Office de la Propriété Intellectuelle du Canada
Un organisme d'Industrie Canada

Canadian Intellectual Property Office
An agency of Industry Canada

CA 2572003 A1 2006/01/12

(21) 2 572 003

(12) DEMANDE DE BREVET CANADIEN
CANADIAN PATENT APPLICATION

(13) A1

(86) Date de dépôt PCT/PCT Filing Date: 2005/07/01
(87) Date publication PCT/PCT Publication Date: 2006/01/12
(85) Entrée phase nationale/National Entry: 2006/12/22
(86) N° demande PCT/PCT Application No.: GB 2005/002590
(87) N° publication PCT/PCT Publication No.: 2006/003412
(30) Priorité/Priority: 2004/07/01 (GB0414832.6)

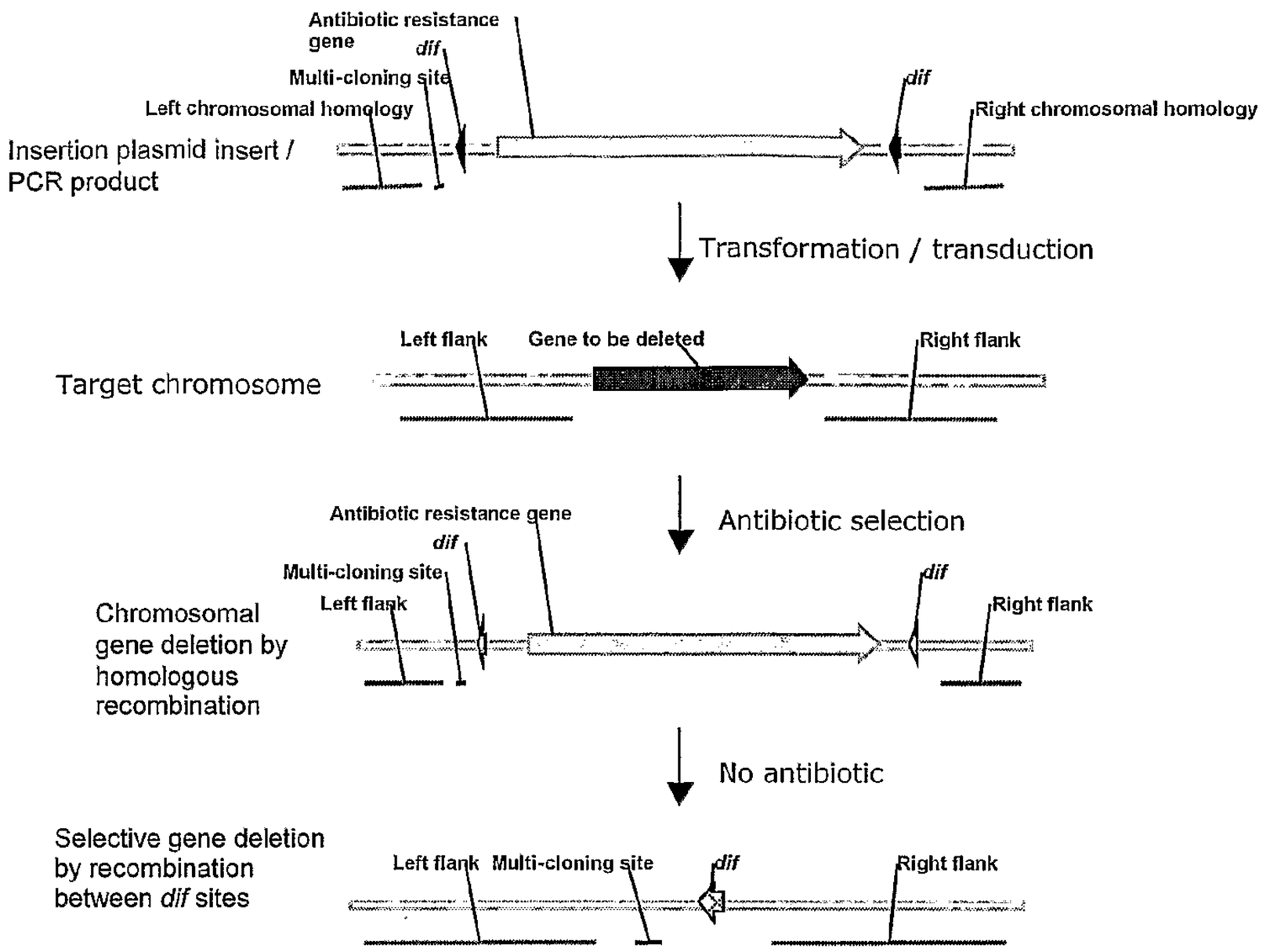
(51) Cl.Int./Int.Cl. *C12N 15/90* (2006.01)

(71) Demandeur/Applicant:
COBRA BIOLOGICS LIMITED, GB

(72) Inventeurs/Inventors:
CRANENBURGH, ROCKY MARC, GB;
BLOOR, ALEXANDRA ELIZABETH, GB

(74) Agent: KIRBY EADES GALE BAKER

(54) Titre : PROCEDE D'ELIMINATION DE SEQUENCES DE GENES SELECTIONNABLES
(54) Title: PROCESS FOR THE REMOVAL OF SELECTABLE MARKER GENE SEQUENCES



(57) Abrégé/Abstract:
The invention relates to a process for the removal of selectable marker gene sequences, in particular antibiotic gene sequences, from nucleic acid molecules, wherein the process makes use of dif-like site-specific recombinase recognition sites. The invention

(21) 2 572 003

(13) A1

(57) Abrégé(suite)/Abstract(continued):
further relates to the application of this process in the unlabelled integration and deletion of chromosomal genes and in controlling gene expression.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
12 January 2006 (12.01.2006)

PCT

(10) International Publication Number
WO 2006/003412 A1

(51) International Patent Classification[7]: C12N 15/90

(21) International Application Number:
PCT/GB2005/002590

(22) International Filing Date: 1 July 2005 (01.07.2005)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
0414832.6 1 July 2004 (01.07.2004) GB

(71) Applicant *(for all designated States except US)*: **COBRA BIOLOGICS LIMITED** [GB/GB]; Stephenson Building, Keele University Science Park, Keele ST5 5SP (GB).

(72) Inventors; and

(75) Inventors/Applicants *(for US only)*: **CRANENBURGH, Rocky, Marc** [GB/GB]; Cobra Biologics Limited, Stephenson Building, Keele University Science Park, Keele ST5 5SP (GB). **BLOOR, Alexandra, Elizabeth** [GB/GB]; Cobra Biologics Limited, Stephenson Building, Keele University Science Park, Keele ST5 5SP (GB).

(74) Agents: **HALLYBONE, Huw, George** et al.; Carpmaels & Ransford, 43-45 Bloomsbury Square, London WC1A 2RA (GB).

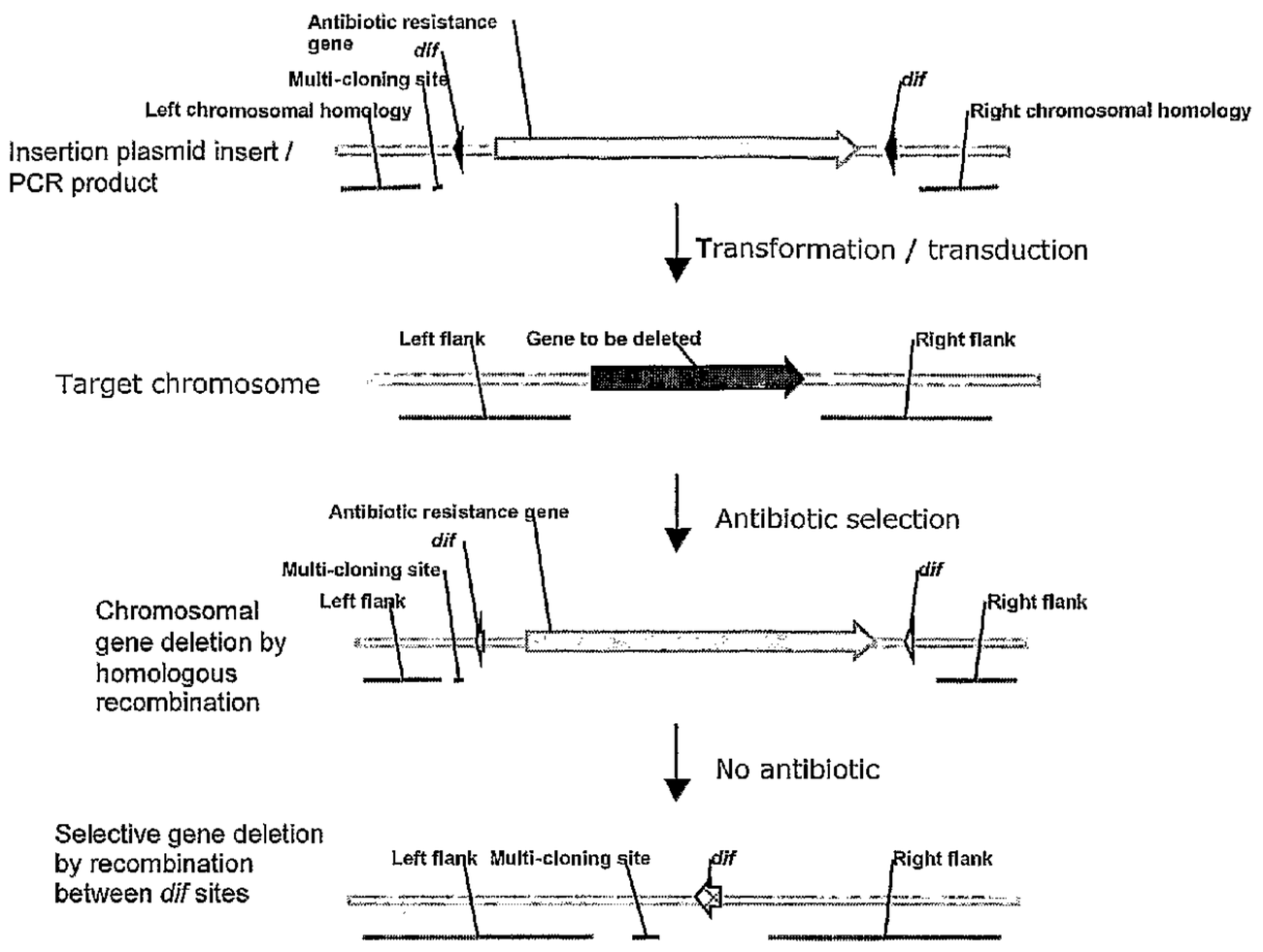
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report*
— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments*

[Continued on next page]

(54) Title: PROCESS FOR THE REMOVAL OF SELECTABLE MARKER GENE SEQUENCES



(57) Abstract: The invention relates to a process for the removal of selectable marker gene sequences, in particular antibiotic gene sequences, from nucleic acid molecules, wherein the process makes use of dif-like site-specific recombinase recognition sites. The invention further relates to the application of this process in the unlabelled integration and deletion of chromosomal genes and in controlling gene expression.

WO 2006/003412 A1

WO 2006/003412 A1

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

PROCESS FOR THE REMOVAL OF SELECTABLE MARKER GENE SEQUENCES

FIELD OF THE INVENTION

The invention relates to a process for the removal of selectable marker gene sequences, in particular antibiotic gene sequences, from nucleic acid molecules. The invention further relates to the application of this process in the unlabelled integration and deletion of chromosomal genes and in controlling gene expression.

All documents referred to herein are incorporated by reference.

BACKGROUND OF THE INVENTION

Antibiotic resistance genes or other selectable marker genes are routinely used to select for the chromosomal insertion of heterologous genes or the deletion of native genes to create new strains of bacteria. These selectable marker genes are also routinely used to select for the presence of plasmids in bacterial cells. However, the retention of selectable marker genes in bacterial host cells, whether they are integrated into the chromosome or present on plasmids, gives rise to a number of problems.

Firstly, the presence of selectable marker genes in the host chromosome reduces the variety of plasmids that can be propagated in a cell, as these also rely on selectable marker genes for their selection and maintenance. Furthermore, genetically modified bacteria containing chromosomal antibiotic resistance genes are undesirable for biotherapeutics production, in particular for DNA vaccine and gene therapy applications, as the chromosomal DNA will represent a low-level contaminant of the final product and carry the risk of antibiotic gene transfer to pathogenic bacteria in the patient or the environment. Antibiotic resistance genes on plasmids are also undesirable, as they constitute a metabolic burden in recombinant protein production and a biosafety concern when the plasmid is being manufactured for use in gene therapy and DNA vaccine applications.

It is thus highly desirable to be able to insert genes into or delete genes from bacterial cell chromosomes without leaving antibiotic resistance or other selectable marker genes behind and to be able to remove these selectable marker genes from plasmids when they are no longer required. To date, a couple of strategies have been developed for unlabelled (*i.e.* selectable marker gene-free) chromosomal gene insertions and deletion in bacterial cells and for deletion of marker genes from plasmids.

One strategy for unlabelled gene insertion and deletion relies on integrating a plasmid containing a selectable marker gene into the bacterial host cell chromosome via a single homologous recombination event, followed by the removal of the plasmid by a second recombination event (resolution) to hopefully produce the desired genotype (Leenhouts *et al.* 1996; Link *et al.*, 1997). A major disadvantage with this approach is that if the insertion or deletion reduces the fitness of the cell in terms of its ability to survive, the resolution event will invariably regenerate the wild type rather than the mutant genotype. This approach is therefore highly inefficient.

An alternative strategy is to use a double recombination event to efficiently integrate an antibiotic resistance gene cassette flanked by regions of chromosomal homology into the bacterial chromosome. Recognition sites for a site-specific recombinase (SSR) immediately flank the antibiotic resistance gene. Following chromosomal integration, a recombinase expressed *in trans* excises the antibiotic resistance gene. Examples of site-specific recombinases/target sites used for antibiotic gene excision include Cre/*loxP* from the bacteriophage P1 (Dale and Ow, 1991), FLP/*FRT* (Datsenko and Wanner, 2000) and R/*RS* (Sugita *et al.,* 2000) from yeast. Alternatively, flanking antibiotic resistance genes with internal resolution sites enables excision by a transposase expressed in *trans* (Sanchis *et al.*, 1997). The disadvantage of this strategy is that it requires an exogenous recombinase or transposase to be expressed in the target cell. The cell must therefore be transformed twice.

Selectable marker genes are currently also removed from plasmids using site-specific recombinases as described for the chromosomal applications above, or, more commonly, by restriction endonuclease digestion. The recombinase approach requires an additional site-specific recombinase gene to be present in *cis* or on a helper plasmid in *trans*. The restriction digest approach requires several extra stages of plasmid DNA manipulation. Both these approaches involve a number of complex manipulations.

Given the increasing importance of generating nucleic acid molecules without selectable marker genes, in particular antibiotic resistance genes, there is a need to develop improved and simpler processes for unlabelled gene insertion and deletion and for removing selectable marker genes from plasmids.

SUMMARY OF THE INVENTION

According to a first aspect of the invention, there is provided a process for removing a selectable marker gene from a nucleic acid molecule in a cell comprising culturing a cell comprising a nucleic acid molecule comprising a selectable marker gene flanked by site-specific recombinase recognition sites under conditions such that an endogenous site-specific recombinase in the cell acts to excise the selectable marker gene by site-specific recombination between the site-specific recombinase recognition sites. Preferably, the site-specific recombinase recognition sites are *dif*-like sites.

Prokaryotic cells contain endogenous site-specific recombinases that resolve chromosomal dimers generated by RecA. These recombinases are XerC/XerD in gram-negative bacteria such as *Escherichia coli* (Leslie and Sherratt, 1995) and RipX/CodV in gram-positive bacteria such as *Bacillus subtilis* (Sciochetti *et al.* 2001). These endogenous site-specific recombinases act at *dif* sites present in the prokarytic chromosome. A single *dif* site is normally present in a prokarotic chromosome. When chromosomal dimers are generated, the site-specific recombinase acts to promote recombination between two *dif* sites to excise the intervening DNA and generate chromosome monomers.

Endogenous prokaryotic site-specific recombinases such as XerC/XerD and RipX/CodV also resolve plasmid dimers generated by RecA by acting at dimer resolution sites that occur naturally in plasmids. For example, the ColE1 plasmid contains a dimer resolution site called *cer* and the pSC101 plasmid contains a dimer resolution site called *psi.* When plasmid dimers are formed, endogenous site-specific recombinases act to excise the DNA between two dimer resolution sites, resulting in plasmid monomers. However, unlike *dif* sites, the site specific recombinases only act on plasmid-borne dimer resolution sites if accessory sequences of ~200 bp are also present on the plasmid (Hayes and Sherratt, 1997).

Eukaryotic cells also contain endogenous site-specific recombinases which act to excise DNA between two site-specific recombinase recognition sites. For example, the Flp recombinase of the yeast two-micron plasmid acts to monomerise concatomers by excising DNA between FRT sites.

Experimental investigations into the mechanism of action of site-specific recombinases at *dif* sites in prokaryotes have shown that these site-specific recombinases also act to excise DNA between tandem *dif* sites present on a plasmid or on a prokaryotic chromosome

(Barre *et al.*, 2000, Recchia *et al.*, 1999). However, there has been no suggestion that endogenous site-specific recombinases could be used in genetic engineering to provide an improved process of unlabelled gene integration and deletion and to provide a simplified process for removing selectable marker genes from plasmids.

The process of the first aspect of the invention exploits the ability of endogenous site-specific recombinases in cells to act on site-specific recombinase recognition sites, preferably *dif*-like sites, to remove selectable marker genes from a nucleic acid molecule without introducing an exogenous recombinase *in trans* as required by current processes for removing selectable marker genes.

The cell in which the process of the first aspect of the invention is carried out may be any cell containing endogenous site-specific recombinases that act at site-specific recombinase recognition sites. The cell may be a prokaryotic cell or a eukaryotic cell. Preferably, the cell is a prokaryotic cell that contains endogenous site-specific recombinases that act at *dif*-like sites. Preferably, the endogenous site-specific recombinases in the prokaryotic cell that act at *dif*-like sites are present in the chromosome of the prokaryotic cell.

Where the cell is a prokaryotic cell, it is preferably a bacterial cell which may be a gram negative bacterial cell or a gram positive bacterial cell. Gram negative bacterial cells useful according to the invention include, but are not limited to cells from *E. coli, Shigella, Vibrio* and *Salmonella, e.g. Salmonella typhimurium.* Preferably, the gram negative bacterial is an *E.coli* cell. *E.coli* cells contain the XerC/XerD site-specific recombinases that act at *dif*-like sites. Gram positive bacterial cells useful according to the invention include, but are not limited to *Bacillus, Streptomyces, Listeria, Mycobacterium, Lactobacillus* and *Lactococcus*. Preferably the gram positive bacterial cell is a *Bacillus subtilis* cell. *B. subtilis* cells contain the RipX/CodV site-specific recombinases that act at *dif*-like sites. Where the cell is a prokaryotic cell, it may be a RecA+ cell or a RecA- cell. Preferably, the cell is a RecA+ cell. Where the cell is a eukaryotic cell, it is preferably a yeast cell.

As used herein, the term "site-specific recombinase recognition site" includes any site which, when present in tandem in a nucleic acid molecule, is capable of being acted on by endogenous site-specific recombinases in the cell in which the process of the first aspect of the invention to excise the portion of the nucleic acid molecule between the tandem sites and produce a nucleic acid molecule containing a single site-specific recombinase site. The

site-specific recombinase recognition sites flanking the selectable marker gene may be the same or different.

Preferably, the site-specific recombinase recognition sites are *dif*-like sites. The term *dif*-like sites" refers to site-specific recombinase recognition sites which are dimer resolution sites that are capable of being acted on by endogenous site-specific recombinases in prokaryotic cells in which the process of the first aspect of the invention is carried out. The *dif*-like sites flanking the selectable marker gene may be the same or different.

A preferred site-specific recombinase recognition site is the FRT site from yeast. Preferred *dif*-like sites include *dif* sites found in bacterial chromosomes, such as the *dif* site from the *E.coli* chromosome (Cornet *et al.*, 1996) and the *dif* site from the *B. subtilis* chromosome (Sciochetti *et al.* 2001). Additional preferred *dif*-like sites include *dif*-like sites found in bacterial plasmids, such as the *cer* site from the *E. coli* plasmid ColEI, or the *psi* site from the *Salmonella* plasmid pSC101 (Cornet *et al.*, 1996). The sequences of these *dif*-like sites are provided in Table 1 below. However, it will be apparent to the skilled person that *dif*-like sites other than those listed in Table 1 from other cell chromosomes and plasmids may also be used in the process of the invention.

The invention also encompasses the use of hybrid *dif*-like sites formed by combining naturally-occurring *dif*-like sites from plasmids and chromosomes. An example of such a hybrid site is the *dif-psi* hybrid site also known as the *pif* site (Cornet *et al.*, 1996), the sequence of which is given in Table 1 below. Further hybrid sites for use in the process of the invention may be developed by generating hybrid sequences and determining the ability of these hybrid sequences to act as *dif*-like sites using simple recombination tests such as those described by Barre *et al*, 2000.

Where the *dif*-like sites used in the process of the invention are derived from a plasmid, the nucleic acid molecule must further comprise accessory sequences that are required for the site-specific recombinases to act. These accessory sequences are 180bp binding sites for the proteins PepA and ArgR which are described in Pham *et al*, 2002 and Bregu et al, 2002.

Table 1: Exemplary dif-like sites for use in the invention

Site name (and origin)	**Sequence (5'-3')**
Ecdif (*E. coli* chromosome)	GGTGCGCATAATGTATATTATGTTAAAT

cer (*E. coli* plasmid ColEI)	GGTGCGTACAATTAAGGGATTATGGTAAAT
psi (*Salmonella* plasmid pSC101)	GTGCGCGCAAGATCCATTATGTTAAAC
pif (*dif-psi* hybrid)	GGTGCGCGCAAGATCCATTATGTTAAAT
Bsdif (*B. subtilis* chromosome)	ACTTCCTAGAATATATATTATGTAAACT

It will be apparent to the skilled person that the nature of the site-specific recombinase recognition sites, preferably *dif*-like sites, included in the nucleic acid molecule in the process of the first aspect of the invention will depend on the site-specific recombinases that are endogenous to the cell in which the process is taking place. The process provides an advantage over prior art processes in that it does not require the introduction of an exogenous recombinase in *trans*. The site-specific recombinase recognition sites must therefore be capable of being acted on by endogenous site-specific recombinases in the cell in which the process is taking place. However, this does not mean that the site-specific recombinase recognition sites must also be endogenous to the cell in which the process is taking place. For example, there is evidence that site-specific recombinases from one species are able to act at *dif*-like sites from other species (Neilson *et al*, 1999). In addition, site-specific recombinases have been shown to resolve *dif*-like sites that are different (Cornet *et al*, 1994), *e.g.* an E.coli *dif* site and a *psi-dif* hybrid. The site-specific recombinase recognition sites, preferably *dif*-like sites, flanking the selectable marker gene may therefore be the same or different. The skilled person will be capable of selecting or developing site-specific recombinase recognition sites, preferably *dif*-like sites, to use to flank the selectable marker gene so that the process of the invention can take place.

The selectable marker gene may be any gene which can be used to detect the presence of the nucleic acid molecule. In general, antibiotic resistance genes are used in the art to identify cells containing a particular nucleic acid molecule, be it a plasmid or a linear DNA cassette integrated into the chromosome. Preferably, the selectable marker gene is therefore an antibiotic resistance gene which allows identification of cells containing the nucleic acid molecule by culture in a medium containing the antibiotic. Antibiotic resistance genes are known in the art and any of these genes may be used. Examples of antibiotic resistance genes which may be used include, but are not limited to, genes which convey resistance to kanamycin, ampicillin, chloramphenicol, tetracycline, neomycin, blasticidin, hygromycin, puromycin and zeocin.

Preferably, the process of the first aspect of the invention comprises culturing the cell in the presence of selective pressure on the selectable marker gene and subsequently removing the selective pressure on the selectable marker gene. Culturing the cell in the presence of a selective pressure on the selectable marker gene allows selection of cells containing the nucleic acid molecule comprising the selectable marker gene. Removal of the selective pressure on the selectable marker gene allows cells in which site-specific recombination to excise the selectable marker gene has taken place to survive. Where the selectable marker gene is an antibiotic resistance gene, the process of the invention thus preferably comprises culturing said cell in the presence of the antibiotic to select for cells containing the nucleic acid molecule and subsequently culturing said cell in the absence of the antibiotic.

According to a further embodiment of the process of the first aspect of the invention, the nucleic acid molecule may further comprise a second gene that allows positive selection of cells from which the selectable marker gene has been excised. Preferably, the second gene that allows positive selection is incorporated adjacent to the selectable marker gene, with both genes flanked by site-specific recombinase recognition sites, preferably *dif*-like sites, such that both genes are excised if site-specific recombination takes place. Where the nucleic acid molecule comprises a second gene that allows positive selection of cells from which the selectable marker gene has been excised, the process preferably further comprises culturing the cell under conditions such that cell death occurs if the second gene (and hence the selectable marker gene) have not been excised.

Suitable genes that allow positive selection include genes that are toxic to the cell under certain conditions. For example, the *sacB* gene expresses the enzyme levansucrase that coverts sucrose into a compound that is toxic to *E. coli* (Link *et al.*, 1997). A cell comprising a nucleic acid molecule comprising an antibiotic resistance gene and a *sacB* gene flanked by site-specific recombinase recognition sites may be cultured in the presence of the antibiotic to select for cells containing the nucleic acid molecule. The cells may subsequently be cultured in the absence of the antibiotic, allowing survival of cells in which the antibiotic resistance gene has been excised as well as cells in which no recombination has taken place. If the cells are then plated onto nutrient agar containing sucrose, any cell that has not lost both the antibiotic resistance gene and the *sacB* gene as a result of recombination between the site-specific recombinase recognition sites will be killed.

However, as shown in the examples, the excision of the marker gene by endogenous site-specific recombinases according to the process of the first aspect of the invention is, surprisingly, efficient enough that it is generally unnecessary to include a second gene for positive selection. This is an additional advantage of the invention.

The process of the first aspect of the invention described above may further comprise the step of introducing the nucleic acid molecule into the cell. Methods of transforming prokaryotic cells and transfecting eukaryotic cells are well known in the art and are described, for example in Sambrook (Molecular Cloning; A Laboratory Manual, Second Edition, 1989).

The nucleic acid molecule comprising the selectable marker gene flanked by site-specific recombinase recognition sites, preferably *dif*-like sites, according to the process of the first aspect of the invention is preferably a linear DNA cassette integrated into the chromosome of the cell or a plasmid.

Where the nucleic acid molecule comprising the selectable marker gene is a linear DNA cassette integrated into the chromosome of the cell, the process of the first aspect of the invention removes the selectable marker gene from the chromosome and can therefore be used as part of a process for unlabelled nucleic acid integration into the chromosome of a cell.

According to a second aspect of the invention, there is provided a process for unlabelled nucleic acid integration into the chromosome of a cell comprising:

a) introducing a linear DNA cassette into a cell, wherein said linear DNA cassette comprises:
    i) a selectable marker gene;
    ii) two site-specific recombinase recognition sites flanking said selectable marker gene; and
    iii) two regions flanking said site-specific recombinase recognition sites which are homologous to the two regions flanking the site of integration in the chromosome of the cell;
b) culturing said cell under conditions such that the linear DNA cassette is integrated into the cell chromosome by homologous recombination; and

c) culturing said cell under conditions such that an endogenous site-specific recombinase in the cell acts to excise the selectable marker gene by site-specific recombination between the site-specific recombinase recognition sites.

The cells, site-specific recombinase recognition sites, site-specific recombinases and selectable marker genes employed in the process of the second aspect of the invention are the same as those described in respect of the process of the first aspect of the invention. In particular, the site-specific recombinase recognition sites are preferably *dif*-like sites.

The process according to the second aspect of the invention may be used for unlabelled gene deletion or unlabelled gene integration and the nature of the nucleic acid molecule will change accordingly. Where the process of the second aspect of the invention is used for deletion of an endogenous gene, the two regions flanking the site-specific recombinase recognition sites, preferably *dif*-like sites, are homologous to the two regions flanking the gene to be deleted. Where the process according to the second aspect of the invention is used for the integration of an exogenous gene, the two regions flanking the site-specific recombinase recognition sites, preferably *dif*-like sites, are homologous to the two regions flanking the site of integration and the linear DNA cassette further comprises the exogenous gene to be integrated, provided that the exogenous gene is not located between the two site-specific recombinase recognition sites. It will be apparent to the skilled person that the exogenous gene to be integrated cannot be located between the site-specific recombinase recognition sites as this would result in the exogenous gene being excised by endogenous site-specific recombinases along with the selectable marker gene.

The process according to the second aspect of the invention may be used not just to delete entire genes but also to delete portions of genes and regulatory regions of genes. Similarly, it may be used to integrate portions of exogenous genes rather than complete exogenous genes.

The research potential of being able to integrate nucleic acid molecules and thus delete endogenous genes or integrate exogenous genes by homologous recombination has been extensively documented in the prior art. The advantage of the process of the invention over prior art methods is that it allows nucleic acid integration to take place without leaving a selectable marker gene behind and without the need to introduce an exogenous recombinase *in trans* to promoter removal of the selectable marker gene, as required by current processes in the art.

The linear DNA cassette used in the process of the second aspect of the invention may be produced by constructing a plasmid comprising the required elements and linearising the plasmid using a restriction endonuclease. Alternatively, the linear DNA cassette may be assembled by PCR, which produces linear DNA, where the PCR primers contain sequence in their 5' ends that is homologous to the chromosomal target. Competent cells of the target strain are then made and transformed or transfected with linearised plasmid DNA or a PCR product.

The conditions required for integration of the DNA cassette by homologous recombination will vary according to the cell used in the process of the invention. In particular, the conditions required for integration of a linear DNA cassette vary in prokaryotic cells. To integrate the linear DNA cassette into the target chromosome of *B. subtilis*, simple transformation and clone selection (*e.g.* by antibiotic resistance) is sufficient. In *E. coli*, however, the RecBCD enzyme rapidly degrades linear DNA, so chromosomal integration into a $RecBCD^-$ strain can be used (Jasin and Schimmel, 1984; Winas *et al.*, 1985) followed by P1 transduction into a $RecBCD^+$ strain if desired (Williams *et al.*, 1998). Where the target strain is $RecA^-$, a helper plasmid expressing *recA* may be necessary. An alternative is to use a helper plasmid expressing the lambda Red functions *bet*, *exo* and *gam*; these inhibit RecBCD and allow chromosomal integration even the absence of RecA (Murphy, 1998).

Preferably, step b) of the process of the second aspect of the invention further comprises culturing the cell in the presence of a selective pressure on the selectable marker gene. Culturing the cell in the presence of selective pressure on the selectable marker gene allows selection of cells in which the linear DNA cassette has integrated into the chromosome. Where the selectable marker gene is an antibiotic resistance gene, this step comprises culturing the cells in the presence of an antibiotic. Preferably, step c) comprises culturing the cell in the absence of any selective pressure, *e.g.* in the absence of antibiotic. Culturing of cells in the absence of selective pressure allows survival of cells from which the selectable marker gene has been excised. Figure 1 provides a summary of a preferred process of the second aspect of the invention. The nucleic acid molecule may further comprise a gene for positive selection of cells in which recombination has taken place, as described in relation to the process of the first aspect of the invention. However, as indicated previously, excision of the selectable marker gene by endogenous site-specific

recombinases is efficient enough that a gene for positive selection of cells in which recombination has taken place is not generally necessary.

According to a third aspect of the invention, the nucleic acid molecule comprising the selectable marker gene referred to in the process of the first aspect of the invention is a plasmid. According to a preferred embodiment of this aspect of the invention, there is provided a process for the removal of a selectable marker gene from a plasmid comprising introducing a plasmid comprising a selectable marker gene flanked by site-specific recombinase recognition sites into a cell and culturing said cell under conditions such that an endogenous site-specific recombinase acts to excise the selectable marker gene from the plasmid by site-specific recombination between the site-specific recombinase recognition sites.

The cells, site-specific recombinase recognition sites, site-specific recombinases and selectable marker genes employed in the process of the third aspect of the invention are the same as those described in respect of the processes of the first aspect of the invention. In particular, the site-specific recombinase recognition sites are preferably *dif*-like sites.

Where the cell according to the third aspect of the invention is a prokaryotic cell, it is preferably a RecA+ cell. In the industrial manufacture of plasmid DNA for applications such as DNA vaccines or gene therapy, there are stringent regulations on the proportion of the product that is supercoiled, monomeric DNA. These requirements mean that plasmid DNA has always been grown in $RecA^-$ strains, as homologous recombination due to RecA generates plasmid multimers that reduce the proportion of monomeric plasmid. However, $RecA^+$ strains are significantly more viable than $RecA^-$ strains, as RecA is essential for repairing stalled replication forks that occur during chromosome replication. In a culture of a $RecA^-$ strain, up to 50% of the cells will not contain a chromosome (Cox *et al.*, 2000). The presence of site-specific recombinase recognition sites, in particular *dif*-like sites, in the plasmids used in the process of the third aspect of the invention allow the plasmid to be produced in RecA+ cells without plasmid multimerisation occurring.

Preferably, the cell is cultured in the presence of selective pressure on the selectable marker gene and is subsequently cultured in the absence of selective pressure on the selectable marker gene. Culturing the cell in the presence of selective pressure allows selection of cells containing the plasmid. The removal of selective pressure on the selectable marker gene allows survival of cells from which the selectable marker gene has

been excised. The nucleic acid molecule may further comprise a gene for positive selection of cells in which recombination has taken place, as described in relation to the process of the first aspect of the invention. However, as indicated previously, excision of the selectable marker gene by endogenous site-specific recombinases is efficient enough that a gene for positive selection of cells in which recombination has taken place is not generally necessary.

Preferably, the process of the third aspect of the invention further comprises maintaining the plasmid in the cell by means of an alternative system not dependent on a selectable marker gene. Preferably, the cell and the plasmid are constructed such that, following removal of the selectable marker gene, the plasmid is capable of being maintained by an alternative maintenance system that is not dependent on the selectable marker gene. Preferably, where the selectable marker gene according to the third aspect of the invention is an antibiotic resistance gene, the process according to the third aspect of the invention allows selection of transformed cells containing the plasmid in the presence of the antibiotic and, following deletion of the antibiotic resistance gene, allows maintenance of the plasmid by an antibiotic-free system.

Preferably, the antibiotic-free system that allows maintenance of the plasmid following removal of the antibiotic resistance gene is operator repressor titration (ORT). Where ORT is used to maintain the plasmid following deletion of the antibiotic resistance gene, the plasmid further comprises an operator susceptible to binding by a repressor and the cell further comprises a first gene present on the chromosome encoding said repressor and a second gene present on the chromosome that is essential for cell growth and is functionally associated with the same operator that is present on the plasmid. In the absence of the plasmid, the repressor binds to the operator upstream of the essential second gene, thereby inhibiting expression of the essential gene such that there is no cell growth. In contrast, when the plasmid is present in sufficient numbers, the operator on the plasmid titrates the repressor such that the essential gene is expressed and the cells grow. ORT is described in detail in WO97/09435, Hanak and Cranenburgh, 2001 and Cranenburgh *et al.*, 2001. Any of the essential genes, operator sequences and repressor sequences referred to in WO97/09435, Hanak and Cranenburgh, 2001 and Cranenburgh *et al.*, 2001 may be used in the cells and plasmids of the present invention to allow maintenance of the plasmid following deletion of the antibiotic resistance gene.

Preferably, the plasmid comprises the *lac* operator and the cell comprises a first gene encoding the *lac* repressor present on the chromosome and a second gene present on the chromosome that is essential for cell growth and is functionally associated with the *lac* operator. The process according to this second aspect of the invention is illustrated in figure 2 where the *lac* operator is included on the plasmid.

An additional application for the processes of the invention is in the regulation of gene expression. The regulation of gene expression is important as a low level of expression prior to induction can lead to a metabolic burden or toxic effects from the recombinant protein, thus reducing or inhibiting cell growth and significantly reducing yield. In prokaryotes and eukaryotes, a promoter must be adjacent to a gene or an operon for effective transcription. Traditionally, regulation of gene expression has been achieved by using a second gene that expresses a repressor protein that binds to an operator in the promoter region of the gene of interest, blocking its expression. Expression of the gene of interest is thus controlled by controlling the expression of the repressor. Alternatively, transcription terminators are inserted between the promoter and the transgene of interest expression preventing expression. The transcription terminators or the gene cassette is flanked by site-specific recombination sites such that expression of the gene in the cassette is prevented until the exogenous site specific recombinase gene is expressed, thus bringing the promoter in close proximity to the transgene and enabling gene expression. However, both these strategies encounter problems with low level expression of the exogenous site-specific recombinase or the repressor. These problems are avoided by the use of endogenous site-specific recombinases that act on site-specific recombinase recognition sites, preferably *dif*-like sites, to control the expression by recombination event when an external selection pressure (e.g. antibiotic selection) is removed.

According to a fourth aspect of the invention, there is therefore provided a process for controlling expression of a gene of interest comprising culturing a cell comprising:

i) a first nucleic acid molecule comprising a gene of interest that is functionally associated with an operator; and

ii) a second nucleic acid molecule comprising a selectable marker gene and a repressor gene flanked by site-specific recombinase recognition sites, wherein said repressor is susceptible of binding to said operator

under conditions such that an endogenous site-specific recombinase in the cell acts to excise the selectable marker gene and said repressor gene by site-specific recombination between the site-specific recombinase recognition sites, thereby permitting expression of a gene of interest.

The first and second nucleic acid molecules used in this process may be plasmid DNA or linear DNA cassettes integrated in the chromosome of the cell. Preferably, both nucleic acid molecules are linear cassettes integrated into the chromosome of the cell. Alternatively, the first nucleic acid molecule may be a linear DNA cassette integrated into the chromosome of the cell and the second nucleic acid molecule may be a plasmid, or vice versa.

According to a second embodiment of the fourth aspect of the invention, there is provided a process for controlling expression of a gene of interest comprising culturing a cell comprising a nucleic acid molecule comprising

i) a gene of interest functionally linked to a promoter; and

ii) a selectable marker gene and a transcription terminator flanked by site-specific recombinase recognition sites, wherein said selectable marker gene and transcription terminator flanked by site-specific recombinase recognition sites are located between the gene of interest and the promoter controlling expression of said gene of interest

under conditions such that an endogenous site-specific recombinase in the cell acts to excise the selectable marker gene and said transcription terminator by site-specific recombination between the site-specific recombinase recognition sites, thereby permitting expression of a gene of interest.

The cells, site-specific recombinase recognition sites, site-specific recombinases and selectable marker genes employed in the process of the fourth aspect of the invention are the same as those described in respect of the process of the first aspect of the invention. In particular, the site-specific recombinase recognition sites are preferably *dif*-like sites. The nucleic acid molecule may be a plasmid or may be a linear DNA cassette integrated in the chromosome.

Preferably, the cell is cultured in the presence of selective pressure on the selectable marker gene followed by removal of the selective pressure. This embodiment is shown in

Figures 3 and 4. The nucleic acid molecule may further comprise a gene for positive selection of cells in which recombination has taken place, as described in relation to the process of the first aspect of the invention.

The processes of the first, second, third and fourth aspects of the invention may also be carried out to remove a selectable marker gene from a nucleic acid molecule *in vitro*. For example, the process may be used *in vitro* to remove a selectable marker gene from a plasmid prior to introducing it into a cell. In these circumstances, the plasmid may contain a second gene that allows positive selection of cells containing the plasmid following its introduction into a cell. According to a fifth aspect of the invention, there is provided a process for removing a selectable marker gene from a nucleic acid molecule *in vitro* comprising supplying a nucleic acid molecule comprising a selectable marker gene flanked by site-specific recombinase recognition sites, preferably *dif*-like sites, with a site-specific recombinase that acts to excise the selectable marker gene by site-specific recombination between the site-specific recombinase recognition sites. The site-specific recombinase supplied in the process of the fifth aspect of the invention may be any of these prokaryotic or eukaryotic recombinases which are known to act at site-specific recombinase recognition sites, preferably *dif*-like sites, as described previously.

The invention further provides host cells and nucleic acid molecules for use in the processes of the invention described above.

According to a sixth aspect of the invention, there is provided a nucleic acid molecule comprising a selectable marker gene flanked by site-specific recombinase recognition sites. Suitable site-specific recombinase recognition sites and selectable marker genes for inclusion in the nucleic acid molecules of the sixth aspect of the invention are discussed above in connection with the process of the first aspect of the invention. Preferably, the site-specific recombinase recognition sites are *dif*-like sites. Preferably the nucleic acid molecule is a linear DNA cassette or a plasmid. Where the nucleic acid molecule is a linear DNA cassette, it preferably further comprises regions of homology that are homologous to regions flanking the chromosomal location at which it is intended to be integrated. The linear DNA cassette may further comprise an exogenous gene. Where the nucleic acid molecule is a plasmid, it may further comprise an operator sequence so that the plasmid can be maintained by ORT following deletion of the selectable marker gene.

According to a seventh aspect of the invention, there is provided a cell comprising a nucleic acid molecule according to the sixth aspect of the invention. The cell may be a prokaryotic cell or a eukaryotic cell. Suitable cells include cells discussed in relation to the process of the first aspect of the invention above. Where the cell comprises a plasmid comprising an operator for maintenance by ORT after deletion of the selectable marker gene, the cell may preferably comprise a first gene present on the chromosome encoding the repressor that binds to the operator on the plasmid and a second gene present on the chromosome that is functionally associated with the same operator and essential for cell growth, as described in more detail above. Where the cell comprising a plasmid is a prokaryotic cell, it may be a RecA+ cell. As noted above, the presence of the site-specific recombinase recognition sites, preferably *dif*-like sites, on the plasmids of the invention enables them to be cultured in RecA+ cells without the problem of multimerisation (Figure 5).

According to an eighth aspect of the invention, there is therefore provided a process for producing a supercoiled, monomeric plasmid DNA in a RecA+ cell comprising culturing a RecA+ cell comprising a plasmid, characterised in that the plasmid comprises a site-specific recombinase recognition site, preferably a *dif*-like site. The presence of the site-specific recombinase recognition site, preferably *dif*-like site, prevents the problem of multimerisation (Figure 5), thereby allowing supercoiled monomeric DNA which meets regulatory requirements to be produced. Suitable site-specific recombinase recognition sites, preferably *dif*-like sites, for including in the plasmid in the process of the eighth aspect of the invention are described above. Preferably, the RecA+ cell is an *E. coli* cell.

BRIEF DESCRIPTION OF THE FIGURES:

Figure 1: Gene deletion and selectable marker excision using site-specific recombination at *dif* sites. For gene insertion, a gene can be cloned into the multi-cloning site and the insertion site can be an intergenic region if required.

Figure 2: Selectable marker excision from a plasmid using site-specific recombination at *dif* sites following the removal of the selection pressure (e.g. antibiotic).

Figure 3: Selectable marker and repressor excision from a plasmid using site-specific recombination at *dif* sites following the removal of the selection pressure (e.g. antibiotic). With the repressor gene removed, the expression of the transgene from the promoter becomes constitutive.

Figure 4: Selectable marker excision from a plasmid using site-specific recombination at *dif* sites following the removal of the selection pressure (e.g. antibiotic) to bring a transgene under the control of an upstream promoter. Transgene expression is prevented by a gene cassette consisting of an antibiotic resistance gene and transcription terminators placed between the promoter and the transgene. With this cassette removed by recombination between *dif* sites, the expression of the transgene from the promoter is enabled.

Figure 5: Resolution of plasmid multimers in $RecA^+$ cells. RecA converts plasmid monomers to dimers by homologous recombination. If a dimer resolution site *dif* (e.g. *cer* and its accessory sequences) is present, the native site-specific recombinases (e.g. XerC and XerD) will convert this dimer back to a monomeric form.

Figure 6: The primers 5DIFCAT and 3DIFCAT were used to amplify *cat* and incorporate flanking *dif* sites. This was cloned into pTOPO to create the precursor deletion plasmid pTOPO-DIFCAT.

Figure 7: A) Diagram of the wild-type, integrant and resolvant loci during the chromosomal deletion of *msbB*. B) Agarose gel of PCR products generated using primers SML and SMR. The wild-type *msbB* locus gives a product of 1428 bp. Integration of the Δ*msbB*-ADifCAT cassette results in an increase in size to 1460 bp, and *cat* excision at *dif* sites results in the final, 462 bp PCR product.

Figure 8: A) Diagram of the wild-type, integrant and resolvant loci during the chromosomal integration of *rbpA*. B) Agarose gel of PCR products generated using primers Int F and Int R. The wild-type integration locus between *ubiB* and *fadR* gives a product of 510 bp. Integration of the rbp-DifCAT cassette results in an increase in size to 1936 bp, and *cat* excision at *dif* sites results in the final, 938 bp PCR product.

The invention will now be described in more detail by way of example with reference to chromosomal gene deletion and integration. It will be appreciated that modifications may be made to the systems described in the Examples.

EXAMPLES

Example 1: Unlabelled chromosomal gene deletion

This example illustrates how the *msbB* gene (Somerville *et al.*, 1995) was deleted from the *E. coli* chromosome to generate a new strain with a reduced endotoxin activity.

Firstly, the chloramphenicol resistance gene *cat* was amplified from the plasmid pKO3 (Link *et al.*, 1997) using primers 5DIFCAT and 3DIFCAT. These 81 nt primers incorporated a 3' region of homology flanking the *cat* gene in pKO3 with a 5' tail that included a 28 bp *dif* site and the restriction sites *Bsu*36I and *Nsi*I. This PCR product was cloned in to pCR2.1 (Invitrogen) using the TOPO cloning method to create a precursor gene deletion plasmid, pTOPO-DifCAT (Figure 6).

To create a strain with an *msbB* gene deletion, the *dif-cat-dif* cassette from pTOPO-DifCAT was amplified by PCR using primers with 5' ends homologous to the chromosomal regions flanking *msbB* (msb.int F and msb.int R) to create the DifCAT PCR product. The *E. coli* strain DH1 was transformed with the tetracycline-selectable plasmid pTP223 that provides the lambda Red gene functions for protection and integration of linear DNA (Murphy, 1998). DH1(pTP223) was then transformed with the DifCAT PCR product, and integrants (DH1::DifCATΔmsbB) were selected on chloramphenicol.

Primers SML and SMR were used to amplify a part of the *msbB* locus by diagnostic PCR (figure 7). In wild-type DH1, this gave a product of 1428 bp for the native *msb*B locus (lane 2). The integrant locus was 1460 bp, but this PCR also amplified a product of 462 bp, indicating an *msbB* deletion, as a proportion of the population underwent XerCD-mediated recombination even in the presence of chloramphenicol (lane 3). Subculture in the absence of antibiotics resulted in the loss of pTP223 and the generation of resolvant, chloramphenicol-sensitive clones with only the 462 bp *msbB* deletion locus detected by PCR (lanes 4-7). The deletion of *msbB* was confirmed by DNA sequencing.

Example 2: Unlabelled chromosomal gene insertion

This example shows the insertion of an exogenous gene, the bovine pancreatic ribonuclease gene *rbpA*, into a chromosomal space between two native genes (*ubiB* and *fadA*) in *E. coli* strain DH1*lacdapD*.

The plasmid prbpA-DifCAT was constructed with *rbpA* adjacent to Dif-CAT from pTOPO-DifCAT. prbpA-DifCAT was used as a PCR template with the 70 nt primers Int F and Int R. The 20 bp at the 3' end of each primer was homologous to the template and there was a 50 bp 5' tail with homology to the target *ubiB-fadA* locus. A PCR integration fragment of 1691 bp was produced and transformed into DH1*lacdapD*(pTP223) to create the integrant DH1*lacdapD*::rbpA-DifCAT(pTP223).

Primers UbiB F and UbiB R were used to amplify a part of the *ubiB-fadA* locus by diagnostic PCR (figure 8). In wild-type DH1*lacdapD*, this gave a product of 510 bp for the native *msb*B locus (lane 2). The integrant locus was 1936 bp, but this PCR also amplified a product of 938 bp, indicating an *rbpA* insertion, as a proportion of the population underwent XerCD-mediated recombination even in the presence of chloramphenicol (lane 3). The integrant strain was cured of the helper plasmid pTP223 and the *cat* gene excised from DH1*lacdapD* by culturing without antibiotics, and the integrated *rbpA* gene detected as a 938 bp PCR product (lanes 4-7). The insertion of *rbpA* was confirmed by DNA sequencing.

Example 3: Estimation of gene excision frequencies

This example determines the frequency of XerCD-mediated recombination at *dif* sites, measured by the excision of *dif*-flanked antibiotic resistance genes integrated into two chromosomal loci of *E. coli* DH1.

Two integrant strains (DH1::ΔmsbB-DifCAT and DH1::rbpA-DifCAT) were used, with *dif*-flanked chloramphenicol resistance genes inserted in different chromosomal loci (*msbB* and *ubiB-fadA* loci respectively). These were inoculated in triplicate into 5 ml LB broth containing 20 μg ml^{-1} chloramphenicol and grown throughout the day until an optical density (OD_{600}) of approximately 0.5. Shake flasks containing 50 ml LB broth were inoculated to a starting OD_{600} of 0.005. The shake flasks were incubated at 37°C with shaking at 200 r.p.m. for a 24-hour period. After the first 24-hour growth period, the OD_{600} was recorded and a calculated volume was used to inoculate another 50 ml shake flask, again to give a starting OD of 0.005. This subculturing procedure was repeated at 24-hour intervals for a total period of 96 hours. The number of generations from each of the six flasks was calculated upon subculture.

After 48 and 96 hours growth, the cultures were serially diluted in LB broth and plated onto LB agar to produce single colonies. To estimate the frequency of XerCD-mediated recombination at the *dif* sites, 100 colonies for each of the six cultures were replica streaked onto LB agar +/- 20 μg ml^{-1} chloramphenicol. Clones that had become chloramphenicol sensitive were screened by PCR using diagnostic PCR primers to amplify the modified region of the locus (SML and SMR primers for the *msbB* locus; UbiB F and UbiB R for the *ubiB-fadA* locus). The resulting data were used to calculate the XerCD-mediated antibiotic resistance gene excision frequencies, as shown in Table 2.

Table 2. Gene excision frequencies by Xer site-specific recombination at *dif* sites. Frequencies are reported at two time-points for the excision of a chloramphenicol resistance gene.

	Gene excision in *msbB* locus		**Gene excision in *ubiB-fadA* locus**	
Time (hours)	48	96	48	96
Generations	19.7	39.2	18.7	37.9
Excision frequency	6.3 %	7.0 %	1.0 %	2.8 %

These data illustrate that after only two days of culturing integrant strains, the excision frequency is sufficiently high (1 – 6 %) that less than 100 colonies need to be screened to identify the desired recombinant.

Appendix: Primers

Primers are written 5' to 3'.

5DIFCAT:

CCTTAGGATGCATGGTGCGCATAATGTATATTATGTTAAATCCCTTATGCGACTCCTGCA
TCCCTTTCGTCTTCGAATAAA

3DIFCAT:

CCTTAGGATGCATATTTAACATAATATACATTATGCGCACCATCCGCTTATTATCACTTA
TTCAGGCGTAGCACCAGGCGT

Msb-int F:

TGCGGCGAAAACGCCACATCCGGCCTACAGTTCAATGATAGTTCAACAGAAGTGTGCTGG
AATTCGCCCT

Msb-int R:

TTGGTGCGGGGCAAGTTGCGCCGCTACACTATCACCAGATTGATTTTTGCATCTGCAGAA
TTCGCCCTTA

Int F:

AAACCCGCCCCTGACAGGCGGGAAGAACGGCAACTAAACTGTTATTCAGTTTGCGCCGAC
ATCATAACGG

Int R:

GCCGGATGCGGCGTGAACGCCTTATCCGGTCTACCGATCCGGCACCAATGGCTACGGTTT GATTAGGGAA

SML:

TGACCTGGTGATTGTCACCC

SMR:

TAAACCAGCAGGCCGTAAAC

UbiB F:

GATCGCCTGTTTGGCGATGC

UbiB R:

GAATCTGATGGAACGCAAAG

REFERENCES:

Barre, F.-X., Aroyo, M., Colloms, S. D., Helfrich, A., Cornet, F. and Sherratt, D. J., 2000. FtsK functions in the processing of a Holliday junction intermediate during bacterial chromosome segregation. *Genes & Dev.* 14: 2976-2988.

Bregu, M., Sherratt, D. J. and Colloms, S. D., 2002. Accessory factors determine the order of strand exchange in Xer recombination at *psi*. *EMBO J.* 21: 3888-3897.

Cornet, F., Mortier, I., Patte, J. and Louarn, J.-M., 1994. Plasmid pSC101 harbors a recombination site, *psi*, which is able to resolve plasmid multimers and to substitute for the analogous chromosomal *Escherichia coli* site *dif*. *J. Bacteriol.* 176, 3188-3195.

Cranenburgh, R. M., Hanak, J. A. J., Williams, S. G., and Sherratt, D. J. (2001). *Escherichia coli* strains that allow antibiotic-free plasmid selection and maintenance by repressor titration. *Nucleic Acids Res.* **29:** e26.

Cox, M. M., Goodman, M. F., Kreuzer, K. N., Sherratt, D. J., Sandler, S. J., Marians, K. J., 2000. The importance of repairing stalled replication forks. Nature 404: 37-41.

Dale, E. C. and Ow, D. W., 1991. Gene transfer with subsequent removal of the selection gene from the host genome. *Proc. Natl. Acad. Sci. USA* 88: 10558-10562.

Datsenko, K. A. and Wanner, B. L., 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. USA* 97: 6640-6645.

Hanak, J. A. J. and Cranenburgh, R. M., 2001. Antibiotic free plasmid selection and maintenance in bacteria. In *Recombinant protein production with prokaryotic and eukaryotic cells*. Kluwer Academic Publishers. p. 111-124.

Jasin,M. and Schimmel,P., 1984. Deletion of an essential gene in *Escherichia coli* by site-specific recombination with linear DNA fragments. *J Bacteriol*. 159: 783-786.

Leenhouts, K., Buist, G., Bolhuis, A., ten Berge, A., Kiel, J., Mierau, I., Dabrowska, M., Venema, G. and Kok, J., 1996. A general system for generating unlabelled gene replacements in bacterial chromosomes. *Mol. Gen. Genet.* 253: 217-224.

Leslie, N. R. and Sherratt, D. J., 1995. Site-specific recombination in the replication terminus region of *Escherichia coli*: functional replacement of dif. *EMBO J.* 14: 1561-1570.

Link, A. J., Phillips, D. and Church, G. M., 1997. Methods for generating precise deletions and insertions in the genome of wild-type *Escherichia coli*: application to open reading frame characterization. *J. Bacteriol*. 1997: 6228-6237.

McCulloch, R., Coggins, L. W., Colloms, S. D. and Sherratt, D. J., 1994. Xer-mediated site-specific recombination at *cer* generates Holliday junctions *in vivo*. *EMBO J.* 13: 1844-1855.

Murphy, K. C., 1998. Use of bacteriophage λ recombination functions to promote gene replacement in *Escherichia coli*. *J. Bactiol.* 180: 2063-2071.

Recchia, G. D., Aroyo, M., Wolf, D., Blakely, G and Sherratt, D. J., 1999. FtsK-dependant and -independent pathways of Xer site-specific recombination. *EMBO J.* 18: 5724-5734.

Sanchis, V., Agaisse, H., Chaufaux, J. and Lereclus, D., 1997. A recombinase-mediated system for elimination of antibiotic resistance gene markers from genetically engineered *Bacillus thuringiensis* strains. *Appl. Environ. Microbiol*. 63: 779-784.

Sciochetti, S.A., Piggot, P.J. and Blakely, G.W., 2001. Identification and characterisation of the *dif* site from *Bacillus subtilis*. *J. Bacteriol*. 183: 1058-1068.

Somerville, J. E., Cassiano, L., Bainbridge, B., Cunningham, M. D. and Darveau, R. P., 1995. A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. *J. Clin. Invest.* 97: 359-365.

Sugita, K., Kasahara, T, Matsunaga, E, Ebinuma, H, 2000. A transformation vector for the production of marker-free transgenic plants containing a single copy transgene at high frequency. *Plant J.* 22:461-469.

Williams, S. G., Cranenburgh, R.M., Weiss, A. M. E., Wrighton, C. J., Sherratt, D. J. and Hanak, J. A. J., 1998. Repressor titration: a novel system for selection and maintenance of recombinant plasmids. *Nucleic Acids Res.*, 26: 2120-2124.

Winans S.C., Elledge S.J., Krueger, J.H., Walker, G.C., 1985. Site-Directed Insertion and Deletion Mutagenesis with Cloned Fragments in *Escherichia coli. J. Bacteriol.,* 161: 1219-1221.

CLAIMS:

1. A process for removing a selectable marker gene from a nucleic acid molecule in a prokaryotic cell comprising culturing a prokaryotic cell comprising a nucleic acid molecule comprising a selectable marker gene flanked by *dif*-like site-specific recombinase recognition sites under conditions such that an endogenous site-specific recombinase in the cell acts to excise the selectable marker gene by site-specific recombination between the *dif*-like site-specific recombinase recognition sites.
2. A process according to claims 1 wherein the cell is a bacterial cell.
3. A process according to claim 2 wherein the cell is a gram negative bacterial cell.
4. A process according to claim 2 wherein the cell is a gram positive bacterial cell.
5. A process according to claim 3 wherein the cell is an *E. coli* cell.
6. A process according to claim 4 wherein the cell is a *B. subtilis* cell.
7. A process according to any one of claims 2 to 6 wherein the cell is a RecA+ cell.
8. A process according to any one of claims 1 to 7 wherein the *dif*-like site-specific recombinase recognition sites flanking the selectable marker gene are the same.
9. A process according to any one of claims 1 to 7 wherein the *dif*-like site-specific recombinase recognition sites flanking the selectable marker gene are different.
10. A process according to any one of claims 1 to 9 wherein at least one of the dif-like sites is selected from the *E.coli dif* site and the *B. subtilis dif* site.
11. A process according to any one of claims 1 to 10 wherein at least one of the *dif*-like sites is selected from the plasmid *dif*-like sites *cer* and *psi* and the nucleic acid molecule further comprises accessory sequences.
12. A process according to any one of claims 1 to 11 wherein at least one of the *dif*-like sites is the hybrid *dif*-like site *pif* and the nucleic acid molecule further comprises accessory sequences.
13. A process according to any one of claims 1 to 12 wherein the selectable marker gene is an antibiotic resistance gene.
14. A process according to any one of claims 1 to 13 comprising culturing said cell in the presence of selective pressure on the selectable marker gene and subsequently removing the selective pressure on the selectable marker gene.
15. A process according to any one of claims 1 to 14 wherein said nucleic acid molecule further comprises a second gene that allows positive selection of cells from which the selectable marker gene has been excised.

16. A process according to any one of claims 1 to 15 further comprising an initial step of introducing the nucleic acid molecule in the cell.

17. A process according to any one of claims 1 to 16 wherein the nucleic acid molecule is a linear DNA cassette integrated into the chromosome of the cell.

18. A process according to any one of claims 1 to 17 wherein the nucleic acid molecule is a plasmid DNA.

19. A process for unlabelled nucleic acid integration into the chromosome of a prokaryotic cell comprising:

a) introducing a linear DNA cassette into a cell, wherein said linear DNA cassette comprises:

i) a selectable marker gene;

ii) two *dif*-like site-specific recombinase recognition sites flanking said selectable marker gene; and

iii) two regions flanking said *dif*-like site-specific recombinase recognition sites which are homologous to the two regions flanking the site of integration in the chromosome of the cell;

b) culturing said cell under conditions such that the linear DNA cassette is integrated into the cell chromosome by homologous recombination; and

c) culturing said cell under conditions such that an endogenous site-specific recombinase in the cell acts to excise the selectable marker gene by site-specific recombination between the *dif*-like site-specific recombinase recognition sites.

20. A process according to claim 19 comprising the features of any one of claims 2 to 13.

21. A process according to claim 19 or 20 for unlabelled gene deletion wherein the two regions flanking said *dif*-like site-specific recombinase recognition sites are homologous to the two regions flanking the gene to be deleted in the chromosome of the cell.

22. A process according to claim 19 or 20 for unlabelled gene integration wherein the two regions flanking the *dif*-like site-specific recombinase recognition sites are homologous to the two regions flanking the site of integration and the linear DNA cassette further comprises the exogenous gene to be integrated, provided that the exogenous gene is not located between the two *dif*-like site-specific recombinase recognition sites.

23. A process of any one of claims 19 to 22 wherein step b) further comprises culturing the cell in the presence of a selective pressure on the selectable marker gene

24. The process according to claim 23 wherein step c) comprises culturing the cell in the absence of any selective pressure.

25. A process according to any one of claims 19 to 24 wherein the nucleic acid molecule further comprises a gene for positive selection of cells in which recombination has taken place.

26. A process for the removal of a selectable marker gene from a plasmid comprising introducing a plasmid comprising a selectable marker gene flanked by *dif*-like site-specific recombinase recognition sites into a prokaryotic cell and culturing said cell under conditions such that an endogenous site-specific recombinase acts to excise the selectable marker gene from the plasmid by site-specific recombination between the *dif*-like site-specific recombinase recognition sites.

27. A process according to claim 26 comprising the features of any one of claims 2 to 15.

28. A process according to claim 26 or 27 further comprising maintaining the plasmid in the cell by means of an alternative system not dependent on the selectable marker gene.

29. A process according to claim 28 wherein the selectable marker gene is an antibiotic resistance gene and the process further comprises maintaining the plasmid by operator repressor titration.

30. A process according to claim 29 wherein the plasmid comprises the *lac* operator and the cell comprises a first chromosomal gene encoding the *lac* repressor and a second chromosomal gene functionally associated with the *lac* operator that is essential for cell growth.

31. A process for controlling expression of a gene of interest comprising culturing a prokaryotic cell comprising:

i) a first nucleic acid molecule comprising a gene of interest that is functionally associated with an operator; and

ii) a second nucleic acid molecule comprising a selectable marker gene and a repressor gene flanked by *dif*-like site-specific recombinase recognition sites, wherein said repressor is susceptible of binding to said operator

under conditions such that an endogenous site-specific recombinase in the cell acts to excise the selectable marker gene and said repressor gene by site-specific recombination between the *dif*-like site-specific recombinase recognition sites, thereby permitting expression of a gene of interest.

32. A process according to claim 31 comprising the features of any one of claims 2 to 15.

33. A process according to claim 31 or claim 32 wherein the first and second nucleic acid molecules are linear DNA cassettes integrated into the chromosome of the cell.

34. A process according to claim 31 or claim 32 wherein the first and second nucleic acid molecules are plasmids.

35. A process according to claim 31 or claim 32 wherein the first nucleic acid molecule is a linear DNA cassette integrated into the chromosome of the cell and the second nucleic acid molecule is a plasmid.

36. A process according to claim 31 or claim 32 wherein the first nucleic acid molecule is a plasmid and the second nucleic acid molecule is a linear DNA cassette integrated into the chromosome of the cell.

37. A process for controlling expression of a gene of interest comprising culturing a prokaryotic cell comprising a nucleic acid molecule comprising

i) a gene of interest functionally linked to a promoter; and

ii) a selectable marker gene and a transcription terminator flanked by *dif*-like site-specific recombinase recognition sites, wherein said selectable marker gene and transcription terminator flanked by *dif*-like site-specific recombinase recognition sites are located between the gene of interest and the promoter controlling expression of said gene of interest

under conditions such that an endogenous site-specific recombinase in the cell acts to excise the selectable marker gene and said transcription terminator by site-specific recombination between the *dif*-like site-specific recombinase recognition sites, thereby permitting expression of a gene of interest.

38. A process according to claim 37 comprising the features of any one of claims 2 to 15.

39. A process according to claim 37 or claim 38 wherein the nucleic acid molecule is a plasmid or is a linear DNA cassette integrated into the chromosome.

40. A nucleic acid molecule comprising a selectable marker gene flanked by *dif*-like site-specific recombinase recognition sites.

41. A nucleic acid molecule according to claim 40 which is a linear DNA cassette.

42. A nucleic acid molecule according to claim 40 which is a plasmid.

43. A nucleic acid molecule according to claim 41 which further comprises regions of homology that are homologous to regions flanking the chromosomal location at which it is intended to be integrated.

44. A nucleic acid molecule according to claim 43 which further comprises an exogenous gene.

45. A nucleic acid molecule according to claim 42 which further comprises an operator sequence.

46. A prokaryotic cell comprising a nucleic acid molecule according to any one of claims 40 to 45.

47. A prokaryotic cell comprising a nucleic acid molecule which is a plasmid according to claim 45 which further comprises a first gene present on the chromosome encoding a repressor that binds to the operator on the plasmid and a second gene present on the chromosome that is functionally associated with the same operator and essential for cell growth.

48. A cell according to claim 46 or claim 47 wherein the cell is a bacterial cell.

49. A cell according to claim 48 wherein the cell is a gram negative bacterial cell.

50. A cell according to claim 48 wherein the cell is a gram positive bacterial cell.

51. A cell according to claim 49 wherein the cell is an *E. coli* cell.

52. A cell according to claim 50 wherein the cell is a *B. subtilis* cell.

53. A cell according to any one of claims 46 to 52 wherein the cell is a RecA+ cell.

54. A nucleic acid molecule or a prokaryotic cell according to any one of claims 40 to 54 wherein the *dif*-like site-specific recombinase recognition sites flanking the selectable marker gene are the same.

55. A nucleic acid molecule or a prokaryotic cell according to any one of claims 40 to 54 wherein the *dif*-like site-specific recombinase recognition sites flanking the selectable marker gene are different.

56. A nucleic acid molecule or a prokaryotic cell according to claim 54 or claim 55 wherein at least one of the *dif*-like sites is selected from the *E.coli dif* site and the *B. subtilis dif* site.

57. A nucleic acid molecule or a prokaryotic cell according to any one of claims 54 to 56 wherein at least one of the *dif*-like sites is selected from the plasmid *dif*-like sites *cer* and *psi* and the nucleic acid molecule further comprises accessory sequences.

58. A nucleic acid molecule or a prokaryotic cell according to any one of claims 54 to 57 wherein at least one of the *dif*-like sites is the hybrid *dif*-like site *pif* and the nucleic acid molecule further comprises accessory sequences.

59. A nucleic acid molecule or a prokaryotic cell according to claims 40-58 wherein the selectable marker gene is an antibiotic resistance gene.

60. A process for producing a supercoiled, monomeric plasmid DNA in a RecA+ cell comprising culturing a RecA+ cell comprising a plasmid, characterised in that the plasmid comprises a *dif*-like site-specific recombinase recognition site.

Figure 1:

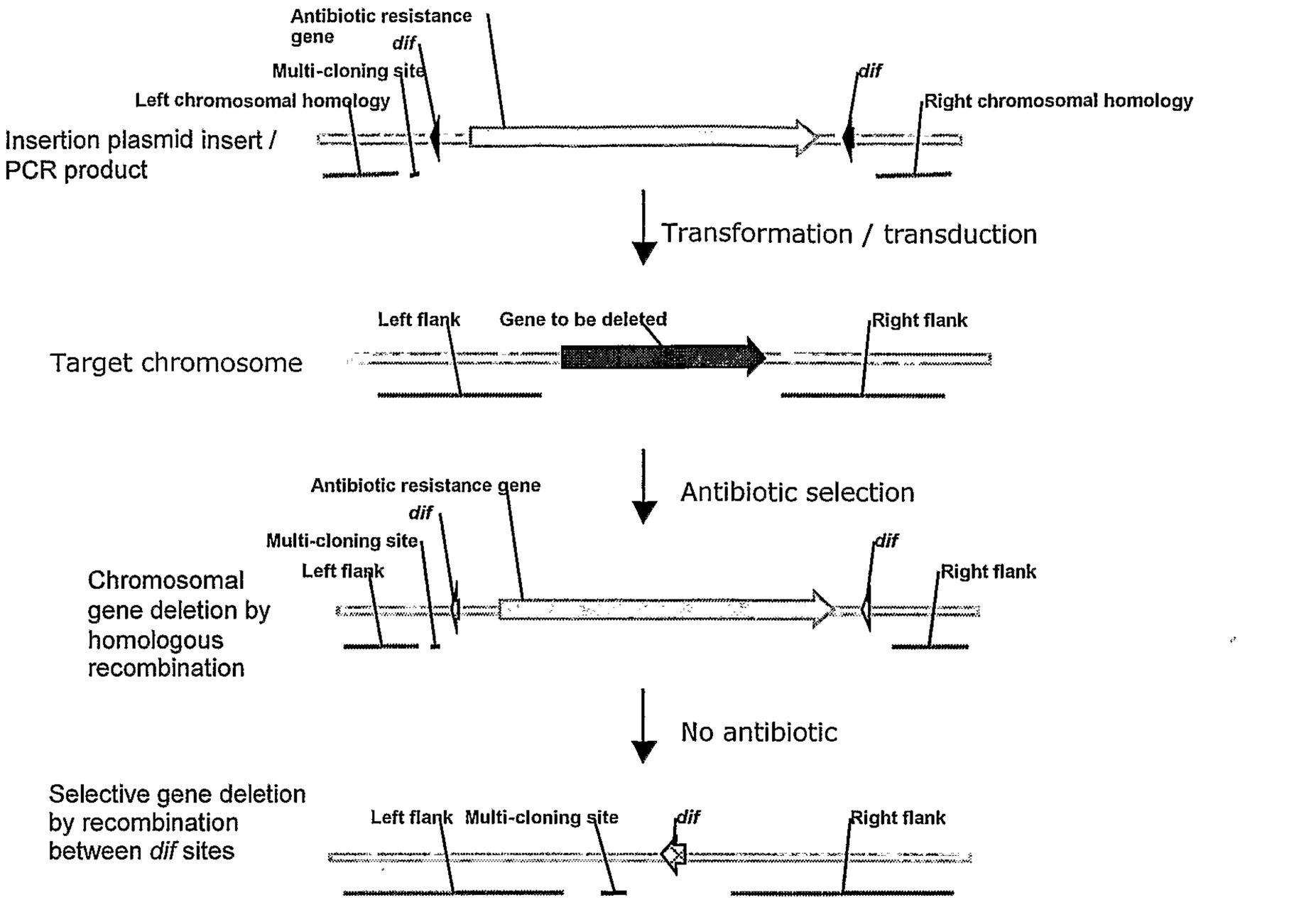


Figure 2:

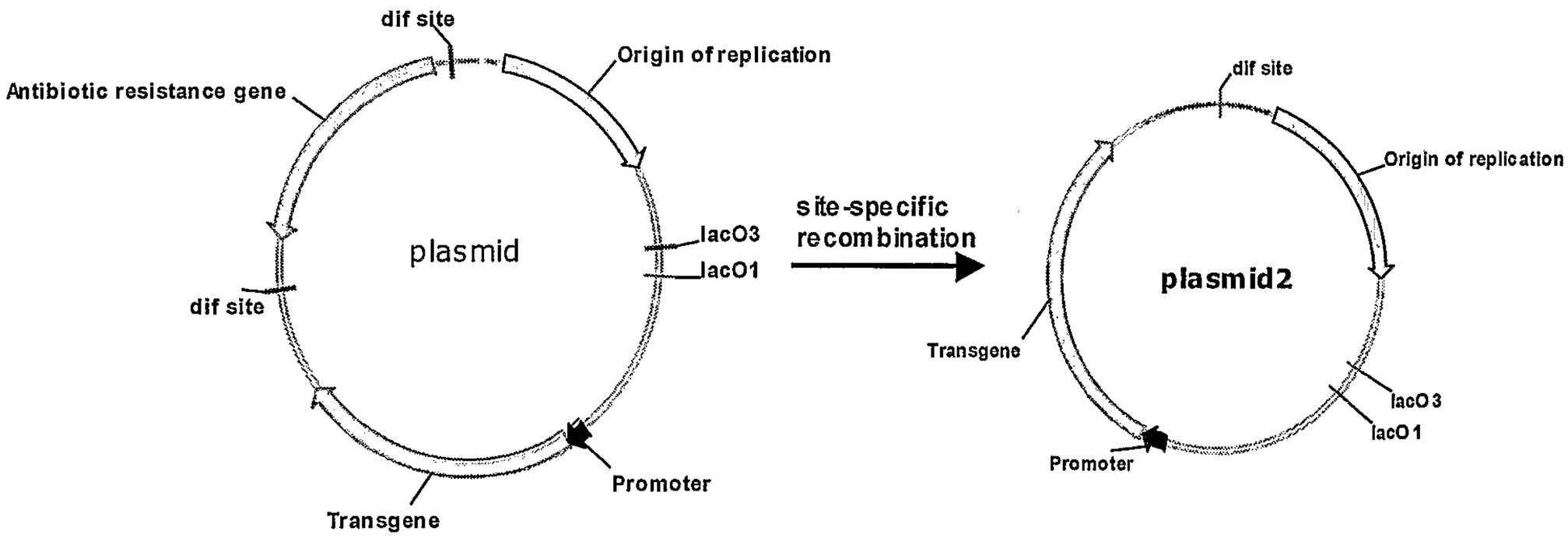


Figure 3:

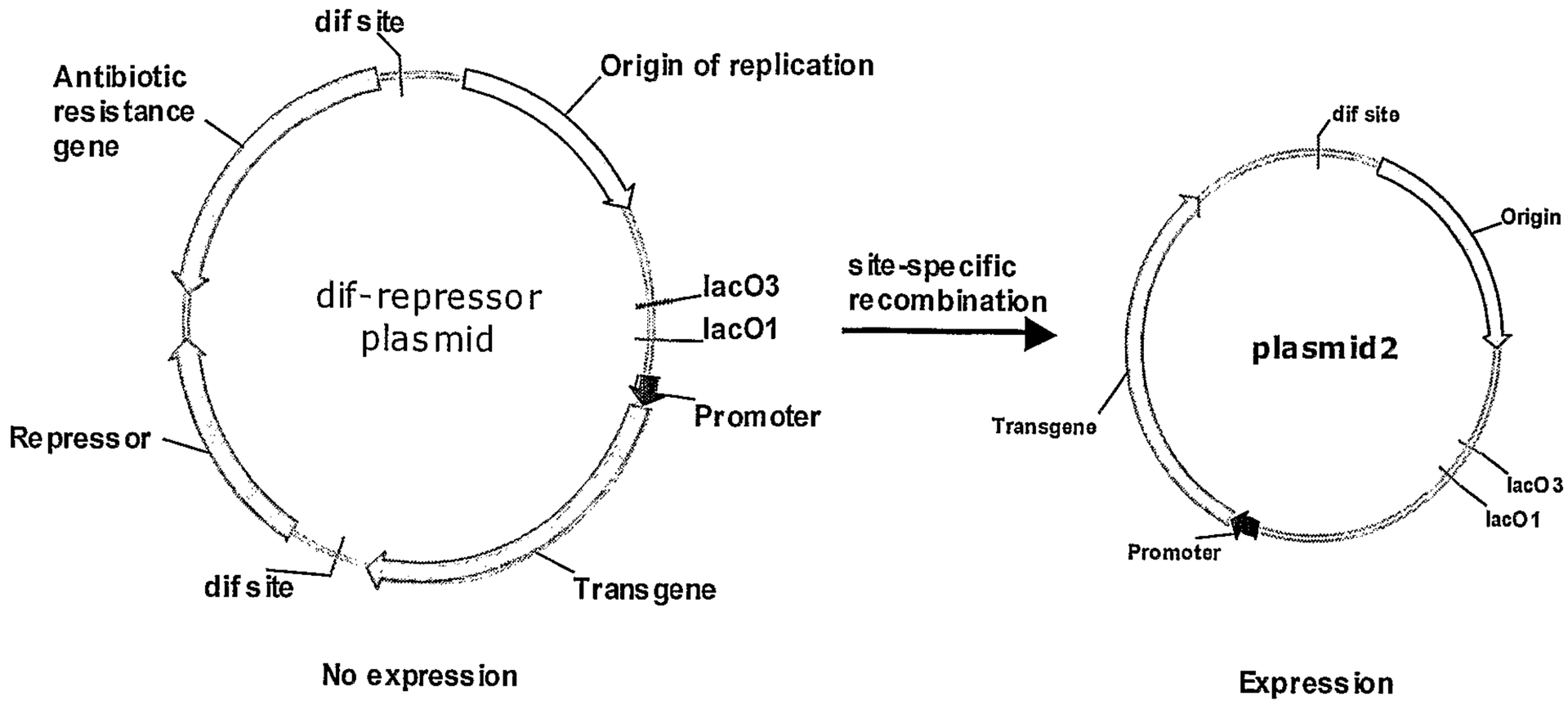


Figure 4:

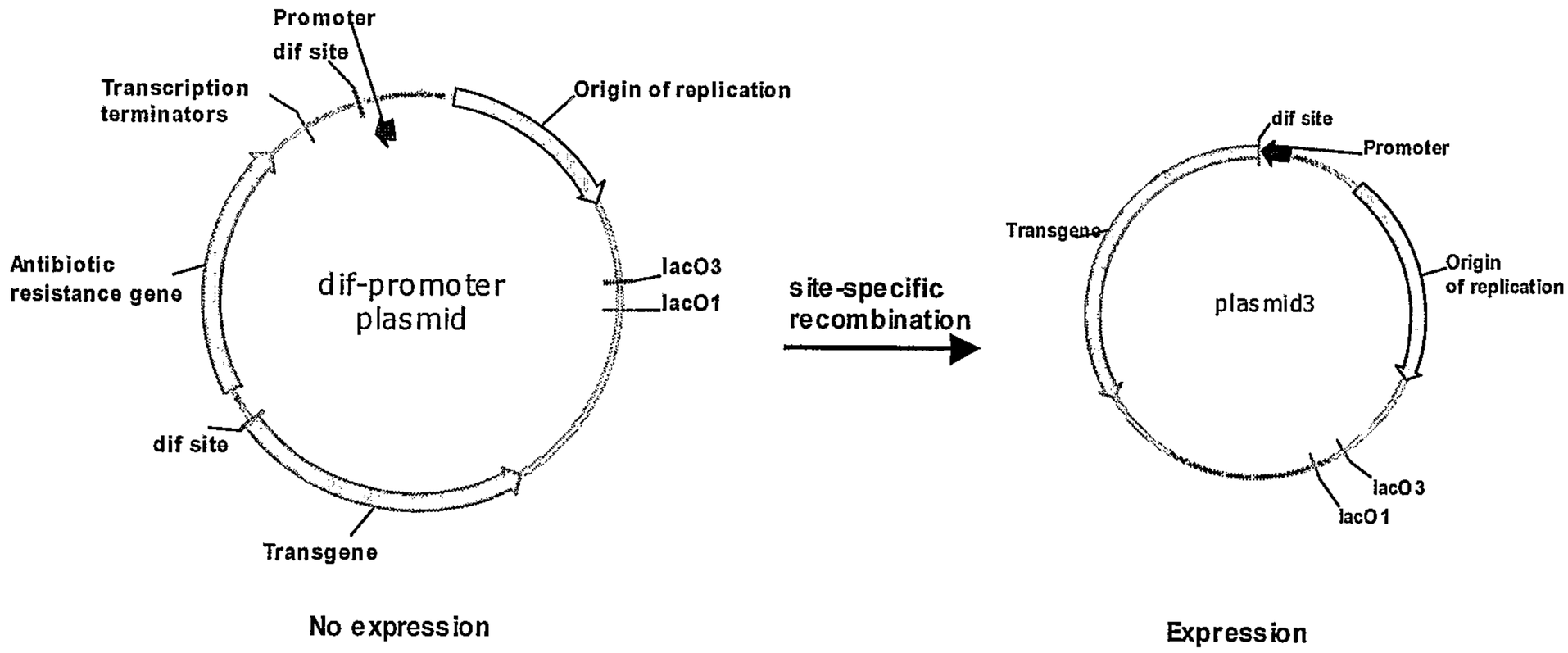


Figure 5:

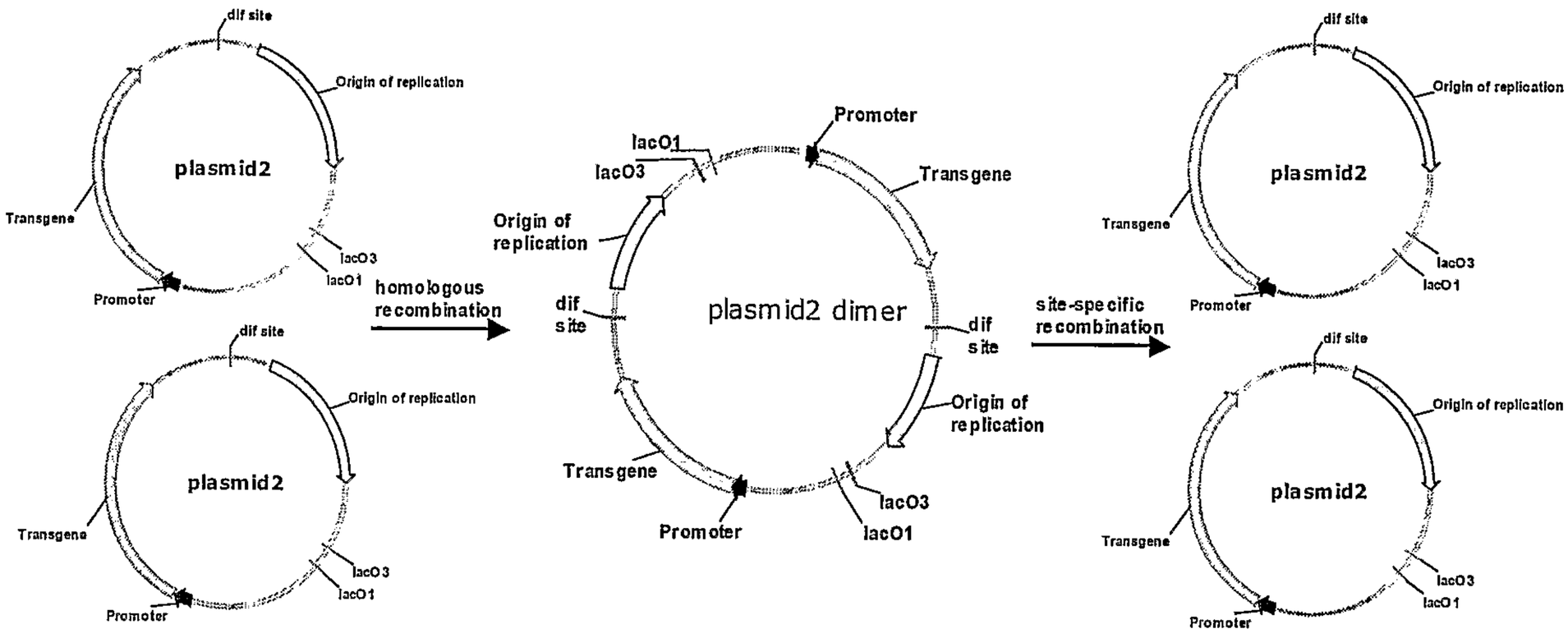


Figure 6:

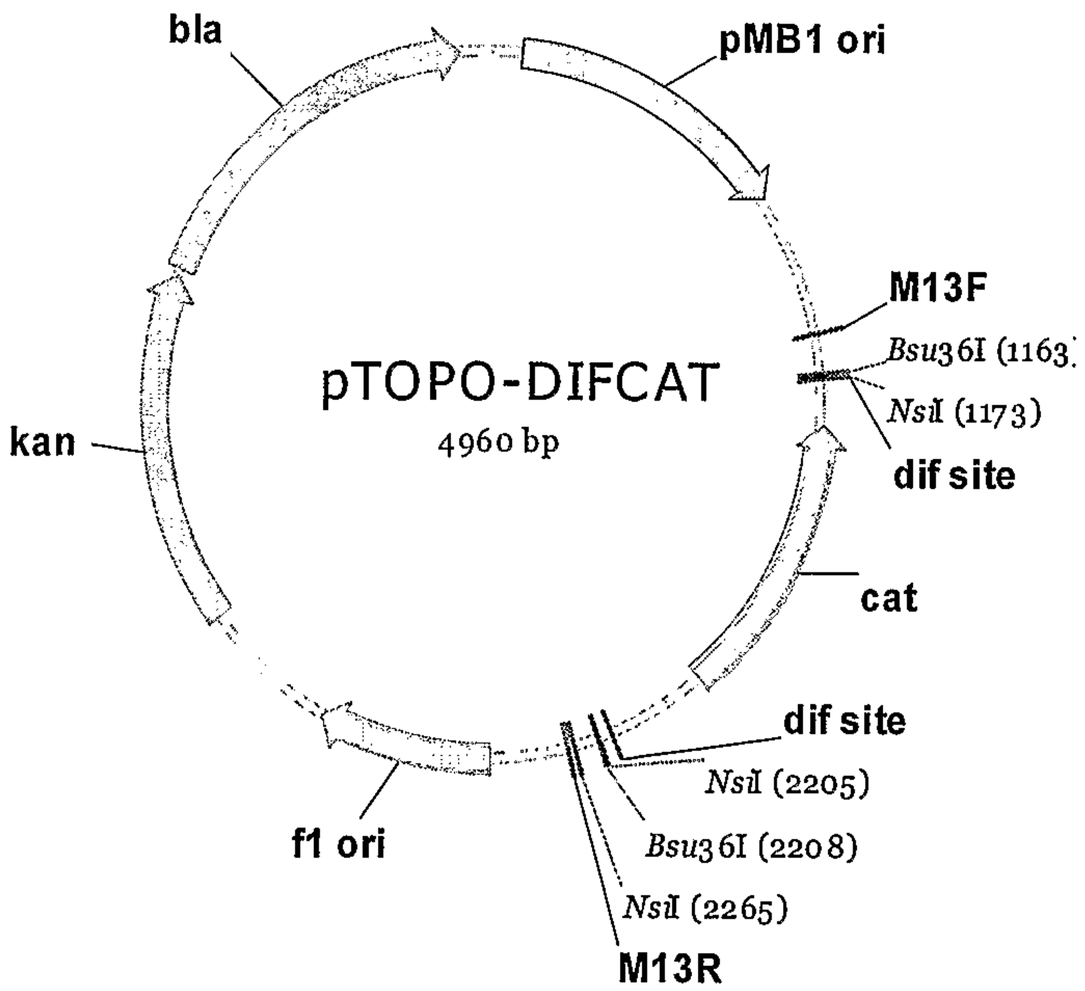
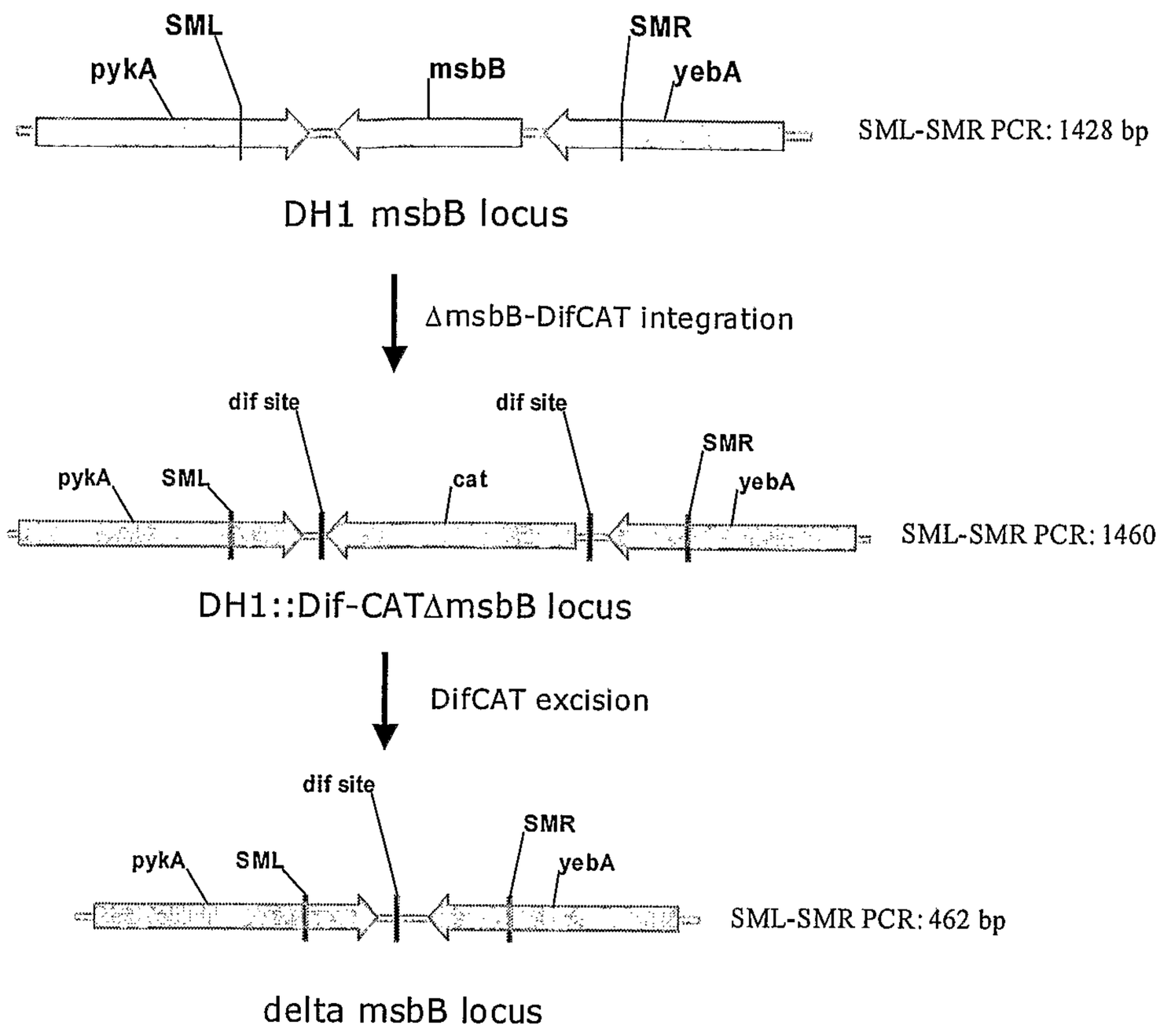
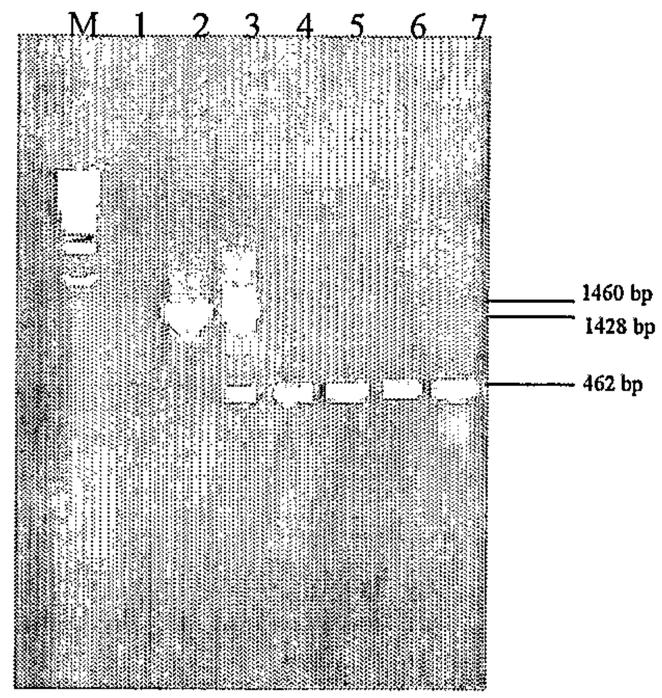


Figure 7:

A)



B)



M. kb DNA ladder

1. Negative control (no DNA)

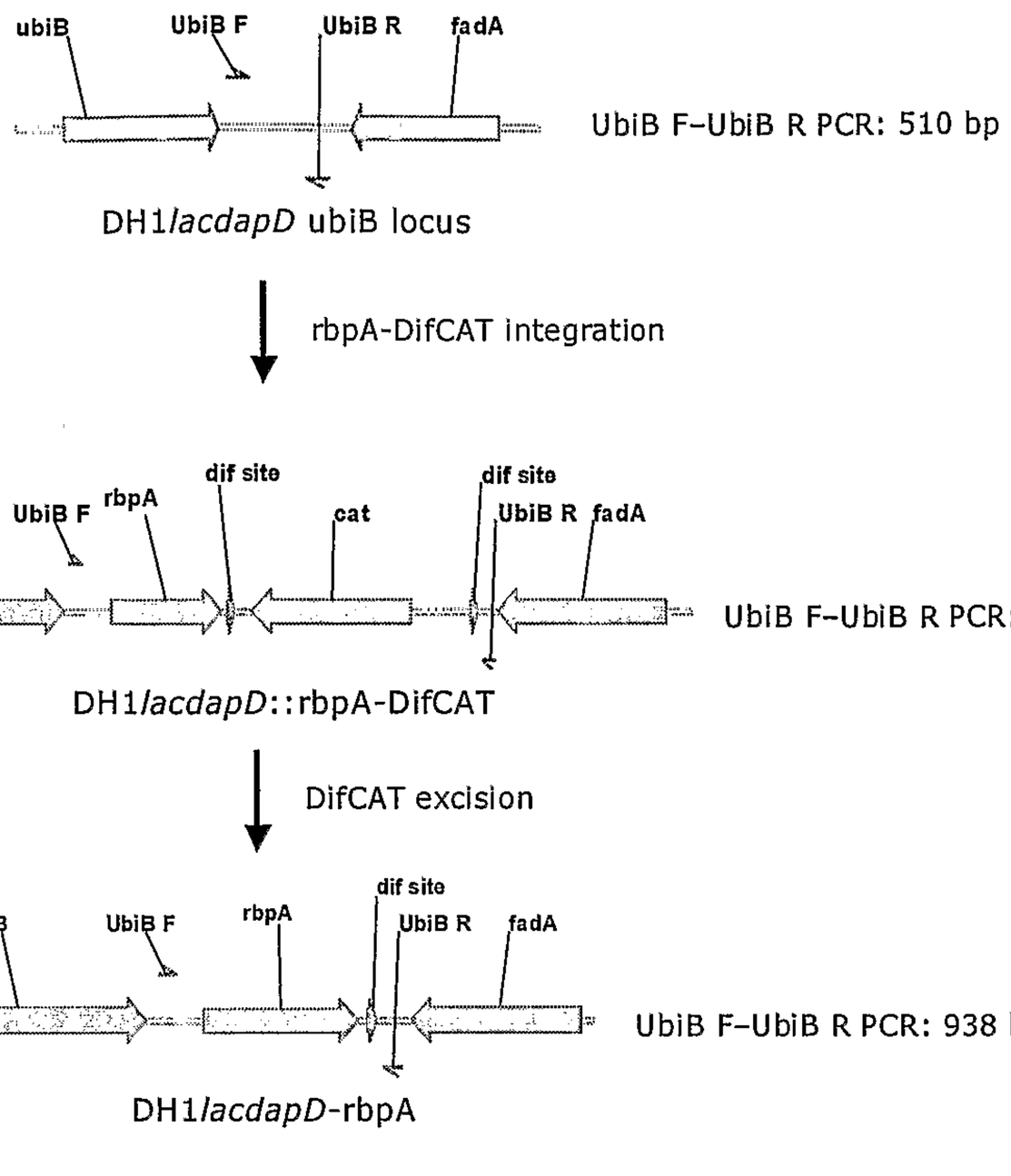
2. Wild type (DH1 gDNA)

3. Integrant gDNA (DH1::ΔmsbB-DifCAT)

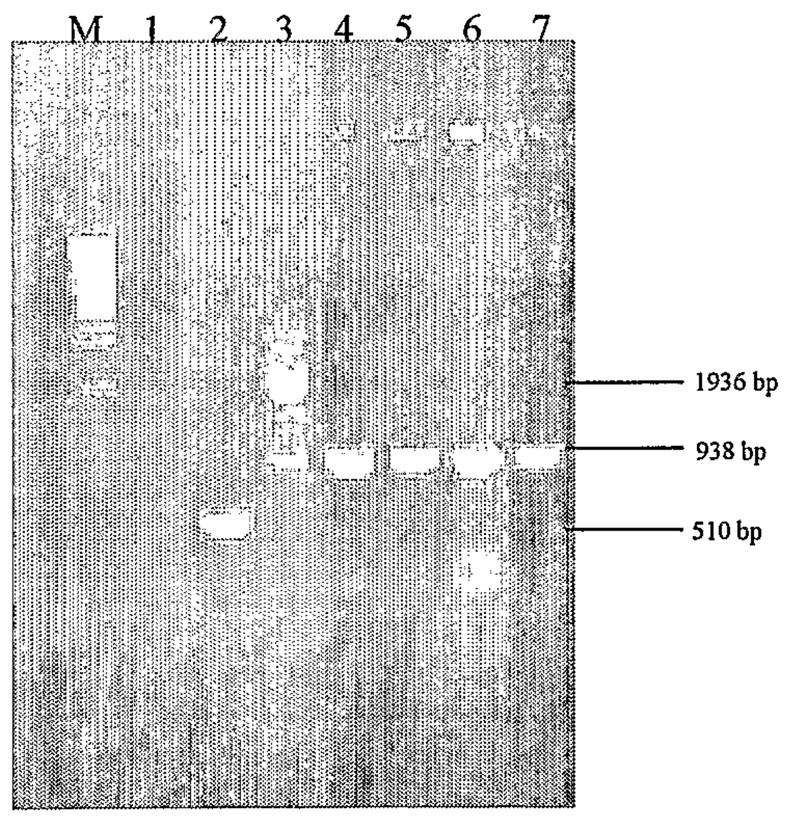
4.-7. Resolvant clones (DH1ΔmsbB)

Figure 8:

A)



B)



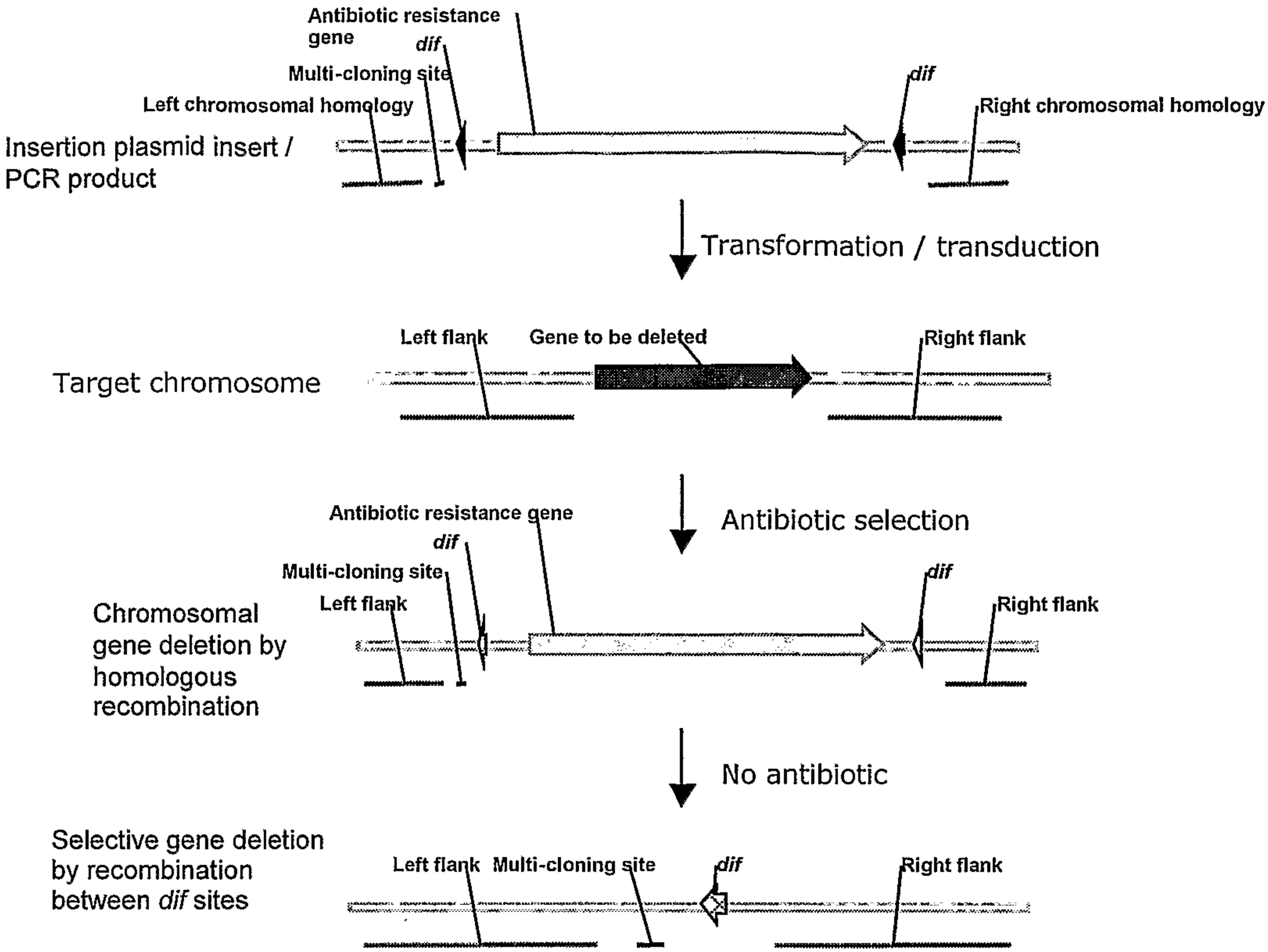
M. kb DNA ladder

1. Negative control (no DNA)

2. Wild type (DH1*lacdapD* gDNA)

3. Integrant gDNA(DH1*lacdapD*::rbpA-DifCAT)

4.-7. Resolvant clones (DH1*lacdapD*-rbpA)


Antibiotic resistance gene
dif
Multi-cloning site
Left chromosomal homology
dif
Right chromosomal homology
Insertion plasmid insert / PCR product
Transformation / transduction
Left flank
Gene to be deleted
Right flank
Target chromosome
Antibiotic resistance gene
dif
Multi-cloning site
Left flank
dif
Right flank
Chromosomal gene deletion by homologous recombination
Antibiotic selection
No antibiotic
Left flank
Multi-cloning site
dif
Right flank
Selective gene deletion by recombination between dif sites