Direct cloning of full-length mouse mitochondrial DNA using a *Bacillus subtilis* genome vector

Izuru Yonemura,1 Kazuto Nakada, Akitsugu Satō, Jun-Ichi Hayashi, Kyoko Fujita, Shinya Kaneko, Mitsuhiro Itaya

**Abstract**

The complete mouse mitochondrial genome (16.3 kb) was directly cloned into a *Bacillus subtilis* genome (BGM) vector. Two DNA segments of 2.06 and 2.14 kb that flank the internal 12 kb of the mitochondrial DNA (mtDNA) were subcloned into an *Escherichia coli* plasmid. Subsequent integration of the plasmid at the cloning locus of the BGM vector yielded a derivative specific for the targeted cloning of the internal 12-kb mtDNA region. The BGM vector took up mtDNA purified from mouse liver and integrated it by homologous recombination at the two preinstalled mtDNA-flanking sequences. The complete cloned mtDNA in the BGM vector was converted to a covalently closed circular (ccc) plasmid form via gene conversion in *B. subtilis*. The mtDNA carried on this plasmid was then isolated and transferred to *E. coli*. DNA sequence fidelity and stability through the BGM vector-mediated cloning process were confirmed.

© 2007 Elsevier B.V. All rights reserved.

**Keywords:** BGM vector; Nicked DNA; Homologous recombination; Megacloning; Genetic competence

1. Introduction

DNA cloning is an important tool for the investigation of genes and gene function. However, there are numerous cases where the desired DNA molecule proves difficult to clone using conventional cloning methods. Moreover, as the size of the desired DNA molecule increases, cloning efficiency becomes increasingly dependent on the preparation of high-quality, unsheared, intact DNA. The development of the *Escherichia coli* Bacterial Artificial Chromosome (BAC) vector increased the size of DNA molecules that could be cloned to far larger than dozens of kilobase pairs (Shizuya et al., 1992; Kim et al., 1996; Frengen et al., 1999).

Mitochondrial DNA (mtDNA) genomes, present in eukaryotic cells, vary in size from about 16 kb in mammals to greater than dozens of kb in plants (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=Pager&DB=genome). The typical animal cell contains hundreds of mitochondria, which produce the majority of the cell’s ATP through oxidative phosphorylation. The ability to clone full-length mtDNA genomes would provide a resource for various molecular biological approaches, such as the creation of recombinant mtDNA haplotypes in mammalian cells. However, a number of attempts to clone mouse and human mtDNAs have been problematic, which might be attributed to the host vector system used to establish stable...
maintenance of the cloned DNA, and not to the difficulty of isolating intact circular mtDNA from mammalian cells and tissues (Bigger et al., 2000).

The cloning of mtDNA from mouse (16.3 kb) has been difficult, as demonstrated in a recent pioneering report by Yoon and Koob (2003). These authors were able to clone full-length mtDNA in plasmid form in E. coli by direct selection for the circular mtDNA carefully isolated from mouse liver into which an E. coli cloning vector had been inserted in vitro. The E. coli vector was randomly inserted into circular mtDNA using in vitro transposition reaction. Three types of clones were obtained; the vectors were inserted into the ND1, COXII and ND5. The recombinant mtDNA was amplified in E. coli and then transferred back into mtDNA-less ρ0 mouse cell line LL/2 for subsequent analyses (Yoon and Koob, 2003). The recombinant mtDNA in LL/2 cell line has transcription ability. Their cloning method required the careful preparation of unsheared, intact circular mtDNA. A further difficulty is that the site at which the mtDNA was interrupted by the cloning vector depends on the transposon-mediated insertion process, which is difficult to regulate.

An alternative protocol for the stable cloning of large DNAs has been reported (Itaya, 1995). The use of the Bacillus subtilis genome as a cloning vehicle is based on unique features of this bacterium, in particular its natural competence development and induced homologous recombination (Itaya et al., 2000). The cloning scheme illustrated in Fig. 1 is categorized as positional cloning, in which two short DNA segments specifying the target region, termed landing pad sequences (LPSs), are prerequisite. The size of DNA that can be cloned between the LPSs, proven to be up to millions of base pairs to date (Itaya et al., 2005), is more than sufficient to encompass the greater than 100-kb fragments of Arabidopsis thaliana mtDNA (Kaneko et al., 2005) and mouse genes (Itaya et al., 2001; Kaneko et al., 2003). The high genetic and structural stability of the cloned DNA in the B. subtilis genome provides for a cloning vehicle

---

Fig. 1. Mouse mtDNA cloning in the BGM vector. The procedure of the BGM cloning is illustrated. Thin lines in each BEST series (iv, vi, ix and xiii) represent the B. subtilis genome and the cell wall. The “V” between the two LPSs indicates a positive selection marker, details of which are shown in Fig. 2 and discussed in Section 2.2. The “X” marks indicate homologous recombination in vivo that occurs between the intermediates shown in large brackets (v, viii and xii). Short vertical bars with “I” indicate I-PpoI sites. Construction of pLPSm102, derived from pCISP310B, was performed in E. coli (steps from i to iii). Two sequences specific to the BGM vector (BEST7003) are GpBR (checkered line and striped line) and the Nm resistance gene (Pr-neo). The cl gene products (curved 3D arrow) repress expression of Pr-neo in BEST7686 (vi). The structure of the circular mouse mtDNA genome with locations of LPS1 and LPS2 are shown (panel B). mtDNA prepared from mouse liver was analyzed by CHEF electrophoresis under the indicated running conditions at 14 °C (panel C, lanes 1 and 2) and by standard agarose gel electrophoresis (panel D, lane 3). Impurities in the range of low-molecular-weight DNA and RNA (asterisk in panel D) are discussed in Section 3.3. The cloned mtDNA in strain BEST7688 was detected as an I-PpoI fragment (arrow in panel E, lanes 4 and 5) resolved by CHEF under the indicated running conditions at 14 °C. Plasmid pYMMM101 was established in BEST7689 using the BReT method. Panel F shows restriction enzyme analysis of pYMMM101 isolated from E. coli using BamHI (lane 6), EcoRI (lane 7), EcoRV (lane 8), HindIII (lane 9), PstI (lane 10), PvuII (lane 11), and XbaI (lane 12). The probe for Southern hybridization (panel C, lane 2; panel E, lane 5) was mouse BALB/c mtDNA.
unparalleled among conventional plasmid-based vectors. However, this one-way cloning protocol remained largely unused until a method was developed to retrieve the cloned DNAs. This method uses the \textit{B. subtilis}-specific Recombinational Transfer (BReT) mechanism to convert the cloned DNA segment to a circular plasmid form (Tsuge and Itaya, 2001). The comprehensive system established to date (Fig. 1A) covers the steps from cloning in the \textit{B. subtilis} genome vector, termed the BGM vector, to isolation of the clone in plasmid form and its transfer to other interesting hosts. In this report we use this system to clone the complete mouse mtDNA genome, which has been otherwise difficult to clone. We also explore parameters affecting BGM cloning efficiency.

2. Materials and methods

2.1. Bacterial strains and plasmids

\textit{B. subtilis} strains used as BGM vector were derived from the genetic cross between 168 \textit{trpC2} and RM125 (\textit{arg leu}) that has no restriction-modification (Itaya, 1997). \textit{E. coli} strains were JA221 (\textit{F} \textit{hsdR hsdM trp leu lacY recA1}), JM109 (\textit{e14} \textit{McrA} \textit{recA1 endA1 gyrA96 thi-1 hsdR17(\textit{rK mK}) supE44 relA1} \Delta(lac-proAB) [\textit{F} \textit{traD36 proAB} \textit{lacPZ\DeltaM15}], and DH5\textalpha\ (\textit{F} \textit{Phi80lacZ\DeltaM15} \Delta(lacZYA-argF) \textit{U169 deoR recA1 endA1 hsdR17(\textit{rK mK}) phoA supE44 \Delta\textit{thi-1 gyrA96 relA1}). Bacteria were grown in Luria–Bertani (LB) broth at 37 °C. Competent \textit{B. subtilis} cells were prepared and transformed as previously described (Itaya, 1999b). Tetracycline (10 \textmu g/ml), chloramphenicol (5 \textmu g/ml), kanamycin (5 \textmu g/ml), spectinomycin (50 \textmu g/ml), and neomycin (Nm, 3 \textmu g/ml) were added to growth medium for selection of transformed \textit{B. subtilis}. Ampicillin (50 \textmu g/ml) was added to the growth medium for selection of transformed JA221, JM109 and DH5\textalpha. Plasmids are described in Fig. 1.

2.2. A \textit{B. subtilis} strain as a BGM vector

BEST7003 has two additional sequences integrated into the \textit{B. subtilis} derivative. One is a pBR322 sequence at the \textit{NotI} site of the \textit{proB} gene, designated \textit{GpBR}, and the other is a Nm resistance gene expressed under the lambda Pr promoter (Pr-neo) at the \textit{ypdQ} gene (Itaya, 1999a). Pr-neo expression confers Nm resistance on BEST7003. The repressor \textit{cl} gene,
3. Results and discussion

3.1. Direct cloning of mouse mtDNA in a BGM vector

The method used for cloning the full-length mouse mtDNA in the BGM vector is outlined in Fig. 1A. The BGM cloning system requires a set of two LPSs flanking and thus specifying the target DNA region to be cloned. To clone full-length mouse mtDNA, we obtained LPS1 (2141 bp, nts 918 to 3058; Fig. 1B) and LPS2 (2058 bp, nts 3059 to 5116; Fig. 1B) from template mtDNA by PCR amplification. LPS1 was amplified using primers 1F 5′-CCCAAGCTTCTCCTCAATTAATTAACCTAACATAAT-3′ and 1R 5′-CCCAAGCTTCAAATCCTAGGGT-TAAATATTATG-3′. The resulting PCR fragment was digested with HindIII and inserted into the HindIII site of pCISP310B, creating plasmid pLPSm101 (Fig. 1A, ii). Similarly, LPS2 was amplified using primers 2F 5′-GGAAAGATCTATTATTTTGCACATCTCAGCAG-3′ and 2R 5′-GGAAAGATCTCGATGTTTTAATCTCAGCAG-3′. The resulting fragment was digested with BgII and inserted into the BamHI site of pLPSm101, creating plasmid pLPSm102 in E. coli (Fig. 1A, iii). A DNA segment including the aligned (LPS1)-(I-spcc)-(LPS2) sequences of pLPSm102 was inserted into the genome of BEST7003 via a double cross-over event with the pBR322 sequences located at the GpBR locus (Fig. 1A, i–vi). The BEST7003 integrated by pLPSm102, namely BEST7686, was selected for by resistance to spectinomycin. Strain BEST7686 (Fig. 1A, vi) exhibited sensitivity to tetracycline, due to loss of the tetracycline resistance gene by gene replacement, and sensitivity to Nm, due to repression of Pr-neo expression by the I repressor protein. Transformation of BEST7686 with 61 μg mtDNA prepared from adult mouse liver produced hundreds of Nm-resistant colonies (Fig. 1A, vii–ix). Screening for spectinomycin sensitivity eliminated the false-positives frequently associated with this counter-selection protocol (Itaya, 1999b; Itaya et al., 2001; Uotsu-Tomita et al., 2005), and finally yielded one clone, BEST7688, as a candidate recombinant (Fig. 1A, ix). In Section 3.3, we examined a reason why only one clone was obtained despite the use of 61 μg mtDNA. In spite of the low frequency of recovery of recombinants, a 16.3-kb DNA clearly resolved by CHEF gel electrophoresis was isolated from the BEST7688 genome upon I-Ppol digestion (Fig. 1E lanes 4 and 5). Southern hybridization of the BamHI, EcoRI, HindIII, and PstI digests of BEST7688 genomic DNA using BALB/c mouse mtDNA as a probe demonstrated that the mtDNA integrated at the GpBR locus of BEST7688 was indeed full-length (data not shown).

3.2. Recovery of mouse mtDNA from BEST7688

3.2.1. Recombinational transfer of mtDNA to a ccc plasmid

The cloned mtDNA integrated into and replicated as part of the 4.2-Mb B. subtilis genome would be of great use if it could be recovered in covalently closed circular (ccc) form and

![Diagram of mtDNA integration and Southern hybridization](image-url)
transferred to another host, such as *E. coli*. A method was established to transfer DNA segments cloned within the GpBR locus to a *B. subtilis* plasmid with θ-type replication (Tsuge and Itaya, 2001). This method, copying DNA from genome to plasmid in *E. coli* in vivo, is briefly outlined in Fig. 1A (x–xii) and is termed BReT, for Bacillus recombinational transfer. The BReT plasmid pGETS109 (Tsuge et al., 2003; Tomita et al., 2004) was linearized with HindIII (Fig. 1A, xi) and taken up by competent BEST7686 cells (Fig. 1A, xii). Linearized pGETS109 is unable to form a replicative structure and therefore can propagate only if the HindIII gap is filled by an *in vivo* marker rescue, illustrated in Fig. 1A xii, analogous to the process described previously (Weinrauch and Dubnau, 1983). Among several colonies selected for tetracycline resistance, at least one clone examined, BEST7689 (Fig. 1A, xiii), harbored an expected plasmid, pYMM101.

### 3.2.2. mtDNA in *E. coli*

Because pGETS109 carries the *E. coli* plasmid pBR322 sequence and is therefore able to replicate in *E. coli*, the 28-kb pYMM101 was introduced into *E. coli* strain JA221 by electroporation (Fig. 1A, xiv). We found no apparent growth reduction of the *E. coli* transformants, consistent with the observation of Yoon and Koob (2003). Restriction enzyme analyses of pYMM101 recovered from *E. coli* confirmed the plasmid's expected structure (Fig. 1F, lanes 6 to 12). Perhaps the *ND1* gene may be an easy site of full-length mtDNA cloning, since cloning vectors used in this study and in the report of Yoon and Koob (2003) were inserted into the *ND1* gene.

To identify where homologous recombination occurred between the mouse mtDNA and the LPS1 and LPS2 target regions upon transformation of BEST7686, we compared the LPS1 and LPS2 nucleotide sequences carried on pYMM101 with those on plPSm102 (Fig. 2). We found several nts on plasmid plPSm102 that differed from the corresponding mouse mtDNA sequence (accession no. AB042432): six differences in LPS1 and five in LPS2 (Fig. 2). These nt differences on plPSm102 may be the result of errors introduced by PCR amplification during LPS construction. Two changed nucleotides (G2591 and G2907) at the distal end of LPS1 were transmitted to pYMM101 prepared in *E. coli*, but four reverted to the published sequences, presumably by gene replacement with mouse mtDNA. Similarly, four changed nts at the distal end of LPS2 were transmitted to pYMM101, whereas C4611 reverted to T. Because homologous recombination occurs within LPS1 and LPS2, these results indicate that resolution of Holliday junctions formed during homologous recombination occurred at regions between 1887 and 2591 in LPS1 and between 4106 and 4611 in LPS2 (Fig. 2).

Mouse mtDNA carries genes that encode 13 proteins, two rRNAs, and 22 tRNAs, all of which are critical components of the electron transport chain and the biogenesis of mouse mitochondria. Nuclear genes encode all of the other genes needed for the biogenesis, maintenance, and regulation of this organelle. We observed no detrimental effects on bacterial growth by the cloned mtDNA carried at single- or low-copy levels in *B. subtilis* strain BEST7688 or BEST7689 or in *E. coli* carrying pYMM101. Upon DNA sequence analysis of pYMM101 isolated from *E. coli*, we found five substituted nts in mtDNA genes *ND1* (nts 2907 and 3225) and *ND2* (nts 4034, 4106, and 4611). Substitutions at the first 4 of these positions result in codon changes of methionine to valine, serine to proline, isoleucine to valine, and threonine to alanine, respectively. The other substituted nts are present in rRNA and tRNA genes. We did not pay particular attention to sequence similarity with sequences in the pubic databases. Rather, application of a pinpoint repair on the newly cloned segment in BGM vector (Itaya et al., 2003, 2005), or preparation of sequence-determined LPS prior to integration will be of great value.

### 3.3. Systematic investigation of cloning efficiency

Mouse mtDNA prepared from liver exhibited multiple bands on CHEF gel electrophoresis (Fig. 1C, lanes 1 and 2), indicating that most of the mtDNA remained circular, some in polymeric form. According to the *B. subtilis* transformation mechanism (for reviews, see Dubnau, 1993, 1999), protein complexes are formed on the surface of a competent cell, bind to added double-stranded DNA, introduce nicks, and transport single-stranded DNA to the cytoplasm, where the DNA is integrated into the genome via the cell’s recombination-proficient genetic pathways. The low frequency of BGM cloning of mouse mtDNA in the present study—only one clone obtained—seemed unusual compared with our previous BGM cloning reports (Itaya et al., 2001, 2005; Kaneko et al., 2003). Moreover, our preparation of mouse mtDNA contained uncharacterized DNA and RNA molecules (Fig. 1D, lane 3, region marked by an asterisk). These observations prompted us to investigate two possible causes for the low frequency of mtDNA cloning: DNA quality and competition by impurities in the DNA preparation.

We first established a standard mtDNA transformation assay in BEST7686 by which transformation efficiency could be quantitatively measured. We used the mtDNA fragment prepared from plasmid pYMM101 by I-Ppo1 digestion (Fig. 1A, xv); using the fragment eliminates the polymeric forms (see Fig. 1C, lanes 1 and 2) and simplifies interpretation of the transformation results. Our preparation of pYMM101 DNA purified from *E. coli* possessed relatively few nicks. *B. subtilis* transformsants equivalent to strain BEST7688 (Fig. 1A, ix) were obtained in a series of transformation experiments using 0.02, 0.2, 2, and 20 μg pYMM101, yielding transformation efficiencies (transfectants per μg DNA used) of 19,589 ± 3056.17 (0.02 μg), 3521.1 ± 524.67 (0.2 μg), 476.9 ± 28.03 (2 μg), and 53.1 ± 5.66 (20 μg). The measured reduction in transformation efficiency with increased DNA amount might be accounted for by the saturation of a limited number of competency-specific protein complexes; a single *B. subtilis* cell produces approximately 50 protein complexes in the membrane during competence development (for reviews, see Dubnau, 1993, 1999). The high transformation efficiency we obtained using DNA with relatively few nicks indicated that the low transformation efficiency we obtained using mtDNA prepared from mouse liver—only one transformant—was attributable to nicks
introduced in the mtDNA by oxidative stress in mouse mitochondria or during purification from mouse liver. This observation is consistent with our previous finding that cloning efficiency is increased by using damage-free DNA (Kaneko et al., 2005).

We next examined the effects of a second possible cause of low transformation efficiency, namely the presence of impurities in the DNA preparation such as those observed in Fig. 1D, lane 3. Using the standard transformation assay described above, we conducted a competition experiment in the presence of E. coli JA221 genomic DNA as an impurity and potential competitor. We transformed BEST7686 with 0.02 μg of E. coli JA221 genomic DNA. However, none of the Nm-resistant colonies we obtained were equivalent to strain BEST7688, being instead false-positives. This result indicates that the efficiency of correct recombinant formation is dramatically reduced by the presence of competitor DNA, although we did not further characterize the impurity in our mouse liver mtDNA preparation.

3.4. Significance of full-length mouse mtDNA cloning

Stable cloning in the BGM vector has been demonstrated for DNA segments larger than 100 kb isolated from, for example, mouse (Kaneko et al., 2003) and Cyanobacterium complete genome (Itaya et al., 2005). Furthermore, the size of DNA that can be cloned using the BRET method also exceeds 100 kb (Tsuge and Itaya, 2001; Kaneko et al., 2003, 2005). Thus, cloning and manipulation via the BGM vector cloning system offers a general strategy for the preparation of far larger reconstructed DNA segments than have been previously considered. Although the unit size of the mouse mtDNA genome is far smaller than 100 kb, its full-length stable cloning in E. coli was only recently accomplished through the pioneering work of Yoon and Koob (2003), who inserted a transposon-cassette into mouse mtDNA in vitro prior to transfer into E. coli. Compared with their transposon-based method, our BGM cloning method has the potential to provide more variation by allowing researchers to choose LPSSs that specify the final construct.

The high fidelity of cloned mtDNA structure through the use of BGM cloning and BRET recovery in the present study is consistent with our previous studies, in which no structural alterations were observed in the cloning of various non-cognate DNAs (Itaya, 1995; Itaya et al., 2000; Tsuge and Itaya, 2001; Kaneko et al., 2003, 2005). The mitochondrial genome remains critical for normal mitochondrial function, and mutations in this genome are known to cause a wide range of human diseases (for reviews, see Wallace, 1999; Simon and Johns, 1999). The complete high-fidelity cloning and flexible manipulation of mammalian mitochondrial genomes would represent an advance in the current experimental systems (Inoue et al., 2000; Ono et al., 2001; Nakada et al., 2001), including computational systems biology of mitochondrial metabolism analysis.

The size and gene content of plant mitochondrial genomes can vary significantly from strain to strain and species to species (for review, see Palmer et al., 2000). In addition, isolation of pure, unsheared circular mtDNA from plants is difficult owing to the high frequency of inter- and intra-mitochondrial re-arrangement (for review, see Palmer et al., 2000). The method developed in this study may be generally suitable for the cloning of complete organelle genomes.

4. Conclusions

The full-length mtDNA from mouse liver was directly cloned into a BGM vector. The complete cloned mtDNA was recovered in ccc plasmid form (BRET method) and transformed into E. coli. We found no apparent growth reduction of the E. coli transformants. The cloned mtDNA was stable in the E. coli. For high efficiency of transformation, DNA with relatively few nicks and without impurities has to be obtained in the method.

Acknowledgement

This work was supported by Grant-in-Aid for Creative Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to J.-I.H.).

References


Itaya, M., 1997. Physical map of the Bacillus subtilis 166 genome: evidence for the inversion of an approximately 1900 kb continuous DNA segment, the translocation of an approximately 100 kb segment and the duplication of a 5 kb segment. Microbiology 143, 3723–3732.


Itaya, M., Tsuge, K., Koizumi, M., Fujita, K., 2005. Combining two genomes in one cell: stable cloning of the Synechocystis PCC6803 genome in the


