# Antibiotic-Free Systems for Production

## Bypassing the Expression of Exogenous Site-Specific Recombinases

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The use of antibiotic markers to select for a successful genetic modification, such as a chromosomal insertion or a plasmid transformation, is common practice. However, with increasing stringency in manufacturing regulations it is essential to ensure that the end product contains no antibiotics and no genes for antibiotic resistance. For this reason a number of strategies have been developed to remove antibiotic selection markers from the biotherapeutic production process.

In the U.S., federal regulations specifically discourage the use of markers that confer resistance to antibiotics in significant clinical use in order to avoid unnecessary risks of spreading antibiotic resistance traits to environmental microbes. In addition to ensuring regulatory approval, removal of resistance markers increases the variety of plasmids that can be propagated within a single cell and removes the risk of altered expression of chromosomal genes adjacent to the antibiotic-resistance marker.

### Xer-cise for Bacteria

Plasmids are usually the best vehicles for gene expression in *E. coli* due to their elevated copy number, but in some species, such as *Bacillus subtilis*, single-copy chromosomal genes provide sufficient levels of secreted protein production. Both plasmid transformation and chromosomal integration usually require the use of resistance markers.

To circumvent the use of antibiotics and marker genes, constructs have traditionally been engineered to first allow selection for the gene insertion and then, in a second step, to remove the marker using site-specific recombinases. Typically these constructs use recombinase systems such as Cre/loxP derived from bacteriophage P1 or Flp/FRT from yeast. However, these systems display varied success rates with much efficiency lost through the need to express exogenous recombinases in a range of bacterial species.

**Cobra Biomanufacturing** (www.cobrabio.com) has developed a new excision system, Xer-cise™, which provides a rapid method for inserting genes into or deleting genes from bacterial chromosomes. The Xer-cise system uses Xer recombinases, naturally present in a wide range of bacteria, to excise antibiotic-resistance genes. This eliminates the need to express exogenous site-specific recombinases.

In a proof-of-concept case study the gene for bovine pancreatic RNase (*rbpA*) was introduced into a plasmid containing the *cat* gene flanked by Xer recombination sites. After chromosomal insertion of the rbpA-*cat* cassette through homologous recombination, the positive transformants were selected by antibiotic resistance. Removal of the antibiotic from the growth medium resulted in the elimination of the marker gene by the endogenously expressed Xer recombinase (*Figure 1*). In contrast to traditional systems, Xer recombinase is endogenously expressed in a majority of prokaryotes, making it ideal for the engineering of difficult strains, or strains that have a limited molecular biology toolkit available.

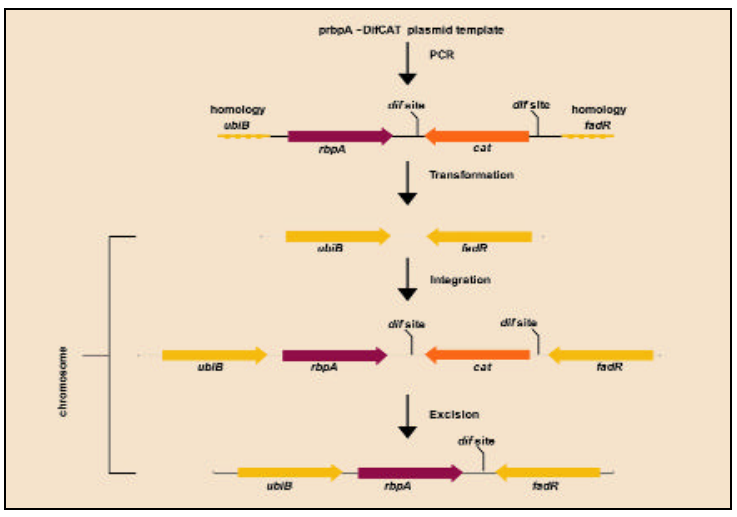


Figure 1. Chromosomal insertion of rbpA using Xer-cise technology. The PCR-product containing the rbpA gene is integrated through homologous recombination with homology to ubiB-fadR .

### Optimized Bacterial Strains

The Xer-cise system can be used to optimize strains that have proven difficult to engineer. Proteases naturally secreted into the growth medium by *B. subtilis* are responsible for the degradation of expressed recombinant proteins, reducing the overall yield in the manufacturing process.

The Xer-cise system was successfully employed to delete two previously described extracellular protease genes, *mpr* and *nprE*, from this organism to increase production efficiency. A gene cassette consisting of the antibiotic-resistance gene flanked by the Xer-recombination sites was inserted directly into the protease genes by homologous recombination.

After selection of the positive transformants, removal of the antibiotics in the medium initiated the Xer recombination events, successfully eliminating the antibiotic-resistance gene. Using this method has enabled Cobra to establish optimized bacterial strains with higher yields, which have the potential to be employed in the GMP production of various therapeutic proteins.

### Maintaining Plasmids without Resistance Markers

While the use of Xer-cise technology to remove resistance markers from chromosomal insertions has clear benefits, the maintenance of plasmid vectors in an organism without the need for antibiotics would offer even greater benefits. Plasmids are the usual vehicles for gene expression in *E. coli* due to their ability to maintain elevated copy number. Antibiotic-resistance genes are commonly used for plasmid selection and maintenance in the laboratory but recently GMP-compliant technologies have been developed for gene expression by plasmid vectors without the requirement for antibiotic markers.

Cobra's operator-repressor titration technology (ORT®) is an established system for high and sustainable gene expression. It provides an effective and efficient method for maintaining plasmids without the use of antibiotic selection and out-performs other strategies such as auxotrophy complementation or post-segregational killing.

ORT is based on an essential bacterial gene, *dapD*, engineered to be under the control of the lac operator. In the absence of an inducer such as lactose, the LacI repressor protein binds to the operator site blocking transcription of the essential gene and eventually leading to cell death. Transformation of the cell with a high copy number plasmid containing the *lac* operator effectively induces *dapD* expression by titrating LacI away from the chromosomal *lac* operator (*Figure 2*). This approach avoids problems associated with the use of antibiotics and resistance genes in therapeutic protein or DNA vaccine production and gene therapy, and ensures the maintenance of a high number of plasmid copies.

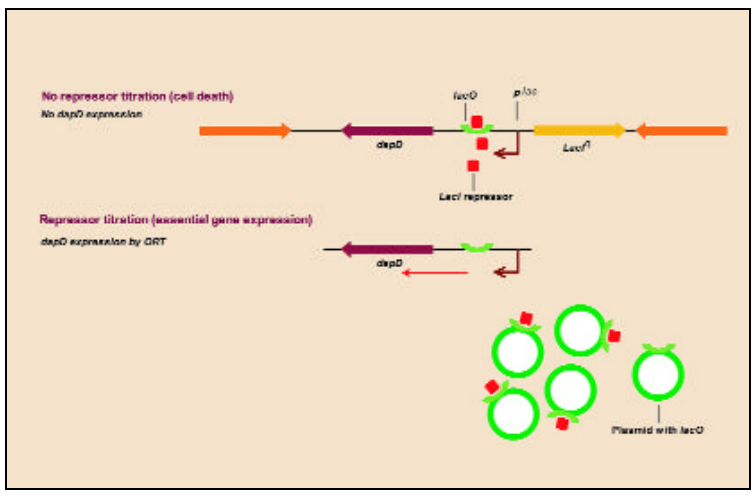


Figure 2. Plasmid maintenance by ORT. The essential gene dapD is under control of the lac operator, which is activated upon titration of the LacI repressor by plasmids coding for lacO.

### ORT in Live Bacterial Vaccines

The use of live attenuated bacteria to deliver recombinant protein vaccine antigens is a promising approach that overcomes the difficulties and cost of vaccine antigen purification and also enhances the presentation of the antigen to the immune system (*Figure 3*).

Although some antigen-expressing plasmids are stable, plasmid loss during cell divisions remains an important problem. It is in the design of live bacterial vaccines that ORT technology has proven its utility. Using the ORT-system to express the antigen of choice eliminates the requirement for antibiotic-resistance markers or any other genes for stable plasmid maintenance. It also has the advantage of potentially giving higher levels of antigen expression than would be possible by single chromosomal integration. The modified ORT-strains can be transformed with plasmids coding for the antigen and containing the *lacO* binding site to titrate the inhibitor from the operator site of the essential gene.

In preclinical studies, an ORT-modified strain of *Salmonella enterica* serovar Typhimurium has been used to consistently maintain a plasmid in mice that was highly unstable in the conventional antibiotic selected strain, demonstrating the potential for an increased, and therefore more potent, vaccine dose from this system. Furthermore, proof-of-principle results showed that mice given a single oral dose of the ORT-modified strain expressing a specific plague antigen achieved a high level of immunity against the disease. ORT-derived vaccines might have potential applications in cancer, HIV, and tuberculosis as well as in newly emerging diseases such as avian flu.

### Compliance with GMP

The development of Xer-cise by Cobra simplifies the excision of selectable marker genes and provides an accelerated method to produce unlabeled mutants in *E. coli* and *B. subtilis*. This system is applicable throughout the ever-increasing number of bacteria for which the native Xer recombination site has been elucidated.

Xer-cise was also used in the development of ORT-strains, which allows the stable maintenance of a high copy number of plasmids without the requirement for antibiotics and antibiotic-resistance markers. Xer-cise for chromosomal and ORT for vector plasmid expression are therefore ideal tools that enable efficient biomanufacturing that comply with regulatory requirements. GEN

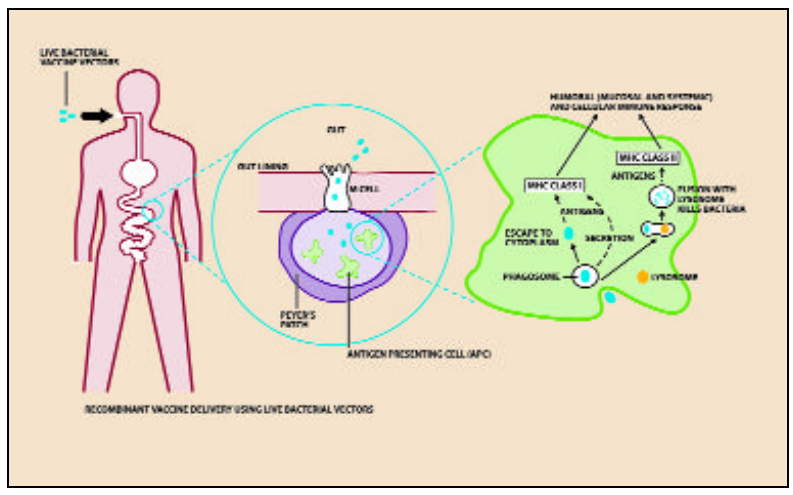


Figure 3. Recombinant vaccine delivery using live bacterial vectors. Bacteria expressing the antigen are engulfed by antigen-presenting cells and activate the immune system after phagocytosis.

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