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(54) Title: TETRACYCLINE-CONTROLLED INSERTION ELEMENT FOR SYSTEMATIC MUTAGENESIS

(57) Abstract: The present invention relates to an insertion element, wherein said insertion element is a recombinant nucleic acid molecule which comprises, in 5' $\diamond$ 3' arrangement: (i) a first recombinant binding site; (ii) a resistance gene, operatively linked to a promoter and, optionally, to transcription control sequences; (iii) a tetracycline-controlled promoter; and (iv) a second recombinant binding site, wherein said tetracycline-controlled promoter is oriented to allow transcription in direction of the second recombinant binding site. The present invention also relates to a method for the generation of a conditional knock-out mutant of a cell, comprising the steps of: (a) introducing a nucleic acid molecule comprising the insertion element of the present invention into a cell, wherein the insertion element is transfected into the cell concomitant with purified recombinant; (b) integrating the insertion element into the cellular genome; and (c) selecting cells for resistance against a substrate for which the resistance gene of the insertion element confers resistance; wherein prior to or concomitant with step (a) an expression construct for a tetracycline-sensitive (poly)peptide is introduced into the cell, said (poly)peptide being capable of binding to the tetracycline-controlled promoter of the insertion element and of modulating its expression. Finally, the present invention relates to a cell containing the insertion element of the present invention, to a method for identifying genetic determinants of pathogen survival and to a method for the generation of a transgenic animal and plant.

Tetracycline-controlled insertion element for systematic mutagenesis

The present invention relates to an insertion element, wherein said insertion element is a recombinant nucleic acid molecule which comprises, in 5' → 3' arrangement: (i) a first recombinase binding site; (ii) a resistance gene, operatively linked to a promoter and, optionally, to transcription control sequences; (iii) a tetracycline-controlled promoter; and (iv) a second recombinase binding site, wherein said tetracycline-controlled promoter is oriented to allow transcription in direction of the second recombinase binding site. The present invention also relates to a method for the generation of a conditional knock-out mutant of a cell, comprising the steps of: (a) introducing a nucleic acid molecule comprising the insertion element of the present invention into a cell, wherein the insertion element is transfected into the cell concomitant with purified recombinase; (b) integrating the insertion element into the cellular genome; and (c) selecting cells for resistance against a substrate for which the resistance gene of the insertion element confers resistance; wherein prior to or concomitant with step (a) an expression construct for a tetracycline-sensitive (poly)peptide is introduced into the cell, said (poly)peptide being capable of binding to the tetracycline-controlled promoter of the insertion element and of modulating its expression. Finally, the present invention relates to a cell containing the insertion element of the present invention, to a method for identifying genetic determinants of pathogen survival and to a method for the generation of a transgenic animal and plant.

Several documents are cited throughout the text of this specification. The disclosure content of the documents cited herein (including any manufacturer's specifications, instructions, etc.) is herewith incorporated by reference.

Despite tremendous advances in our comprehension of the molecular basis of infectious diseases or diseases such as cancer, substantial gaps remain both in our understanding of disease pathogenesis and in the development of effective strategies for early diagnosis and for treatment. Proteomic approaches to investigate diseases will overcome some of the limitations of other approaches. The opportunities as well as the challenges facing disease proteomics are formidable. Particularly promising areas of research include: delineation of altered protein

expression, not only at the whole-cell or tissue levels, but also in subcellular structures, in protein complexes and in biological fluids; the development of novel biomarkers for diagnosis and early detection of disease; and the identification of new targets for therapeutics and the potential for accelerating drug development through more effective strategies to evaluate therapeutic effect and toxicity.

Despite earlier predictions to the contrary, infectious diseases remain a leading cause of worldwide death. A complicating factor in therapy for infectious diseases is the development of resistance to commonly used drugs (for example, as has occurred in tuberculosis), which heightens the need for developing effective new therapies. Interest in the application of proteomics to microbiology goes back at least two decades, with the pioneering work of Fred Neidhardt to characterize protein expression patterns in *Escherichia coli* under different growth conditions (VanBogelen, R. A., Schiller, E. E., Thomas, R. D. & Neidhardt, F. C. Diagnosis of cellular states of microbial organisms using proteomics. *Electrophoresis* 20, 2149-2159 (1999)).

At present, the characterization of genes involved in bacterial pathogenesis or of genes involved in cancer onset is primarily based on biochemical analysis of individual proteins or protein sets [see Hanash, 2003 and Jain, 2000 for reviews and references cited therein]. For proteome analysis, pathogenic microorganisms are often grown *in vitro* (in a chemostat, for example) under conditions known or assumed to mimic conditions in a host organism [*Streptococcus mutans* - (Len et al., 2003); *Salmonella typhimurium* - (Deiwick et al., 2002); *Pseudomonas aeruginosa* - (Guina et al., 2003); *Leishmania infantum* - (El Fakhry et al., 2002); *Mycobacterium tuberculosis* - (Rosenkrands et al., 2002)]. The quest for identifying new tissue- or disease-specific proteins is hampered by the fact that methods like tissue micro-dissection, Protein Chip Array technologies, MALDI-TOF mass spectrometry or mass spectrometric analysis of *in situ* tissue sections are time-consuming, expensive and technically demanding (Hanash, 2003; von Eggeling et al., 2000).

In contrast to protein based analysis systems, analytic systems based on randomized genetic approaches are generally more efficient and versatile. It would therefore be desirable to have such a genetic system at hands, allowing to randomly

modify the genes of a cell to control their gene expression by external control elements. This would help to understand the impact of these genes and their gene products on pathogenicity.

A number of control elements have previously been described Fussenegger, Biotechnol. Prog. 2001, 17, 1-51. The tetracycline-dependent gene regulation system is based upon a tetracycline resistance determinant discovered in Gram-negative and Gram-positive bacteria. Thereby, a repressor regulates transcription of the resistance gene *tetA* and its own gene by binding to two operators. The tetracycline repressor (TetR) reacts very efficiently to tetracycline (or its derivatives like anhydrotetracycline, etc), by binding two molecules of the drug and detaching from its operator sequence mediated by a conformational change. Thereby transcription of *tetR* and *tetA* is relieved (Figure 1). Meanwhile, TetR mutants are known which display the contrary phenotype (2). These, so called reverse Tet repressors allow turning off genes upon addition of tetracycline or its analogues, thereby opening new possibilities for application. The properties of this system are applied to gain stringent and highly sensitive transcriptional control over both pro- and eukaryotic genes of choice (3).

Numerous transposon based mutagenesis systems, which can be utilised in different organisms, have been described in the art. Most of these systems contain a vector transferring the donor-DNA together with a transposase gene into the target cell, where transposition of the donor-DNA into the chromosome takes place (4). These systems are disadvantageous in mostly being apt just for a specific target organism and in not ensuring genetic stability, as the transposase encoding gene is present in the cell. By contrast, in *in vitro* systems transposase is added to the donor- and target-DNA, and the modified target-DNA is brought back into the chromosome of the target cell (5). Goryshin *et al.* have developed an *in vitro* transposition system based upon the Tn5 transposon, where a stable complex of transposase and insertion element is electroporated into a target cell. This system can be applied in a broad range of different organisms (6). In 2000, Judson *et al.* published the first *in vitro* mutagenesis system vested with a regulable promoter to gain regulatory control over downstream genes (7). This system is based upon the arabinose-inducible promoter pBAD. The inducer arabinose, however, is not

suitable for investigations in mammalian infection models, as it is metabolised there. In addition, not all bacteria possess an arabinose uptake system, therefore the choice of bacteria to be examined is limited. This is also the case in the transposon Tn10 based *in vivo* mutagenesis system T-POP, which has been described elsewhere (8). The Tn10 transposon has the disadvantage not to insert randomly but having insertion hot spots in the chromosome. This interferes with the successful creation of a totally randomized mutant pool. The T-POP system consists of a deletion variant of the whole Tn10 transposon so all *tet* promoters (P_{tetR1} , P_{tetR2} and P_{tetA}) are active. Moreover the gene for tetracycline resistance *tetA* is present. So transcription from the *tetA* promoter has to exceed a long DNA fragment, before it reaches its destination i.e. the chromosomal DNA downstream of the insertion element. More important is the fact, that the *tetA* gene, if expressed unnecessarily, decreases the bacterial fitness by imbalancing the proton gradient of the cell wall. This is another crucial point for a versatile genetic tool not to influence the host cell's fitness by expressing unnecessary genes.

The complete sequencing of a number of microbial genomes has provided a framework for characterizing the role of various proteins and their relevance for pathogenic persistence or for disease progression. A crucial aspect of the continuing fight against pathogenic organisms is the question of whether or not it will be possible to identify those proteins within the pathogenic proteome, the expression of which is essential for the survival of the pathogen. To this end, it will be important to have a versatile system allowing to screen a large number of genetic components within a manageable time frame. In particular, it will be important to be able to selectively switch on (or off) genes, since this would allow to study their impact on disease onset or disease progression.

Thus, the technical problem underlying the present invention was to provide means and methods for generating conditional knock-out mutants of cells. The solution to this technical problem is achieved by providing the embodiments characterized in the claims.

Accordingly, the present invention relates to an insertion element, wherein said insertion element is a recombinant nucleic acid molecule which comprises, in 5' → 3' arrangement: (i) a first recombinase binding site; (ii) a resistance gene,

operatively linked to a promoter and, optionally, to transcription control sequences; (iii) a tetracycline-controlled promoter; and (iv) a second recombinase binding site, wherein said tetracycline-controlled promoter is oriented to allow transcription in direction of the second recombinase binding site.

The term "insertion element" as used herein refers to a nucleic acid molecule which is incorporated into a larger nucleic acid molecule, particularly into the chromosome of a eukaryotic or bacterial cell.

As used herein, the term "nucleic acid molecule" refers to DNA and RNA which may be single stranded or double stranded, circular or linear. The term "recombinant" refers to the fact that the subunits of the insertion element have been combined by recombinant means.

The term "5' → 3' arrangement" refers to the polarity of DNA and RNA molecules as described for example in standard textbooks of genetics such e.g. Genes, B. Lewin (ed.), 1983, Wiley and Sons, Inc. New York.

The term "transcription control sequences" comprises elements such as e.g. promoter, enhancer, terminator, operator or silencer.

The insertion element of the present invention is flanked by a 5' and a 3' recognition sequence for a recombinase. As used herein, the term "recombinase" refers to transposases as well as recombinases. These recognition sequences are also called "first" and "second" recombinase binding site", wherein the 5' recognition sequence is the "first" and the 3' recognition sequence is the "second" recombinase binding site. Recombinase is the enzyme which catalyses a recombination reaction. In the context of the present invention, a recombinase activity is required to insert the insertion element of the present invention into the host cell genome which is usually made of DNA or RNA. A recombinase which is in accordance with the present invention must have the minimal functions of (1) inserting a break into a genomic nucleic acid molecule of the host cell and (2) of joining the resulting free ends with the free ends of the insertion element of the present invention. This activity is carried out, inter alia, by transposases or recombinases. However, any other protein, riboprotein or RNA-enzyme with this activity may be used in accordance with the teaching of the present invention.

The term "resistance gene" refers to a gene encoding a protein conferring resistance to a compound, wherein said compound is usually an antibiotic. Therefore, the term "resistance gene", as used herein, can also be understood as a gene encoding a protein conferring antibiotic resistance. "Antibiotic resistance" is the ability of a microorganism or of a cell to withstand the effects of an antibiotic.

The term "tetracycline-controlled promoter" refers to a promoter which is modulated by the presence and activity of one or more control elements which are located in the vicinity of said promoter. This control element, which is also called "tet operator" (*tetO*), represents the binding site for a tetracycline-sensitive (poly)peptide. Preferably a single *tetO* is selected from any one of SEQ ID NOs: 1 to 6. the following sequence motifs.

The term "promoter" as used throughout the specification means binding site of the RNA polymerase enabling transcription.

The term "(poly)peptide" refers alternatively to peptide or to polypeptide. Peptides conventionally are covalently linked amino acids of up to 30 residues, whereas polypeptides (also referred to as "proteins") comprise 31 and more amino acid residues.

In one embodiment of the present invention, the tetracycline-sensitive (poly)peptide is the tetracycline repressor (TetR) which is well known in the art (Berens 2003). It is envisaged by the teaching of the present invention that any tetracycline sensitive (poly)peptide may be used, provided that it is capable of (1) binding to a nucleic acid based binding site and (2) modulating the level of transcription of the nearby promoter, wherein modulation of transcription refers to an upregulation or downregulation of transcription, including shut-off of transcription. Useful tetracycline sensitive (poly)peptides and binding sites for such peptides are disclosed herein.

The insertion element of the present invention contains in its simplest embodiment one, two or three *tetO* sequences. However, in order to improve control by tetracycline sensitive (poly)peptides it is possible to add further *tetO* sequences. The *tetO* sequences need not be the same, as long as they provide a functional binding site for tetracycline sensitive (poly)peptides.

The liquid media screening systems described in the examples of the present invention yields a plethora of phenotypes of different *E. coli* transponants. This is due to the differences in growth of the candidates with or without *atc*, which can hardly be differentiated on solid media plates. Besides auxotrophs, candidates are found that display a reverse regulable phenotype, i.e. growth only in the absence of *atc*. During all screenings, 202 different genes were detected where the element had inserted (Fig. 19). These include mutants that could not be verified on solid medium.

The utilisation of two regulators with different phenotypes, wtTetR and revTetR, resulted in a different spectrum of mutants: While in the wtTetR screening reverse regulable mutants were found, the revTetR screen yielded essential genes. This demonstrates that different kinds of regulators can be applied to reach the goal of the screening. The analysis of the 15 regulable candidates described above revealed that some of them are already published, such as *mraZ*, the first gene in the *dcw* cluster, responsible for cell division and cell wall synthesis. The transcription of this cluster has previously been put under the control of a regulable promoter and analysed (13). By contrast, the *yrbK* gene was identified, whose function is unknown so far. This mutant could now be subjected to further analysis to elucidate this gene's function and to possibly obtain a novel drug target. Table 1 (see below) summarises all found regulable auxotroph mutants as well as the assigned function, product or biosynthesis pathway of the respective gene. The numbers presented in the Examples of the present invention clarify that the present invention's technique can be applied to generate numerous mutants with different phenotypes. Recently, element one has been improved by adding additional features. In these additional experiments, the kanamycine resistance cassette is now flanked by two FRT sites to optionally excise the cassette by using Flp recombinase. This opens the possibility to insert an additional element into the chromosome of a distinct mutant utilizing the "recycled" selection marker. A R6K γ -ori was also added to the element, which can initiate replication only when the π -protein, which was deleted from this ori, is present elsewhere in a suitable strain. This ori simplifies the recovery of the insertion site by the so called "rescue cloning" technique. The last feature added to the element was a T7 promoter. This is widely

used for in vitro transcription to obtain transcripts from the chromosome for further analysis e.g. in microarrays. This modified element improves versatility as tool for the search and examination of new drug targets.

The term "oriented to allow transcription in direction of the second recombinase binding site" means the tetracycline dependent promoter enables transcription directly out of the insertion element.

In a preferred embodiment of the present invention, at least one stop codon is located between the first recombinase binding site and the resistance gene. This stop codon has the function to suppress translation of open reading frames (ORF) located 5' of the resistance gene. In the case of bacterial cells, stop codons may be selected from UAA, UAG, UGA, in the case of eukaryotic cells, stop codons may be selected from UAA, UAG, UGA. In order to block translation of all open reading frames, it is preferred to have three neighboring stop codons which are positioned within all possible reading frames. However, sometimes it may be sufficient that the insertion element only comprises two or even one stop codon. Stop codons may overlap or be separated by additional nucleotides.

In another preferred embodiment of the present invention, at least one terminator of transcription is located 5' of the resistance gene. In most cases, it is sufficient to use one or two terminators of transcription. However, it is conceivable to position two, three, four or even five terminators next to each other. The terminator sequences do not need to be the same and any known terminator sequences may be used at the indicated position within the insertion element. In fact, it is also possible to use artificial terminator sequences. Generally any sequence is suitable in this regard, as long as it results in a reduction of the level of transcription. The skilled person can determine transcription termination activity of a given nucleotide sequence by conventional assays such as Northern Blot analysis. Typical examples of terminator sequences which are in accordance with the present invention are the Transposon Tn10 transcriptional terminator sequence = SeqID No. 372 (aaatataatgaccctcttgataacccaagagggcattttta) (NAR, Vol.13, No.12 (1985), Schollmeier K. et.al.); Bacteriophage Lambda T0 transcriptional terminator = SeqID No. 373.

(gactcctgttgatagatccagtaatgacctcagaactccatctggattgttcagaacgctcgggtgccgccggggcggtt
ttattggtgagaatccaagcta) (Ref: NCBI Nucleotide entry: AY737006); Escherichia coli
rrnB T1 transcriptional terminator =SeqID No.374
(aggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgtttatctgttggttgcg) (JMB, 269
(1997), Lyakhof D.L. et al.); Escherichia coli rrnB T2 transcriptional terminator
=SeqID No.375 (ttaagcagaaggccatcctgacggatggccttttgcgtttctac) (JMB, 269 (1997),
Lyakhof D.L. et al.); Bacteriophage T7 transcription terminator =SeqID No. 376
(ctagcataacccttggggcctctaaacgggtcttgaggggtttttgctgaaag) (JMB, 269 (1997),
Lyakhof D.L. et al.).

In another preferred embodiment of the present invention, the resistance gene is selected from the group consisting of *cat*, *aph*, *bla*, *ermB*, *rpsL*, *aada5*, *hph*, *rpoB*. The following table shows the proteins encoded by the aforementioned genes and the corresponding antibiotic resistance.

antibiotic resistance	gene	product
Chloramphenicol	<i>cat</i>	chloramphenicol acetyl transferase
Kanamycin	<i>aph</i>	aminoglycosyl transferases
Ampicillin, Penicillin G	<i>bla</i>	beta-lactamases
Erythromycin	<i>ermB</i>	erythromycinmethylase
Streptomycin	<i>rpsL</i> , <i>aada5</i>	modified ribosomal protein S12, aminoglycoside-3'-adenyltransferase
Hygromycin B	<i>hph</i>	hygromycin B phosphotransferase
Rifampicin	<i>rpoB</i>	modified RNA polymerase subunit beta

In another preferred embodiment of the present invention, the resistance gene is flanked by additional recombinase binding sites which are different from the first and second recombinase binding site. The presence of such additional recombinase binding sites allows to selectively remove the resistance gene at later stages of the present invention's method. For example, after selecting cells with the desired properties, recombinase activity specific for the third and fourth recombinase binding site may be used to excise the resistance gene. Particularly in cases when undesired reactions of the host environment against the presence of the resistance

gene are to be expected, removal of the resistance gene can prevent such reactions.

In a more preferred embodiment of the present invention, said additional recombinase binding sites are selected from FLP recombinase dependent FRT sites and Cre recombinase dependent loxP sites.

In yet another preferred embodiment, the insertion element of the present invention comprises a prokaryotic origin of replication. Generally, any ori of replication may be used in accordance with the teaching of the present invention. R6K γ is a preferred ori.

In another preferred embodiment of the present invention, at least one viral promoter is located upstream of the tetracycline controlled promoter. Such a promoter may be obtainable from a virus of a eukaryotic or prokaryotic cell. Preferably, the viral promoter is a promoter obtainable from a eukaryotic virus or from a prokaryotic virus. Preferred promoters are promoters obtainable from the bacteriophages T7, T3 or SP6. Examples of such promoters are the T7-promoter (SeqID No.377: CCTCCCTATAGTGAGTCGTATTA); the T3-promoter (SeqID No.378: GGAATTAACCCTCACTAAAGGG) and the SP6-promoter: (SeqID No.379: ATTTAGGTGACACTATAGAA).

In another preferred embodiment, the insertion element of the present invention may comprise an additional coding sequence for a tetracycline repressor, wherein the coding sequence is (a) operatively linked to a promoter and, optionally, to transcription control sequences; or (b) operatively linked to an internal ribosomal entry site and arranged 3' to a promoter sequence of the insertion element, allowing expression of the repressor from a multicistronic RNA. As pointed out above, the present invention's teaching relates to insertion elements comprising a tetracycline-controlled promoter. It is envisaged by the present invention that the tetracycline sensitive (poly)peptide, controlling the promoter by binding to one or more *tetO* sites, may be encoded by an open reading frame located either on the same nucleic acid molecule as the *tetO* (located in *cis*) or located on a different nucleic acid molecule (located in *trans*). A suitable promoter may be any of the promoters described herein above. A suitable transcription control sequence may be any of the

transcription control sequences described herein above. The term "ribosomal entry site" (IRES) refers to a nucleic acid sequence on them RNA which mediates internal entry and start of ribosomal translation. Such sites are particularly useful for enabling translation of (poly)peptides encoded by open reading frames located 3' of the 5' proximal open reading frame, i.e. for expressing the open reading frames of a multicistronic RNA. A multicistronic RNA is an RNA which contains more than one open reading frame. IRES elements may, however, also be used for expressing the open reading frame of a monocistronic RNA. Preferably, IRES elements are selected from the group consisting of poliovirus-IRES and EMCV-IRES (see SEQ ID NO.s 392 and 393).

In another preferred embodiment of the present invention, the promoter is a prokaryotic promoter. Preferably, said prokaryotic promoter is selected from the group consisting of T7, T3, xylA, tetA or SP6. Examples of such promoters are the T7-promoter (SeqID No.377: CCTCCCTATAGTGAGTCGTATTA); the T3-promoter (SeqID No.378: GGAATTAACCCTCACTAAAGGG) and the SP6-promoter: (SeqID No.379: ATTTAGGTGACACTATAGAA).

In a more preferred embodiment of the present invention, the promoter is controlled by at least one tet-operator binding site sequence selected from (a) SEQ ID NO: 1 to 6; or from (b) SEQ ID NO: 7 to 13; or permutations thereof. The sequences of the tet-operators are as follows (see also SEQID NOs: 1 to 6):

tetO1 ACTCTATCATTGATAGAGT
 TGAGATAGTAACTATCTCA

tetO2 TCCCTATCAGTGATAGAGA
 AGGGATAGTCACTATCTCT

tetO1-4C ACTCTGTCATTGACAGAGT
 TGAGACAGTAACTGTCTCA

tetO1-6C ACTGTATCATTGATACAGT
 TGACATAGTAACTATGTCA

tetO2-4C TCCCTGTCAGTGACAGAGA

AGGGACAGTCACTGTCTCT
tetO2-6C TCCGTATCAGTGATACAGA
 AGGCATAGTCACTATGTCT

The other sequences SEQ ID NOs: 7 to 13 represent complete promoters containing tet-operators.

In another preferred embodiment of the present invention, the promoter is a eukaryotic promoter.

In a more preferred embodiment of the present invention, the eukaryotic promoter is selected from the group consisting of CMV, RSV, SV40, Cadherin and hAPC and β -Act, AFP, CD43, CD45, CD68, c-erbB2, EF1, E2F-1, EGR1, FerL, GAPDH, HSP70, IFNB, Mb, PGK-1, TRP1.

In a more preferred embodiment of the present invention, the eukaryotic promoter is selected from any one of SEQ ID NOs: 14 to 21.

In another more preferred embodiment of the present invention, the recombinase binding site is a binding site obtainable from the transposases Tn5, Tn10, Tn7, or from phage Mu.

Tn5 binding sites: =SeqID No. 380/381 [CTGTCTCTTATACACATCT (binding site 1), agatgtgtataagagacag (binding site 2)]; Tn10 binding sites: =SeqID No.382/383 [CTGATGAATCCCCTAATGATTTT (BINDING SITE 1), AAAATCATTAGGGGATTCATCAG (BINDING SITE 2)]; Tn7 binding sites: =SeqID No.384/385

[TGTGGGCGGACAATAAAGTCTTAAACTGAACAAAATAGATCTAAACTATGACAA TAAAGTCTTAAACTAGACAGAATAGTTGTAAACTGAAATCAGTCCAGTTATGCT GTGAAAAAGCATACTGGACTTTTGTATGGCTAAAGCAAACCTCTTCATTTTCTGA AGTGCAAATTGCCCCGT CGTATTAAAGAGGGGCGTGG (BINDING SITE 1), AACCAGATAAGTGAAATCTAGTTCCAAACTATTTTGTCAATTTTAATTTTCGTATT AGCTTACGACGCTACACCCAGTTCCCATCTATTTTGTCACTCTTCCCTAAATAAT CCTTAAAACTCCATTTCCACCCCTCCAGTTCCCAACTATTTTGTCCGCCAC A (BINDING SITE 2)]; Phage Mu binding sites: SeqID No.386/387

[TGAAGCGGCGCACGAAAAACG (BINDING SITE 1),
CGTTTTTCGTGCGCCGCTTCA (BINDING SITE 2)].

In yet another more preferred embodiment of the present invention, the recombinase binding site is selected from the group consisting of *tc1*/mariner and minos. Himar1 binding sites: =SeqID No.388/389 comprise the nucleotide sequences TAACAGGTTGGCTGATAAGTCCCCGGTCT (binding site 1) and AGACCGGGGACTTATCAGCCAACCTGTTA (binding site 2), NCBI Nucleotide entry: AY680862. Acmar1 binding sites =SeqID No.390/391 comprise the nucleotide sequences ATACGTATTATATATTTT (binding site 1) and AAAATATATATATACTAT (binding site 2), NCBI Nucleotide entry: AB081476. These binding sites are more or less perfect inverted terminal repeats of the transposable elements.

The present invention also relates to a vector, comprising an insertion element of the present invention. The term "vector", as used here, refers to prokaryotic and eukaryotic vectors. Such vectors may be specific for prokaryotic or eukaryotic expression. However, the insertion element of the present invention may also be part of a vector functional in prokaryotes and eukaryotes.

A typical prokaryotic expression vector usually contains a promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription of the transcript. Transcription of DNA is dependent upon the presence of a promoter which is a DNA sequence that directs the binding of RNA polymerase and thereby promotes mRNA synthesis. The DNA sequences of eukaryotic promoters differ from those of prokaryotic promoters. Furthermore, eukaryotic promoters and accompanying genetic signals may not be recognized in or may not function in a prokaryotic system, and, further prokaryotic promoters are not recognized and do not function in eukaryotic cells. Similarly, translation of mRNA in prokaryotes depends upon the presence of the proper prokaryotic signals which differ from those of eukaryotes. Promoters vary in their "strength" (i.e. their ability to promote transcription). In some cases it may be desirable to use strong promoters in order to obtain a high level of transcription and, hence, expression of a protein. Depending upon the host cell system utilized, any one of a number of suitable promoters may be used. For instance, when using *E.*

coli, its bacteriophages, or plasmids, promoters such as the T7 phage promoter, *lac* promoter, *trp* promoter, *recA* promoter, ribosomal RNA promoter, the P_R and P_L promoters of coliphage lambda and others, including but not limited, to *lacUV5*, *ompF*, *bla*, *lpp*, and the like, may be used to direct high levels of transcription of adjacent DNA segments. Additionally, a hybrid *trp-lacUV5* (*tac*) promoter or other *E. coli* promoters produced by recombinant DNA or other synthetic DNA techniques may be used to provide for transcription of the inserted gene. Specific initiation signals are also required for efficient gene transcription and translation in prokaryotic cells. These transcription and translation initiation signals may vary in "strength" as measured by the quantity of gene specific messenger RNA and protein synthesized, respectively. The DNA expression vector or nucleic acid molecule encoding a (poly)peptide, which contains a promoter, may also contain any combination of various "strong" transcription and/or translation initiation signals. For instance, efficient translation in *E. coli* requires an SD sequence about 7-9 bases 5' to the initiation codon ("ATG") to provide a ribosome binding site. The SD sequences are complementary to the 3'-end of the 16S rRNA (ribosomal RNA) and probably promote binding of mRNA to ribosomes by duplexing with the rRNA to allow correct positioning of the ribosome. For a review on maximizing gene expression, see Roberts and Lauer, *Methods in Enzymology*, 68:473 (1979), which is hereby incorporated by reference. Thus, an SD-ATG combination that can be utilized by host cell ribosomes may be employed. Such combinations include but are not limited to the SD-ATG combination from the *cro* gene or the N gene of coliphage lambda, or from the *E. coli* tryptophan E, D, C, B or A genes. Additionally, any SD-ATG combination produced by recombinant DNA or other techniques involving incorporation of synthetic nucleotides may be used. Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pET expression vectors (Novagen), pQE expression vectors (Qiagen), pASK expression vectors (IBA), pBAD expression vectors (Invitrogen), pWH1950 (Ettner et al., 1996), pWH1520 (MoBiTec), pWH353 with a Xyl/Tet promoter (Geissendörfer and Hillen, 1990), pLZ vectors with SPAC, Xyl and Xyl/Tet promoter systems (Zhang et al., 2000) or pNZ8008 with a *nisA* promoter (Eichenbaum et al., 1998).

A typical eukaryotic expression vector contains the promoter element, which mediates the initiation of transcription of mRNA, the protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription can be achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin, EF-1 α and ubiquitin C promoters). Suitable expression vectors for use in practicing the present invention include, for example, vectors such as pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146) and pBC12MI (ATCC 67109). Host cells that could be used include human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells. Alternatively, the encoded (poly)peptides can be expressed in stable cell lines that contain a nucleic acid molecule of interest integrated into a chromosome. The co-transfection with a selectable marker such as dhfr, gpt, neomycin, hygromycin allows the identification and isolation of the transfected cells. The transfected gene can also be amplified to express large amounts of the encoded protein. The DHFR (dihydrofolate reductase) marker is useful to develop cell lines that carry several hundred or even several thousand copies of the gene of interest. Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., *Biochem J.* 227:277-279 (1991); Bebbington et al., *Bio/Technology* 10:169-175 (1992)). Using these markers, the cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins. The expression vectors pC1 and pC4 contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen *et al.*, *Molecular and Cellular Biology*, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart *et al.*, *Cell* 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites *Bam*HI, *Xba*I and *Asp*718, facilitate the cloning of the gene of interest. The vectors contain in addition the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene. As indicated

above, the vectors of the present invention will preferably include at least one selectable marker. Such markers include dihydrofolate reductase, Herpes simplex virus thymidine kinase, G418 or puromycin or zeocin resistance for eukaryotic cell culture and tetracycline, hygromycin, apramycin, zeocin, kanamycin or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Appropriate culture mediums and conditions for the above-described host cells are known in the art.

The present invention also relates to a method for the generation of a conditional knock-out mutant of a cell, comprising the steps of: (a) introducing a nucleic acid molecule comprising the insertion element of the present invention into a cell; wherein (i) the insertion element is transfected into the cell concomitant with purified recombinase ; (b) integrating the insertion element into the cellular genome; and (c) selecting cells for resistance against a substrate for which the resistance gene of the insertion element confers resistance, wherein prior to or concomitant with step (a) an expression construct for a tetracycline-sensitive (poly)peptide is introduced into the cell, said (poly)peptide being capable of binding to the tetracycline-controlled promoter of the insertion element and of modulating its expression.

The term "conditional knock-out mutant" refers to cells which can only express the affected gene under controlled conditions.

The term "introducing a nucleic acid molecule" means bringing DNA or RNA molecules into the cell. This can be achieved by various methods which comprise transfection, transformation, infection, transduction or electrotransformation.

The term "concomitant with purified recombinase", as used herein, means that at the same time the nucleic acid is introduced into the cell, recombinase proteins are introduced. To this end, the nucleic acid and recombinase molecules may be mixed and pre-incubated prior to the transfection step.

The term "selecting cells for resistance against a substrate" means identifying cells with a particular phenotype, i.e. those cells which can grow in the presence of a substrate which is a cytotoxic substrate or a cytostatic substrate for the cell or which reduces growth and/ or cell division.

The term "binding" ... "and" "modulating its expression" means, that in dependence of the presence of inducer the expression of downstream genes can be modulated by binding or by a lack of binding of TetR to its operator sequence

This method may also be used within a method for generating a transgenic animal. In this case, the cell used for the generation of the conditional knock-out mutant of a cell is an embryonic stem cell, an adult stem cell or a cell of an established cell line and from such a cell a viable organism is grown. A typical method for the generation of a transgenic mammal may comprise the steps of (a) injecting e.g. a selected embryonic stem cell into blastocysts, (b) transfer of blastocysts into the uterus of a mammal and (c) delivering the transgenic mammal.

In a preferred embodiment of the present invention, the tetracycline-sensitive (poly)peptide is a repressor of transcription. The term "tetracycline-sensitive repressor of transcription", as used herein, is a (poly)peptide which, in the absence of tetracycline, is capable of binding to a Tet operator and as a consequence thereof represses transcription from a nearby promoter sequence. In contrast, whenever sufficient amounts of tetracycline are present, the repressor binds to tetracycline and its affinity to the tet operator sequence is reduced so that transcription from the nearby promoter can proceed.

In a more preferred embodiment of the present invention, the tetracycline-sensitive (poly)peptide comprises the amino acid sequence of any one of (a) SEQ ID NOs: 22 to 41; (b) SEQ ID NOs: 42 to 63; (c) SEQ ID NOs: 64 to 256; (d) SEQ ID NOs: 257 to 266; (e) SEQ ID NOs: 267 to 311 or (f) SEQ ID NOs: 312 to 316 or a tetracycline-sensitive mutant of this sequence, wherein said mutant has a sequence identity of at least 92%, is capable of binding to the operator sequence and is inducible by a tetracycline.

In a further preferred embodiment of the present invention, the tetracycline-sensitive (poly)peptide is an activator of transcription. The term "tetracycline-sensitive activator of transcription", as used herein, is a (poly)peptide which, in the absence of tetracycline, is capable of binding to a Tet operator and as a consequence thereof activates transcription from a nearby promoter sequence. Whenever sufficient amounts of tetracycline are present, however, the activator binds to tetracycline and

its affinity to the tet operator sequence is reduced so that transcription from the nearby promoter is no longer activated and vice versa in the case of the reverse TetR alleles (activating transcription in the presence of tetracycline).

In a more preferred embodiment of the present invention, the tetracycline-sensitive (poly)peptide comprises the amino acid sequence of any one of SEQ ID NOs: 317 to 357.

In a preferred embodiment of the present invention, the cells are grown in the presence of tetracycline or of a functional homolog thereof, wherein said homolog is selected from the group consisting of Tetracycline, Doxycycline, Minocycline, Sancycline, Anhydrotetracycline, Chlorotetracycline, 4-DDMA-Anhydrotetracycline, Oxytetracycline, 4-DDMA-tetracycline, 2-Nitrilo-tetracycline, 4-Dedimethyl-aminosancycline, 4-DDMA-chlor-tetracycline, 4-DDMA-11,12-pyrazolo-tetracycline, 4-DDMA-4-hydroxy-tetracycline, 4-DDMA-12a-dehydroxy-tetracycline, 4-DDMA-doxycycline.

Moreover, any derivative may be used provided that it can induce the tetracycline sensitive (poly)peptide described in the embodiments of the present invention. The functional homologue may also be a (poly)peptide capable of binding to the tet repressor

In another preferred embodiment of the present invention, the cells are grown in the presence of a peptide-based functional homolog of tetracycline or in the presence of a (poly)peptide comprising said peptide, wherein said peptide-based homolog or said (poly)peptide comprises any one of SEQ ID NOs: 358 to 371. The "peptide-based functional homologue" may be a short peptide or be part of a polypeptide comprising the amino acid sequence of such a peptide.

The present invention also relates to a cell containing the insertion element or the vector of the present invention or which is obtainable by the method of the present invention.

In a preferred embodiment of the present invention, said cell is a eukaryotic or prokaryotic cell.

In a more preferred embodiment of the present invention, said cell is selected from the group consisting of vertebrate and invertebrate cell. Preferably, said cell is a cell

obtainable from *Fugu*, *C.elegans*, *Leishmania*, *Plasmodium*, *Toxoplasma*, an insect cell obtainable from *Drosophila*, *Anopheles*, a fungal cell obtainable from *Candida*, *Aspergillus*, *Dictyostelium* or a bacterial cell.

In an even more preferred embodiment of the present invention, the vertebrate cell is a cell of xenopus, zebrafish or a mammalian cell.

In a most preferred embodiment of the present invention, the mammalian cell is an embryonic stem cell, an adult stem cell or a somatic cell.

In another more preferred embodiment of the present invention, the eukaryotic cell is a plant cell. Preferably, said plant cell is from *Arabidopsis*, *Zea mais*, *Oryza*.

In another more preferred embodiment of the present invention, the bacterial cell is selected from the group consisting of *Acinetobacter*, *Actinobacillus*, *Actinomyces*, *Aeromonas*, *Bacillus*, *Bacteroides*, *Bdellovibrio*, *Bordetella*, *Borrelia*, *Branhamella*, *Brucella*, *Burkholderia*, *Campylobacter*, *Capnocytophaga*, *Clamydia*, *Citrobacter*, *Clostridia*, *Corynebacterium*, *Coxiella*, *Eikenella*, *Erysipelothrix*, *Escherichia*, *Francisella*, *Fusobacterium*, *Haemophilus*, *Helicobacter*, *Klebsiella*, *Legionella*, *Leptospira*, *Listeria*, *Meningococcus*, *Moraxella*, *Mycobacteria*, *Mycoplasma*, *Neisseriaceae*, *Nocardia*, *Pasteurella*, *Peptostreptococcus*, *Pneumococcus*, *Proteus*, *Providencia*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Selenomonas*, *Serratia*, *Shigella*, *Spirillum*, *Staphylococcus*, *Streptococcus*, *Treponema*, *Veillonella*, *Vibrio*, *Wolinella*, *Yersinia*.

The present invention also relates to a method for identifying genetic determinants of pathogen survival, comprising the steps of: (a) infecting an organism with a pathogen, wherein the pathogen is the cell of the present invention or a multi-cellular organism comprising said cell; (b) growing the cell in the presence and absence of tetracycline or in the presence or absence of a functional homolog thereof, wherein said homolog is (i) a peptide-based functional homolog of tetracycline or a (poly)peptide comprising said peptide, wherein said peptide-based homolog or said (poly)peptide comprises any one of SEQ ID NOs: 358 to 371; or (ii) a homolog which is selected from the group consisting of Tetracycline, Doxycycline, Minocycline, Sancycline, Anhydrotetracycline, Chlorotetracycline, 4-DDMA-Anhydrotetracycline, Oxytetracycline, 4-DDMA-tetracycline, 2-Nitrilo-tetracycline, 4-

Dedimethyl-aminosancycline, 4-DDMA-chlor-tetracycline, 4-DDMA-11,12-pyrazolo-tetracycline, 4-DDMA-4-hydroxy-tetracycline, 4-DDMA-12a-dehydroxy-tetracycline, 4-DDMA-doxycycline; (c) selecting cells, the growth of which is affected by the presence of tetracycline or the functional homolog thereof; and (d) determining which gene or gene product of the cell of (c) is effected by the insertion of the insertion element, wherein the gene or gene product identified in step (d) is a genetic determinant for the survival of the pathogen in the infected organism.

The term "identifying genetic determinants of pathogen survival" means identifying those nucleic acid sequences within the genome of the pathogenic cell which have an impact on its survival within the host. In most cases, the genetic determinant will be a gene or the gene product such as a regulatory RNA or a (poly)peptide.

The term "pathogen" refers to organisms or particles that can lead to a more or less severe disease of an organism.

The term "selecting cells ..." means identifying and isolating individual cells, the growth of which is affected by the presence of tetracycline or its homolog.

The term "determining which gene or gene product ..." means establishing by sequencing the insertion region in the chromosome.

The presence of the tetracycline-controlled promoter sequence located within the insertion element of the present invention provides an external means of control of expression of said genetic determinant. For example, if the insertion element of the present invention is integrated between the native promoter sequence of said gene and its coding sequence, transcription from the native promoter will generally be impaired and, be halted if a transcription terminator sequence is located within the insertion element 5' to the tetracycline-controlled promoter. On the other hand, transcription of said coding sequence can be induced e.g. by adding tetracycline to the host environment.

In a preferred embodiment of the present invention, the infected organism is a eukaryotic or prokaryotic cell, for example a pathogenic bacterial cell infecting a eukaryotic cell.

In another preferred embodiment of the present invention, the infected organism is a vertebrate or invertebrate animal. Preferably, vertebrates are selected from the

group consisting of *Xenopus*, zebrafish, human cells, mouse. Preferably, the invertebrate is *C. elegans*.

In a more preferred embodiment of the present invention, the vertebrate is selected from mammalian, *xenopus* and zebrafish. Preferred mammals are human, *sus scrofa*, mouse, *equus*.

The present invention also relates to a method for the generation of transgenic non-human animal, comprising the step of propagating the cell of the present invention and developing from said cell a viable organism, wherein said cell is non-human.

As described above, the cell of the present invention may be used to develop a viable organism. Such a cell may be for example an embryonic stem cell or an adult stem cell. A typical method for the generation of a transgenic mammal may comprise, for example, the steps of (a) injecting e.g. a selected embryonic stem cell of the present invention into blastocysts, (b) transfer of blastocysts into the uterus of a mammal and (c) delivering the transgenic mammal.

In a preferred embodiment of the present invention, said animal is a mammalian animal.

The present invention also relates to a transgenic non-human animal produced by the method of the present invention. Such an animal may be a vertebrate or an invertebrate. It may be for example a mammalian, a *xenopus* or zebrafish. Preferred mammals are selected from the group consisting of *sus scrofa*, human, mouse, *equus*. Such animals may be useful in various areas: The transgenic non-human animal of the present invention may be used, for example, to investigate how the animal reacts (tetracycline-dependently) on different pathogen invasion. Another example is to create conditionally lethal embryonic mutations to investigate important functions of development. The present invention also relates to a method for the production of a vaccine composition, comprising the steps of (a) growing the bacterial cell of the present invention in the presence and absence of tetracycline and selecting cells which display an attenuated phenotype in the absence of tetracycline; and optionally (b) formulating the bacteria with a pharmaceutically acceptable carrier. The steps of this method may be combined with other steps or modified. For example, it is conceivable that not the initially identified cell is used in

the vaccine composition but rather a mutant thereof. To generate such a mutant, the specific genetic determinant(s) of pathogenicity might be identified. If the genetic determinant is a gene, for example, various specific mutants of this gene may be generated. Such a mutant may be e.g. a more efficient knock-out mutant. Alternatively, it is conceivable to generate mutants with various degrees of gene expression of a pathogenic gene. In this way the degree of attenuation might be modulated.

By "vaccine" is meant an agent used to stimulate the immune system of an individual so that protection is provided against one or more antigen(s) of the bacterial cell, not recognized as a self-antigen by the immune system. The term "immune system" refers to the organ responsible for the process of inducing a continuing high level of antibody and/or cellular immune response in which T-lymphocytes can either kill the invading microbe and/or activate other cells (e.g., phagocytes) to do so in an individual, which is directed against a microbe to which the organism has been previously exposed. The phrase "immune system" is intended to refer to the anatomical features and mechanisms by which an individual produces antibodies against an antigenic material which invades the cells of the individual or the extra-cellular fluid of the individual and is also intended to include cellular immune responses. In the case of antibody production, the antibody so produced can belong to any of the immunological classes, such as immunoglobulins, A, D, E, G or M. Vaccines of the nature described herein are likely to produce a broad range of immune responses, for example cellular and humoral immunity. Immune responses to microbes and antigens are well studied and widely reported. A survey of immunology is provided in Elgert, Klaus D., *Immunology*, Wiley Liss, Inc., (1996); Stites et al., *Basic & Clinical Immunology*; 7th Ed., Appleton & Lange, (1991) the entirety of which are incorporated herein by reference.

An individual treated with a vaccine of the present invention is intended to mean one of the organisms described in the embodiments of the present invention. The vaccine can be prepared by growing the vaccine strain in suitable growth media and then used as is or formed into a vaccine composition by combining the growing culture, or the cells therefrom, with a suitable diluent. Suitable diluents are preferably liquids and are more preferably a liquid that does not adversely effect the

stability and vitality of the vaccine culture. The diluent is preferably free of chlorine, antibiotics, antimicrobials, or any other agent that may be harmful to the live vaccine organisms. Vaccine should be dispersible in the diluent so that no solid lumps or chunks of vaccine remain and the diluent should be at a temperature that is not harmful to the live vaccine microbes. Examples of suitable diluents include water, distilled water, de-ionized water, skim milk, water containing Marek's vaccine stabilizer, buffered saline with gelatin, and similar compositions that are well-known to persons of skill in the art. The vaccine is preferably introduced into the diluent while the diluent is at a temperature of approximately room temperature or cooler more preferably from about 34° C. to about 15° C.

The present invention also relates to a vaccine composition prepared by the method of the present invention.

The present invention also relates to the use of the bacterial cell of the present invention for the preparation of a vaccine composition for ameliorating or preventing diseases caused by the bacterial cell. The methods described herein are used to generate attenuation mutants of bacterial cells. Typically, such mutants will contain an insertion element of the present invention, located in the vicinity of a genetic determinant of pathogenicity. Such a mutant may be e.g. a mutant of *Actinobacillus pleuropneumoniae* (APP) or a derivative thereof which may be used for the preparation of a vaccine composition for ameliorating or preventing a disease caused by the naturally occurring *Actinobacillus pleuropneumoniae* (APP). In fact, any of the bacteria described herein may be used for the preparation of a vaccine composition comprising an attenuation mutant as described above, for ameliorating or preventing a disease caused by the naturally occurring bacteria.

The present invention also relates to a method for generating a transgenic plant, comprising the steps of: (a) cultivating the cell of the present invention or a protoplast derived therefrom in suitable media; and (b) regeneration of plants.

Moreover, the present invention also relates to a plant or a product of the plant produced by the method of the present invention.

Finally, the present invention relates to a method of cultivating a plant, comprising the step of growing the plant of the present invention.

The figures show:

Figure 1: Diagram of the tetracycline determinants in Gram-negative bacteria. The upper part displays activities at the cytoplasmic membrane, while the lower part depicts the genetic organisation and induction of the *tet* genes. Tetracycline (tc) diffuses in its neutral form across the cytoplasmic membrane into the cell (left upper part). There it is complexed by divalent cations (Me^{2+}), and can then act as the substrate for the proton-tc-antiporter TetA, depicted as a 12- α -helical membrane bound protein (right upper part). The same tc-complex is the molecular inducer (black boxes) of Tet repressor (TetR, blue filled circles), encoded by *tetR* (blue arrow). In the absence of tc, TetR binds as a dimer to the tandem operators O_1 and O_2 , located between the two genes *tetR* and *tetA*. These divergently oriented genes are transcribed by overlapping promoters (not shown). The $[\text{tc-Me}]^+$ -complex binds to TetR and thereby causes a conformational change in the protein so that it can no longer bind to *tet*-operator but quickly dissociates from the DNA (1).

Figure 2: Schematic depiction of the insertion element one. Between the specific binding sites for transposase (ME), stop codons (red upper case "S"es), a transcriptional terminator (stem loop), the kanamycine resistance gene (Km^R) and the tetracycline dependent promoter (right directed grey arrow) containing one tet operator (grey box) are located. The tetracycline dependent promoter drives transcription outward of the element.

Figure 3: Schematic depiction of the insertion element two. Between the specific binding sites for transposase (ME), stop codons (red upper case "S"es), a transcriptional terminator (stem loop), the kanamycine resistance gene (Km^R), flanked by two asymmetric loxP sites (yellow boxes) are located. As a major difference to element one, the tetracycline repressor gene is located on the element, transcribed by an autoregulated promoter (left directed grey arrow). The tetracycline

dependent promoter driving transcription (right directed grey arrow) contains one tet operator in this example (or two, see text).

Figure 4 : β -galactosidase activity measurement to assay the regulatory function of the insertion element. The ordinate shows the relative β -galactosidase activity w/o TetR displays the 100%-value. TetR(BD)wt, revTetR_1 and revTetR_2 are three measured TetR variants with (yellow bars) or without anhydrotetracycline (blue bars) as effector.

Figure 5: β -galactosidase activity measurement to assay the regulatory function of insertion element two. The ordinate shows the absolute β -galactosidase activity. depicted are measurements of two different elements, containing one or two tet operators within the outward promoter (as indicated). Measurements were conducted with (yellow bars) or without anhydrotetracycline (blue bars) as effector.

Figure 6 : Plot of molar excess of transposase against the number of kanamycine resistant colonies.

Figure 7: Insertion loci (blue lines) of the insertion element into the target plasmid.

Figure 8 : Cyclic representation of the *E.coli*-genome with insertion loci (black lines).

Figure 9: Depiction of the insertion loci of the auxotroph candidates in the genome of *E.coli*-Genom. The gene products are given and the regulable candidates are marked.

Figure 10: Minimal media plates to confirm the regulable auxotroph candidates. The two auxotroph candidates with disruptions of *argG* and *serA* served as control, as they do not grow on either plate. The two regulable candidates with insertion near *cysP* and *thiC* do not grow without, but with atc. The incubation time was 36 h at 37°C

Figure 11. Depiction of the locus and the direction of insertion of the element

(black arrow) in the regulable auxotroph mutant *cysP*.

- Figure 12: Depiction of the locus and the direction of insertion of the element (black arrow) in the regulable auxotroph mutant *thiC*
- Figure 13: Minimal media plates to confirm the regulable auxotroph *Bacillus* candidates. A prototroph bacillus strained, grwing on either plate served as control. The three regulable candidates with insertions near *kinA* and *yhaO* only grow in the absence of *atc*. Te insertion locus of the third auxotroph was not determined. The incubation time was 48 h at 37°C.
- Figure 14: Depiction of the locus and the direction of insertion of element two in the first regulable auxotroph *Bacillus* strain. The putatively regulated target gene may be the gene *yhaM* that displays similarity to similar to CMP-binding factors.
- Figure 15: Depiction of the locus and the direction of insertion of element two in the second regulable auxotroph *Bacillus* strain. The putatively regulated target gene may be the gene *patA* that encodes an aminotransferase or *kinA* whose gene product is part of a two-component system.
- Figure 16: Depiction of the different phenotypes in liquid medium. "WT" corresponds to *E.coli* MG1655 pWH1411BD+. Highlighted in yellow are regulable candidates without auxotroph phenotype, candidates in orange display a regulable (white characters) or a constitutive growth defect (black characters). The candidates highlighted red comprise regulable auxotrophy (white characters) or constitutive auxotrophy (black characters). "F" denotes the ratio in OD₅₉₅ between growth with and without *atc*, as a means for regulation.
- Figure 17: Cyclic depiction of the *E.coli*-genome and the loci of insertion (black lines).
- Figure 18: Distribution of the insertion loci concerning the insertion sequence.

Given are the base pairs, the insertion element has inserted into, as well as the frequency.

Figure 19: Cyclic depiction of the *E.coli*-genoms and the 202 different insetion loci (black lines). The randomised insertion was clearly observed.

Figure 20: Depiction of the plasmid containing the enhanced element one. In between the two Mosaic elements (ME) there are the R6Ky-ori (light blue), the kanamycine resistance cassette (dark blue) flanked by two FRT sites (orange), the T7 promoter (pT7, pink), two transcriptional terminators (T, green) and the tetracycline dependent promoter (*ptetA*, blue). Additionally the plasmid contains a ampicillin resistance cassette (*bla*, red).

The Examples illustrate the invention:

Example I: Construction of insertion elements

Two different insertion elements were constructed. While element one is intended to be used in Gram negative bacteria, element two is supposed to be applied in Gram positive organisms.

Construction of insertion element one: The construction of the first insertion element was accomplished by oligonucleotide hybridisation and subsequent cloning into a suitable vector. After successful cloning of the plasmid, a selectable resistance cassette was cloned into the insertion element, now consisting of 1350 bp. It contains the following components (see Fig. 2): Between two specific transposase binding sites (ME), there are three staggered stop-codons at the 5' end followed by a transcriptional terminator to oppress possible upstream translation and transcription effects. Downstream there is a kanamycine resistance cassette and directly at the 3' end in front of the Mosaic Element there is the tetracycline dependent promoter transcribing outward of the insertion element. The entire element can be cut out of the carrier-vector by *PvuII*, allowing the application as *in vitro* system.

Construction of insertion element two: The construction of the second element was accomplished likewise. In contrast to element one, the resistance cassette is flanked by asymmetric *loxP* sites which are the targets for cre recombinase (9). These will allow the excision of the selection marker. Furthermore, the *tetR* gene is positioned on the element, which is transcribed by an autoregulative promoter (Fig. 3).

There are two versions of the tetracycline dependent promoter transcribing outward of the element: One contains a single *tet* operator (see Fig. 3) and corresponds to *p_{xyl/tet}* of plasmid pWH353 (10), while the other has two of these TetR binding sites (not shown) and resembles the promoter of pWH354 (10). Like element one, element two can be cut out of its carrier vector with *PvuII*.

Example II: Tests of the regulable promoters

Test of the outward promoter of element one: to test the regulable promoter of the element one, the DNA fragment was cloned upstream of an episomal promoterless *lacZ* gene. The resulting reporter plasmid was cotransformed into the strain *E. coli* DH5 α together with an expression plasmid for TetR. Thereby, different *tetR* alleles with different phenotypes were used. By means of β -galactosidase assays, the anhydrotetracycline dependent regulation was demonstrated and quantified (Fig.4). As shown in the figure, the regulatory window with a factor of 888 for wild type TetR and 524, or 105, respectively for the two analysed reverse TetR variants is very broad.

Test of the outward promoter of element two: To test the regulation capacities of element two, the respective DNA fragments were cloned upstream of a promoterless *lacZ* gene flanked by up- and downstream *amyE* sequences of *Bacillus subtilis*. The resulting reporter plasmid was transferred into *B. subtilis* 168 (wild type) and was partially integrated into the *amyE* locus by double homologous recombination. Via β -galactosidase assays, the anhydrotetracycline dependent regulation was demonstrated and quantified (Fig.5).

Example III: Functionality test of the system by *in vitro* insertion experiments

Functionality of element one: To test for the functionality of the systems, *in vitro* insertion experiments were conducted. As target DNA the plasmid pUC19 was used. The cut and purified insertion element and the transposase were applied in different ratios, to gain optimal yield of insertion mutants. Subsequently, *E. coli* DH5 α was transformed with the reaction mixtures and plated on LB media plates containing kanamycine. The different obtained colony numbers were plotted against the molar excess of transposase over insertion element (Fig.6).

The highest yield of kanamycine resistant colonies was achieved at a molar ratio of insertion element to transposase of 1:5. Of cells of some colonies, plasmids were extracted to determine the site of insertion. Of 30 examined candidates, 24 showed a different insertion locus (Fig. 7). Therefore, the activity of the transposase could be

confirmed and indication was gained for random integration instead of certain preferred loci of the plasmid.

Functionality of element two: As element two also contains Mosaic Elements and in general shares a quite common architecture with element one, tests for its functionality were not so extensive. Applying the optimised reaction set-ups (see in the figure), it was demonstrated that the element can integrate into a vector *in vitro*. Of three prepared new plasmids of the respective *E. coli* transformants, restriction patterns confirmed different insertion sites (data not shown).

Example IV: Test of the insertion activity into bacterial genomes

Assembly of the insertion complex: For the assembly of the protein-DNA complex, purified transposase is added to the respective insertion element and the reaction mixture is incubated 30 min at room temperature. No magnesium ions must be present in the mixture to avoid undesired insertion reactions.

Electroporation of the complex into *E. coli* cells: Electroporation of element one into *E. coli* was performed according to the instruction of Sambrook (11). 1 µl of the protein-DNA-complex and an aliquot of the electrocompetent cell suspension was used. The recipient strain was *E. coli* MG1655, bearing pWH1411BD+. MG1655 is the reference strain of the nucleotide database whose complete sequence is known. The plasmid pWH1411BD+ constitutively expresses TetR(BD) (2). Afterwards the cells were streaked out on plates containing kanamycine and chloramphenicol as selection agents for the insertion element and the plasmid. Plates were incubated over night at 37°C and colonies were counted the next day. Of the deployed cells, approximately 0.3% survived the electroporation procedure. Among these, around 0.6% carried the insertion element.

Electroporation of the complex into *B. subtilis* cells: Electroporation of element two into *B. subtilis* was performed according to an instruction described in the PhD thesis of Fuchsbauer (12). The reaction set-up again somewhat reflects the method applied for *E. coli*. The entire reaction mixture of the protein-DNA-complex was added to 300 µl of electrocompetent cells of *B. subtilis* 168, whose complete

sequence is known. Possible transponants were streaked out on plates containing 15 µg/l kanamycine for selection.

Characterisation of insertion *E. coli* candidates: Of cultures of some of the kanamycine resistant *E. coli* candidates putatively containing the insertion element one, chromosomal DNA was prepared without pre-selection. After precipitation with polyethylene glycol, the DNA was restricted with *EcoRI* and sequenced via the cycle-sequencing reaction. The sequences again displayed random integration into the genome. A total of eleven candidates' DNA was assayed, whereas no insertion locus appeared twice (Fig. 8).

Example V: Screening system for *E. coli* on solid medium

Construction of mutant pool of *E. coli* by insertion of the DNA fragments: For detailed examination of the insertion reaction, a mutant pool of *E. coli* strains apt for a selection system should be generated. Therefore, electroporation was performed and the total number of kanamycine resistant colonies was determined to be around 7,200.

The screening system: first a screen on minimal agar plates with glucose as sole carbon source with or without anhydrotetracycline was conducted. A total of 2,400 colonies were streaked out. After over night incubation of the plates at 37°C they were analysed for growth of the candidates. 35 auxotroph candidates were found, two of which with a regulable auxotrophy (red in Fig. 9). All auxotroph candidates were streaked out on LB-agar plates, inoculated in liquid media and their chromosomal DNA was extracted using the Qiagen DNA-Mini kit. After restriction with *EcoRI* a sequencing reaction was performed and the resulting sequences were subjected to BLASTN analyses to determine the insertion locus in the genome. Like before, the random manner of insertion was confirmed.

The two regulable candidates were re-streaked on minimal media plates to verify their phenotype (Fig. 10). Clearly, the two regulable candidates can not grow in the absence (left part of Fig. 10) but in the presence of atc (Fig. 10 right part). The two other not regulable auxotroph candidates served as controls. The insertion locus of the two regulable candidates was determined by sequencing their chromosomal

DNA. One carries the insertion element downstream of the start codon of the gene *cysP* (Fig. 11), whereby the natural start codon is replaced by the GTG sequence of the Mosaic Element. Thus the tetracycline dependent promoter has gained transcriptional control over the *cys*-operon *cysPUWAM*, encoding the thiosulphate-ABC-transporter.

The other mutant carries the insertion element at the 3'-end of the gene *thiC* (Fig.12). Therefore, the rest of the *thi*-operon is controlled by the tetracycline dependent promoter. The *thi*-operon encodes proteins for biosynthesis of thiamine.

A massive constraint of this kind of screening system is the need to quantify the colony size by eye.

Example VI: Screening system for *B. subtilis* on solid medium

Construction of mutant pool of *B. subtilis* by insertion of the DNA fragments: a similar approach was taken for the creation of a mutant bank of *B. subtilis*. Upon electroporation a total of approximately 390 kanamycine resistant candidates, putatively harbouring element two were obtained.

Like for *E. coli*, a screen on minimal agar plates with glucose as sole carbon source with and without 0.4 μ M atc was conducted to screen for auxotroph bacilli. However, CSK instead of M9 minimal medium was used. All 387 obtained candidates were streaked out. After several days of incubation at 37°C they were analysed for growth of the candidates. 11 auxotroph candidates were found, three of which with a regulable auxotrophy (red in Fig. 13).

The procedure for detecting the insertion loci was essentially like for *E. coli*. Of the three candidates' DNA only two sequences could be obtained. The two insertion loci are around 400 kb apart (data not shown). In the first candidate the element had inserted into the *yhaO* gene (see Fig. 14). The putatively regulated target gene may be the gene *yhaM* that displays similarity to CMP-binding factors.

The second candidate has the element integrated in the ATG start codon of *kinA* (Fig. 15). As is the case in one of the regulable *E. coli* candidates, the GTG sequence of the Mosaic Element may compensate for the disrupted start codon.

Example VII: Screening system for *E. coli* in liquid medium

We have developed a 96-well plate liquid culture broth screening system for auxotroph *E. coli* strains. Thus the growth of the candidates can be monitored by measuring the optical density (OD₆₀₀). Minimal medium with glucose as sole carbon source, and kanamycine (Km) and chloramphenicol (Cm) as selection markers was used. For preparation, half of the well plates were filled with 200µl of minimal medium per well and stored at 4°C. Furthermore, a stock culture of the mutant pool was streaked onto LB/Km/Cm-plates such that single colonies appeared after incubation. At the next day, each well was inoculated with one colony. Each well-plate also contained minimal medium without culture and the parent strain as references. After inoculation wells were mixed using a multi-channel pipette and half of the inoculated medium was transferred into well containing 25µl of a 2µM atc solution as inducer. Finally 25µl of fresh minimal medium was transferred into the wells without atc to equalise the volumes. Plates were then incubated over night at 37°C in an incubator. The next day, after mixing of the cultures, the well plates could be evaluated in a TECAN Multiplate-Reader by measuring the optical density OD₅₉₅. The following phenotypes were encountered (Fig. 16):

The candidates highlighted yellow display regulation without auxotroph phenotype. The candidates given in orange display growth defect. Among them, regulables are given in white characters and non-regulables are in black characters. Finally the red highlighted candidates display an auxotroph phenotype, separated into non-regulable (black characters) and regulable (white characters). Of the latter phenotype, four candidates were identified. "F" denotes the ratio of OD₅₉₅ with and without atc, as a means for regulation. The most interesting candidates, i.e. all auxotrophs and the best of the other regulables were inoculated and their chromosomal DNA was prepared. After restriction with *EcoRI* the insertion locus was determined by sequencing. The sequence analysis again confirmed the random fashion of insertion into the genome (Fig. 17). Likewise, the sequence of the insertion loci was determined in all candidates. No preference of the insertion for a particular base pair was detected (Fig. 18).

Example VIII: Accomplishment of further screens in liquid medium and verification of the results

Meanwhile, further mutant pools of *E. coli* MG1655 were created and screened for regulable auxotroph candidates, as described above. For this purpose, also tetracycline repressors with a reverse phenotype (revTetR) were utilised to possibly yield further phenotypes, e.g. regulability of essential genes. The total number of mutants in the "WT-pool" has now expanded to 25,000, while the total number of the "revTetR-pools" is 105,000. 7,520 candidates were subjected to screening in liquid medium. It turned out that not all phenotypes were consistent in this screen. This was assayed on solid minimal medium, where the interesting candidates were streaked out. It turned out that only around 20% of the candidates could be confirmed according to their phenotype. Eventually, of 396 candidates streaked out, 75 could be confirmed (including the mutants of the solid medium screen described elsewhere herein). Of these, 15 candidates displayed a regulable phenotype that can be divided into two subgroups: The auxotroph regulable phenotype was found 11 times and the auxotroph regulable phenotype four times. The growth defect phenotypes could not be confirmed on solid media plates.

Example IX: Summary and future perspectives

The liquid media screening systems yields a plethora of phenotypes of different *E. coli* transponants. This is due to the differences in growth of the candidates with or without atc, which can hardly be differentiated on solid media plates. Besides auxotrophs, candidates are found that display a reverse regulable phenotype, i.e. growth only in the absence of atc. During all screenings, 202 different genes were detected where the element had inserted (Fig. 19). These include mutants that could not be verified on solid medium.

The utilisation of two regulators with different phenotypes, wtTetR and revTetR, resulted in a different spectrum of mutants: While in the wtTetR screening reverse regulable mutants were found, the revTetR screen yielded essential genes. This demonstrates that different kinds of regulators can be applied to reach the goal of the screening. The analysis of the 15 regulable candidates described above

revealed that some of them are already published, such as *mraZ*, the first gene in the *dcw* cluster, responsible for cell division and cell wall synthesis. The transcription of this cluster has previously been put under the control of a regulable promoter and analysed (13). By contrast, the *yrbK* gene was identified, whose function is unknown so far. This mutant could now be subjected to further analysis to elucidate this gene's function and to possibly obtain a novel drug target. Table 1 summarises all found regulable auxotroph mutants as well as the assigned function, product or biosynthesis pathway of the respective gene.

Table 1: List of all regulable mutants obtained from the liquid- and solid media screenings. Given are genes directly affected by insertion. "Regaux" denotes auxotroph regulable mutants, while "revreg" reverse regulable mutants.

gene	function, gene product or biosynthesis pathway	kind of regulation
<i>aroB</i>	3-Dehydroquinate synthase	regaux
<i>cysP</i>	thiosulphate ABC transporter	regaux
<i>cysQ</i>	sulphite synthesis	regaux
<i>ilvY</i>	transcriptional dual regulator	regaux
<i>mraZ</i>	involved in cell wall synthesis and cell division	regaux
<i>nirC</i>	nitrite FNT transporter	regaux
<i>pyrF</i>	orotidine-5'-phosphate-decarboxylase	regaux
<i>rplM</i>	50S ribosomal subunit protein L13	regaux
<i>thiC</i>	thiamine biosynthesis	regaux
<i>trpE/trpL</i>	subunit of anthranilate synthetase	regaux
<i>yrbK</i>	conserved hypothetical protein	regaux
<i>b3557</i>	IS5 protein	revreg
<i>b3557</i>	IS5 protein	revreg
<i>b3557</i>	IS5 protein	revreg
<i>cspA</i>	transcriptional activator CspA	revreg

These numbers clarify that this technique can be applied to generate numerous mutants with different phenotypes. In the time coming up, we are trying to establish even more efficient methods. Furthermore, this insertion analysis is planned to be performed in pathogen bacteria like *Salmonella typhimurium*. Recently, element one has been improved by adding additional features (Fig. 20). The kanamycine resistance cassette is now flanked by two FRT sites to optionally excise the cassette

by using Flp recombinase. This opens the possibility to insert an additional element into the chromosome of a distinct mutant utilizing the "recycled" selection marker. A R6K γ -ori was also added to the element, which can initiate replication only when the π -protein, which was deleted from this ori, is present elsewhere in a suitable strain. This ori simplifies the recovery of the insertion site by the so called "rescue cloning" technique. The last feature added to the element was a T7 promoter. This is widely used for *in vitro* transcription to obtain transcripts from the chromosome for further analysis e.g. in microarrays. These features are currently being tested. So this element may have gained a lot more versatility as tool for the search and examination of new drug targets.

For *Bacillus*, the efficiency of electroporation has to be enhanced. In addition, the application of *in vitro* transposition followed by transformation of recombinant DNA may be a promising approach. Despite the relatively low amount of transposant *Bacilli*, three auxotroph strains with a regulable phenotype were discovered. It is planned to exchange components of the second element to gain a broader range of regulation as well as to exploit revTetR regulation, as in *E. coli*. The functionality of the asymmetric loxP sites for cre-recombinase mediated excision of the selection marker has already been confirmed in *E. coli* but will have to be established for *B. subtilis*. Furthermore application of element two in pathogen Gram positive bacteria like *Listeria*, *Staphylococcus*, or *Streptococcus* are envisioned.

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CLAIMS

1. Insertion element, wherein said insertion element is a recombinant nucleic acid molecule which comprises, in 5' → 3' arrangement:
 - (i) a first recombinase binding site;
 - (ii) a resistance gene, operatively linked to a promoter and, optionally, to transcription control sequences;
 - (iii) a tetracycline-controlled promoter; and
 - (iv) a second recombinase binding site;wherein said tetracycline-controlled promoter is oriented to allow transcription in direction of the second recombinase binding site.
2. The insertion element of claim 1, wherein at least one stop codon is located between the first recombinase binding site and the resistance gene.
3. The insertion element of claim 1 or 2, wherein at least one terminator of transcription is located 5' of the resistance gene.
4. The insertion element of any one of claims 1 to 3, wherein the resistance gene is selected from the group consisting of cat, aph, bla, ermB, rpsL, aada5, hph, rpoB.
5. The insertion element of any one of claims 1 to 4, wherein the resistance gene is flanked by additional recombinase binding sites which are different from the first and second recombinase binding site.
6. The insertion element of claim 5, wherein said additional recombinase binding sites are selected from FLP recombinase dependent FRT sites and Cre recombinase dependent loxP sites.
7. The insertion element of any one of claims 1 to 6, comprising a prokaryotic origin of replication.
8. The insertion element of any one of claims 1 to 7, wherein at least one viral promoter is located upstream of the tetracycline controlled promoter.
9. The insertion element of any one of claims 1 to 8, comprising an additional coding sequence for a tetracycline repressor, wherein the coding sequence is

- (a) operatively linked to a promoter and, optionally, to transcription control sequences; or
 - (b) operatively linked to an internal ribosomal entry site and arranged 3' to a promoter sequence of the insertion element, allowing expression of the repressor from a multicistronic RNA.
10. The insertion element of any one of claims 1 to 9, wherein the promoter is a prokaryotic promoter.
11. The insertion element of claim 10, wherein the promoter is controlled by at least one tet-operator binding site sequence selected from
- (a) SEQ ID NO: 1 to 6; or from
 - (b) SEQ ID NO: 7 to 13;
- or permutations thereof.
12. The insertion element of any one of claims 1 to 6, wherein the promoter is a eukaryotic promoter.
13. The insertion element of claim 12, wherein the eukaryotic promoter is selected from the group consisting of CMV, RSV, SV40, Cadherin and hAPC and β -Act, AFP, CD43, CD45, CD68, c-erbB2, EF1, E2F-1, EGR1, FerL, GAPDH, HSP70, IFNB, Mb, PGK-1, TRP1.
14. The insertion element of claim 13, wherein the eukaryotic promoter is selected from any one of SEQ ID NOs: 14 to 21.
15. The insertion element of claim 10 or 11, wherein the recombinase binding site is a binding site obtainable from the transposases Tn5, Tn10, Tn7, or from phage Mu.
16. The insertion element of claim 13 or 14, wherein the recombinase binding site is selected from the group consisting of tc1/mariner and minos.
17. A vector, comprising an insertion element of any one of claims 1 to 16.
18. A method for the generation of a conditional knock-out mutant of a cell, comprising the steps of:
- (a) introducing a nucleic acid molecule comprising the insertion element of any one of claims 1 to 16 into a cell;

wherein the insertion element is transfected into the cell concomitant with purified recombinase

- (b) integrating the insertion element into the cellular genome; and
- (c) selecting cells for resistance against a substrate for which the resistance gene of the insertion element confers resistance;

wherein prior to or concomitant with step (a) an expression construct for a tetracycline-sensitive (poly)peptide is introduced into the cell, said (poly)peptide being capable of binding to the tetracycline-controlled promoter of the insertion element and of modulating its expression.

- 19. The method of claim 18, wherein the tetracycline-sensitive (poly)peptide is a repressor of transcription.
- 20. The method of claim 19, wherein the tetracycline-sensitive (poly)peptide comprises the amino acid sequence of any one of
 - (a) SEQ ID NOs: 22 to 41;
 - (b) SEQ ID NOs: 42 to 63;
 - (c) SEQ ID NOs: 64 to 256;
 - (d) SEQ ID NOs: 257 to 266;
 - (e) SEQ ID NOs: 267 to 311 or
 - (f) SEQ ID NOs: 312 to 316

or a tetracycline-sensitive mutant of this sequence, wherein said mutant has a sequence identity of at least 92%, is capable of binding the operator sequence and is inducible by a tetracycline.

- 21. The method of claim 18, wherein the tetracycline-sensitive (poly)peptide is an activator of transcription.
- 22. The method of claim 21, wherein the tetracycline-sensitive (poly)peptide comprises the amino acid sequence of any one of SEQ ID NOs: 317 to 357.
- 23. The method of any one of claims 18 to 22, wherein the cells are grown in the presence of tetracycline or of a functional homolog thereof, wherein said homolog is selected from the group consisting of Tetracycline, Doxycycline, Minocycline, Sancycline, Anhydrotetracycline, Chlorotetracycline, 4-DDMA-Anhydrotetracycline, Oxytetracycline, 4-DDMA-tetracycline, 2-Nitrilo-

tetracycline, 4-Dedimethyl-aminosancycline, 4-DDMA-chlor-tetracycline, 4-DDMA-11,12-pyrazolo-tetracycline, 4-DDMA-4-hydroxy-tetracycline, 4-DDMA-12a-dehydroxy-tetracycline, 4-DDMA-doxycycline.

24. The method of any one of claims 18 to 22, wherein the cells are grown in the presence of a peptide-based functional homolog of tetracycline or in the presence of a (poly)peptide comprising said peptide, wherein said peptide-based homolog or said (poly)peptide comprises any one of SEQ ID NOs: 358 to 371.
25. A cell containing the insertion element of any one of claims 1 to 16, the vector of claim 17 or which is obtainable by the method of any one of claims 18 to 24.
26. The cell of claim 25, wherein said cell is eukaryotic or prokaryotic cell.
27. The cell of claim 26, wherein said cell is selected from the group consisting of vertebrate and invertebrate cell, insect cell, fungal cell or bacterial cell.
28. The cell of claim 27, wherein said vertebrate cell is a cell of xenopus, zebrafish or a mammalian cell.
29. The cell of claim 28, wherein said mammalian cell is an embryonic stem cell, an adult stem cell or a somatic cell.
30. The cell of claim 26, wherein said eukaryotic cell is a plant cell.
31. The cell of claim 27, wherein said bacterial cell is selected from the group consisting of *Acinetobacter*, *Actinobacillus*, *Actinomyces*, *Aeromonas*, *Bacillus*, *Bacteroides*, *Bdellovibrio*, *Bordetella*, *Borrelia*, *Branhamella*, *Brucella*, *Burkholderia*, *Campylobacter*, *Capnocytophaga*, *Chlamydia*, *Citrobacter*, *Clostridia*, *Corynebacterium*, *Coxiella*, *Eikenella*, *Erysipelothrix*, *Escherichia*, *Francisella*, *Fusobacterium*, *Haemophilus*, *Helicobacter*, *Klebsiella*, *Legionella*, *Leptospira*, *Listeria*, *Meningococcus*, *Moraxella*, *Mycobacteria*, *Mycoplasma*, *Neisseriaceae*, *Nocardia*, *Pasteurella*, *Peptostreptococcus*, *Pneumococcus*, *Proteus*, *Providencia*, *Pseudomonas*, *Rickettsia*, *Salmonella*, *Selenomonas*, *Serratia*, *Shigella*, *Spirillum*,

Staphylococcus, Streptococcus, Treponema, Veillonella, Vibrio, Wolinella, Yersinia.

32. A method for identifying genetic determinants of pathogen survival, comprising the steps of:
 - (a) infecting an organism with a pathogen, wherein the pathogen is the cell of any one of claims 25 to 31 or a multi-cellular organism comprising said cell;
 - (b) growing the cell in the presence and absence of tetracycline or in the presence or absence of a functional homolog thereof, wherein said homolog is
 - (i) a peptide-based functional homolog of tetracycline or a (poly)peptide comprising said peptide, wherein said peptide-based homolog or said (poly)peptide comprises any one of SEQ ID NOs: 358 to 371; or
 - (ii) a homolog which is selected from the group consisting of Tetracycline, Doxycycline, Minocycline, Sancycline, Anhydrotetracycline, Chlorotetracycline, 4-DDMA-Anhydrotetracycline, Oxytetracycline, 4-DDMA-tetracycline, 2-Nitrilo-tetracycline, 4-Dedimethyl-aminosancycline, 4-DDMA-chlor-tetracycline, 4-DDMA-11,12-pyrazolo-tetracycline, 4-DDMA-4-hydroxy-tetracycline, 4-DDMA-12a-dehydroxy-tetracycline, 4-DDMA-doxycycline;
 - (c) selecting cells, the growth of which is affected by the presence of tetracycline or the functional homolog thereof; and
 - (d) determining which gene or gene product of the cell of (c) is effected by the insertion of the insertion element;

wherein the gene or gene product identified in step (d) is a genetic determinant for the survival of the pathogen in the infected organism.
33. The method of claim 32, wherein the infected organism is a eukaryotic or prokaryotic cell.
34. The method of claim 32, wherein the infected organism is a vertebrate or invertebrate animal.

35. The method of claim 34, wherein the vertebrate is selected from mammalian, zebrafish and xenopus.
36. A method for the generation of transgenic non-human animal, comprising the step of propagating the cell of claim 28 or 29 and developing from said cell a viable organism, wherein said cell is non-human.
37. The method of claim 36, wherein said animal is a mammalian animal.
38. A transgenic non-human animal produced by the method of claim 36 or 37.
39. A method for the production of a vaccine composition, comprising the steps of:
 - (a) growing the bacterial cell of claim 27 or 31 in the presence and absence of tetracycline or a homolog thereof and selecting cells which display an attenuated phenotype in the absence of tetracycline; and optionally
 - (b) formulating the bacteria with a pharmaceutically acceptable carrier.
40. A vaccine composition prepared by the method of claim 39.
41. Use of the bacterial cell of claim 31 for the preparation of a vaccine composition for ameliorating or preventing diseases caused by the bacterial cell.
42. A method for generating a transgenic plant, comprising the steps of:
 - (a) cultivating the cell of claim 30 or a protoplast derived therefrom in suitable media; and
 - (b) regeneration of plants.
43. A plant or a product of the plant produced by the method of claim 42.
44. A method of cultivating a plant, comprising the step of growing the plant of claim 43.

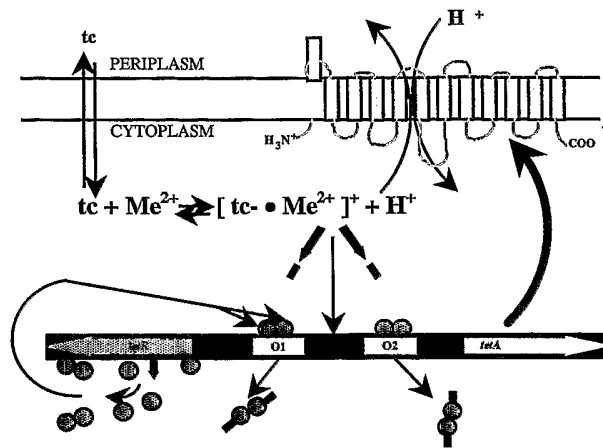


Figure 1

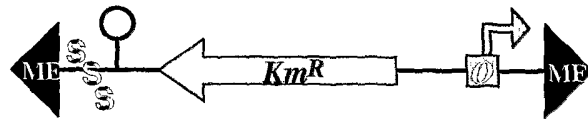


Figure 2

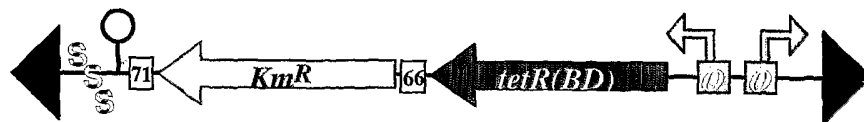


Figure 3

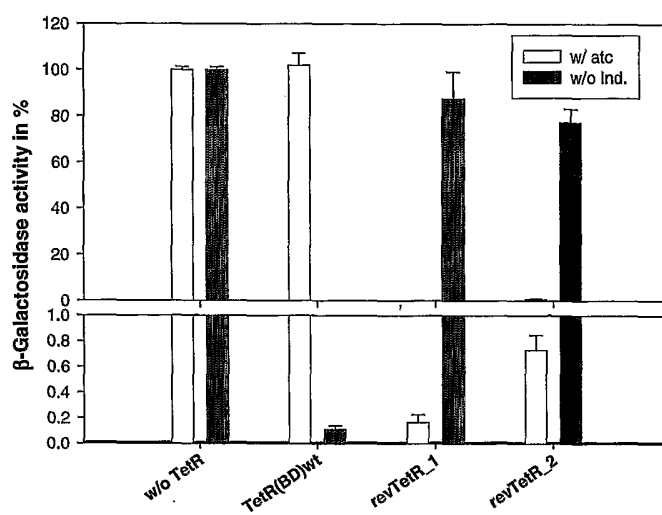


Figure 4

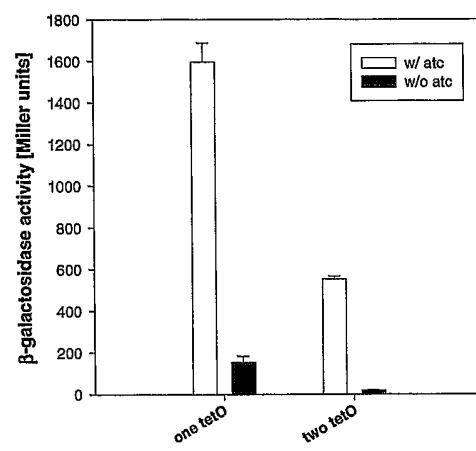


Figure 5

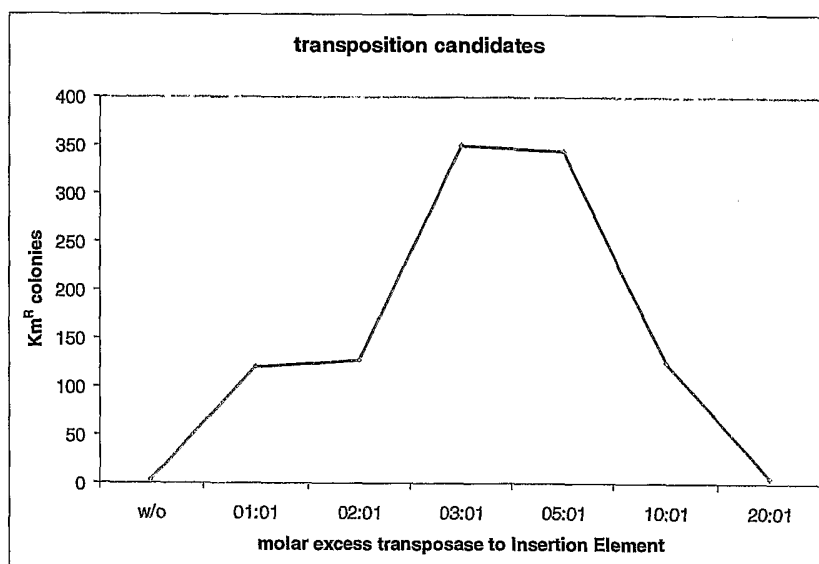


Figure 6

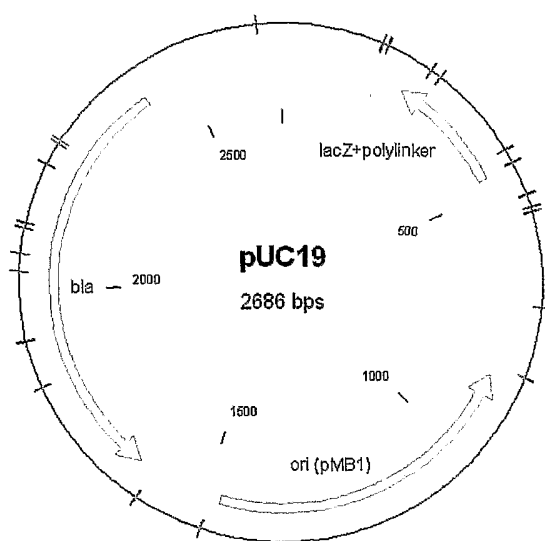


Figure 7

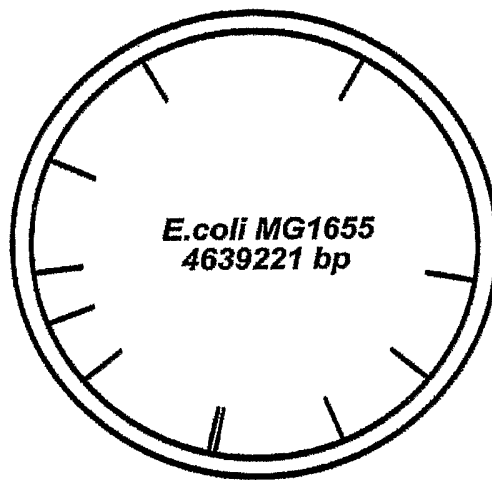


Figure 8

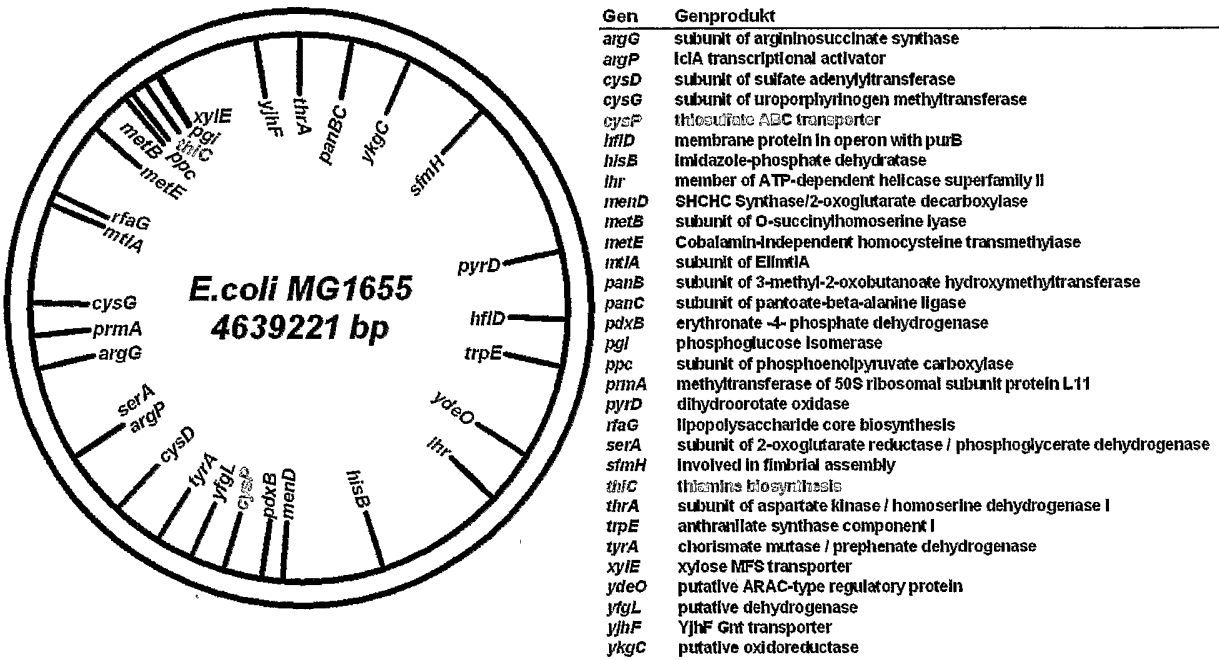


Figure 9

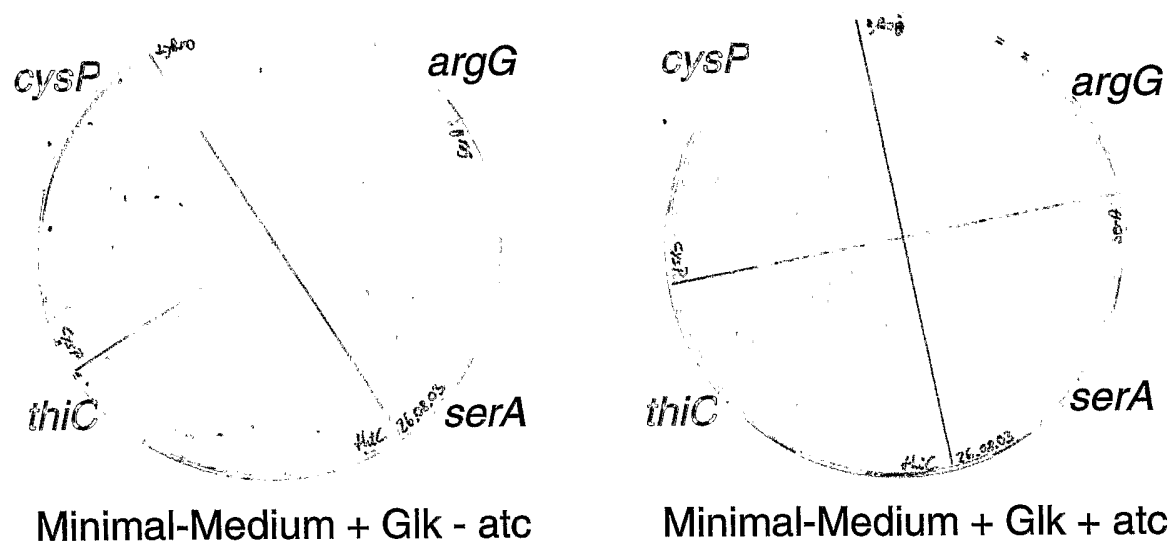


Figure 10

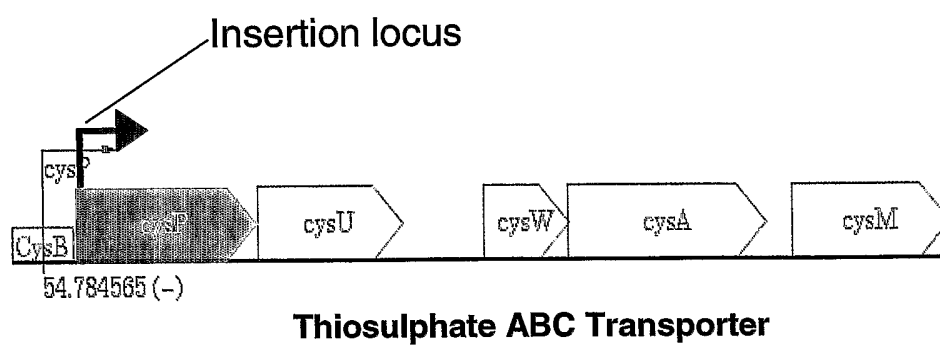


Figure 11

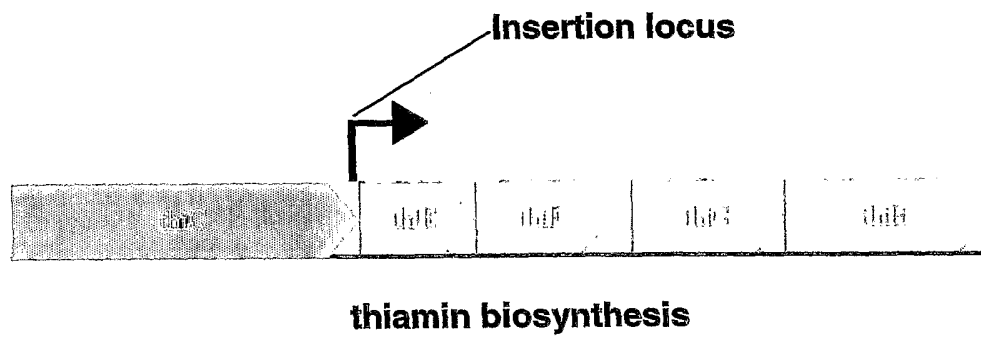


Figure 12

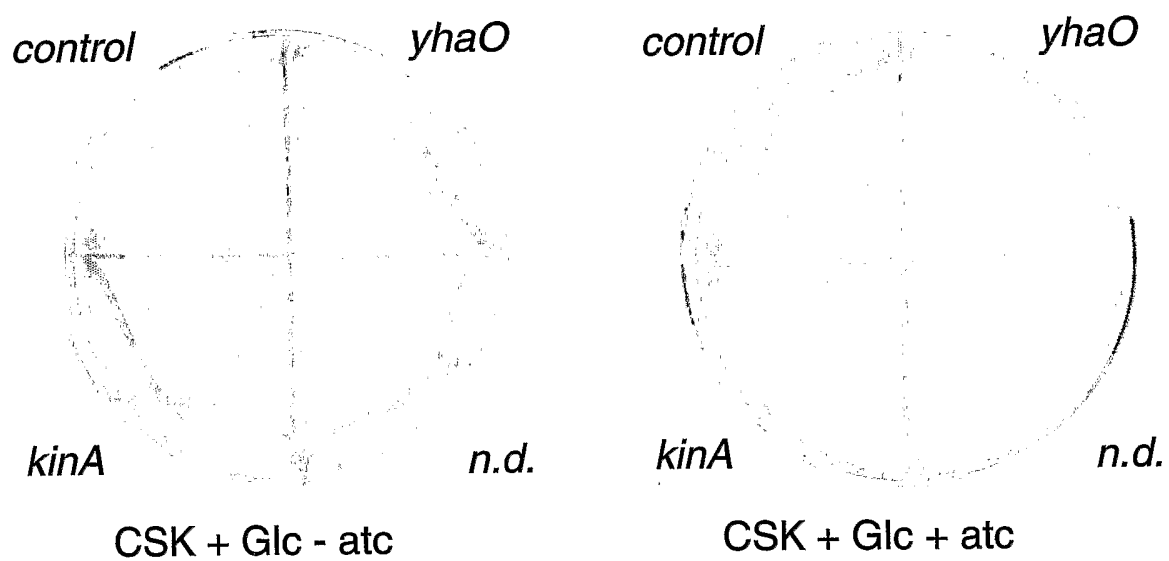


Figure 13

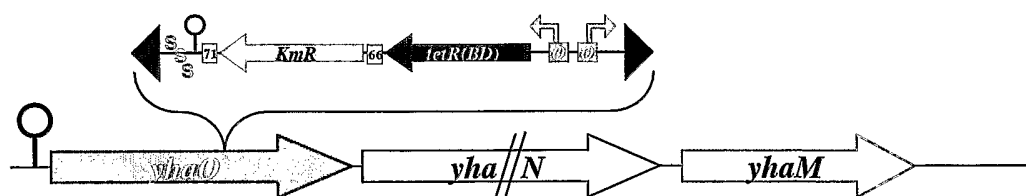


Figure 14

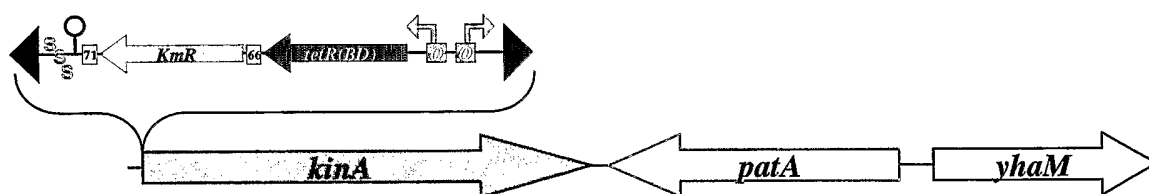


Figure 15

Candidate	OD ₅₉₅ -atc	OD ₅₉₅ +atc	auxotroph	regulable	F
WT	0,2579	0,3067	x	x	
A5	0,2534	0,0973	no	yes	2,6
A7	0,1502	0,1242	growth deficient	no	
G11	0,1492	0,0455	growth deficient	yes	3,3
D8	0,0451	0,0141	yes	no	
G6	0,0209	0,2360	yes	yes	10,9

Figure 16



Figure 17

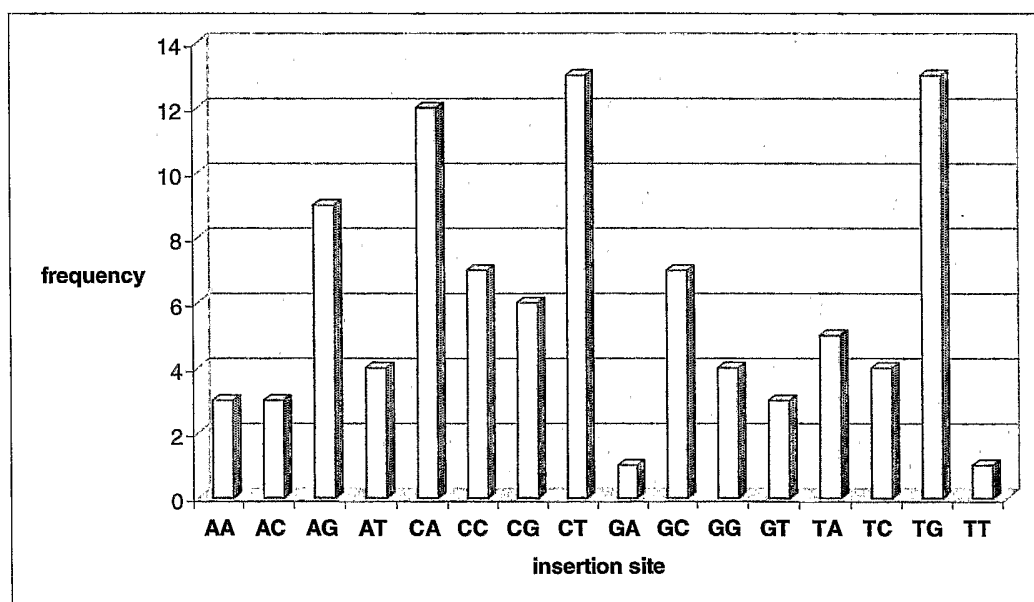


Figure 18



Figure 19

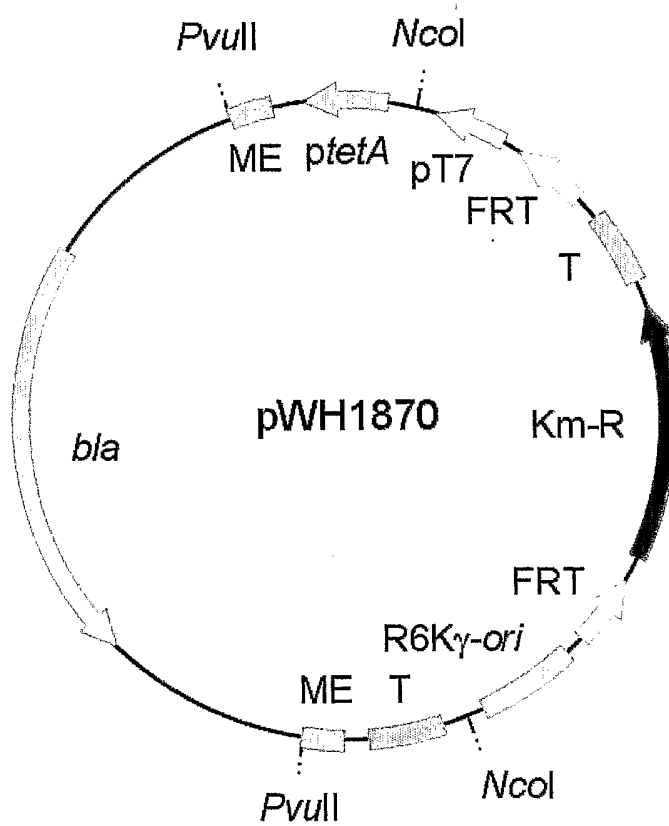


Figure 20