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Research Article

Xer recombination for the automatic deletion of selectable marker genes from plasmids in enteric bacteria

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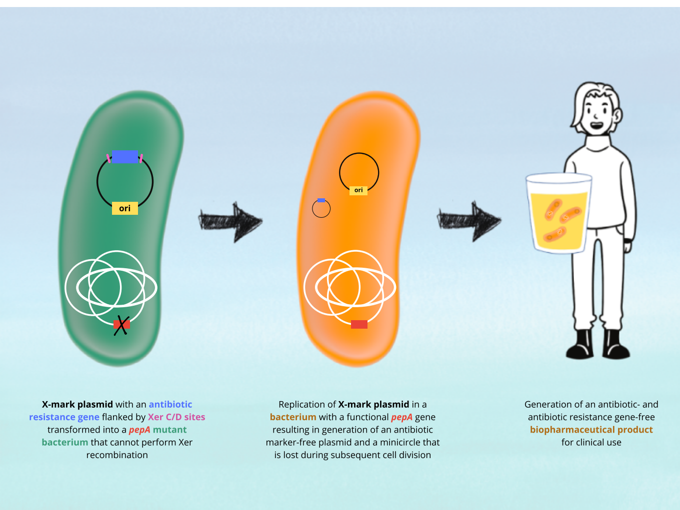
Abstract

Antibiotic resistance genes are widely used to select bacteria transformed with plasmids and to prevent plasmid loss from cultures, yet antibiotics represent contaminants in the biopharmaceutical manufacturing process, and retaining antibiotic resistance genes in vaccines and biological therapies is discouraged by regulatory agencies. To overcome these limitations, we have developed X-mark™, a novel technology that leverages Xer recombination to generate selectable marker gene-free plasmids for downstream therapeutic applications. Using this technique, X-mark plasmids with antibiotic resistance genes flanked by XerC/D target sites are generated in *Escherichia coli* cytosol aminopeptidase (*E. coli pepA*) mutants, which are deficient in Xer recombination on plasmids, and subsequently transformed into enteric bacteria with a functional Xer system. This results in rapid deletion of the resistance gene at high resolution (100%) and stable replication of resolved plasmids for more than 40 generations in the absence of antibiotic selective pressure. This technology is effective in both *Escherichia coli* and *Salmonella enterica* bacteria due to the high degree of homology between accessory sequences, including strains that have been developed as oral vaccines for clinical use. X-mark effectively eliminates any regulatory and safety concerns around antibiotic resistance carryover in biopharmaceutical products, such as vaccines and therapeutic proteins.

Key words: plasmid; antibiotic resistance; *E. coli*; *Salmonella*; X-mark

Graphical Abstract



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1. Introduction

Plasmids are self-replicating DNA sequences that exist naturally in bacteria and other microorganisms and are widely used in the biotechnology industry to create products such as therapeutic proteins and DNA vaccines in bacterial cultures. These plasmids typically encode an antibiotic resistance gene to efficiently identify and retain transformed bacteria using antibiotic-containing media (1). However, the use of antibiotics in biopharmaceutical applications has become strictly controlled by regulatory agencies, and the presence of certain classes of antibiotics, such as beta-lactams, is highly discouraged (2, 3). Similarly, maintaining antibiotic resistance genes in applications where the downstream therapy or vaccine involves live bacteria is not recommended (2, 4). These concerns are predominantly centered around the potential for horizontal gene transfer of remaining plasmid selection markers and how this could contribute to the emergence of antibiotic-resistant bacteria in the clinical setting, which represents a serious global healthcare issue (5).

These regulatory constraints have encouraged the development of numerous alternative plasmid selection technologies that do not rely on antibiotic selection (5–7). Many of these technologies involve introducing a mutation into a chromosomal gene (typically encoding an enzyme involved in a metabolic pathway) within a host bacterial strain and supplying a functional copy of that gene from the plasmid to complement host auxotrophy, with transformants selected using a medium lacking the metabolite or the use of post-segregational killing systems that rely on toxin/antitoxin pairs such as *hok/sok* and *ccdA/ccdB*. There are two key issues with these approaches. Firstly, the burden of constitutive expression of selectable marker gene can lead to plasmid loss (8, 9); and secondly, the rate of plasmid loss can be disguised by the post-segregational killing of plasmid-free cells (10, 11). With auxotrophy complementation, there is also the potential impact of issues such as cross-feeding (12).

To our knowledge, there are currently only two published technologies that allow plasmids to be selected without the expression of any additional marker genes: Operator-Repressor Titration (ORT) and oriSELECT. ORT uses a short plasmid-encoded operator sequence to titrate a repressor protein that would otherwise prevent the expression of an essential chromosomal gene (13–15). oriSELECT takes advantage of an antisense RNA (RNAI) that is naturally produced by the pMB1 origin of replication to regulate its copy number. This antisense RNAI binds to and inhibits a hybrid messenger RNA (mRNA) comprising a complimentary sequence of pMB1 RNAII and the cistron of the repressor of an essential gene (16–18). Both ORT and oriSELECT require prior genetic modification of the host bacterial chromosome, which may be technically challenging as well as time-consuming.

Here, we describe a novel approach to plasmid selection that allows selectable marker genes to be retained for initial plasmid construction and transformation, but deleted in the final bacterial host without the need for genetically modifying the final host cell. This technology, named X-mark, takes advantage of the native Xer site-specific recombination mechanisms that are ubiquitous in bacteria that possess circular replicons (19), and relies on a *pepA* mutant of *E. coli* that cannot perform Xer recombination on plasmids. *pepA* encodes the hexameric protein aminopeptidase-A, which is involved in the metabolism of exogenous and endogenous peptides, and has a secondary role in Xer recombination. Importantly, this gene is nonessential, and the peptidase activity is not required for successful chromosomal Xer recombination (20). In this work, we report the stable construction of an X-mark plasmid possessing an antibiotic resistance gene flanked by Xer recombinase target sites in a *pepA* mutant, subsequent excision of this gene following transformation into Xer-competent bacteria by the native recombinases XerC and XerD and the extended stability of the resultant plasmid.

2. Materials and methods

2.1 Biological resources

Strains. *E. coli* strain DH1 (Coli Genetic Stock Center (CGSC), Yale University, Connecticut, USA), *E. coli* strain DH1PEPA (DH1 ΔpepA; created in this study) and *Salmonella enterica* Typhimurium (*S.* Typhimurium) strain WT05 (TML Δ*aroC,* Δ*ssaV*) (21) were used in this study. All *E. coli* strains were cultured in LB medium (LB Lennox: 10 g/L Phytone™ Peptone (Becton Dickinson), 5 g/L yeast extract (Becton Dickinson),0 5 g/L sodium Chloride (Sigma-Aldrich)) and *S. enterica* Typhimurium WT05 was cultured in LB–aro mix (LB supplemented with a mixture of aromatic amino acids (1 μg/ml 4-aminobenzoic acid; 1 μg/ml 2,3 dihydroxybenzoic acid; 4 μg/ml L-phenylalanine; 4 μg/ml L-tryptophan; 4 μg/ml L-tyrosine; all from Sigma-Aldrich, Gillingham, Dorset, UK)). Antibiotic-resistant strains were cultured in media supplemented with 20 μg/ml chloramphenicol (Sigma-Aldrich, Gillingham, Dorset, UK).

DH1PEPA and plasmid construction. The Xer-cise system (22) was initially used to delete the *pepA* gene from the chromosome of *E. coli* DH1 (23). Briefly, a 1090 base-pair (bp) *dif-cat-dif pepA* deletion cassette was generated by polymerase chain reaction (PCR) using the pTOPO-DifCAT plasmid as a template (22) and primers 5pepAXer and 3pepAXer, both of which include 50 nucleotides at the 5′ end complimentary to the upstream and downstream sequences of the *pepA* coding region on the DH1 chromosome, respectively. Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Hitchin, Herts, UK), 10 ng of DNA plasmid template and primers at 200 nM each were used in a 50 μl reaction. A Bio-Rad T100 thermal cycler was used with the following conditions: initial denaturation at 98°C for 1 min, 30 cycles at 98°C for 20 s and 72°C for 90 s, followed by a final elongation at 72°C for 5 min. The 1090 bp amplicon was gel extracted and transformed by electroporation using a Bio-Rad micropulser (1 mm cuvette, 1.8 kV) into electrocompetent DH1 cells containing a helper plasmid (pRed; Cobra Biologics, Keele, Staffordshire, UK) expressing the lambda Red genes *beta, exo* and *gam* to prevent degradation of the linear PCR product and enable homologous recombination. The *pepA* deletion mutant was selected based on antibiotic resistance following culture in LB medium containing 20 μg/ml chloramphenicol (Sigma-Aldrich, Gillingham, Dorset, UK), and subsequently cultured in the absence of antibiotic selection to permit the excision of the *cat* gene and curing of the helper plasmid, pRed. *pepA* gene deletion was confirmed by PCR with primers 5pepA and 3pepA, and the resultant strain was named **DH1PEPA**.

The plasmid, **pBRT1Nc**, was constructed by synthesizing two fragments: A-1c&2c and B-1c (Genewiz, South Plainfield, New Jersey, USA) and cutting them out from the plasmids in which they were supplied using ApaLI and NcoI restriction enzymes (New England Biolabs, Hitchin, Herts, UK) (the latter being present inside the *cat* gene). This was followed by purification using agarose gel electrophoresis. Both fragments were ligated using T4 DNA ligase (New England Biolabs, Hitchin, Herts, UK). This approach prevented premature resolution at *psi* during DNA synthesis. The ligation product was used to transform electrocompetent DH1PEPA cells, which were plated onto LB agar plates containing 20 μg/ml chloramphenicol (Sigma-Aldrich, Gillingham, Dorset, UK). Single colonies were selected and the resultant

DH1PEPA(pBRT1Nc) cells were cultured in LB-chloramphenicol broth (Sigma-Aldrich, Gillingham, Dorset, UK). pBRT1Nc-derived plasmids, pLTBST, pCF5, pCF10 and pRFP, were generated via NdeI-SalI restriction digest and subcloning of the transgenes downstream of inducible promoter P_{ssaG} in pBRT1Nc. LTBST is a chimeric fusion of heat labile toxin subunit B and heat stable toxin from entero-toxigenic *Escherichia coli* ETEC (456 bp); CF5 is a fusion protein of epitopes from five ETEC colonization factors (893 bp); CF10 is a fusion protein of epitopes from ten ETEC colonization factors (1329 bp); and RFP is red fluorescent protein (678 bp).

All incubation stages were carried out overnight at 37°C, with liquid media shaken at 200 revolutions per minute (RPM). Cells were cryopreserved at –80°C following the addition of 20% glycerol (Fisher Scientific, Loughborough, Leicestershire, UK). All primers were obtained from Sigma-Aldrich (Gillingham, Dorset, UK).

Plasmid maps were created using SnapGene 4.3.7 software. More details on primers, plasmids and bacterial strains/genotypes are provided in Table 1.

Growth rate studies. Three individual colonies of *E. coli* strains DH1 and DH1PEPA were cultured in 5 ml of LB medium for 16 h at 37°C and 200 RPM. These overnight cultures were used to reinoculate 0.15 ml of LB in duplicate wells at a starting cell density of OD_{600nm} and in 96-well U-bottom plates. Bacterial cell growth was monitored for 20 h by recording absorbance at OD_{600nm} at hourly intervals using a multi-well plate reader (Spark® multimode microplate reader, Tecan, Männedorf, Switzerland) set at 37°C in continuous rotation mode.

Xer recombination frequency. The plasmid pBRT1Nc was purified from the DH1PEPA strain cultured in LB-chloramphenicol and transformed into *E. coli* DH1 or *S. enterica* Typhimurium WT05 electrocompetent cells. Transformants were selected on LB or LB–aro mix agar plates, respectively, containing 20 μg/ml chloramphenicol (Sigma-Aldrich, Gillingham, Dorset, UK). One colony each of DH1PEPA(pBRT1Nc), DH1(pBRT1Nc) or WT05(pBRT1Nc) were picked from chloramphenicol selective plates and reinoculated into 5 ml of LB or LB–aro mix without antibiotic selection and cultured for approximately 16 h at 37°C and 200 RPM. Overnight bacterial cultures were subsequently serially diluted using 10-fold dilution factors in phosphate-buffered saline (PBS; Life Technologies, Loughborough, Leicestershire, UK) and 0.1 ml of dilutions in the 10^{-6} to 10^{-8} range were plated out on LB or LB–aro mix agar plates to obtain single colonies. To calculate *cat* gene resolution frequency after growth in the absence of antibiotic selective pressure, 50 colonies each of DH1(pBRT1Nc), DH1PEPA(pBRT1Nc) or WT05(pBRT1Nc) were picked from the serial dilution plates and replicated onto an LB versus LB-chloramphenicol plate (plus aro-mix supplements for the WT05 strain, as required). The experiment was repeated for three independent biological replicates, giving a total of 150 colonies per strain (50×3) screened by replica plating. The number of colonies growing in the presence and absence of chloramphenicol were enumerated and compared.

Table 1. Details of primers (nucleotide sequences), plasmids and bacterial strains. The pepA locus homology is underlined where it is present in primer sequences

Name	Details	Reference
Primers		
5pepAXer	ATTCTATCTGTAGCCACCGCCGTTGTCTTTAA-GATTCAGGAGCGTAGTGCctgcagaattcgcccttcct	This work
3pepAXer	GGATAAGGCGTTCACGCCGCATCCGGCAATAACAGCCTTGCCT-GACGCAAagtgtgctggaattcgccct	This work
5pepA	GCGGACATGAGTTACGAAAG	This work
3pepA	ATCAGGCCTACGAGTTCAGT	This work
pMB-L	GCTCACGCTGTAGGTATCTC	This work
pMB-R	CAACTCTTTTTCCGAAGGTA	This work
Plasmids		
pACYC184	Source of *cat* gene	NEB (Hitchin, Hertfordshire, UK)
pTOPO-DifCAT	Source of *dif-cat-dif cassette* sequence	Bloor & Cranenburgh 2006 (Ref. 22)
pRed	Helper plasmid with lambda-Red genes, *beta*, *exo* and *gam*	This work
pBRT1Nc	X-mark™ plasmid carrying *cat* gene flanked by directly repeated *psi* and accessory sequences	This work
pBRT1N	Antibiotic resistance gene-free plasmid derived from pBRT1Nc	This work
pLTBST	pBRT1Nc-derived plasmid carrying the 456 bp transgene LTBST	This work
pCF5	pBRT1Nc-derived plasmid carrying the 893 bp transgene CF5	This work
pCF10	pBRT1Nc-derived plasmid carrying the 1329 bp transgene CF10	This work
pRFP	pBRT1Nc-derived plasmid carrying the 678 bp transgene RFP	This work
Bacterial strains & genotypes		
E. coli DH1	F^-, *endA1*, *hsdR17* (r_k^-, m_k^+), λ^-, *glnV44*, *thi-1*, *recA1*, *gyrA96*, *relA1*	Hanahan 1983 (Ref. 23)
E. coli DH1PEPA	F^-, *endA1*, *hsdR17* (r_k^-, m_k^+), λ^-, *glnV44*, *thi-1*, *recA1*, *gyrA96*, *relA1*, *pepA*	This work
S. enterica serovar Typhimurium WT05	TML *aroC*, *ssaV*	Hindle 2002 (Ref. 21)

Abbreviations: *cat*, chloramphenicol acetyltransferase; CF5, fusion protein of epitopes from five ETEC colonization factors; CF10, fusion protein of epitopes from ten ETEC colonization factors; *E. coli*, *Escherichia coli*; ETEC, Enterotoxigenic *Escherichia coli*; LTBST: chimeric protein fusion of heat-labile enterotoxin beta and heat-stable toxin from ETEC; NEB, New England Biolabs; RFP, red fluorescent protein; *S. enterica*, *Salmonella enterica*.

Of the 150 colonies screened for antibiotic resistance by replica plating, a total of 25 chloramphenicol-sensitive colonies per strain were further tested by colony–PCR to confirm the presence of the plasmid. Colony–PCR reactions were set up in a 20 μl reaction volume using MyTaq™ HS Red DNA Polymerase (Bioline-Meridian Bioscience, London, UK) and primers pMB-L and pMB-R. 5 μl of each PCR reactions was run on a 1% agarose gel with the HyperLadder™ 1kb DNA size marker (Bioline-Meridian Bioscience, London, UK) for comparison. A positive control PCR reaction using 10 ng of plasmid pBRT1Nc purified from DH1PEPA as a template was included.

Assessment of plasmid stability. The plasmid pBRT1Nc was purified from DH1PEPA(pBRT1Nc) cells and transformed into *E. coli* DH1 or *S. enterica* Typhimurium WT05 electrocompetent cells. Transformants were selected on LB agar plates containing 20 μg/ml chloramphenicol (Sigma-Aldrich, Gillingham, Dorset, UK) or 20 μg/ml chloramphenicol plus aro-mix for the WT05 strain. One colony each of DH1(pBRT1Nc) or WT05(pBRT1Nc) transformants were picked and inoculated into 5 ml of LB broth plus 20 μg/ml chloramphenicol (Sigma-Aldrich, Gillingham, Dorset, UK), supplemented with aro-mix as necessary and cultured for approximately 18 h at 37°C. Plasmid DNA was extracted from a volume of overnight culture equivalent to an optical density with absorbance at $OD_{600nm} = 4$ (measured using an Ultrospec™ 2100, GE Healthcare, Chalfont St Giles, Buckinghamshire, UK). Fresh LB medium (5 ml) without antibiotics was reinoculated using a starting optical density of $OD_{600nm} = 0.001$ obtained from overnight cultures. Plasmid extraction and subculturing in LB medium without antibiotic selection was repeated for a total of 5 days.

Normalization of the amount of DNA loaded per well was performed by extracting the plasmid from an equivalent biomass per sample. Briefly, OD_{600nm} values from all samples were used to determine the volume of each culture to harvest to ensure equivalent number of cells across all samples. For example, if a culture had reached $OD_{600nm} = 1$ and another had reached $OD_{600nm} = 2$, then 2 ml of the first culture and 1 ml of the second culture were harvested, respectively, to generate the equivalent number of total cells to an OD_{600nm} value of 2 across both samples. Plasmid purification was performed using a QIAprep® Spin Miniprep Kit (QIAGEN, Manchester, UK). Daily plasmid preparations (50 μl) were collected and stored at –20°C until use. 10 μl aliquots of plasmid were digested with NdeI (New England Biolabs, Hitchin, Herts, UK) and assessed using 1% agarose gel electrophoresis with the HyperLadder™ 1kb DNA size marker for comparison. The plasmid pBRT1Nc was purified from DH1PEPA cultured in LB-chloramphenicol broth and used as a reference strain.

Plasmid resolution and plasmid stability experiments were each independently repeated more than three times with reproducible results.

To determine the effect of transgene cloning and expression on plasmid stability, four plasmid derivatives of pBRT1Nc (pLTBST, pCF5, pCF10 and pRFP) were isolated from the DH1PEPA strain and transformed into *S. enterica* Typhimurium WT05 electrocompetent cells. Transformed WT05 cells were selected on LB–aro mix agar plates containing 20 μg/ml chloramphenicol. Chloramphenicol-resistant WT05 colonies were subcultured for 16 h in LB–aro mix to allow *cat* gene excision. Chloramphenicol-sensitive clones were isolated for each strain by replica plating on LB–aro mix and LB–aro mix chloramphenicol plates. One antibiotic-sensitive colony for each strain was inoculated in 5 ml of LB–aro mix and grown for ∼18 h at 37°C and 200 RPM. The LB–aro mix overnight cultures (day 1) were used to reinoculate 5 ml of either fresh LB–aro mix medium or 5 ml of inducing medium, PCN pH 5.8 supplemented with aromatic amino acids (same aro-mix concentration as in LB–aro mix), at a starting optical density of $OD_{600nm} = 0.001$. The composition of PCN pH 5.8 has previously been described (24). Subculturing in LB–aro mix and in PCN aro-mix medium was repeated for a total of 4 days. Plasmid DNA was extracted from cultures collected after each round of subculturing. A volume of overnight culture equivalent to an optical density with absorbance at $OD_{600nm} = 1$ was used to isolate the plasmids using QIAprep® Spin Miniprep Kit (QIAGEN, Manchester, UK) and DNA was loaded onto agarose gels to assess plasmid stability over time.

3. Results

3.1 Overview and development

We have developed a new method for excising selectable marker genes from plasmids using Xer recombination (Figure 1). In this approach, plasmids containing a selectable marker gene flanked by directly repeated XerC/D recombination target sites (*psi*) and cognate accessory sequences are initially cultured in a host cell environment where recombination cannot proceed (i.e. in a bacterial strain that cannot perform Xer recombination). Plasmids are subsequently cultured in a different host cell environment where recombination can proceed (i.e. in a bacterial strain with a functional Xer recombination system), which leads to site-specific recombination and excision of the selectable marker gene. This creates a self-deleting 'minicircle' antibiotic resistance gene sequence with no origin of replication, which is lost upon cell division, and maintains a plasmid in the final host that contains a transgene of interest along with a single *psi* site and accessory sequences without the need for antibiotic selection (Figure 1).

Initially, we used the Xer-cise™ system (22) to delete the *pepA* gene from the chromosome of *E. coli* DH1 (23) to create a host strain named **DH1PEPA**. *pepA* encodes the hexameric protein aminopeptidase-A, which has a primary role in the metabolism of exogenous and endogenous peptides and a secondary role in Xer recombination (20).

The *E. coli pepA* mutant DH1PEPA was used to construct the plasmid **pBRT1Nc**, which possesses the medium copy number pMB1 origin of replication in addition to an X-mark cassette (Figure 2A). The X-mark cassette consists of two *psi* sites and accessory sequences in the same orientation flanking a selectable marker gene, in this case the chloramphenicol antibiotic resistance gene, *cat* (chloramphenicol acetyltransferase).

Importantly, the *E. coli pepA* mutant, DH1PEPA, maintains functional Xer recombination on chromosomes, and exhibits no apparent decrease in viability compared to the parental DH1 strain when cultured in nutrient broth (Supplementary Figure S1).

Culturing the pBRT1Nc plasmid in *E. coli* DH1PEPA, which has a defective Xer system, prevents recombination events at *psi* sites and loss of the *cat* gene. Culturing the same pBRT1Nc plasmid in the DH1PEPA parent strain (DH1), or another enteric bacterium with a functional Xer system, results in *cat* gene excision by the native recombinases, XerC and XerD. This generates an antibiotic resistance gene-free plasmid, **pBRT1N**, that is designed to be stably maintained in the absence of antibiotics (Figure 2B). Ultimately, downstream clonal selection of an antibiotic resistance gene-free clone would subsequently be performed to generate the final biopharmaceutical product for manufacturing and clinical use.

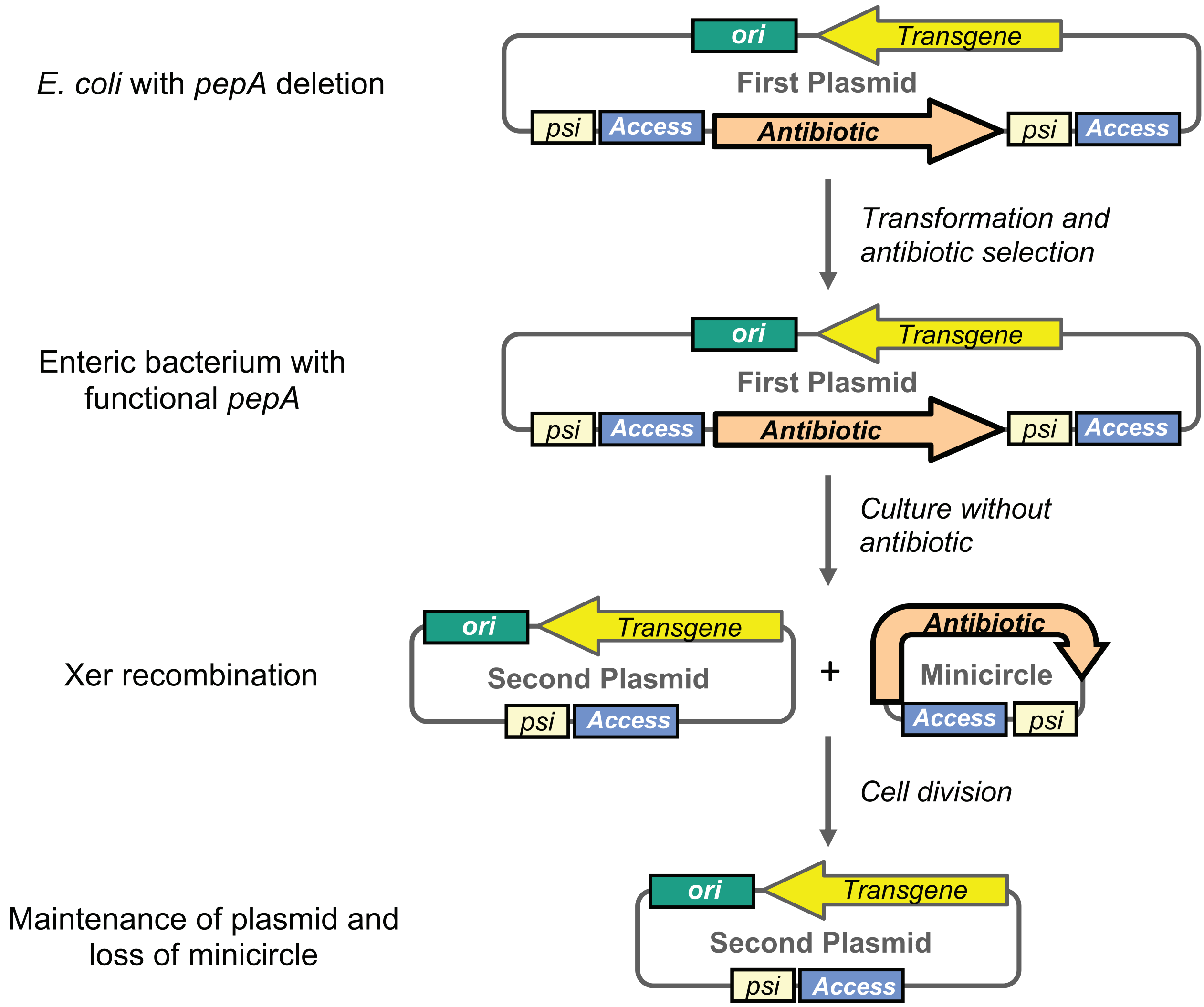

Figure 1. The X-mark mechanism of selectable marker gene deletion by Xer recombination. A plasmid with a selectable marker gene, such as an antibiotic resistance gene, flanked by directly repeated site-specific XerC/D recombination target sites (*psi*) and cognate accessory sequences is cultured in an *E. coli pepA* mutant that is incapable of performing Xer recombination. Subsequent culture of the plasmid in an enteric bacterium with a functional *pepA* gene allows the antibiotic resistance gene to be excised as a 'minicircle', which has no origin of replication and is subsequently lost upon cell division, and maintains a plasmid in the final host that contains a transgene of interest along with a single *psi* site and accessory sequences without the need for antibiotic selection. Abbreviations: Access, accessory sequences; antibiotic, selectable marker gene, such as an antibiotic resistance gene; *E. coli*, *Escherichia coli*; *ori*, origin of replication; *pepA*, cytosol aminopeptidase; *psi*, *psi* site; transgene, recombinant gene cassette.

3.2 Testing gene resolution and plasmid maintenance

We assessed resolution of the *cat* gene in two bacterial strains with a functional Xer system: *E. coli* DH1 and *S. enterica* Typhimurium WT05. The latter strain was developed as an oral live bacterial vaccine against emerging infectious and malignant diseases (21, 25). We also included the Xer-deficient *E. coli* DH1PEPA as a reference strain.

After transforming the pBRT1Nc plasmid into each of the three test strains, we observed that *cat* gene resolution occurred rapidly and at a high frequency in both bacterial strains with a functional Xer system. After approximately 16 h of culturing these bacteria in the absence of antibiotic selection, 100% of both DH1(pBRT1N) and WT05(pBRT1N) screened colonies were sensitive to chloramphenicol (Figure 3A). This suggested efficient resolution of the *cat* gene through Xer recombination. As expected, 100% of the DH1PEPA(pBRT1Nc) screened colonies retained chloramphenicol resistance, confirming the inability of the *pepA* mutant to perform Xer recombination to delete the *cat* gene (Figure 3A). Colony–PCR confirmed the presence of the plasmid in 100% of the PCR-tested chloramphenicol-sensitive colonies for both DH1(pBRT1N) and WT05(pBRT1N) (Figure 3A). pBRT1N plasmid maintenance was subsequently assessed over a period of 5 days of repetitive subculture (approximately 40 generations) in the absence of antibiotic selection in *E. coli* DH1 and *S. enterica* Typhimurium WT05. DNA extraction confirmed that the antibiotic marker-free plasmid, pBRT1N, was maintained in both bacterial strains over 5 days of sequential subculturing with no significant loss throughout the study (Figure 3B). Importantly, plasmids with active transgenes ranging in size from 456 to 1329 bp were also stably maintained with no significant loss over time in *S. enterica* Typhimurium WT05 grown in aro-mix-supplemented PCN medium to induce transgene expression (Supplementary Figure S2). This suggested stable, long-term maintenance of antibiotic marker gene-free plasmids in both the presence and absence of active transgenes.

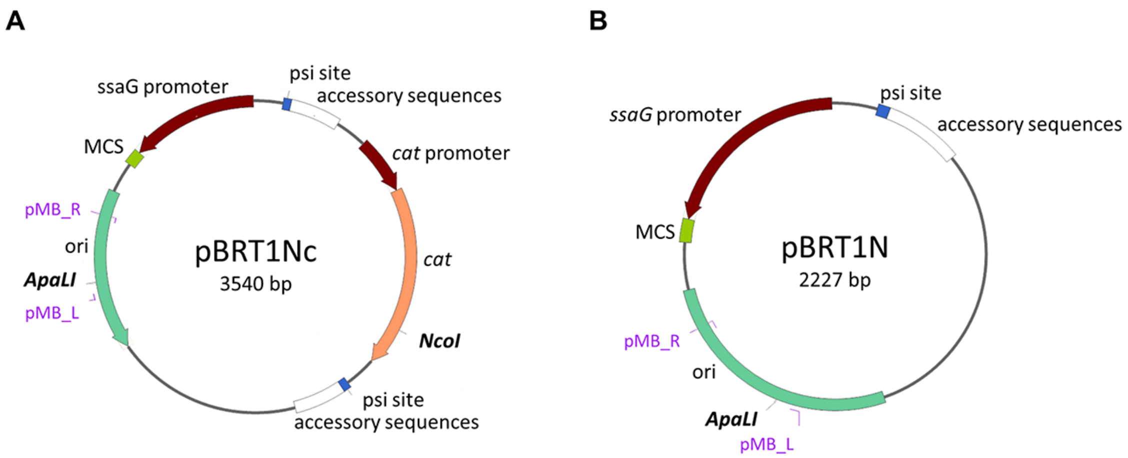

Figure 2. Generating a selectable marker gene-free plasmid in *E. coli*. A) Plasmid map of the X-mark plasmid, pBRT1Nc, containing an X-mark cassette comprising direct repeats of XerC/D recombination target sites (*psi*) and cognate accessory proteins ArcA/PepA binding site (accessory sequences) flanking the chloramphenicol antibiotic resistance gene. B) Plasmid map of pBRT1Nc's resolved derivative, pBRT1N, where the X-mark cassette has been lost following Xer recombination. Abbreviations: bp, base pairs; *cat*, chloramphenicol acetyl transferase gene; MCS, multiple cloning site; *ori*, origin of replication; *psi*, pSC101 stabilized inheritance site; *ssaG*, SPI2 secretion system apparatus protein SsaG.

4. Discussion

In this study, we have described the development of a recombination technology that is positioned to provide a method for generating a variety of downstream biopharmaceutical products for clinical use that fully comply with regulatory recommendations around the use of antibiotics and antibiotic resistance genes in final applications. Using X-mark, constitutive selectable marker gene expression is rapidly eliminated in the final bacterial host in the absence of antibiotics and without the need for *a priori* genetic modification of the host strain. Of note, the excision frequency of an X-mark cassette following transformation into a *pepA*-positive strain is such that transformant colonies can easily be selected in the presence of antibiotics, yet the resistance gene is rapidly excised during further culture in the absence of antibiotics. The advantages of antibiotic selection for plasmid construction and transformation are also retained in the upstream steps of the cloning process. Importantly, the X-mark technology restores the single *psi* and accessory sequences in the final host plasmid to provide greater stability in RecA-positive bacterial strains, including *E. coli* and *Salmonella*, which are commonly used for recombinant protein expression or vaccine development. This is achieved by preventing the generation of plasmid multimers through RecA that could otherwise result in plasmid loss through the 'dimer catastrophe' (26).

The X-mark technology relies on the natural bacterial process of Xer recombination, which converts chromosome and plasmid dimers generated by replication or homologous recombination back to monomers (27). This process requires the 28 bp target site *dif* plus XerC and XerD site-specific recombinases together with the cell division translocase protein, FtsK, in *E. coli* and *Salmonella* species (28) or RipX and CodV in *Bacillus* species (29). The excision of an antibiotic resistance gene flanked by *dif* sites was first demonstrated by Recchia *et al.* to investigate the role of FtsK in Xer recombination. It has since been utilized in the Xer-cise™ technology for antibiotic resistance gene excision following host bacterial chromosomal genetic modification in a variety of bacterial species (22, 30, 31).

On the plasmids of the Enterobacteriaceae family, ∼180 bp accessory sequences and proteins are also required in addition to XerC and XerD. *E. coli* plasmids, such as ColEI and pMB1, possess the *cer* target site (32) and recombination also requires the host accessory proteins, PepA and ArgR (the arginine repressor) (33). *Salmonella* plasmids, such as pSC101, possess the *cer* homologous target sequence *psi* (34) and require accessory proteins PepA and ArcA (the DNA binding protein of a two-component system that regulates gene expression in anaerobic conditions) (35). Initial strand exchange at the 28 bp *psi* sites is catalyzed by XerC to form a Holliday junction, upon which XerD acts to complete the recombination reaction and generate two covalently closed circular DNA molecules (36). On plasmids, the accessory sequences and accessory proteins function to ensure that Xer recombination is an exclusively intramolecular reaction, enabling dimer resolution but preventing the intermolecular recombination that would otherwise convert monomers into dimers (20). PepA and ArgR interact directly on an *E. coli* ColE1 plasmid dimer with two directly repeated *cer* sites to form a complex, with the accessory sequence DNA wrapping around PepA and ArgR in three negative supercoils. This aligns the *cer* sites and enables XerC and XerD to catalyze strand exchange (37).

Stirling *et al.* first demonstrated the excision of a *cat* gene flanked by *cer* sites in plasmid pKS455 to select *xer* mutants (38). X-mark relies on an *E. coli* strain in which Xer recombination on plasmids cannot take place; otherwise, premature excision of the antibiotic resistance gene would occur, resulting in a mixed population even in the presence of antibiotics. An alternative approach would be to mutate *xerC* or *xerD*; however, this results in a phenotype that is not amenable for plasmid selection, since the failure of correct chromosomal partitioning results in bacteria with a filamentous cellular morphology and poor viability (39). In contrast, the *E. coli pepA* mutant, DH1PEPA, maintains functional Xer recombination on chromosomes, and exhibits no apparent decrease in viability compared to the parental DH1 strain when cultured in nutrient broth, making it suitable for use in the X-mark system. The X-mark plasmid described in this study (pBRT1Nc), which comprises an antibiotic resistance gene flanked by *psi* and accessory sequences, functions in both *E. coli* and *Salmonella* bacteria due to the high degree of sequence homology between accessory proteins (40). Specifically, ArcA is able to

A

	E. coli DH1PEPA	*E. coli* DH1	*S. enterica* WT05
Number of colonies screened	150	150	150
Chloramphenicol-sensitive colonies	0/150 (0%)	150/150 (100%)	150/150 (100%)
Chloramphenicol-resistant colonies	150/150 (100%)	0/150 (0%)	0/150 (0%)
Resolution frequency	0%	100%	100%
Plasmid-positive colonies by colony PCR*	25/25 (100%)	25/25 (100%)	25/25 (100%)

B

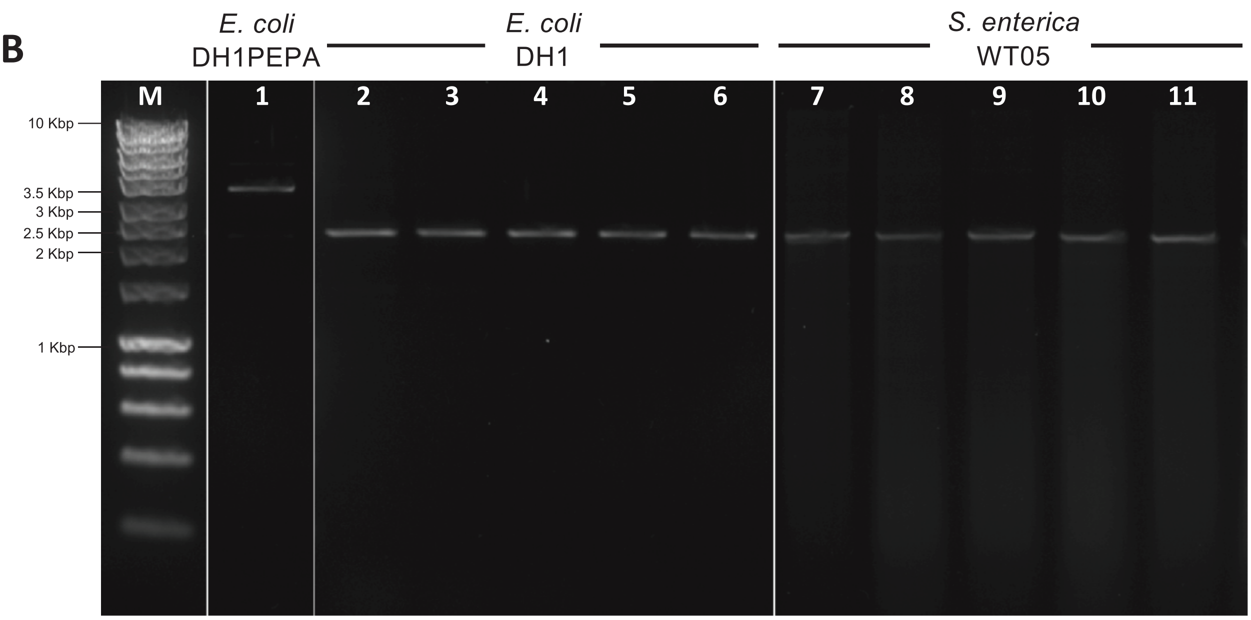

Figure 3. Chloramphenicol acetyltransferase (*cat*) gene resolution study. A) *cat* gene resolution frequency and percentage of plasmid-positive colonies in *E. coli* DH1PEPA (Xer recombination-deficient), *E. coli* DH1 (the parental strain; Xer recombination-proficient) or S. enterica Typhimurium WT05 (Xer recombination-proficient). This was based on three independent experimental repeats with 50 colonies tested each time. B) NdeI-cut pBRT1Nc plasmid extracted from *E. coli* DH1PEPA (lane 1), *E. coli* DH1 (lanes 2–6) or *S. enterica* Typhimurium WT05 (lanes 7–11) cultured for a total of 5 days without antibiotic selection. Each lane represents a different day of culture (day 1 = lanes 1, 2 & 7; day 2 = lanes 3 & 8; day 3 = lanes 4 & 9; day 4 = lanes 5 & 10; and day 5 = lanes 6 & 11). Abbreviations: *E. coli*, *Escherichia coli*; Kbp, kilobase pairs; M, HyperLadder™ 1Kbp marker; PCR, polymerase chain reaction; *S. enterica*, *Salmonella enterica* Typhimurium WT05. The gel in part B) is representative of three independent experimental repeats. Images were cropped from the same gel run in the same experiment. * of PCR-tested chloramphenicol-sensitive colonies.

substitute for ArgR to enable Xer recombination at *psi*. Cloning genes into the pBRT1Nc plasmid using a *pepA* mutant is a straightforward process using standard molecular biology techniques that can be easily adapted to suit a wide range of downstream applications.

Site-specific recombination for gene excision is currently used in minicircle technology, where a non-replicating DNA minicircle carrying a gene of interest together with its regulatory elements is generated by the induction of an exogenous recombinase gene and subsequent purification from the mini-plasmid (41). X-mark differs from this approach in its use of a two-cell strategy, endogenous site-specific recombinases and its ultimate goal of creating a mini-plasmid containing a transgene of interest that is maintained plus a minicircle containing a selectable marker gene that is subsequently lost from the cell. From a downstream processing perspective, a key difference between X-mark and minicircle technology is that with X-mark, the molecule of interest for transgene expression is not the minicircle (i.e. the nonreplicative circular DNA molecule) but the mini-plasmid (containing the origin of replication and transgene) obtained after excision of the antibiotic resistance gene. Thus, there is no need for downstream purification to isolate the mini-plasmid from the minicircle. With minicircle technology, affinity chromatography is still required to separate the mini-plasmid that is carrying the 'unwanted' antibiotic resistance marker gene. X-mark also simplifies the isolation and propagation of bacterial cells that only contain the mini-plasmid of interest through clonal selection of bacteria that have lost antibiotic resistance. These mini-plasmid-carrying bacteria can subsequently be used as live vectors for recombinant vaccine delivery.

To our knowledge, X-mark represents the first recombination technology to allow selectable marker genes to be retained for plasmid construction and transformation but automatically deleted in the final bacterial host without the need for genetically modifying the final host cell. The versatility of this technology, which can be used in multiple host strains given sufficient sequence homology of accessory proteins without the need for prior genetic modification, represents a major advance in the ability to quickly and easily manufacture biopharmaceutical-grade products for downstream clinical applications, including vaccines and therapeutic proteins, without the need for antibiotics or the retention of antibiotic resistance gene.

Supplementary data

Supplementary data are available at SYNBIO Online.

Material availability

The material and resources presented in this study are available subject to MTA.

Data availability

The original data contributions presented in this study are all included in the article.

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Author contributions

P.S., M.W.L., B.H. and R.M.C. contributed to the conception and design of the work; P.S., M.W.L. and B.H. contributed to the acquisition, analysis and interpretation of data; P.S. contributed to drafting the work; and all authors contributed to critically revising the work, finally approving it for submission and agreeing to be held accountable for all aspects of the work.

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