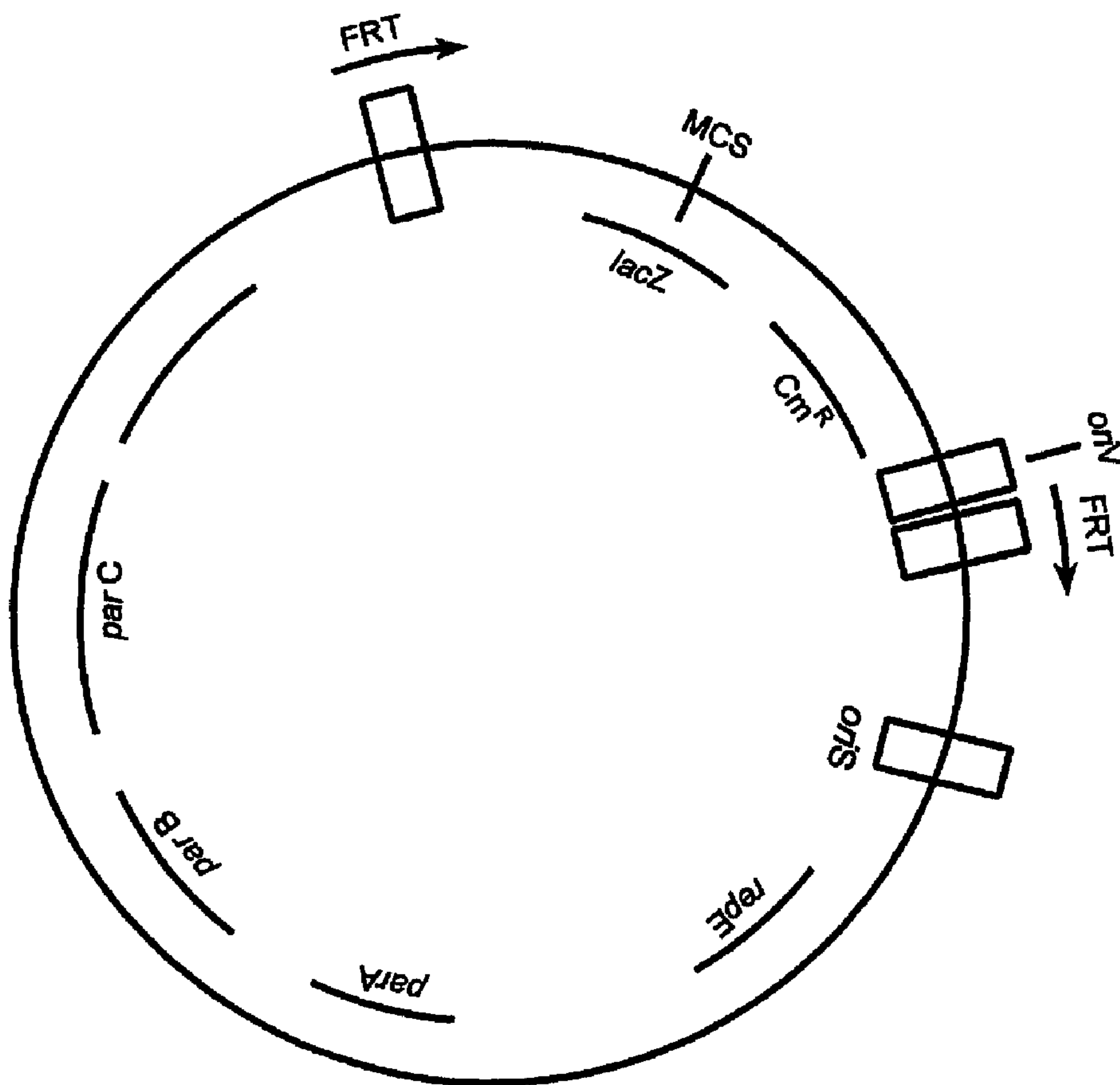




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 (72) Inventeurs/Inventors:  
SZYBALSKI, WACLAW, US;  
WILD, JADWIGA, US;  
HRADECNA, ZDENKA, US  
 (73) Propriétaire/Owner:  
WISCONSIN ALUMNI RESEARCH FOUNDATION, US  
 (74) Agent: BORDEN LADNER GERVAIS LLP

(54) Titre : VECTEUR DE CHROMOSOME BACTERIEN ARTIFICIEL AMPLIFIABLE A TITRE CONDITIONNEL  
 (54) Title: CONDITIONALLY AMPLIFIABLE BACTERIAL ARTIFICIAL CHROMOSOME (BAC) VECTOR



(57) Abrégé/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.



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 (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).

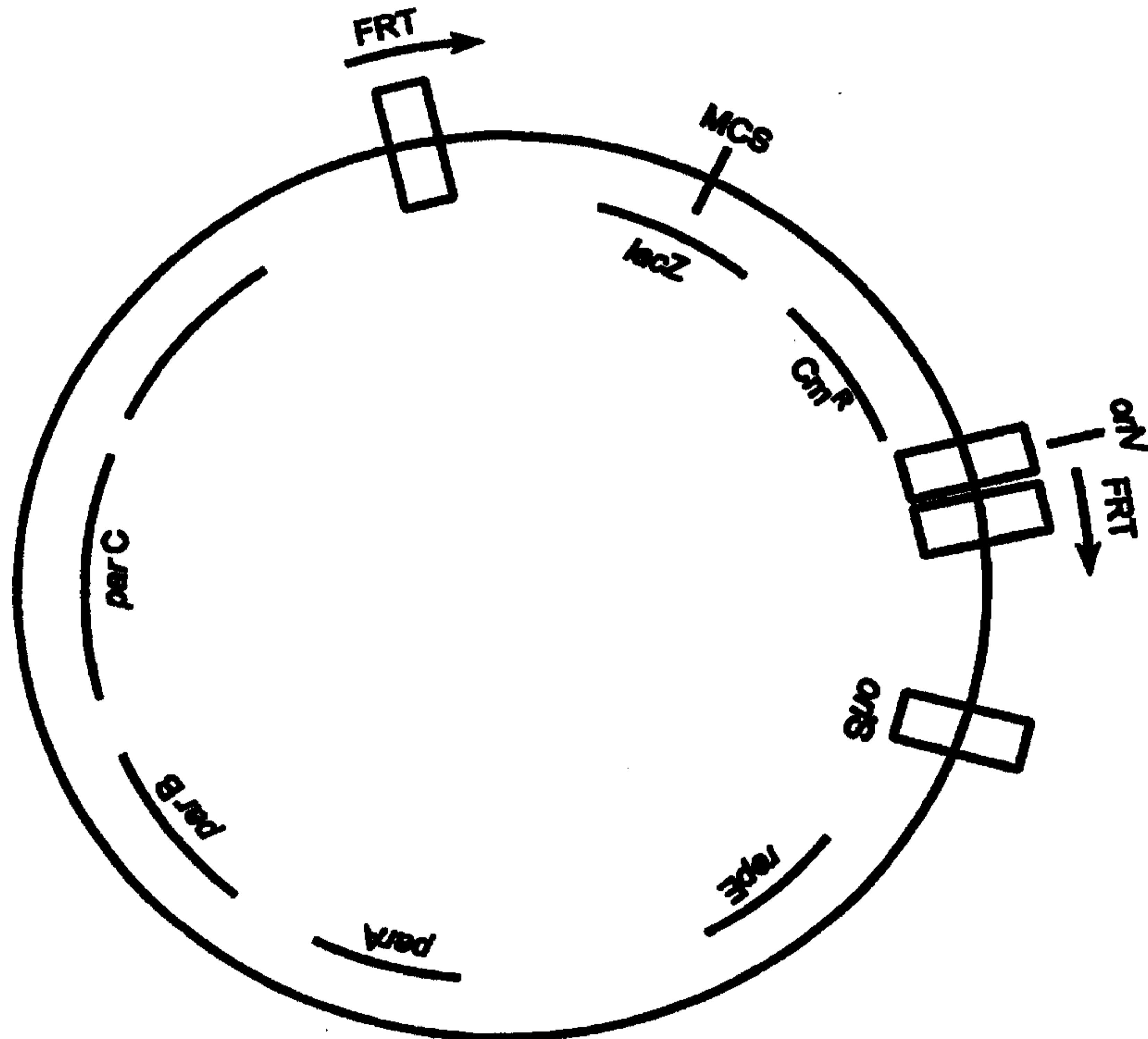
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(54) Title: CONDITIONALLY AMPLIFIABLE BACTERIAL ARTIFICIAL CHROMOSOME (BAC) VECTOR

(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

5 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  
10 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.  
15 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  
20 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  $\alpha$  complementation of the *lacZ* gene to distinguish insert-containing recombinant molecules from  
25 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  
30 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  $\lambda$  att,  $2\mu$  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

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.

5 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  
10 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  
15 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.

20 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*). Modifications in accordance with the present invention are described relative to pBeloBac11, although one skilled in  
25 the art can readily make the same changes to other plasmids including, but not limited to, pBac108L.

The invention also provides a bacterial artificial chromosome comprising a conditional origin of replication responsive to an amplification protein.

30 The invention further provides 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  
5 fragment can be cloned, which fragment is excisable to produce a circular plasmid; and a conditional origin of replication responsive to an amplification protein on the excisable fragment.

In a second aspect, the invention is a host cell  
10 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,  
15 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  
20 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. 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 *XhoI* 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 *XhoI* site which had been made blunt ended. The orientation of the two joined fragments is such that when the fragment is cloned into the *XhoI* 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 *HpaI*-cleaved pBeloBAC11 that had previously been blunted using Klenow fragment. In the preferred embodiment, the *EMS* was provided on a *HpaI-SalI* fragment where the *SalI* 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.

5 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  
10 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  
15 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  
20 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  
25 *trfA278D* 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  
30 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  
35 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.

5           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  
10 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* DH5 $\alpha$ . The cells  
15 are grown at a permissive temperature and then are shifted to a replication-limiting temperature such as 42°C. In the presence of  $\lambda$  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  
20 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  
25 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  
30 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  
35 be introduced into a host cell, typically an *E. coli* cell, and preferably *E. coli* DH5 $\alpha$  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.

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.

Briefly, size selected high-molecular-weight DNA is partially or completely digested with a rare-cutting restriction endonuclease such as NotI, 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-

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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  
5 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/  
10 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  
15 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.

#### 20 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  $p_{tet}$  promoter. The cells were  
25 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 *NcoI*.

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

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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  
5 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  
10 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.

15

## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Wisconsin Alumni Research Foundation
- (ii) TITLE OF INVENTION: Conditional Amplifiable Bacterial Artificial Chromosome (BAC) Vector
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Borden Ladner Gervais LLP
  - (B) STREET: 60 Queen Street
  - (C) CITY: Ottawa
  - (D) PROVINCE: Ontario
  - (E) COUNTRY: Canada
  - (F) POSTAL CODE: K1P 5Y7
- (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
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  - (B) FILING DATE: 21-NOV-1997
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Conn, David L.
  - (B) REGISTRATION NUMBER: 3960
  - (C) REFERENCE/DOCKET NUMBER: PAT 46912W-1
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (613) 237-5160
  - (B) TELEFAX: (613) 787-3558

## (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:

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CAAGGCGATT	AAGTTGGGTA	ACGCCAGGGT	TTTCCCAGTC	ACGACGTTGT	AAAACGACGG	300
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TGTTTGACAG	CTTATCATCG	AATTTCTGCC	ATTCATCCGC	TTATTATCAC	TTATTCAGGC	720
GTAGCAACCA	GGCGTTTAAG	GGCACCAATA	ACTGCCTTAA	AAAAATTACG	CCCCGCCCTG	780
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AACGGCATGA	TGAACCTGAA	TCGCCAGCGG	CATCAGCACC	TTGTGCGCCTT	GCGTATAATA	900
TTTGCCCATG	GTGAAAACGG	GGGCGAAGAA	GTTGTCCATA	TTGGCCACGT	TTAAATCAAA	960
ACTGGTGAAA	CTCACCCAGG	GATTGGCTGA	GACGAAAAC	ATATTCTCAA	TAAACCCTTT	1020
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CTGCCGAAA	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	CAAAAATAC	GCCCGGTAGT	GATCTTATTT	CATTATGGTG	1500
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CTTCCCGGTA	TCAACAGGGA	CACCAGGATT	TATTTATTCT	GCGAAGTGAT	CTTCCGTCAC	1620

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CCGCCGCTGC	TGCTGACGGT	GTGACGTTCT	CTGTTCCGGT	CACACCACAT	ACGTTCCGCC	1800
ATTCCTATGC	GATGCACATG	CTGTATGCCG	GTATACCGCT	GAAAGTTCTG	CAAAGCCTGA	1860
TGGGACATAA	GTCCATCAGT	TCAACGGAAG	TCTACACGAA	GGTTTTTGCG	CTGGATGTGG	1920
CTGCCC GGCA	CCGGGTGCAG	TTTGCGATGC	CGGAGTCTGA	TGCGGTTGCG	ATGCTGAAAC	1980
AATTATCCTG	AGAATAAATG	CCTTGGCCTT	TATATGGAAA	TGTGGAAGTG	AGTGGATATG	2040
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TCGGGAAAAT	CTCCATTAT	CGTAGAGATC	CGCATTATTA	ATCTCAGGAG	CCTGTGTAGC	2160
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TCAGCAATGG	ACAGAACAAC	CTAATGAACA	CAGAACCATG	ATGTGGTCTG	TCCTTTTACA	2340
GCCAGTAGTG	CTCGCCGAG	TCGAGCGACA	GGGCGAAGCC	CTCGAGTGAG	CGAGGAAGCA	2400
CCAGGGAACA	GCACTTATAT	ATTCTGCTTA	CACACGATGC	CTGAAAAAAC	TTCCCTTGGG	2460
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CTTTTTTAGA	GCGCCTTGTA	GGCCTTTATC	CATGCTGGTT	CTAGAGAAGG	TGTTGTGACA	2580
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GATGTGTTTG	GTACGCGATT	GCGACGTGCT	GAAGACGTAT	TTCCACCGGT	GATCGGGGTT	4440
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GTAATGCCAT	TACTTTGCCT	GTATGTGGTC	GGGATGTGAA	GTTTACTCTT	GAAGTGCTCC	5460
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CCTGATGCGA	CCACGTTTTA	GTCTACGTTT	ATCTGTCTTT	ACTTAATGTC	CTTTGTTACA	6300
GGCCAGAAAG	CATAACTGGC	CTGAATATTC	TCTCTGGGCC	CACTGTTCCA	CTTGTATCGT	6360
CGGTCTGATA	ATCAGACTGG	GACCACGGTC	CCACTCGTAT	CGTCGGTCTG	ATTATTAGTC	6420
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TCGTATCGTC	GGTCTGATAA	TCAGACTGGG	ACCACGGTCC	CACTCGTATC	GTCGGTCTGA	6540
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TCGGTCTGAT TATTAGTCTG GGACCACGGT CCCACTCGTA TCGTCGGTCT GATTATTAGT	6720
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CAAGTATTGA CATGTCGTCG TAACCTGTAG AACGGAGTAA CCTCGGTGTG CGGTTGTATG	6900
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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 TTCCTTCTC TGTTTTTGTC CGTGGAATGA	7440
ACAATGGAAG TCCGAGCTCA TCGCTAATAA CTTCGTATAG CATAATTAT 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 CGGAAACTT GGCCCTCACT GACAGATGAG GGGCGGACGT	60
TGACACTTGA GGGGCCGACT CACCCGGCGC GCGTTGACA 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

**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  
a conditional origin of replication responsive to an amplification protein on the excisable fragment.
2. A bacterial artificial chromosome according to claim 1 wherein the pair of excision-mediating sites are *FRT*,  $\lambda$ att 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 of replication is *oriV*.
5. A cell comprising in its interior the bacterial artificial chromosome as defined in any one of claims 1 to 4;  
a gene encoding an excision-mediating protein capable of inducing the excisable fragment to excise from the

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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 chromosomes are *FRT* sites and the gene encoding the excision-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 a mutant thereof that encodes an amplification-mediating protein.

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.

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

11. A host cell comprising a bacterial artificial chromosome, said bacterial artificial chromosome comprising a conditional origin of replication responsive to an amplification signal provided to the host cell.

12. A bacterial artificial chromosome comprising at least two distinct origins of replication wherein at least one of said distinct origins of replication is a conditional origin of replication responsive to an amplification protein.

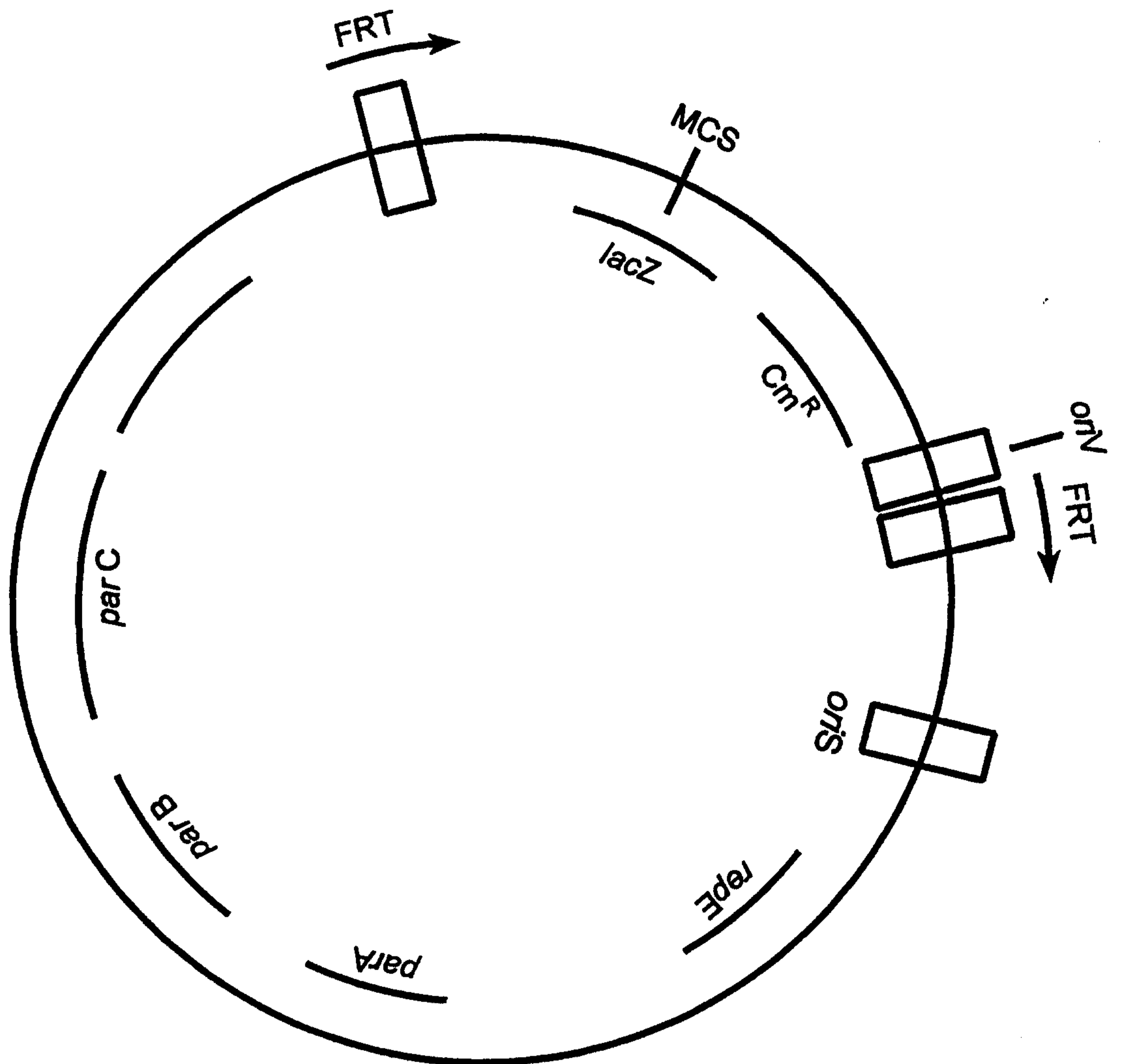


FIG 1

