bio-therapeutic agents. This role can be played by antibacterial peptides, bacteriophages and mobile elements, mainly plasmids which can be delivered in a natural way to unwanted bacteria resulting in their death. Bacteroiphages and bacteriophage encoding enzymes have been explored and appear to be effective in the treatment of certain pathogenic *E. coli* and *S. pneumoniae* strains (Chibani-Chennoufi *et al*., 2004; Jado *et al*., 2003). Bacteriophages as bio-therapeutic agents are being used in the USA, Poland, Georgia (for review see Carlton, 1999). The major limitations of this therapy are narrow host-specificity and relatively quick development of bacteria resistant to phage infection. The very new strategy of pathogenic

bacteria elimination based on recombinant plasmids transferred to pathogenic recipient, preferentially by conjugation is the scope of presented minireview.

Horizontal DNA transfer in bacteria

Horizontal DNA transfer between various bacterial species and even genera is often observed in the environment. Transfer of DNA to recipient bacteria can be executed by one of the tree mechanisms – conjugation, transformation and transduction among which the first one has the main impact on bacterial variability, diversity and evolution (Davidson, 1999; Wolska, 2003). Conjugation, first described by Lederberg and Tatum (1946) denotes the transfer of DNA from cell to cell by direct contact. It requires a specific type of DNA replication in which one strand of replicated molecule is retained in the donor and the second is transferred to the recipient in 5'→3' direction (Lanka and Wilkins, 1995).

Conjugation functions in gram-negative bacteria are usually plasmid encoded however horizontal transfer of conjugative transposons or genomic islands has also been reported (Schubert *et al*., 2004). Plasmids are small DNA molecules, mainly circular, which are stably maintained in bacterial population. Plasmids replicate extra-chromosomally and a lot of them can transfer their DNA by conjugation. Some plasmids can integrate themselves into the chromosome and are then able to promote transfer of chromosomal markers. Conjugative transfer are quite efficient in the environment.

Briefly, the DNA sequences that control the extra-chromosomal replication of plasmids are called vegetative origins (*oriV*) and *rep* genes, those controlling conjugative transfer are the origins of transfer (*oriT*) and *tra* genes. Gram-negative bacterial conjugative systems demand the presence of special structure – pilus – on the donor cell surface. These systems are grouped together into the type IV secretion system (Christie, 2001) composed of multi component transporters adapted to functions as diverse as conjugative DNA transfer or the delivery of effector proteins into eukaryotic target cells in pathogenesis (Seubert *et al*., 2003). No pili are present in gram-positive conjugation systems in which cell-to-cell contact is facilitated by pheromones (Clewell, 1993).

Conjugative transfer was described in a variety of ecosystems among which human and animal bodies are of major importance in relation to the spread of antibiotic resistance. Conjugative transfer of plasmids encoding multiple drug resistance was reported for coliform bacteria living in human and animal intestine tracts (Balis *et al*., 1996) and for bacteria of human and bovine origins in a farm environment (Oppregaard *et al*., 2001). Transfer of plasmids was also observed in insects *e.g*. in digestive tract of cutworm *Peridroma saucia* (Armstrong *et al*. 1990) and lepidopterous larvae *Galleria mellonella* and *Spodoptera littoralis* (Jarret and Stephenson, 1990).

Horizontal DNA transfer events in the natural environments by transformation and transduction have also been reported (Nielsen *et al*., 1997; Jiang and Paul, 1998) however they are not as common as conjugation, especially in animal ecosystems and therefore do not play the crucial role in the antibiotic resistance spread.

Recombinant plasmid as the elimination agents

Research design. Recently, studies have been intensified on the construction of recombinant palsmids encoding anti-bacterial functions and optimization of their conjugative transfer in order to create the new possibility of pathogenic bacteria killing after plasmid delivery from benign host to pathogenic recipient. Two main killing strategies are explored, *e.g*. killing by unabated (run-away) replication and killing by expression of lethal functions.

Plasmids are present within host cell in characteristic copy number controlled in part by repressor-like molecules (Helinski *et al*., 1996; Filutowicz and Rakowski, 1998). Therefore, mutations that disrupt the repressor function cause plasmids over-replication leading to an increase in their copy number (Blasina *et al*., 1996). These copy-up mutations result in run-away replication due to the loss of copy-control mechanisms, what in turn stops the replication of bacterial chromosomes because of titration of multi-protein complexes required for DNA synthesis. Thus run-away replication can result in cell death. The method of killing demands the use of self-transmissible, preferentially broad-host-range plasmids able to replicate in diverse assortment of bacteria (Sakai and Komano, 1996). Special precautions should be taken to avoid further spread of newly delivered plasmids to other pathogenic/nonpathogenic recipients. To ensure that,

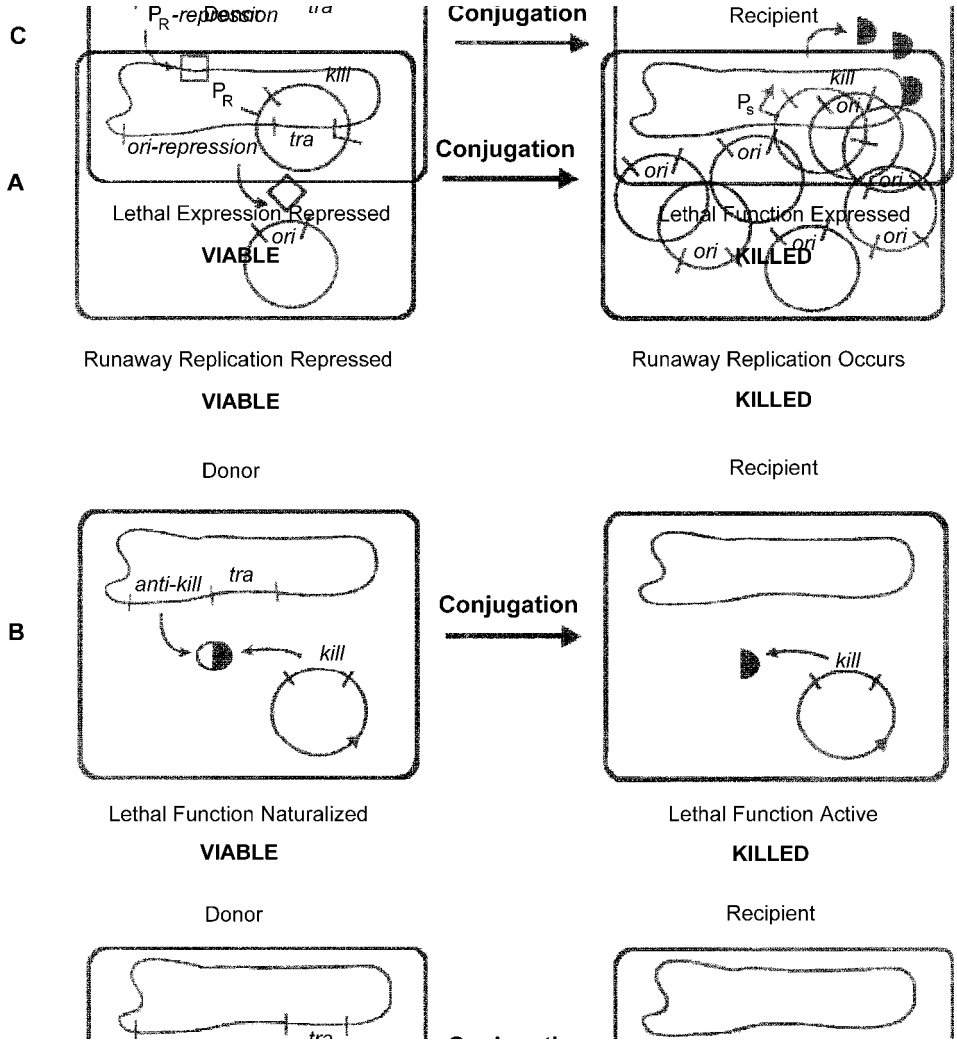

Fig. 1. Approach to killing of recipient cell.
Three methods of plasmid-based killing of bacteria are shown: A) killing by run-away replication,
B) killing by activation of bacteriocin (lack of its neutralization), C) killing by derepression of gene encoding bacteriocin.
◊ – repressor of *oriV*, □ – repressor of bacteriocin transcription, ◖ – bacteriocin, ◗ – antidote.

plasmid minireplicons devoid of *tra* genes are used. Transfer of minireplicons to the recipients demands cloning of *tra* region in chromosome of donor cells. The use of runaway plasmid replication has a great advantage – resistance to over-replication based killing is unlikely to occur and spread horizontally.

Another mechanism of killing is based on plasmid-encoded bacteriocin activity. Cells producing bacteriocin produce also a bacteriocin-specific antidote, typically a peptide or RNA (Engelberg-Kulka and Glaser, 1999). As a result of transfer of the gene encoding bacteriocin to antidote-deprived recipient cell death can occur. In another approach a donor, but not recipient, might be rendered insensitive to bacteriocin by using a tightly regulated promoter-operator system preventing repressor synthesis in donor cells.

The approaches described above are diagrammed in Fig. 1.

Killing of unwanted recipient bacteria demands very efficient conjugative transfer of plasmids so the conditions should be worked out to ensure the maximal frequency of this process. As biofilms provide ideal

niches for conjugation the proposed approaches are especially suitable to use against biofilm-forming pathogens (Ghigo, 2001; Hausner and Wuertz, 1999). It should be mentioned here that biofilms of certain pathogens are thought to be the major obstacle for conventional antibiotics, preventing them from reaching and killing pathogenic bacteria (Mah and O'Toole, 2001).

Reduction of biohazard. Special attention should be drawn to reduce biohazard connected with using live bacteria for delivering killing agents. Environmentally safe bacteria must be used as donors, such as *E. coli* F18 or various *Lactobacillus* and *Lactococcus* strains. Application of live bacteria is nothing new in therapy and agriculture. For example, the use of live attenuated bacterial vaccine strains allows the targeted delivery of macromolecules to mammalian cells and tissues and recently the ability of attenuated strains of *Salmonella*, *Shigella* and *Yersinia* spp., as well as *Listeria monocytogenes* and invasive *E. coli*, to deliver eukaryotic expression plasmids into mammalian cells *in vivo* and *in vitro* has been determined (Loessner and Weiss, 2004). *Pseudomonas fluorescens* and *Erwinia herhicola* are used to control the fire blight. These strains compete for nutrients with the pathogenic *E. amylovora* thus inhibiting its growth (Johnson and Stockwell, 2000).

Another strategy recommends application of non-growing donors cells such as minicells and maxicells instead of living cells. Minicells lack chromosomal DNA but may contain plasmids, they neither divide nor grow (Frazer and Curtiss, 1975). Maxicells are obtained from a strain of *E. coli* that carries mutations in the key DNA repair pathways, *recA, uvrA* and *phr* (Sancar *et al*., 1979). This strain dies upon exposure to low doses of UV but plasmid molecules can replicate and plasmid-directed transcription and translation can occur efficiently.

It is also very important, as mentioned before, to use plasmids which are mobilizable by conjugative machinery but not self-transmissible. Moreover antibiotic resistance markers for selection should be avoided. Of special advantage is the use of conditionally suicidal donors and/or plasmids systems, for example conditionally replicated plasmids which can replicate in the donor but are not able to replicate in the recipient (not in run-away replication strategy). It should be also mentioned that even for plasmids transferred by conjugation, the host range can be modulated by restriction-modification systems, ubiquitous in bacteria (Roberts and Macelis, 1996). By using a donor strain producing none, one or many methylases it would be possible to evade cleavage by none, one or many restriction enzymes. The plasmid DNA can also be engineered by site-directed mutagenesis in order to avoid of specific restriction sites or introduce the new ones.

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