OPIC
Office de la propriété intellectuelle du Canada

CIPO
Canadian Intellectual Property Office

(12) (19) (CA) **Demande-Application**

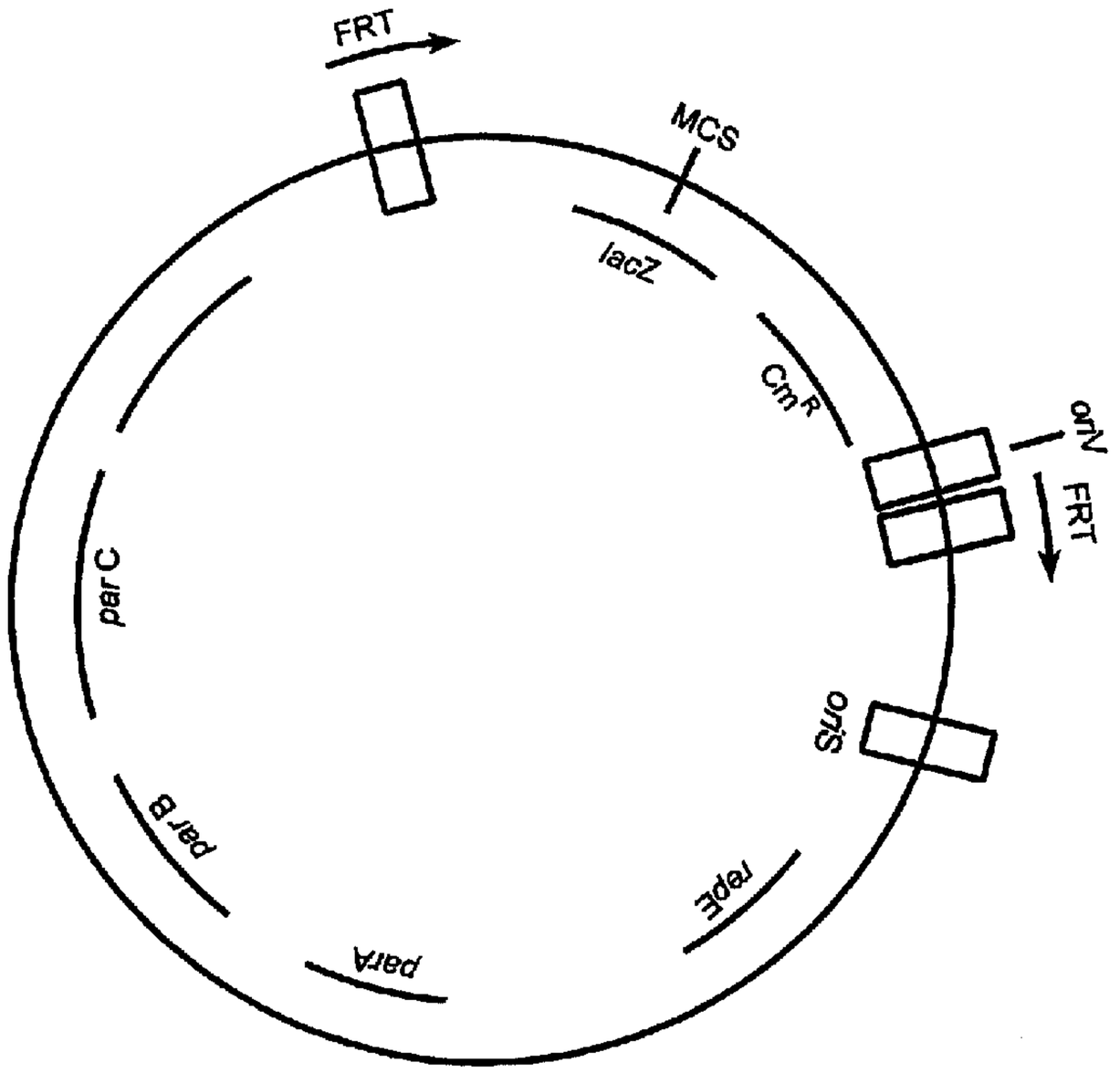
(21) (A1) **2,308,608**
(86) 1998/11/17
(87) 1999/06/03

(72) SZYBALSKI, WACLAW, US
(71) WISCONSIN ALUMNI RESEARCH FOUNDATION, US

(51) Int.Cl.[6] C12N 15/74, C12N 15/70, C12N 15/64
(30) 1997/11/21 (08/975,763) US

(54) **VECTEUR DE CHROMOSOME BACTERIEN ARTIFICIEL AMPLIFIABLE A TITRE CONDITIONNEL**

(54) **CONDITIONALLY AMPLIFIABLE BACTERIAL ARTIFICIAL CHROMOSOME (BAC) VECTOR**




(57) L'invention concerne un système permettant d'obtenir de grandes quantités de fragment d'ADN génomique à partir d'un chromosome bactérien artificiel. Ce système comprend un vecteur doté d'un site dans lequel le clonage du fragment génomique est possible. Des sites médiateurs d'excision jouxtent le site considéré. Le vecteur comprend en outre une origine de réplication contrôlable, entre les sites médiateurs d'excision et à proximité de l'un des sites.

(57) A system for obtaining large amounts of a genomic DNA fragment from a bacterial artificial chromosome includes a vector that has a site into which the genomic fragment can be cloned and, flanking the site are excision mediating sites. The vector also includes, between the excision mediating sites and near one of the sites, a controllable origin of replication.

**PCT** WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/74, 15/70, 15/64

A1

(11) International Publication Number: WO 99/27118

(43) International Publication Date: 3 June 1999 (03.06.99)

(21) International Application Number: PCT/US98/24612

(22) International Filing Date: 17 November 1998 (17.11.98)

(30) Priority Data:
08/975,763 21 November 1997 (21.11.97) US

(71) Applicant: WISCONSIN ALUMNI RESEARCH FOUNDATION [US/US]; P.O. Box 7365, Madison, WI 53707–7365 (US).

(72) Inventor: SZYBALSKI, Waclaw; 1124 Merrill Springs Road, Madison, WI 53705 (US).

(74) Agent: BERSON, Bennett, J.; Quarles & Brady LLP, P.O. Box 2113, Madison, WI 53701–2113 (US).

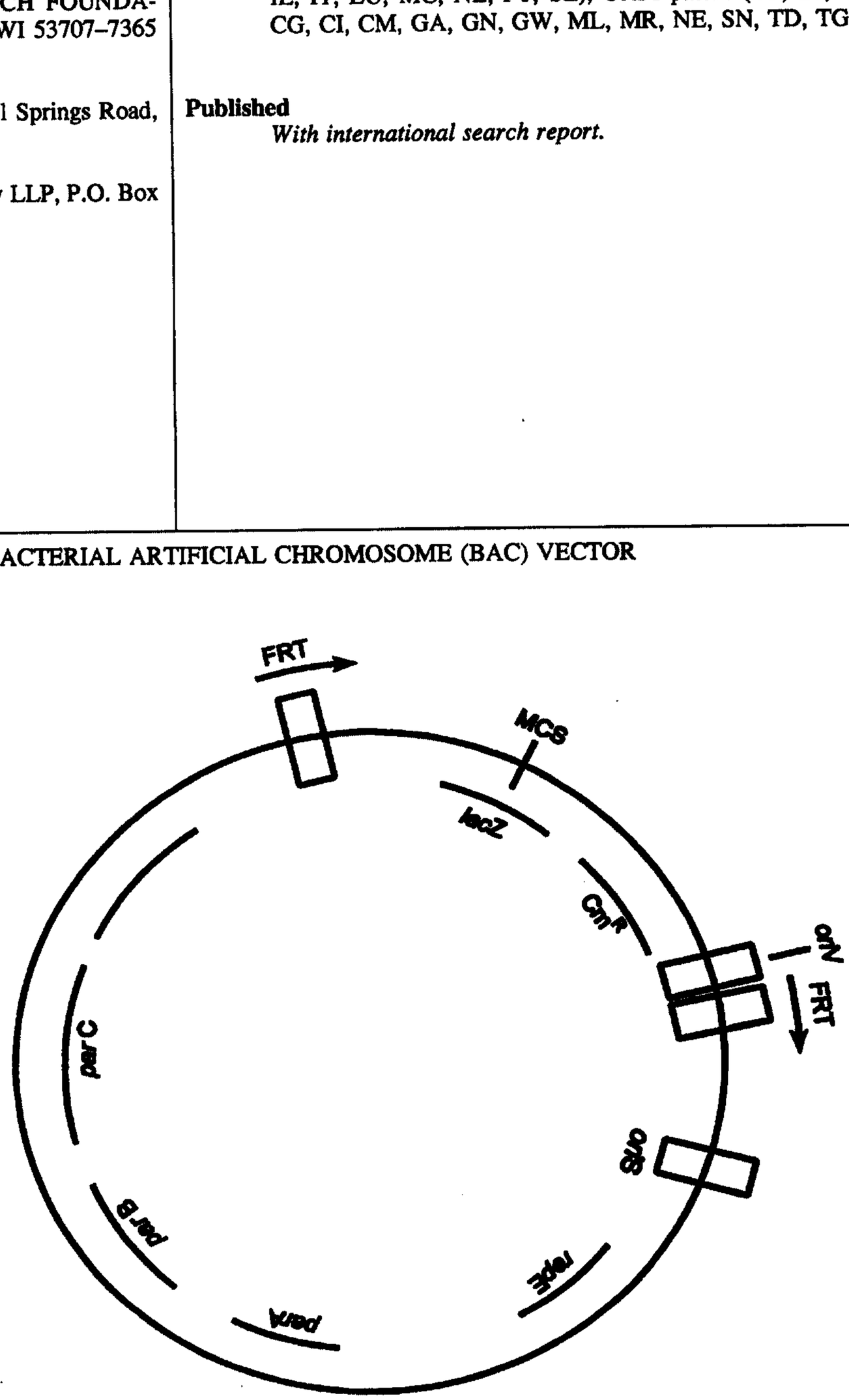
(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: CONDITIONALLY AMPLIFIABLE BACTERIAL ARTIFICIAL CHROMOSOME (BAC) VECTOR

(57) Abstract

A system for obtaining large amounts of a genomic DNA fragment from a bacterial artificial chromosome includes a vector that has a site into which the genomic fragment can be cloned and, flanking the site are excision mediating sites. The vector also includes, between the excision mediating sites and near one of the sites, a controllable origin of replication.



# CONDITIONALLY AMPLIFIABLE BACTERIAL ARTIFICIAL CHROMOSOME (BAC) VECTOR

## CROSS-REFERENCE TO RELATED APPLICATIONS

Not applicable.

## STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

Not applicable.

## BACKGROUND OF THE INVENTION

Efforts to determine the nucleotide sequence of complete genomes, or a large portion thereof, have traditionally taken a so-called bottom-up approach, including the steps of mapping the genome, preparing a library of random large clones in yeast artificial chromosomes (YACs), bacterial artificial chromosomes (BACs), P1 or cosmids, followed by random subcloning in M13-like vectors. Among such systems, BACs are at present the preferred vector for maintaining large genomic DNA fragments. BACs are preferred because individual DNA fragments are maintained stably in a single copy vector in the host cells, even after 100 or more generations of serial growth. In contrast, the DNA fragments cloned into YACs tend to be unstable and can yield chimeric clones. It is difficult to recover DNA clones from YACs in a pure form.

BAC (or pBAC) vectors typically accommodate inserts in the range of approximately 30 to 300 kilobase pairs. A widely used BAC vector, pBeloBac11, uses α complementation of the *lacZ* gene to distinguish insert-containing recombinant molecules from colonies carrying the BAC vector, by color. When a DNA fragment is cloned into the *lacZ* gene of pBeloBac11, insertional activation results in a white colony on X-Gal/IPTG plates after transformation. Kim, U-J et al., "Construction and Characterization of a Human Bacterial Artificial Chromosome Library," Genomics 34:213-218 (1996). Thus, it is now possible to distinguish those colonies that contain BACs with inserts

from those that lack inserts. A similar prior vector, pBAC108L, lacked the ability to distinguish insert-containing BACs. Shizuya, H., "Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector," P.N.A.S. U.S.A. 89:8794-8797 (1992).

Although these single-copy vectors are advantageously used to clone large genomic DNA fragments for subsequent analysis, especially sequence analysis, the single-copy nature of these vectors is also a limitation in that large numbers of cells containing a BAC clone of interest must be grown to produce a sufficient quantity for subsequent analysis. It is, of course, possible to amplify portions of a BAC clone of interest using, for example, PCR, but simple amplification of the entire insert from a BAC vector has not previously been possible.

In 1993, Szybalski proposed a system for fragment-by-fragment sequencing of entire bacterial genomes by *in vivo* excision and subsequent amplification of the excised genomic fragments. Szybalski, W., "From the Double-Helix to Novel Approaches to the Sequencing of Large Genomes," *Gene* 135:279-290 (1993). One aspect of the Szybalski (1993) system is that the excision from the bacterial chromosome and the amplification of the excised genomic fragment be controlled very stringently so that excision is induced only on command and amplification is initiated only upon induction.

According to the proposed system, excision-mediating sites (***EMS***) are placed at 500-100 kb intervals throughout a well mapped genome in places where the ***EMS*** do not interfere with viability or genomic stability. The ***EMS*** are placed throughout the system either by (1) randomly inserting plasmids carrying transposons, ***EMS***, and selective markers, or (2) by targeting insertions that inactivate specific, non-essential genes. ***EMS*** identified by Szybalski include λ *att*, 2*μ* ***FRT***, and P1 *lox*.

The net result of adding ***EMS*** to a genome in the Szybalski (1993) system is a library of strains, each of which carries one ***EMS*** at a physically mapped site. Using genetic crosses between strains having neighboring ***EMS*** at a suitable distance from one another, a set of strains are produced where each



*rB

strain possesses exactly two neighboring *EMS*. From such strains, the intervening segment (e.g., 50-100 kb), can be excised. Accordingly to the Szybalski 1993 system, if a cis-acting *ori* element is positioned together with and next to the excision elements, the *ori* element will be present on the excised DNA circle and can promote the amplification of the DNA circle.

Szybalski (1993) does not contemplate employing an amplification and excision system in conjunction with a bacterial artificial chromosome. Such a system would provide additional benefit in that it would not require the steps of interspersing *EMS* throughout the genome and crossing *EMS*-containing strains, but could instead rely upon the use of rare-cutting restriction enzymes to generate genomic fragments of suitable size for cloning into a BAC vector.

## BRIEF SUMMARY OF THE INVENTION

In a first aspect, the present invention is an improved bacterial artificial chromosome (BAC) cloning vector (pBAC) that comprises a pair of excision-mediating sites (*EMS*) provided in parallel orientation to one another and flanking a site into which a genomic fragment insert can be cloned (which can be a multiple cloning site) one *EMS* being 5' and a second *EMS* being downstream of the cloning site. In the presence of a suitable signal in a suitable host cell, the *EMS* of an insert-containing BAC clone are conditionally activated to recombine with one another and thereby excise the nucleic acid therebetween to produce a circular plasmid that comprises the genomic fragment insert. Both the *EMS* and origin of replication (ori) are responsive to *trans*-acting signals that direct the fragment to be excised from the vector and to be amplified by replication.

Also provided on the same fragment of the BAC vector between the *EMS*, and close to one of the *EMS*, is an *ori* that can be conditionally activated in the host cell in which the BAC vector is maintained. The conditional *ori* is positioned such that it resides on the plasmid excised from the BAC clone

in the excision step. In response to a suitable signal in the host cell, replication is initiated at the *ori* and the excised plasmid is amplified and accumulates in the cell at high-copy number.

Any BAC vector can be improved in accordance with the present invention. Before being improved, the BAC vector must be capable of independent replication in the host cells. The vector should therefore include an *ori* that functions in the host cells, as well as any other genes that encode proteins required for plasmid replication, maintenance, and partitioning. The vector can contain a selectable marker such as a chloramphenicol-resistance gene, and at least one cloning site into which the genomic fragments can be cloned. The vector also preferably contains a gene that can be disrupted by inserting a genomic fragment into the cloning site, thereby imparting an ability to distinguish clones that contain a genomic insert from those that lack a genomic insert, preferably on the basis of color.

A preferred starting vector that can be modified in accordance with the invention is either pBAC108L (Shizuya, H., et al., *supra*) or pBeloBAC11 (Kim, U-J, et al., *supra*), both publications being incorporated herein by reference. Modifications in accordance with the present invention are described relative to pBeloBac11, although one skilled in the art can readily make the same changes to other plasmids including, but not limited to, pBac108L.

In a second aspect, the invention is a host cell comprising in its interior the improved BAC vector of the present invention, where the host cell can conditionally provide the signals required to excise and amplify the insert from the BAC vector. The host cell is preferably bacterial, such as *E. coli*, but can be a plant, yeast, or animal cell, including a mammalian cell, as long as the *EMS* and the *ori* in the BAC vector, and the activating functions in the host cell are selected so as to function in the selected host. Hamilton, C.M., "A Binary-BAC system for plant transformation with high-molecular weight DNA," Gene 200:107-116 (1997) describes a BAC

vector for use in plant cells.

In a third aspect, the invention is a method for amplifying in a host cell a genomic DNA insert in a BAC vector modified in accordance with the invention. The method includes the steps of (1) providing in the host cell a first signal that directs excision of the genomic DNA insert at the *EMS*s to form an first plasmid that comprises the excised insert and an *ori* competent in the host cell, and a second plasmid that comprises the BAC vector backbone, and (2) providing in the host cell a second signal that directs amplification of the excised plasmid to replicate, thereby producing multiple copies of itself.

Existing pBAC vectors could alternatively also be improved simply by providing a conditional *ori* on the vector. Such a modified vector (with insert) would be maintained at a single copy, but could be amplified in its entirety upon command. Such a vector could be prepared as described herein, except there would be no need to provide the *EMS* or the excision-mediating protein. A conditional activator of the *ori* would still be required, and could be provided as described herein.

It is an object of the present invention to retain the advantageous properties of existing BAC vectors.

It is another object of the present invention to provide a bacterial artificial chromosome that can, in the presence of an appropriate first signal, excise a sequence of interest from the BAC which can, in the presence of a second signal, amplify the insert to a copy number of between 10 and 500 copies or higher.

It is yet another object of the present invention to provide a vector that can be conditionally amplified in the presence of an appropriate signal.

It is a feature of the present invention that the BAC vector includes a pair of excision mediating sites (*EMS*) flanking a site into which genomic DNA fragments can be cloned and furthermore contain a conditional *ori* near one *EMS* and in any event between the pair of *EMS* that flank the cloned gene.

It is an advantage of the present invention that an abundance of a target insert in a BAC can be selectively

replicated by providing appropriate signals to the host cell.

Other objects, advantages, and features of the present invention will become apparent upon consideration of the following detailed description taken in conjunction with the accompanying drawings.

## BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Figure 1 schematically depicts a vector prepared in accordance with the present invention.

## DETAILED DESCRIPTION OF THE INVENTION

Shown in SEQ ID NO:1 is the sequence of starting vector pBeloBAC11 as reported at GenBank Accession Number U51113, the record of which is incorporated herein by reference. SEQ ID NO:1 is a circular plasmid sequence that includes *oriS*, the repE gene that produces a protein that initiates replication at *oriS*, and partition genes *parA*, *B*, and *C*. For selection, pBeloBAC11 includes a chloramphenicol-resistance-encoding gene ($Cm^R$). The vector also includes a *lacZ* gene that can be disrupted or eliminated from the vector when an insert is cloned.

In accordance with the improvement, a first insertion, a combined *EMS/ori* element, can be made at the unique *Xho*I site of pBeloBAC11 at nucleotide 2381. It is not essential that the *EMS* and the *ori* be immediately adjacent to one another, although this can be convenient in the laboratory. It is essential, however, that the *ori* element is more proximal than the *FRT* element to the MCS so that the conditional *ori* element is provided on the same segment of the BAC vector as the cloning site. In the preferred embodiment, the selected *EMS* was an *FRT* sequence element derived from the 2$\mu$ yeast plasmid. A yeast 2$\mu$ *FRT* element (SEQ ID NO:3) includes a pair of specific 13-base pair nearly-perfect inverted repeats with an 8-base pair spacer between the inverted repeats. This short sequence is the target for the yeast Flp protein. Other *EMS* are also equally well suited to be used to modify the BAC vector in accordance with the invention. Among others, the $\lambda$

*att* and P1 *lox* sequences are also suitable.

The preferred conditional *ori* is *oriV*, although the conditional *ori* could be any *ori* that functions in the host cell and is responsive to the amplification-mediating protein(s). It is preferable, for the sake of simplicity that the *ori* respond to a single protein, although this is not essential, and multi-protein replication systems are known. It is preferable that the *ori* amplify the plasmid to high copy number where the goal is to produce large quantities of DNA. The *oriV* is preferred because of its broad host range, its known capacity to replicate DNA fragments of 100-kb or larger, its high copy number and its requirement for only one inducing protein.

To make this insertion in the preferred embodiment, the *oriV* element of SEQ ID NO:2 and the *FRT* element of SEQ ID NO:3 were ligated together and the resulting fragment was made blunt ended and then ligated into the *Xho*I site which had been made blunt ended. The orientation of the two joined fragments is such that when the fragment is cloned into the *Xho*I site, the *ori* is physically located between the nearby *FRT* site and the insert cloning site.

A second *EMS* can be inserted by blunt end ligation into *Hpa*I-cleaved pBeloBAC11 that had previously been blunted using Klenow fragment. In the preferred embodiment, the *EMS* was provided on a *Hpa*I-*Sal*I fragment where the *Sal*I end was blunted. It is important that the *EMS* be inserted in parallel orientation to the *EMS* inserted to the opposite side of the cloning site. Since excision will occur only if the two *EMS* are in parallel orientation, one can readily determine whether the proper insertion has been made by performing the excision assay noted below. If excision is not noted, then the second *EMS* is not in parallel with the first and is not appropriate for further use according to the invention.

The resulting improved vector, depicted schematically in Fig. 1, can receive genomic DNA fragments to produce libraries in exactly the same manner as for pBeloBac11. Insert-containing clones of interest can be obtained for further

analysis from the resulting libraries using the same techniques as are described in Kim, U-J., et al. supra, incorporated herein by reference.

An advantage of the present invention is that in addition to its capacity to maintaining (and yielding) only a single copy of the cloned fragment, multiple copies can be easily produced without requiring additional cloning steps. Once an insert-containing clone of interest is identified, the conditional excision and amplification of the present invention can be induced, but only on command. To excise the fragment of interest, the cells containing the BAC vector are induced to produce the excision-mediating protein. If the *FRT* sequence is the *EMS*, the yeast Flp protein is provided. In the presence of Flp, which interacts with the pair of parallel *FRT* sites, the fragment of interest is excised and circularized. Since the excised insert-containing plasmid contains the conditional *ori*, in the presence of the amplification-mediating replication protein(s) the plasmid is induced to replicate from the conditional *ori* until it reaches its maximal copy number, which can typically range between 10 and 500. In the preferred embodiment, employing the *oriV*, the plasmid is induced to replicate to high copy number in the presence of TrfA protein. The sequence of TrfA is known as is the sequence of the gene that encodes TrfA. In the preferred embodiment, copy-up mutant trf278D was employed, although others could be used.

Several suitable means exist for providing the excision-mediating and amplification-mediating functions in the host cell that contains the modified BAC with insert. Since the excision and amplification are only desired at a certain circumstance or stage in the process, the genes that encode the required proteins are under tight regulatory control, without regard to the means by which the functions are provided. In the exemplified embodiment, the *FLP* and *trfA* genes were provided adjacent to one another in a cassette under the transcriptional control of the $p_{tet}$ promoter which can itself be repressed by the TetR protein. The *tetR* gene was provided, to ensure the presence of TetR. When the TetR repressor activity

is eliminated, in the presence of autoclaved chlortetracycline, the genes that encode the excision- and amplification-mediating proteins are transcribed and translated, whereupon excision and amplification of the cloned fragment can proceed.

A first means for providing the controlled activation requires the host cell chromosome to include the genes that encode both functions, and this method is considered preferred by the inventor, since it does not require the maintenance of a delivery plasmid. To insert the above-noted cassette into the host genome, the cassette can simply be provided on a plasmid that also includes an *attP* integration site, a temperature-sensitive *ori*, and an antibiotic-resistance-encoding gene such as a gene encoding kanamycin resistance. The plasmid can be transformed into a host cell such as *E. coli* DH5α. The cells are grown at a permissive temperature and then are shifted to a replication-limiting temperature such as 42°C. In the presence of λ Int integration protein (encoded by the same or a second plasmid), recombination can proceed between the *attP* site of the plasmid and the *attB* site of the bacterial chromosome of the host cell. Colonies that contain a chromosome with the integrated cassette can be obtained by selecting antibiotic-resistant colonies that can grow at 42°C. The unintegrated plasmid will not persist at the elevated temperature, since it can only replicate at the permissive temperature, and thus it will become diluted.

If the genes that activate the excision and amplification functions are not provided in the host cell chromosome, a comparable regulated cassette can be provided on a low-, medium-, or high-copy number plasmid that resides within the host cell. The cassette can readily be ligated into a backbone of, for example, low-copy-number plasmid RSF1010, medium-copy-number plasmid pBR322, or high-copy-number plasmid pBBR1 in a manner known to one skilled in the art, where the backbone provides the plasmid replication functions. The plasmids can be introduced into a host cell, typically an *E. coli* cell, and preferably *E. coli* DH5α by a standard transformation procedure. In addition to varying copy numbers, the delivery plasmid that

supplies the *trans*-activating functions can be chosen on the basis of host range. Procedures for preparing such plasmids and for delivering the plasmids into host cells are described in Wild, J. et al., Gene 179:181-188 (1997), and particularly the references cited in the legend accompanying Fig. 4 thereof, all of which are incorporated herein by reference.

In the method of the present invention, BAC libraries are prepared using the vector of the present invention using the same techniques as are now presently employed using e.g., pBeloBac11 or other pBAC vectors. See, Shizuya, H. et al., supra and Kim, U-J., et al., supra, both incorporated herein by reference. Briefly, size selected high-molecular-weight DNA is partially or completely digested with a rare-cutting restriction endonuclease such as *Not*I, and the size-selected DNA is ligated into the vector of the present invention at an approximately 10:1 molar ratio of insert DNA:vector in the presence of a DNA ligase such as T4 DNA ligase. After overnight incubation at 16°C, the ligations are dialyzed for two hours at room temperature against 0.5 x TE buffer and are transferred into host cells (capable of providing the *trans*-acting functions upon suitable induction), preferably by electroporation. The mixture of transformed cells is allowed to recover and is then plated, preferably in the presence of a color indicator system such as X-Gal/IPTG.

Library screening is accomplished as previously described in the above-incorporated papers. When a clone of interest is identified for further analysis, the cells of the clone are grown overnight in LB medium supplemented with appropriate antibiotics to ensure that the cell maintains both the BAC clone as well as the delivery plasmid (if required).

The overnight cultures are used to inoculate fresh M9 minimal medium [Sambrook, J., et al., "Molecular Cloning: A Laboratory Manual," Second Edition CSHL Press, Cold Spring Harbor, New York (1989)] supplemented with 2% glycerol (as a carbon source)/0.1% casamino acids, and antibiotics (ampicillin, chloramphenicol, and kanamycin). Cultures are grown at 30°C in a water bath shaker until the $A_{590}$ reached 0.4-

0.5. To induce synthesis of the *trans*-activating genes, 10 $\mu$g of chlortetracycline, which is autoclaved for thirty minutes prior to use, is added (per ml), and the incubation is continued overnight. During this incubation, the fragment between the *FRT* sites that contains the cloning site and the conditional *ori* is excised from the BAC clone and amplified to the levels described.

DNA is extracted from the overnight cultures by alkaline lysis. Samples are purified by extraction with phenol/ chloroform/isoamyl alcohol, and are precipitated with ethanol. DNA from a 3 ml culture was resuspended in 30 $\mu$l of TE buffer. Although it is preferred that the resulting DNA be somewhat purified before further analysis, especially if the DNA will be fragmented for use in shotgun cloning, purification is not considered an essential step of the process. Purification may not be required for subsequent use of the DNA for sequence analysis by primer walking.

The present invention will be better understood upon consideration of the following non-limiting example.

## Example

In a test of the method and improved vector of the present invention, the modified BAC vector was introduced into DH5 host cells that comprised in their genome the *FLP* and *trfA* genes under control of the repressed $_{ptet}$ promoter. The cells were either not induced (control) or induced with autoclaved chlortetracycline (cTc). DNA was obtained from the control and induced host cells as described herein, and was cleaved with *Nco*I.

*Nco*I-digested uninduced vector produced only two fragments, long and short, which can be resolved visually from one another after electrophoresis. As a result of the location of the two *Nco*I sites and the two *FRT* sites in the test vector, the resulting excised plasmid and the BAC backbone plasmid each contain a single *Nco*I site. After induction, both of the newly created plasmids were clearly visible after electrophoresis as new bands in addition to the residual long and short bands

noted above (see control) which are maintained because the excision reaction is not 100% efficient. Notably, the band that corresponds to the fragment that contains the *oriV* and the cloning site is wider and brighter than any other band, even despite its smaller size. This is indicative of targeted amplification of that one *oriV*-containing and excised plasmid, as would be expected.

Thus it is shown that a system for excising and amplifying an insert from a BAC clone in a tightly controlled manner is an advantageous improvement over existing pBAC systems and a vector that includes excision mediating sites flanking both a site into which a genomic clone can be inserted and a tightly controlled *ori*, is also an improvement over existing pBAC vectors.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Szybalski, Waclaw

(ii) TITLE OF INVENTION: Conditionally Amplifiable BAC Vector

(iii) NUMBER OF SEQUENCES: 3

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Quarles & Brady
(B) STREET: 1 South Pinckney Street
(C) CITY: Madison
(D) STATE: WI
(E) COUNTRY: US
(F) ZIP: 53703

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Berson, Bennett J
(B) REGISTRATION NUMBER: 37094
(C) REFERENCE/DOCKET NUMBER: 960296.95068

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 608-251-5000
(B) TELEFAX: 608-251-9166

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7507 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: circular

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "pBeloBac11"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGGCCGCAA GGGGTTCGCG TCAGCGGGTG TTGGCGGGTG TCGGGGCTGG CTTAACTATG 60

CGGCATCAGA GCAGATTGTA CTGAGAGTGC ACCATATGCG GTGTGAAATA CCGCACAGAT 120

GCGTAAGGAG AAAATACCGC ATCAGGCGCC ATTCGCCATT CAGGCTGCGC AACTGTTGGG 180

AAGGGCGATC GGTGCGGGCC TCTTCGCTAT TACGCCAGCT GGCGAAAGGG GGATGTGCTG 240

CAAGGCGATT AAGTTGGGTA ACGCCAGGGT TTTCCCAGTC ACGACGTTGT AAAACGACGG 300

CCAGTGAATT GTAATACGAC TCACTATAGG GCGAATTCGA GCTCGGTACC CGGGGATCCT 360

CTAGAGTCGA CCTGCAGGCA TGCAAGCTTG AGTATTCTAT AGTGTCACCT AAATAGCTTG 420

GCGTAATCAT GGTCATAGCT GTTTCCTGTG TGAAATTGTT ATCCGCTCAC AATTCCACAC 480

AACATACGAG CCGGAAGCAT AAAGTGTAAA GCCTGGGGTG CCTAATGAGT GAGCTAACTC 540

ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG GAAACCTGTC GTGCCAGCTG 600

CATTAATGAA TCGGCCAACG CGAACCCCTT GCGGCCGCCC GGGCCGTCGA CCAATTCTCA 660

TGTTTGACAG CTTATCATCG AATTTCTGCC ATTCATCCGC TTATTATCAC TTATTCAGGC 720

GTAGCAACCA GGCGTTTAAG GGCACCAATA ACTGCCTTAA AAAAATTACG CCCCGCCCTG 780

CCACTCATCG CAGTACTGTT GTAATTCATT AAGCATTCTG CCGACATGGA AGCCATCACA 840

AACGGCATGA TGAACCTGAA TCGCCAGCGG CATCAGCACC TTGTCGCCTT GCGTATAATA 900

TTTGCCCATG GTGAAAACGG GGGCGAAGAA GTTGTCCATA TTGGCCACGT TTAAATCAAA 960

ACTGGTGAAA CTCACCCAGG GATTGGCTGA GACGAAAAAC ATATTCTCAA TAAACCCTTT 1020

AGGGAAATAG GCCAGGTTTT CACCGTAACA CGCCACATCT TGCGAATATA TGTGTAGAAA 1080

CTGCCGGAAA TCGTCGTGGT ATTCACTCCA GAGCGATGAA AACGTTTCAG TTTGCTCATG 1140

GAAAACGGTG TAACAAGGGT GAACACTATC CCATATCACC AGCTCACCGT CTTTCATTGC 1200

CATACGGAAT TCCGGATGAG CATTCATCAG GCGGGCAAGA ATGTGAATAA AGGCCGGATA 1260

AAACTTGTGC TTATTTTTCT TTACGGTCTT TAAAAAGGCC GTAATATCCA GCTGAACGGT 1320

CTGGTTATAG GTACATTGAG CAACTGACTG AAATGCCTCA AAATGTTCTT TACGATGCCA 1380

TTGGGATATA TCAACGGTGG TATATCCAGT GATTTTTTTC TCCATTTTAG CTTCCTTAGC 1440

TCCTGAAAAT CTCGATAACT CAAAAAATAC GCCCGGTAGT GATCTTATTT CATTATGGTG 1500

AAAGTTGGAA CCTCTTACGT GCCGATCAAC GTCTCATTTT CGCCAAAAGT TGGCCCAGGG 1560

CTTCCCGGTA TCAACAGGGA CACCAGGATT TATTTATTCT GCGAAGTGAT CTTCCGTCAC 1620

AGGTATTTAT TCGCGATAAG CTCATGGAGC GGCGTAACCG TCGCACAGGA AGGACAGAGA 1680

AAGCGCGGAT CTGGGAAGTG ACGGACAGAA CGGTCAGGAC CTGGATTGGG GAGGCGGTTG 1740

CCGCCGCTGC TGCTGACGGT GTGACGTTCT CTGTTCCGGT CACACCACAT ACGTTCCGCC 1800

ATTCCTATGC GATGCACATG CTGTATGCCG GTATACCGCT GAAAGTTCTG CAAAGCCTGA 1860

TGGGACATAA GTCCATCAGT TCAACGGAAG TCTACACGAA GGTTTTTGCG CTGGATGTGG 1920

CTGCCCGGCA CCGGGTGCAG TTTGCGATGC CGGAGTCTGA TGCGGTTGCG ATGCTGAAAC 1980

AATTATCCTG AGAATAAATG CCTTGGCCTT TATATGGAAA TGTGGAACTG AGTGGATATG 2040

CTGTTTTTGT CTGTTAAACA GAGAAGCTGG CTGTTATCCA CTGAGAAGCG AACGAAACAG 2100

TCGGGAAAAT CTCCCATTAT CGTAGAGATC CGCATTATTA ATCTCAGGAG CCTGTGTAGC 2160

GTTTATAGGA AGTAGTGTTC TGTCATGATG CCTGCAAGCG GTAACGAAAA CGATTTGAAT 2220

ATGCCTTCAG GAACAATAGA AATCTTCGTG CGGTGTTACG TTGAAGTGGA GCGGATTATG 2280

TCAGCAATGG ACAGAACAAC CTAATGAACA CAGAACCATG ATGTGGTCTG TCCTTTTACA 2340

GCCAGTAGTG CTCGCCGCAG TCGAGCGACA GGGCGAAGCC CTCGAGTGAG CGAGGAAGCA 2400

CCAGGGAACA GCACTTATAT ATTCTGCTTA CACACGATGC CTGAAAAAAC TTCCCTTGGG 2460

GTTATCCACT TATCCACGGG GATATTTTTA TAATTATTTT TTTTATAGTT TTTAGATCTT 2520

CTTTTTTAGA GCGCCTTGTA GGCCTTTATC CATGCTGGTT CTAGAGAAGG TGTTGTGACA 2580

AATTGCCCTT TCAGTGTGAC AAATCACCCT CAAATGACAG TCCTGTCTGT GACAAATTGC 2640

CCTTAACCCT GTGACAAATT GCCCTCAGAA GAAGCTGTTT TTTCACAAAG TTATCCCTGC 2700

TTATTGACTC TTTTTTATTT AGTGTGACAA TCTAAAAACT TGTCACACTT CACATGGATC 2760

TGTCATGGCG GAAACAGCGG TTATCAATCA CAAGAAACGT AAAAATAGCC CGCGAATCGT 2820

CCAGTCAAAC GACCTCACTG AGGCGGCATA TAGTCTCTCC CGGGATCAAA AACGTATGCT 2880

GTATCTGTTC GTTGACCAGA TCAGAAAATC TGATGGCACC CTACAGGAAC ATGACGGTAT 2940

CTGCGAGATC CATGTTGCTA AATATGCTGA AATATTCGGA TTGACCTCTG CGGAAGCCAG 3000

TAAGGATATA CGGCAGGCAT TGAAGAGTTT CGCGGGGAAG GAAGTGGTTT TTTATCGCCC 3060

TGAAGAGGAT GCCGGCGATG AAAAAGGCTA TGAATCTTTT CCTTGGTTTA TCAAACGTGC 3120

GCACAGTCCA TCCAGAGGGC TTTACAGTGT ACATATCAAC CCATATCTCA TTCCCTTCTT 3180

TATCGGGTTA CAGAACCGGT TTACGCAGTT TCGGCTTAGT GAAACAAAAG AAATCACCAA 3240

TCCGTATGCC ATGCGTTTAT ACGAATCCCT GTGTCAGTAT CGTAAGCCGG ATGGCTCAGG 3300

CATCGTCTCT CTGAAAATCG ACTGGATCAT AGAGCGTTAC CAGCTGCCTC AAAGTTACCA 3360

GCGTATGCCT GACTTCCGCC GCCGCTTCCT GCAGGTCTGT GTTAATGAGA TCAACAGCAG 3420

AACTCCAATG CGCCTCTCAT ACATTGAGAA AAAGAAAGGC CGCCAGACGA CTCATATCGT 3480

ATTTTCCTTC CGCGATATCA CTTCCATGAC GACAGGATAG TCTGAGGGTT ATCTGTCACA 3540

GATTTGAGGG TGGTTCGTCA CATTTGTTCT GACCTACTGA GGGTAATTTG TCACAGTTTT 3600

GCTGTTTCCT TCAGCCTGCA TGGATTTTCT CATACTTTTT GAACTGTAAT TTTTAAGGAA 3660

GCCAAATTTG AGGGCAGTTT GTCACAGTTG ATTTCCTTCT CTTTCCCTTC GTCATGTGAC 3720

CTGATATCGG GGGTTAGTTC GTCATCATTG ATGAGGGTTG ATTATCACAG TTTATTACTC 3780

TGAATTGGCT ATCCGCGTGT GTACCTCTAC CTGGAGTTTT TCCCACGGTG GATATTTCTT 3840

CTTGCGCTGA GCGTAAGAGC TATCTGACAG AACAGTTCTT CTTTGCTTCC TCGCCAGTTC 3900

GCTCGCTATG CTCGGTTACA CGGCTGCGGC GAGCGCTAGT GATAATAAGT GACTGAGGTA 3960

TGTGCTCTTC TTATCTCCTT TTGTAGTGTT GCTCTTATTT TAAACAACTT TGCGGTTTTT 4020

TGATGACTTT GCGATTTTGT TGTTGCTTTG CAGTAAATTG CAAGATTTAA TAAAAAAACG 4080

CAAAGCAATG ATTAAAGGAT GTTCAGAATG AAACTCATGG AAACACTTAA CCAGTGCATA 4140

AACGCTGGTC ATGAAATGAC GAAGGCTATC GCCATTGCAC AGTTTAATGA TGACAGCCCG 4200

GAAGCGAGGA AAATAACCCG GCGCTGGAGA ATAGGTGAAG CAGCGGATTT AGTTGGGGTT 4260

TCTTCTCAGG CTATCAGAGA TGCCGAGAAA GCAGGGCGAC TACCGCACCC GGATATGGAA 4320

ATTCGAGGAC GGGTTGAGCA ACGTGTTGGT TATACAATTG AACAAATTAA TCATATGCGT 4380

GATGTGTTTG GTACGCGATT GCGACGTGCT GAAGACGTAT TTCCACCGGT GATCGGGGTT 4440

GCTGCCCATA AAGGTGGCGT TTACAAAACC TCAGTTTCTG TTCATCTTGC TCAGGATCTG 4500

GCTCTGAAGG GGCTACGTGT TTTGCTCGTG GAAGGTAACG ACCCCCAGGG AACAGCCTCA 4560

ATGTATCACG GATGGGTACC AGATCTTCAT ATTCATGCAG AAGACACTCT CCTGCCTTTC 4620

TATCTTGGGG AAAAGGACGA TGTCACTTAT GCAATAAAGC CCACTTGCTG GCCGGGGCTT 4680

GACATTATTC CTTCCTGTCT GGCTCTGCAC CGTATTGAAA CTGAGTTAAT GGGCAAATTT 4740

GATGAAGGTA AACTGCCCAC CGATCCACAC CTGATGCTCC GACTGGCCAT TGAAACTGTT 4800

GCTCATGACT ATGATGTCAT AGTTATTGAC AGCGCGCCTA ACCTGGGTAT CGGCACGATT 4860

AATGTCGTAT GTGCTGCTGA TGTGCTGATT GTTCCCACGC CTGCTGAGTT GTTTGACTAC 4920

ACCTCCGCAC TGCAGTTTTT CGATATGCTT CGTGATCTGC TCAAGAACGT TGATCTTAAA 4980

GGGTTCGAGC CTGATGTACG TATTTTGCTT ACCAAATACA GCAATAGTAA TGGCTCTCAG 5040

TCCCCGTGGA TGGAGGAGCA AATTCGGGAT GCCTGGGGAA GCATGGTTCT AAAAAATGTT 5100

GTACGTGAAA CGGATGAAGT TGGTAAAGGT CAGATCCGGA TGAGAACTGT TTTTGAACAG 5160

GCCATTGATC AACGCTCTTC AACTGGTGCC TGGAGAAATG CTCTTTCTAT TTGGGAACCT 5220

GTCTGCAATG AAATTTTCGA TCGTCTGATT AAACCACGCT GGGAGATTAG ATAATGAAGC 5280

GTGCGCCTGT TATTCCAAAA CATACGCTCA ATACTCAACC GGTTGAAGAT ACTTCGTTAT 5340

CGACACCAGC TGCCCCGATG GTGGATTCGT TAATTGCGCG CGTAGGAGTA ATGGCTCGCG 5400

GTAATGCCAT TACTTTGCCT GTATGTGGTC GGGATGTGAA GTTTACTCTT GAAGTGCTCC 5460

GGGGTGATAG TGTTGAGAAG ACCTCTCGGG TATGGTCAGG TAATGAACGT GACCAGGAGC 5520

TGCTTACTGA GGACGCACTG GATGATCTCA TCCCTTCTTT TCTACTGACT GGTCAACAGA 5580

CACCGGCGTT CGGTCGAAGA GTATCTGGTG TCATAGAAAT TGCCGATGGG AGTCGCCGTC 5640

GTAAAGCTGC TGCACTTACC GAAAGTGATT ATCGTGTTCT GGTTGGCGAG CTGGATGATG 5700

AGCAGATGGC TGCATTATCC AGATTGGGTA ACGATTATCG CCCAACAAGT GCTTATGAAC 5760

GTGGTCAGCG TTATGCAAGC CGATTGCAGA ATGAATTTGC TGGAAATATT TCTGCGCTGG 5820

CTGATGCGGA AAATATTTCA CGTAAGATTA TTACCCGCTG TATCAACACC GCCAAATTGC 5880

CTAAATCAGT TGTTGCTCTT TTTTCTCACC CCGGTGAACT ATCTGCCCGG TCAGGTGATG 5940

CACTTCAAAA AGCCTTTACA GATAAAGAGG AATTACTTAA GCAGCAGGCA TCTAACCTTC 6000

ATGAGCAGAA AAAAGCTGGG GTGATATTTG AAGCTGAAGA AGTTATCACT CTTTTAACTT 6060

CTGTGCTTAA AACGTCATCT GCATCAAGAA CTAGTTTAAG CTCACGACAT CAGTTTGCTC 6120

CTGGAGCGAC AGTATTGTAT AAGGGCGATA AAATGGTGCT TAACCTGGAC AGGTCTCGTG 6180

TTCCAACTGA GTGTATAGAG AAAATTGAGG CCATTCTTAA GGAACTTGAA AAGCCAGCAC 6240

CCTGATGCGA CCACGTTTTA GTCTACGTTT ATCTGTCTTT ACTTAATGTC CTTTGTTACA 6300

GGCCAGAAAG CATAACTGGC CTGAATATTC TCTCTGGGCC CACTGTTCCA CTTGTATCGT 6360

CGGTCTGATA ATCAGACTGG GACCACGGTC CCACTCGTAT CGTCGGTCTG ATTATTAGTC 6420

TGGGACCACG GTCCCACTCG TATCGTCGGT CTGATTATTA GTCTGGGACC ACGGTCCCAC 6480

TCGTATCGTC GGTCTGATAA TCAGACTGGG ACCACGGTCC CACTCGTATC GTCGGTCTGA 6540

TTATTAGTCT GGGACCATGG TCCCACTCGT ATCGTCGGTC TGATTATTAG TCTGGGACCA 6600

CGGTCCCACT CGTATCGTCG GTCTGATTAT TAGTCTGGAA CCACGGTCCC ACTCGTATCG 6660

TCGGTCTGAT TATTAGTCTG GGACCACGGT CCCACTCGTA TCGTCGGTCT GATTATTAGT 6720

CTGGGACCAC GATCCCACTC GTGTTGTCGG TCTGATTATC GGTCTGGGAC CACGGTCCCA 6780

CTTGTATTGT CGATCAGACT ATCAGCGTGA GACTACGATT CCATCAATGC CTGTCAAGGG 6840

CAAGTATTGA CATGTCGTCG TAACCTGTAG AACGGAGTAA CCTCGGTGTG CGGTTGTATG 6900

CCTGCTGTGG ATTGCTGCTG TGTCCTGCTT ATCCACAACA TTTTGCGCAC GGTTATGTGG 6960

ACAAAATACC TGGTTACCCA GGCCGTGCCG GCACGTTAAC CGGGCTGCAT CCGATGCAAG 7020

TGTGTCGCTG TCGACGAGCT CGCGAGCTCG GACATGAGGT TGCCCCGTAT TCAGTGTCGC 7080

TGATTTGTAT TGTCTGAAGT TGTTTTTACG TTAAGTTGAT GCAGATCAAT TAATACGATA 7140

CCTGCGTCAT AATTGATTAT TTGACGTGGT TTGATGGCCT CCACGCACGT TGTGATATGT 7200

AGATGATAAT CATTATCACT TTACGGGTCC TTTCCGGTGA TCCGACAGGT TACGGGGCGG 7260

CGACCTCGCG GGTTTTCGCT ATTTATGAAA ATTTTCCGGT TTAAGGCGTT TCCGTTCTTC 7320

TTCGTCATAA CTTAATGTTT TTATTTAAAA TACCCTCTGA AAAGAAAGGA AACGACAGGT 7380

GCTGAAAGCG AGCTTTTTGG CCTCTGTCGT TTCCTTTCTC TGTTTTTGTC CGTGGAATGA 7440

ACAATGGAAG TCCGAGCTCA TCGCTAATAA CTTCGTATAG CATACATTAT ACGAAGTTAT 7500

ATTCGAT 7507

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 520 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CCGGCGTTGT GGATACCACG CGGAAAACTT GGCCCTCACT GACAGATGAG GGGCGGACGT 60

TGACACTTGA GGGGCCGACT CACCCGGCGC GGCGTTGACA GATGAGGGGC AGGCTCGATT 120

TCGGCCGGCG ACGTGGAGCT GGCCAGCCTC GCAAATCGGC GAAAACGCCT GATTTTACGC 180

GAGTTTCCCA CAGATGATGT GGACAAGCCT GGGGATAAGT GCCCTGCGGT ATTGACACTT 240

GAGGGGCGCG ACTACTGACA GATGAGGGGC GCGATCCTTG ACACTTGAGG GGCAGAGTGA 300

TGACAGATGA GGGGCGCACC TATTGACATT TGAGGGGCTG TCCACAGGCA GAAAATCCAG 360

CATTTGCAAG GGTTTCCGCC CGTTTTTCGG CCACCGCTAA CCTGTCTTTT AACCTGCTTT 420

TAAACCAATA TTTATAAACC TTGTTTTTAA CCAGGGCTGC GCCCTGGCGC GTGACCGCGC 480

ACGCCGAAGG GGGGTGCCCC CCCTTCTCGA ACCCTCCCGG 520

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 34 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GAAGTTCCTA TTCTCTAGAA AGTATAGGAA CTTC 34

QBMAD\147375

## CLAIMS

1. A bacterial artificial chromosome comprising:

a site into which an DNA fragment can be cloned;

a pair of inducible excision-mediating sites flanking the site into which the DNA fragment can be cloned, the excision-mediating sites being provided in parallel orientation relative to one another and defining a fragment that comprises the site into which the DNA fragment can be cloned, which fragment is excisable to produce a circular plasmid; and

an inducible origin of replication on the excisable fragment.

2. A bacterial artificial chromosome according to claim 1 wherein the pair of excision-mediating sites are *FRT*, λatt or P1 *lox* sites.

3. A bacterial artificial chromosome according to claim 2 wherein the pair of excision-mediating sites each comprise a sequence as shown in SEQ ID NO:3.

4. A bacterial artificial chromosome as claimed in claim 1, 2 or 3 wherein the inducible origin or replication is *oriV*.

5. A cell comprising in its interior a bacterial artificial chromosome as defined in any one of the preceding claims;

a gene encoding an excision-mediating protein capable of inducing the excisable fragment to excise from the bacterial artificial chromosome and circularise to form an excised plasmid; and

a gene encoding an amplification-mediating protein capable of inducing the excised plasmid to replicate.

6. A cell according to claim 5 wherein the excision-mediating sites on the bacterial artificial chromosome are *FRT* sites and the gene encoding the excision-

AMENDED SHEET

mediating protein is a *FLP* gene.

7. A cell according to claim 5 or 6 wherein the gene encoding the amplification-mediating protein is selected from a group consisting of a *trfA* gene and mutants thereof.

8. A cell according to any one of claims 5 to 7 which is an *E. coli* cell.

9. A method for amplifying in a cell an insert in a bacterial artificial chromosome, the insert being provided on an excisable fragment between a pair of inducible excision-mediating sites, the excisable fragment further comprising an inducible origin of replication, the method comprising the steps of:

providing in the host cell an excision-mediating protein that induces the excisable fragment to excise from the bacterial artificial chromosome and to circularise to form an excised plasmid; and

providing in the host cell an amplification-mediating protein that induces the excised plasmid that comprises the insert to replicate.

10. A method according to claim 9 wherein the excised plasmid is replicated to a copy number from 10 to 500.

11. A bacterial artificial chromosome comprising an inducible origin of replication.

AMENDED SHEET

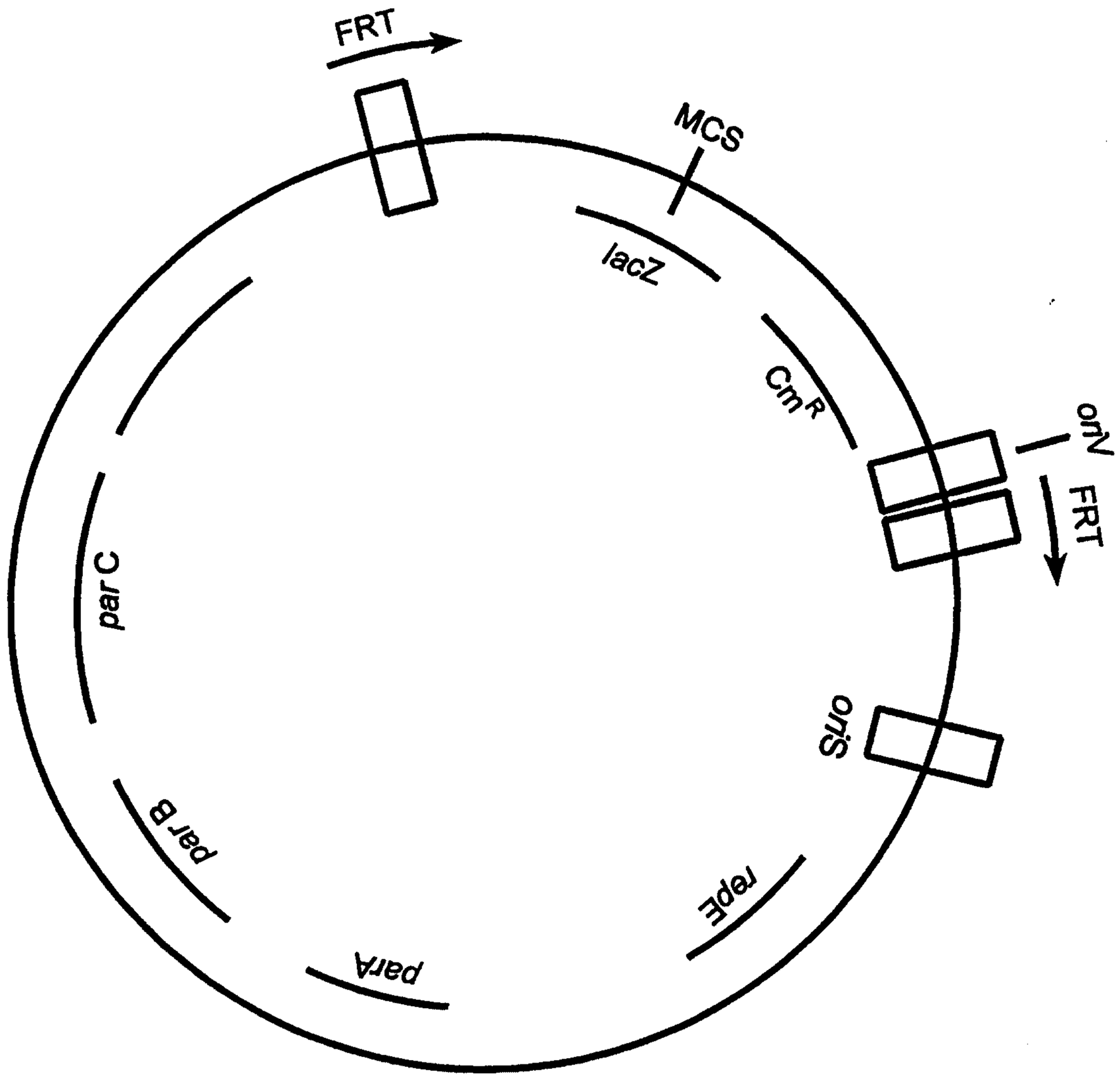



FIG 1