Engineering Bacteria for DNA Medicine Production

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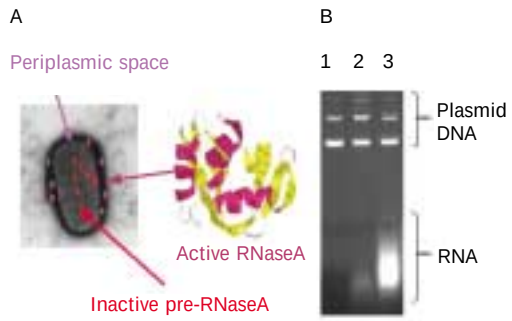
Plasmids prepared from Escherichia coli are fundamental tools in molecular biology, and their most recent applications as DNA medicines for gene therapy and DNA vaccines is a field that has expanded rapidly in the last decade. DNA vaccine candidates are in clinical trials against important diseases such as HIV, tuberculosis, malaria and various cancers; plasmids are also important in gene therapy and anti-cancer therapies. Unlike DNA vaccines, gene therapies can require relatively large doses of purified plasmid DNA.

Plasmids are routinely purified at laboratory scale for research use, but scaling-up the process to generate therapeutic quantities of plasmid requires the removal of host-derived contaminants. Plasmids are often lost from cells in a culture unless antibiotics are added to the growth medium, but antibiotics represent an additional contaminant and their use is discouraged by regulatory agencies. One approach currently being employed genetically engineers the bacterial cell to enable optimum plasmid selection, maintenance and purification. An alternative approach to consider involves using live bacteria as vaccine delivery systems to avoid downstream purification and efficiently target DNA vaccines to antigen-presenting cells.

REMOVAL OF HOST RNA

RNA is a significant contaminant in plasmid purification as it comprises about a fifth of the dry mass of an *E coli* cell and binds to the resins used in DNA purification. For laboratory-scale plasmid preparations, bovine-derived RNase A is added to degrade the RNA. However, regulatory guidelines recommend that bovine-derived materials should be avoided in the manufacture of

Figure 1: Degradation of Cellular RNA by *E coli* JMRNaseA



A. RNase A is sequestered to the periplasm where it folds into the correct conformation. B. An agarose gel showing plasmid preparations from E coli strains. Lane 1: exogenous RNase A added to the lysate of JM107. Lane 2: endogenous production of RNase A by JMRNaseA. Lane 3: no RNase A added to the lysate of JM107.

biotherapeutics due to the risk of transmissible spongiform encephalopathies such as new variant Creutzfeld-Jakob disease, and there is no commercially available source of recombinant RNase A. To solve this problem, the RNase A gene (rpbA) can be integrated into the *E coli* chromosome under the control of a trc promoter. When induced, either by the addition of IPTG or by repressor titration, a pre-RNase A is expressed. The native RNase A leader sequence enables targeting to the periplasm, where it folds to the active conformation in the oxidising environment but is segregated from the cytoplasm. When the cells are lysed during downstream processing, the active RNase A is released to degrade the contaminating cellular RNA (see Figure 1). Originally, JM107 was modified to create a strain called JMRNaseA used to demonstrate this technology in collaboration with John Ward and Dunstan Cooke at University College, London (1) and improved antibiotic gene-free strains based on the recA- DH1 for commercial plasmid production have been generated.

REDUCTION OF ENDOTOXIN

The lipid A portion of the lipopolysaccharide from gram-negative bacteria, also known as endotoxin, is a potent immunostimulatory molecule that can cause anaphylactic shock, and great care is taken during product purification to remove it prior to formulation. Representing an integral part of the bacterial cell membrane, mutants with an altered lipid A are generally temperature sensitive or non-viable. One exception are mutants in the msbB gene, which codes for an enzyme involved in the addition of a myristoyl fatty acid moiety to lipid A. This non-myristoylated endotoxin has a much-reduced immunostimulatory potential (by 1,000 to 10,000-fold) but with no corresponding loss of viability compared to the wild-type strain (2).

Generating msbB- strains for the production of DNA vaccines and recombinant proteins takes advantage of this. While the products made in new strains will present the same levels of lipid A as if they were produced in conventional strains, the endotoxin remaining in the final product after purification will be far less potent, thus greatly increasing product safety.

ANTIBIOTIC-FREE PLASMID PRODUCTION BY ORT

A high plasmid yield is key to cost-effective therapies, and most therapeutic plasmids utilise the high copy number pMB1 origin of replication that enables concentrations of several hundred copies per cell. However, plasmid loss can still be a problem due to the metabolic burden of replicating a high copy number plasmid and the absence in current cloning vectors of sequences that ensure correct segregation. The most common strategy for plasmid selection and maintenance involves plasmid-borne antibiotic resistance genes with the selective antibiotic added to the growth medium. This approach has a number of disadvantages. Antibiotics are labile in culture due to the growth temperature and presence of the resistance gene product in the culture medium, and this is of particular concern in the large volume, high biomass fermentations used in therapeutic plasmid manufacture. The regulatory authorities discourage the use of antibiotics related to those in clinical use, and (-lactams are already banned due to the risk of anaphylaxis. There is a requirement to demonstrate the removal of any antibiotic added for fermentation, but even

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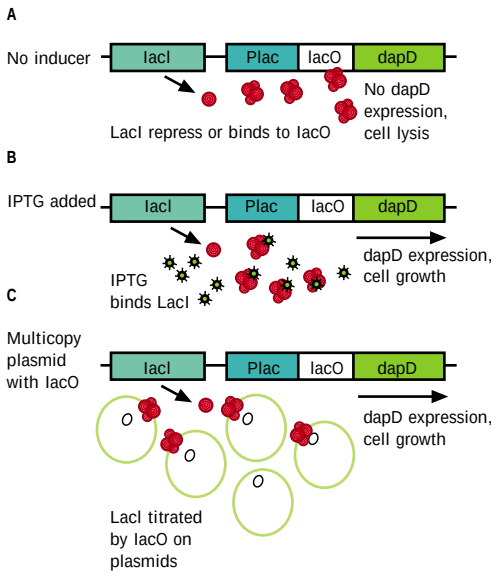
Figure 2: The Mechanism of Operator-Repressor Titration



A. With no inducer or plasmid present, the LacI repressor binds to lacO, preventing the expression of the essential gene dapD and causing cell lysis. B. Prior to transformation, the ORT strain can be cultured by the addition of the inducer IPTG, which prevents LacI from binding to lacO. C. When transformed with a multi-copy plasmid that also contains lacO, LacI is titrated, enabling dapD expression.

when antibiotics are omitted, the presence of the resistance gene itself is still a cause for concern. Therapeutic plasmids carry the risk of transfer of antibiotic resistance genes to microbes living in the patient or in the environment. Many species of bacteria will readily take up foreign DNA, and plasmids with the pMB1 origin of replication are able to replicate in a range of enteric bacteria, including pathogenic *E coli* strains and Salmonella. Given the increasing problem of multi drug-resistant pathogens, alternatives are essential to eliminate the requirement for antibiotics and their resistance genes. Alternative strategies such as post-segregational killing (PSK) mechanisms and complementation of a host auxotrophy either still require antibiotic resistance genes or another expressed bacterial gene as the selectable marker (3).

An operator-repressor titration (ORT) system invented by David Sherratt at the University of Oxford, for antibiotic-free plasmid selection and maintenance (see Figure 2), demonstrates the mechanism by which ORT works (4). An ORT strain is constructed with an essential gene regulated by the lac operator/promoter. The lac repressor protein (produced by the lacI gene) binds to the lac operator (lacO) and prevents expression of the essential gene. Therefore, unless an inducer such as IPTG is present to remove the repressor, the cell will die. However, if the cell is transformed with a multicopy plasmid that also contains lacO, this will titrate the repressor away from the chromosomal operator regulating the essential gene, allowing the cell to grow. The lacO sequence is short (21 bp), non-expressed and found in many plasmids in common use. The essential gene in the principal ORT strain DH1lacdapD is dapD, which is required for bacterial cell wall integrity by cross-linking of peptidoglycan (5). This strain has already been used to produce a significant number of the antibiotic-free DNA vaccines in clinical trials (6). In addition to the high copy number plasmids for DNA medicines, DH1lacdapD is also able to select and maintain low copy number plasmids that are often advantageous in recombinant protein production.

ANTIBIOTIC-FREE VACCINE DELIVERY BY ORT-VAC

For a successful protective immune response following vaccine delivery, there are significant difficulties to overcome. These include the delivery of sufficient quantities of the therapeutic product to antigen-presenting cells, the requirement of additional adjuvants to stimulate a cytotoxic T lymphotye response and the necessity of multiple rounds of inoculation by injection (a 'prime-boost' strategy). To overcome these problems, the use of attenuated pathogenic bacteria for intracellular delivery of recombinant protein and DNA vaccines is of special interest. The most suitable candidate organisms as intracellular vaccine vectors are those that naturally invade the gut mucosa (Listeria, Salmonella and Shigella) or the lung mucosa (Mycobacteria) (7). Attenuated strains of these organisms are still able to invade recipient cells where they express and release antigens or DNA vaccines that stimulate an immune response by a variety of pathways. Plasmid loss is an important problem during this period; measures to avoid this have included integration of the gene expression cassette into the host chromosome and PSK

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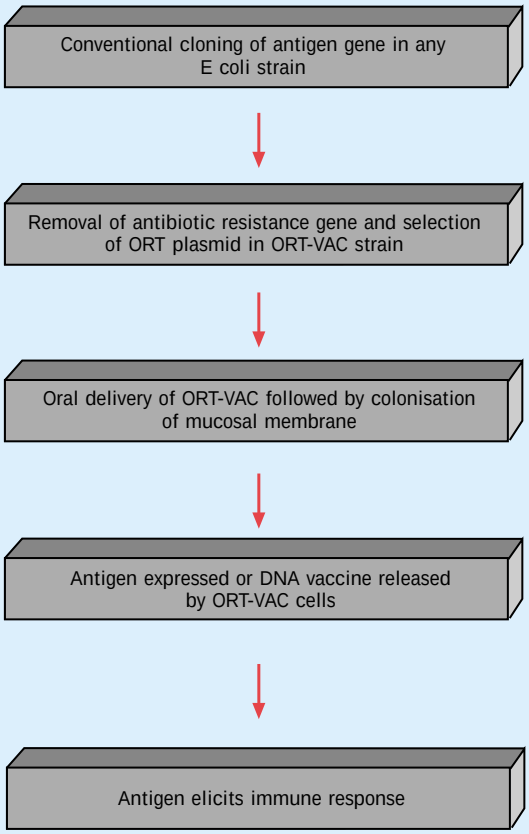
Figure 3: Antibiotic-Free Vaccine Delivery by ORT-VAC



mechanisms. Gene integration is difficult and time-consuming, and is clearly only suitable for recombinant protein delivery as DNA vaccine applications require many copies of a plasmid to be released into a recipient cell for antigen expression driven by a eukaryotic promoter. Stabilising plasmids using PSK mechanisms is of little use as these systems do nothing to enhance plasmid stability: they simply kill cells that have already lost a plasmid. The principle disadvantage of most *in vivo* vaccine delivery systems is the presence of the antibiotic resistance gene, frequently needed for initial selection during transformation. As the route of colonisation is across mucosal membranes, the vaccine-delivery strain will initially be in an environment populated by other microbes, an ideal opportunity for plasmid transfer to occur.

For example, one new ORT strain of attenuated Salmonella typhimurium has been engineered, which acts as the equivalent to the E coli strain DH1lacdapD, using dapD as its essential gene. It has been shown to enable successful maintenance in mice of a plasmid that was lost in a conventional Salmonella strain. This technology is easily applicable to convert any suitable attenuated strain to an ORT-VAC strain for vaccine delivery in humans. The mechanism for recombinant protein or DNA vaccine delivery by ORT-VAC is outline in Figure 3. ◆

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