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ANTIBIOTIC-FREE PLASMID SELECTION AND MAINTENANCE IN BACTERIA

Key words. Antibiotic, antibiotic resistance gene, plasmid selection, plasmid maintenance, bacteria, complementation, post-segregational killing, repressor titration

Abstract. The use of antibiotics and antibiotic resistance genes is rapidly becoming unacceptable in many areas of biotechnology, such as recombinant protein production, engineering of transgenic organisms, DNA vaccine and gene therapy applications. Plasmid-borne antibiotic resistant genes cause a considerable metabolic burden to the host bacterial cell. The resistance gene product, or even residual antibiotic contamination, can induce an immune response or cytotoxicity in patients during therapeutic applications. The risk of antibiotic resistant genes spreading from genetically-modified organisms to environmental pathogens is also of concern. Here we discuss the alternative methods of maintaining recombinant plasmids in bacteria. These include the complementation of a host auxotrophy, post-segregational killing mechanisms, the generation of minicircles by recombination, and the technique that we have developed involving the use of repressor titration. Some of these systems use antibiotics for selection of transformants but have alternative mechanisms of plasmid maintenance, whilst others are completely independent of antibiotics and their resistance genes.

1. THE ANTIBIOTIC PROBLEM

Antibiotics and their resistance genes have been important tools in the development of molecular biology. The presence of antibiotic resistance genes on recombinant plasmids allows for the very efficient selection of these plasmids following transformation of competent bacterial cells. Subsequent maintenance of the plasmid often relies on the presence of the selective antibiotic in the growth media to kill plasmid-free segregants. However, the presence of these genes and the antibiotics themselves has become undesirable in many areas of biotechnology.

1.1 Gene therapy and DNA vaccine applications

Gene therapy applications involve the direct delivery of DNA into patients to replace faulty gene functions or kill tumours, and DNA vaccines consist of plasmid-encoded antigens which function to elicit an immune response. Certain antibiotic resistance genes such as *bla* (β-lactamase, providing ampicillin resistance) contain immunostimulatory sequences called CpG dinucleotides. These can stimulate interferon-α production, which is disadvantageous to gene-replacement therapies as it may inhibit mRNA and protein synthesis, and cause an immune response to the recombinant protein (Sato *et al*. 1996). In mice injected with expression vectors

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containing *lacZ* (which expresses β-galactosidase) and either *bla* or *kan* (encoding kanamycin resistance), a much stronger antibody response to β-galactosidase was seen where *bla* was also present (Sato *et al.* 1996).

The replacement of *bla* with the *kan* gene in a luciferase reporter plasmid resulted in a two-fold increase in luciferase activity following murine skeletal muscle injection, and subsequent removal of other non-essential bacterial DNA (such as the *lacZ* α–peptide) increased this activity by a further 1.5 times (Hartikka *et al.* 1996). Although the amino 3'-glycosyl phosphotransferase gene, *neo* (conferring resistance to the antibiotic G481) is the most commonly used selectable marker gene in mammalian cell transfection, the phosphotransferase activity can affect the cellular phosphorylation state. When NIH-3T3 fibroblast cells expressing *neo* were transfected with plasmids, a 50 % reduction (as compared to non-*neo* expressing cells) was seen in fructose 2,6-bisphosphate and lactate production, indicating that these cells were less glycolytic (Valera *et al.* 1994). Altered levels of mRNA where also detected, with a marked decrease in expression of procollagen 1α and fibronectin genes, and an increase in c-*myc* gene expression. The c-*myc* gene product, Myc, functions as a transcription modulator and can induce the suppression of certain genes, so may have been responsible for the decrease in procollagen 1α mRNA. Modification of gene expression was also seen in FTO-2B rat hepatoma cells when *neo* was introduced (Valera *et al.* 1994). This indicates that cells expressing *neo* may have altered gene expression that could be deleterious to certain therapeutic applications.

Even when under the control of a bacterial promoter which does not function in the target host or cell line, there is still the possibility that the antibiotic resistance gene product may be expressed in the target cells due to read-through from the therapeutic cassette, or from upstream eukaryotic promoters if the plasmid is integrated into the host chromosome. This could also induce an immune response or have cytotoxic effects. The presence of the antibiotic itself if co-purified with the plasmid may lead to immune sensitisation and anaphylaxis. In the 'Guidance for Human Somatic Cell Therapy and Gene Therapy' published by the Food and Drug Administration (FDA) in the USA (Murphy 1998),

> "it is recommended that penicillin and other β–lactam antibiotics be avoided during production, due to the risk of serious hypersensitivity reactions in patients".

FDA guidelines also state that:

> "it is preferable not to use selection markers which confer resistance to antibiotics in significant clinical use, in order to avoid unnecessary risk of spread of antibiotic resistance traits to environmental microbes".

Many of the antibiotics in clinical use today are either derived from or related to those commonly used in molecular biology.

1.2 Genetic engineering to create transgenic organisms

There has been concern that the antibiotic resistance genes present in genetically modified organisms (GMOs) may be transferred to microbes present in the environment or the intestines of animals and humans, which could include pathogenic organisms. The Advisory Committee on Novel Foods and Processes in the UK declared that the presence of a gene conferring resistance to β–lactam antibiotics in a strain of genetically modified maize posed an "unacceptable risk" due to the chance that bacteria in the gut could take up and express the gene (Wadman 1996). There are several studies that indicate the horizontal transfer of antibiotic resistance genes between different species of bacteria (gram positive and negative) in the human and animal intestine, as many bacteria are capable of plasmid transfer by conjugation, or are naturally competent (reviewed in Davidson 1999). Although there is currently little if any evidence of intestinal bacteria becoming transformed with genes present in food, this would become more likely in animals and humans treated with antibiotics and eating the produce of genetically-modified crops. Tetracycline is widely used both as an animal growth promoter, and a treatment for acne in humans. The tetracycline resistance gene *tetQ* is virtually identical in *Bacteriodes* species that inhabit the human gut and in the distantly related *Prevotella ruminicola* from the digestive tract of farm animals, suggesting that tetracycline has stimulated the conjugal transfer of the resistance determinant (Davidson 1999). Thus although the risk of initial transfer of antibiotic resistance gene to bacteria is small, it can be increased by the presence of a selection pressure, and once a strain of bacteria has taken up the gene, it becomes a reservoir for transfer to other species.

There is also concern that horizontal gene transfer can occur between soil bacteria and transgenic crop plants. A number of antibiotic resistance genes have been used, the most common being *nptII* (kanamycin, neomycin and gentomycin resistance) which is present in over 30 transgenic plants (Dröge *et al.* 1998). Several species of soil bacteria are naturally competent throughout or at certain stages in their life cycle. DNA has been shown to persist in soil by adsorption to sand and clay particles, becoming 100- to 1000- fold more resistant to nucleases and retaining the ability to transform competent bacteria for several weeks (Davidson 1999). In practice, the risk of such horizontal gene transfer occurring is extremely small, and studies looking for evidence of horizontal gene transfer have detected no transfer. In their review on horizontal gene transfer, Dröge *et al.* (1998) question the use of *nptII* in transgenic plants, and conclude that:

> "effort should be put into the development of new strategies to remove antibiotic resistance genes from transgenic constructs".

Despite the small risk of horizontal gene transfer, the presence of the resistance gene is unnecessary as it serves no function in the GMO, but is a relic of the earlier cloning process. This fact, together with public concern over genetic engineering and the availability of alternative plasmid selection systems, increases the possibility of legislation to prevent the use of antibiotic resistance genes in GMOs.

1.3 Industrial production of plasmid DNA and recombinant protein

The use of antibiotics in the fermenters used to produce large amounts of clinical-grade DNA and recombinant protein results in a loss of selection pressure due to antibiotic degradation, which is accelerated by the presence of the antibiotic resistance gene product in the media, either from secretion or cell lysis. This will result in the accumulation of plasmid-free cells, with the resulting loss of specific yield.

The metabolic burden of plasmid replication reduces the growth rate of plasmid-bearing bacterial cells, which are out-competed by plasmid-free cells in the absence of a selection pressure (Corchero and Villaverde 1998). A high-copy number plasmid may be present at up to 1000 copies per cell in a high-biomass *Escherichia coli* fermentation, and thus a 5 kilobase-pair plasmid would represent an equivalent amount of DNA to the host chromosome. Thus the presence of an antibiotic resistance gene, a constitutively expressed sequence that produces the resistance product greatly in excess of that required for plasmid selection or maintenance, can contribute to a significant metabolic burden on the bacterial host cell during DNA or protein production. Indeed, it is the plasmid-encoded gene that is the principle factor in the metabolic burden of recombinant bacteria (Bentley *et al.* 1990). When the *kan* promoter on a recombinant plasmid had its promoter function reduced by site-directed mutagenesis such that the kanamycin phosphotransferase was produced at the minimal level required for resistance, this resulted in a corresponding two-fold increase in the production of the recombinant protein (Panayotatos 1988).

2. ALTERNATIVE GENES FOR PLASMID MAINTENANCE

Some of the problems of antibiotic selection are shared by the plasmid maintenance systems discussed below, which rely on the presence of a plasmid-borne gene. These include the metabolic burden of gene expression and the possibility of expression *in vivo* during gene therapy and DNA vaccine applications. However, these systems do reduce the proliferation of plasmid-free cells.

2.1 Complementation of a host auxotrophy

These systems rely on a plasmid-borne function that complements a lethal chromosomal mutation, thus allowing the transformed cells to survive whilst plasmid-free cells die. This is distinct from the post-segregational killing mechanisms (discussed below) in that plasmid-free segregants die as a result of losing the essential gene function present on the plasmid, rather than being killed by a plasmid-borne gene product. Unlike the post-segregational killing mechanisms, complementation of a host auxotrophy can be used as a method of transformant selection as well as plasmid maintenance.

To overcome the problem of a pBR322-derived plasmid that contained the tryptophan operon being lost during culture, the gene encoding valyl-tRNA

synthetase, *valS*, was cloned into the plasmid. A thermosensitive *valS* mutant of *E. coli* was used as the host strain, with a nonpermissive temperature of 37 °C and a permissive temperature of 30 °C. The *valS*$^{+}$ plasmid, pSGS21, was lost at a rate of 1.2 % per generation at 30 °C in this mutant, whilst at 37 °C it was stably maintained for over 200 generations (Skogman and Nilsson 1984). The selection in this case was on ampicillin as the *bla* gene was present on pSGS21, but the principle of plasmid maintenance is antibiotic-free at the nonpermissive temperature. The disadvantage of complementing this type of auxotrophy is that valine is present in nutrient broth so minimal media must be used, thus limiting the plasmid yield.

Miwa *et al.* (1994) have designed a system that uses an *E. coli* 4D host that is dependant on the antibiotic streptomycin being present in the growth media. This mutation is complemented by a plasmid that carries the streptomycin-resistance gene *rpsL*, so when the streptomycin-dependant strain is transformed with this plasmid, transformants are able to grow in streptomycin-free media. Although an antibiotic resistance gene is present on the plasmid, a rich, antibiotic-free growth media can be used for plasmid production.

The *dapD* gene encodes tetrahydrodipicolinate *N*-succinyltransferase, which catalyses a step in the lysine / diaminopimelic acid (DAP) pathway (Richaud *et al.* 1984). Therefore *dapD* mutants are lysine and DAP auxotrophs, and although lysine is present in nutrient broth such as LB, DAP is not. Without DAP to cross-link peptidoglycan in the cell wall, *dapD*$^{-}$ mutants will lyse. A completely antibiotic-free plasmid maintenance system is based on the *dapD* gene (Degryse 1991). This allows the selection and maintenance of the *dapD*-containing plasmid in the *dapD*$^{-}$ mutant strain in LB media. Two plasmids consisting of the same origin of replication and expressing the human γ–interferon gene were compared. One contained *bla* and the other *dapD* as selectable markers, and they were grown in the parental and derived *dapD*$^{-}$ *E. coli* hosts respectively. Maintenance of the *dapD* plasmid was greater than that of the *bla* plasmid, as the overgrowth of the culture by plasmid-free cells was prevented by the obligate requirement for DAP of the mutant strain.

DAP auxotrophy has also been used in plasmid maintenance in avirulent *Salmonella typhimurium* mutants that are designed for live delivery of vaccines to stimulate an immune response. These are mutants in the *asd* gene in the DAP biosynthesis pathway, which encodes aspartate β–semialdehyde dehydrogenase. As with *dapD* mutants, *asd* mutants will lyse if DAP is not supplied, and DAP is not present in the target mammalian tissues. The *asd* gene was cloned into a plasmid which was normally very unstable with antibiotic selection, and this was grown in *asd*$^{-}$ *S. typhimurium* with and without the addition of DAP to the media (Galán *et al.* 1990). Without DAP, the plasmid was maintained in 100 % of the colonies tested. With DAP present, and therefore no selective advantage to maintain the plasmid, it was lost in 70 % of colonies. Plasmids expressing *asd* have also been used in attenuated, *asd*$^{-}$ *S. typhi* for the live delivery of plasmid-encoded hepatitis B antigen (Tacket *et al.* 1997).

Vectors containing the *thyA* gene (encoding thymidylate synthetase) from *E. coli* have been used to enable plasmid maintenance in *thyA*$^{-}$ mutants of *E. coli* and attenuated *S. typhimurium* and *S. typhi* strains (Morona *et al.* 1991). This system has

been used to maintain a plasmid also expressing the *rbf* genes, which encode O-antigen biosynthesis in *Vibrio cholerae* O1. The plasmid selection pressure is retained in minimal media, but nutrient media contain thymine. The selection pressure may be present for *in vivo* vaccine delivery, as human body fluids are low in thymine, so the bacteria may not be able to scavenge sufficient thymine from their hosts and therefore be reliant on the $thyA^+$ plasmid (Morona *et al.* 1991).

An attenuated vaccine delivery strain of *Vibrio cholerae* had a mutation in *glnA* (glutamine synthetase) introduced to allow antibiotic-free plasmid maintenance (Ryan *et al.* 2000). This strain, Peru2Δ*glnA*, is unable to grow in minimal media unless supplemented with glutamine or transformed with a plasmid expressing *glnA* (the *glnA* gene in this case was from *S. typhimurium*). Peru2Δ*glnA* did not have this selection pressure *in vivo*, however, as enough glutamine was present in the intestinal lumen of the mice to which they had been delivered to complement the auxotrophy. The *glnA*-expressing plasmids did contain antibiotic resistance genes, but antibiotics were not used *in vivo*, and the plasmids were maintained and appeared to confer a survival advantage on Peru2Δ*glnA*.

The pCOR plasmid utilises a suppressor tRNA gene for plasmid selection in minimal media, and replicates in *E. coli* strain XAC-1*pir116* that has a mutation in the argenine biosynthesis gene *argE* (Soubrier *et al.* 1999). This mutation introduced an amber stop codon (TAG) into the *argE* open reading frame, and pCOR contains an amber suppressor tRNA gene which incorporates phenylalanine at this codon, allowing transcription of *argE* and plasmid selection on minimal media lacking argenine. In addition, the pCOR origin of replication (R6K γ) is dependant on a host-encoded π initiator protein, which prevents pCOR from replicating in any other *E. coli* strain. pCOR was stably maintained for over 50 generations in a fed-batch fermentation, but it has no mechanism for plasmid maintenance in complex media.

2.2 Post-segregational killing mechanisms

Post-segregational killing mechanisms involve killing of the host bacterial cell following the loss of a recombinant plasmid that encodes a protein or RNA that inhibits the killing mechanism. This mechanism either exists on the plasmid itself, or on the host chromosome. The *c*Its857 gene of bacteriophage λ encodes the thermosensitive λ repressor. This was cloned into a series of plasmids that also encoded human insulin, and were easily lost from cultures in the absence of a selection pressure. These plasmids were transformed into *E. coli* RV308 that had been lysogenised with repressor-defective λ *c*I90 (Rosteck and Hershberger 1983). Loss of the plasmids resulted in loss of the repressor, causing the cells to lyse as the prophage entered the lytic cycle. A high percentage of plasmid retention was seen when compared to the control, non-lysogenic strains.

The plasmid-derived post-segregational killing (PSK) systems consist of plasmids encoding a toxin-antitoxin pair, where the toxin is more stable than the antitoxin. Thus the toxin molecules, following the degradation of the antitoxin, will kill any

plasmid-free daughter cell of a PSK plasmid-containing parental cell. This requires no modification of the host cell.

The *hok*/*sok*, *srnB* and *pnd* PSK systems operate in the plasmids R1, F and R483 respectively, and involve a killer protein encoded by stable mRNA and regulated by small, unstable antisense RNA that binds to the killer RNA and inactivates it irreversibly. The killer RNA is retained in plasmid-free segregants after the antisense RNA has degraded, and is translated into the lethal protein (Thisted *et al.* 1994). An expression vector was constructed containing the *hok*/*sok* locus *parB*, under the control of the *phoA* (alkaline phosphatase) promoter (Schweder *et al.* 1992). In addition to the PSK application, this allows an inducible suicide system to operate under conditions of phosphate limitation, addressing the biosafety issue of transgenic bacteria entering the environment, where phosphate is often limiting. *E. coli* containing plasmids with and without the *hok*/*sok* locus were compared in a chemostat and after 50 generations only 3 % loss was detected from cells containing *hok/soc* plasmids, but 97 % of those without *hok/soc* lost their plasmids.

Plasmid maintenance using the *hok*/*sok* system was investigated in the attenuated live vector vaccine strain *Salmonella typhi* CDV 908-*htrA* (Galen *et al.* 1999). The *hok/soc* genes were present on a series of plasmids that also contained the *bla* gene, and the gene for green fluorescent protein (GFPuv) under the control of the osmolarity-induced *ompC* promoter. Flow cytometry was used to quantify the relative number of plamid-containing and plasmid-free cells by fluorescence following growth for 24 hours. CDV 908-*htrA* containing a plasmid with the *hok*/*sok* system had over 80 % of cells retaining this plasmid, but an equivalent plasmid without *hok/soc* was present in only 13 % of cells. The percentage of plasmid loss increased with increasing NaCl concentration which induced *ompC*-GFPuv, contributing to the metabolic burden. When the passive partition locus *par* and the active partition locus *parA* were introduced into the *hok*/*sok* plasmid, the proportion of plasmid-containing cells was further increased (around 95 %), and was independent of increasing osmolarity. However, after growing these cultures for a further 24 hours in the absence of antibiotic, over 95 % of the cells lost their plasmids.

The *ccd* locus from the *E. coli* F plasmid consists of two protein components encoded by *ccdA* and *ccdB*. CcdB is a lethal protein that acts by preventing the religation of DNA following cleavage by DNA gyrase, and CcdA binds to CcdB to prevent it from targeting the DNA-gyrase complex (Holčík and Iyer 1997). The half-life of CcdA is shorter than that of CcdB, enabling post-segregational killing of F-plasmid-free cells following cell division. Other *E. coli* PSK systems that have proteins as the toxin / antitoxin pair and work by a similar mechanism to *ccd* include the *parD* operon from the R1 plasmid and the *parDE* operon from plasmid RK2 (Holčík and Iyer 1997). *parD* encodes the cytotoxic Kid and the antidote Kis, the target of Kid being DnaB, which is involved in the initiation of DNA replication. The *parDE* operon encodes the toxic ParE protein and its antidote ParD. ParD inhibits ParE by forming a complex with it, and also acts as a negative regulator of the *parDE* operon.

These plasmid-borne PSK mechanisms were thought to increase vertical plasmid transmission, but recent research has suggested that PSK has not evolved to increase plasmid stability, but as a consequence of inter-plasmid competition. Cooper and Heinemann (2000) compared equivalent plasmids with and without the *parDE* PSK system (psk^+_p and psk^-_p respectively). When comparing *E. coli* cultures starting from psk^+_p and psk^-_p cells, the psk^+_p culture contained a higher proportion of plasmid-containing cells as expected. However, plasmid stability is also a measure of the accumulation of plasmid-containing cells, but a comparison of these two cultures revealed that the total number of plasmid-containing cells remained constant over 200 generations, which is not consistent with a mechanism proposed to enable plasmid maintenance. When comparing conjugation frequencies from donor psk^+_p and psk^-_p –containing cells, both were transmitted with equal frequency within a plasmid-free host culture, and transmission of psk^+_p was not affected by the presence of psk^-_p. However, psk^-_p was unable to invade a host population of cells containing psk^+_p. As both plasmids are incompatible, psk^-_p invading a cell containing psk^+_p would lead to plasmid mis-segregation, with the daughter cell containing only psk^-_p being killed. Also, plasmid-free cells do accumulate due to occasional 'escape' of the PSK mechanism (Cooper and Heinemann 2000).

3. MINICIRCLES

The plasmid pXL2650 is designed to produce DNA minicircles for gene therapy and DNA vaccine applications, and replicates in *E. coli* host D1210HP (Darquet *et al.* 1997). pXL2650 contains the luciferase gene *luc* flanked by *attP* and *attB* (recognition sites for bacteriophage λ integrase), in addition to *bla* and the ColEI origin of replication. D1210HP contains a thermosensitive λ lysogen that is disabled for the lytic functions that result in cell lysis, but a temperature shift to 42 °C induces the expression of the λ integrase and results in recombination between *attP* and *attB*. Following decatenation by *E. coli* DNA topoisomerase IV, the two products are a minicircle containing *luc*, and a miniplasmid containing *bla* and the ColEI *ori*. The *luc*-minicircle was separated from the miniplasmid by density gradient centrifugation and used in transfection assays of mammalian cell lines. There was a significantly higher luciferase activity for the *luc*-minicircle than for pXL2650. This system is designed to carry a therapeutic gene in place of *luc*, and the product is a minicircle with no antibiotic resistance gene, but ampicillin is still required for the parental plasmid selection and maintenance.

4. ANTIBIOTIC AND MARKER GENE-FREE PLASMID MAINTENANCE

The system that we have developed enables the selection and maintenance, in complex media in the absence of antibiotic, of a plasmid that does not contain an expressed sequence as the selectable marker. This is operator-repressor titration (ORT), which utilises the natural phenomenon of titration of a chromosomally encoded repressor protein by a multicopy plasmid that contains the binding site of

the repressor (the operator). This out-competes the same operator sequence regulating a chromosomal gene, which is therefore derepressed and so can be transcribed. In ORT systems, the chromosomal gene is made conditionally essential and put under the control of the lactose operator / promoter (*lacO*/*P*), so that the altered *E. coli* cells are not able to grow unless induced by IPTG or allo-lactose, or transformed with a plasmid that competitively binds all the cellular repressor protein, allowing the essential gene to be expressed and so enabling cell growth. The repressor binding to the chromosomal operator and switching off the essential gene will kill any cells that lose the *lacO*-containing plasmid (Figure 1).

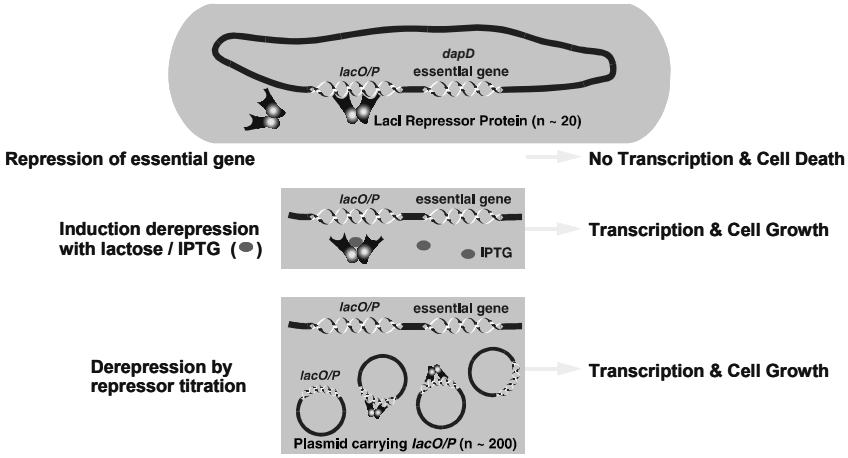

Figure 1. Derepression of chromosomal lacO/P-dapD by induction and repressor titration.

The first ORT strain, DH1*lackan*, was built to test the system and is able to replicate plasmids that do not contain an antibiotic resistance gene (Williams *et al.* 1998). The antibiotic resistance gene *kan* was placed under the control of *lacO*/*P* and inserted into the chromosome of *E. coli* DH1 to create DH1*lackan*. Transformants can be selected and plasmids maintained in the presence of kanamycin by repressor titration.

For a completely antibiotic-free ORT system, the *dapD* gene was deleted from DH1, and then a copy of *dapD* regulated by *lacO*/*P* was inserted to create DH1*lacdapD* (Cranenburgh *et al.* 2001). This strain can be transformed with a *lacO*-containing plasmid and transformants selected simply by their ability to grow on nutrient agar plates with no other additives necessary, as untransformed DH1*lacdapD* is unable to form single colonies on plates. Transformation efficiencies by ORT selection are equivalent to those by antibiotic selection in this strain. However, untransformed DH1*lacdapD* is able to grow in liquid culture due to

leakiness from the *lac* promoter, so the –10 region of the promoter was mutated to reduce its activity. This created strain DH1*lacP2dapD*, which is not able to grow on solid or in liquid media unless induced by IPTG or derepressed by a *lacO*-containing plasmid.

Another advantage of this system is that many commercially available plasmids contain the required $lacO_1$ and $lacO_3$ sequences (e.g. pUC, pGEM, pBluescript) to regulate the expression of the *lacZ* α–peptide required for blue / white selection of plasmid clones. Therefore the only modification that would be required to convert a therapeutic plasmid to an ORT plasmid (defined as antibiotic gene-free and possessing *lacO*) would be the removal of the antibiotic resistance gene. We have also developed a series of plasmids (pORT) that allow initial DNA manipulations, such as cloning of a therapeutic cassette into the multi-cloning site (MCS), to be carried out in any host strain using ampicillin resistance as the selection. Then *bla* is removed as a final step prior to transformation into an ORT host strain. This is achieved by exploiting the six restriction endonuclease recognition sites ('excision sites' XS) that flank *bla* in direct repeat. Three of these (*Avr*II, *Sfi*I, and *Fse*I) have rare cleavage sites, so the probability of all being present in an insert is very low. This allows the construction of plasmids with a minimal vector backbone. Figure 2 shows the removal of *bla* from pORT1 to generate the ORT plasmid pORT1a.

5. CONCLUSIONS

There are several disadvantages in using antibiotics and antibiotic resistance genes in recombinant protein and therapeutic DNA production. The immunostimulatory CpG dinucleotides and the expression of certain antibiotic resistance genes *in vivo* can be a problem in gene therapy. Antibiotic resistance genes in transgenic organisms carry the small but unnecessary risk of transfer to environmental microbes. In recombinant protein production, the principal cause of the metabolic burden that significantly reduces the yield of the required product is the presence of the antibiotic resistance gene. Also, there is the risk of residual antibiotic contaminating the final product. These difficulties make the use of alternative systems highly desirable.

The various plasmid maintenance systems are summarised in Table 1. The selectable markers are those genes or elements that enable the selection of transformants, but are not always the same as those used for plasmid maintenance. The post-segregational killing mechanisms do not enable plasmid selection following transformation but are dependant on antibiotic resistance, thus the antibiotic resistance gene must remain on the plasmid. This makes the PSK systems unsuitable for the *in vivo* delivery of therapeutic DNA. Of the systems that complement a host auxotrophy, those using *rpsL* (Miwa *et al.* 1994) and *dapD* (Degryse 1991) allow plasmid maintenance in complex media, with the latter being independent of antibiotic resistance genes. The pCOR suppressor tRNA approach (Soubrier *et al.* 1996) allows a small plasmid to be selected that does not express a protein-encoding gene, but is specifically designed for therapeutic DNA applications rather than recombinant protein production due to the requirement of growth in minimal media.

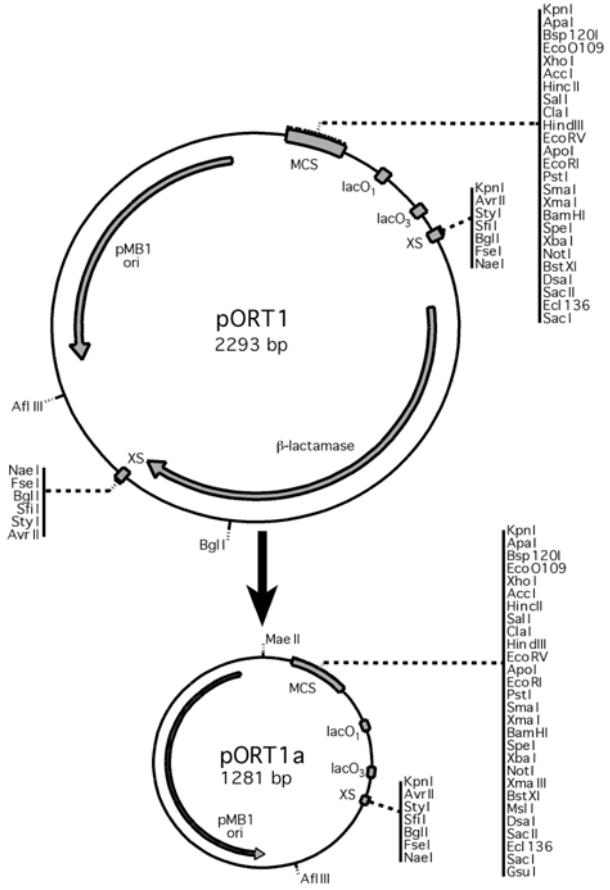

Figure 2. Conversion of cloning vector pORT1 to ORT vector pORT1a by removal of the bla gene.

The precursor plasmid of the minicircle requires ampicillin, enabling growth in complex media, but the *bla* gene is removed by a recombination event to generate the smallest therapeutic DNA molecule available (Darquet *et al.* 1997). Again, this is a specialist application for therapeutic DNA production. Our ORT system depends only on the short, non-expressed *lacO* sequence that is present on many plasmids, and enables antibiotic-free plasmid selection and maintenance in complex media (Cranenburgh *et al.* 2001). This allows the production of very small plasmids that can be used for completely antibiotic-free recombinant protein production, or for gene therapy and DNA vaccine applications.

Table 1. Summary of mechanisms of antibiotic-free plasmid maintenance.
Antibiotic abbreviations are Ap: ampliclin, Sm: streptomycin, Tc: tetracycline and Cm: chloramphenicol.

Mechanism	*Strain*	*Selectable marker*	*Antibiotics needed for selection*	*Plasmid maintenance gene(s)*	*Maintenance in complex media*	*Possible applications*	*Reference*
Complementation	*E. coli*	*bla*	Ap	*valS*	No	Protein	Skogman and Nilsson 1984
Complementation	*E. coli*	*rpsL*	Sm	*rpsL*	Yes	Protein	Miwa*et al.* 1994
Complementation	*E. coli*	*dapD*	-	*dapD*	Yes	Protein/DNA	Degryse 1991
Complementation	*S. typhimurium*	*asd*	-	*asd*	Yes	Vaccine delivery	Galán *et al.* 1990
Complementation	*S. typhi*	*asd*	-	*asd*	Yes	Vaccine delivery	Tacket *et al.* 1997
Complementation	*S. typhimurium*, *S. typhi*	*thyA*	-	*thyA*	No	Vaccine delivery	Morona *et al.* 1991
Complementation	*Vibrio cholerae*	*bla, tet, cat*	Ap, Tc, Cm	*glnA*	No	Vaccine delivery	Ryan *et al.* 2000
Complementation	*E. coli*	*sup*Phe	-	*sup*Phe	No	DNA	Soubrier *et al.* 1996
PSK	*E. coli*	*tet*	Tc	*c*Its857	Yes	Protein	Rosteck and Hershberger 1983
PSK	*E. coli*	*bla*	Ap	*hok/sok*	Yes	Protein	Schweder *et al.* 1992
PSK	*S. typhi*	*bla*	Ap	*hok/sok*	Yes	Vaccine delivery	Galen *et al.* 1999
PSK	*E. coli*	*cat*	Cm	*parD, parE*	Yes	Protein	Cooper and Heinemann 2000
Minicircle	*E. coli*	*bla*	Ap	*bla*	Yes	DNA	Darquet *et al.* 1997
ORT	*E. coli*	*lacO*	-	-	Yes	Protein/DNA	Cranenburgh *et al.* 2001

6. AFFILIATIONS

The authors are employees of Cobra Therapeutics, The Science Park, Keele, Staffordshire, ST5 5SP, U.K. The ORT system was developed in collaboration with David J. Sherratt, Division of Molecular Genetics, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, U.K. Figure 2 is reproduced from Cranenburgh *et al*. 2001 by permission of Oxford University Press.

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