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Production of Multi-Purpose BAC Clones in the Novel *Bacillus subtilis* Based Host Systems

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1. Introduction

1.1 A brief history of BACs

The BAC vector that replicates in *Escherichia coli* K-12 was first introduced in 1992 by Shizuya et al. More detailed information on current BAC applications may be referred to other chapters of this book. Because BACs can accommodate much longer DNA stretches than the plasmids for *E. coli* available at that time, as large as >100 kbp, they were suitable for the preparation of DNA libraries covering entire cellular genomes regardless of their original size (Fig. 1a). The initial goal for BACs was to provide materials for whole genome-sequencing projects and
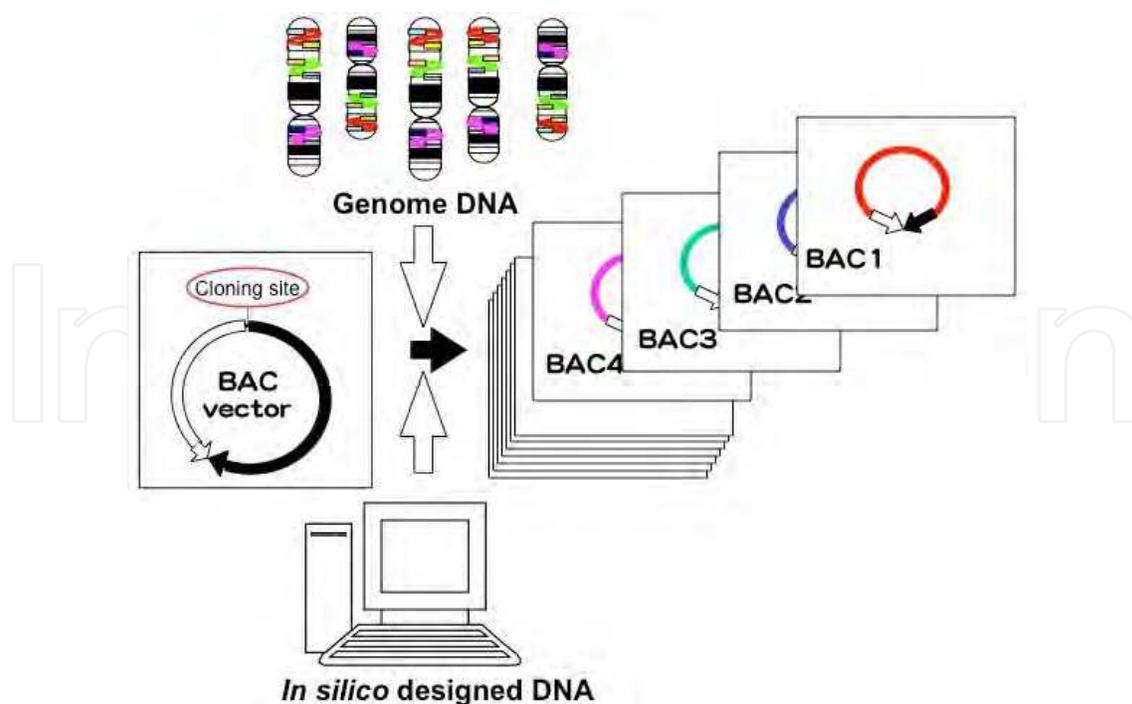

Fig. 1a. BAC library for the contiguous genomic DNA. The ability to carry large DNA (>100kb) is useful for not only the direct analysis of genomic regions in reverse genetics, but also for the *de novo* synthesis of genomic DNA in the field of synthetic biology.

their contribution to long-range sequence determinations has been demonstrated (Frengen et al., 1999; Osoegawa et al., 2001). Cutting-edge technologies that facilitate direct genome sequencing have dramatically reduced the need for BAC libraries as a sequencing resource.

1.2 BAC applications

The *de novo* synthesis of small DNA fragments has become routine although it remains expensive (Gibson et al., 2008, 2010; Itaya et al., 2008; Itaya, 2010). In that process, commercial enterprises customize desired DNA fragments based on nucleotide sequence information provided by the end-user. Larger DNA, equivalent to lengths clonable in BACs, can be prepared routinely by assembling small DNA fragments (Gibson 2011; Itaya & Tsuge, 2011). Reverse genetics methods applied in studies involving cultured cells and model animals are increasingly important in mutation research (Yang et al., 1997; Hardy et al., 2010). DNA can now be obtained by *de novo* synthesis using designed sequences or by flexible engineering of cloned DNA in BACs. Although BACs can accommodate DNA fragments longer than 100 kbp, the intrinsic physicochemical features of long-stretched polymer molecules render them fragile and their handling difficult. Due to the shearing force in liquids, DNA fragments easily break into small pieces and nuclease contamination may be introduced in the course of biochemical isolation procedures. Therefore care must be taken in the isolation and purification of large DNAs (Kaneko et al., 2005) and appropriate host cell systems are needed to nurture and protect the fragile DNA fragments to facilitate the preparation of undamaged DNA samples regardless of their size.
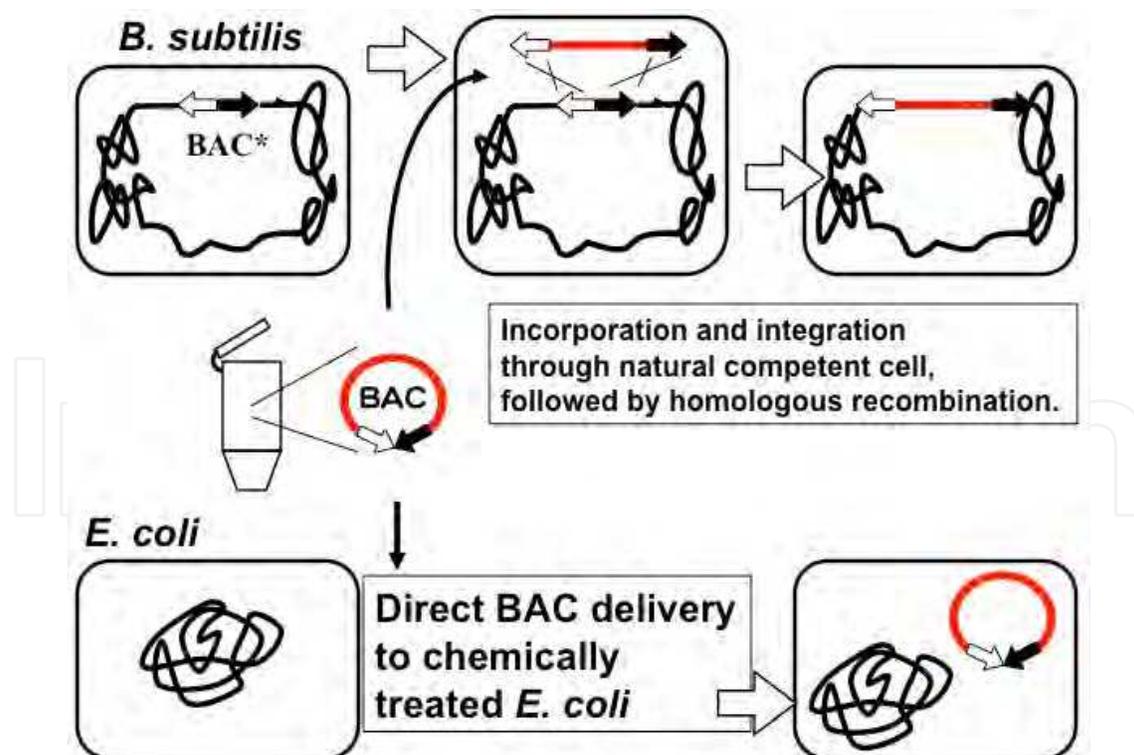

Fig. 1b. *B. subtilis* as a new BAC dealer.
The integration of BAC inserts into the genome of *B. subtilis* is a starting point for subsequent manipulations. The BAC vector region (**BAC*)** preinstalled in the host genome provides the cloning site for guest BAC clones via homologous recombination (identified by X).

1.3 *E. coli* - **An original host and an engineering system for BACs: Size limits for stable cloning**

Of particular import with respect to the direct application of the BACs carrying genes of interest is their modification to render them useful for in-depth reverse genetics research. Some examples on current application and molecular engineering of BAC vectors can be found in other chapters of this book. Currently-available systems for handling large DNA in *E. coli* make it possible to carry out pin-point engineering on DNA carried in BACs. The BAC clone is initially maintained in autonomously-replicating plasmid form in *E. coli*. The prepared BACs are then used for delivery to an *E. coli* BAC-engineering system (Fig. 1b). Genetic manipulations on BACs are summarized in Fig. 2. The maintenance of BACs in *E. coli* requires selection by antibiotics during all stages, as is the case with other plasmid vectors.
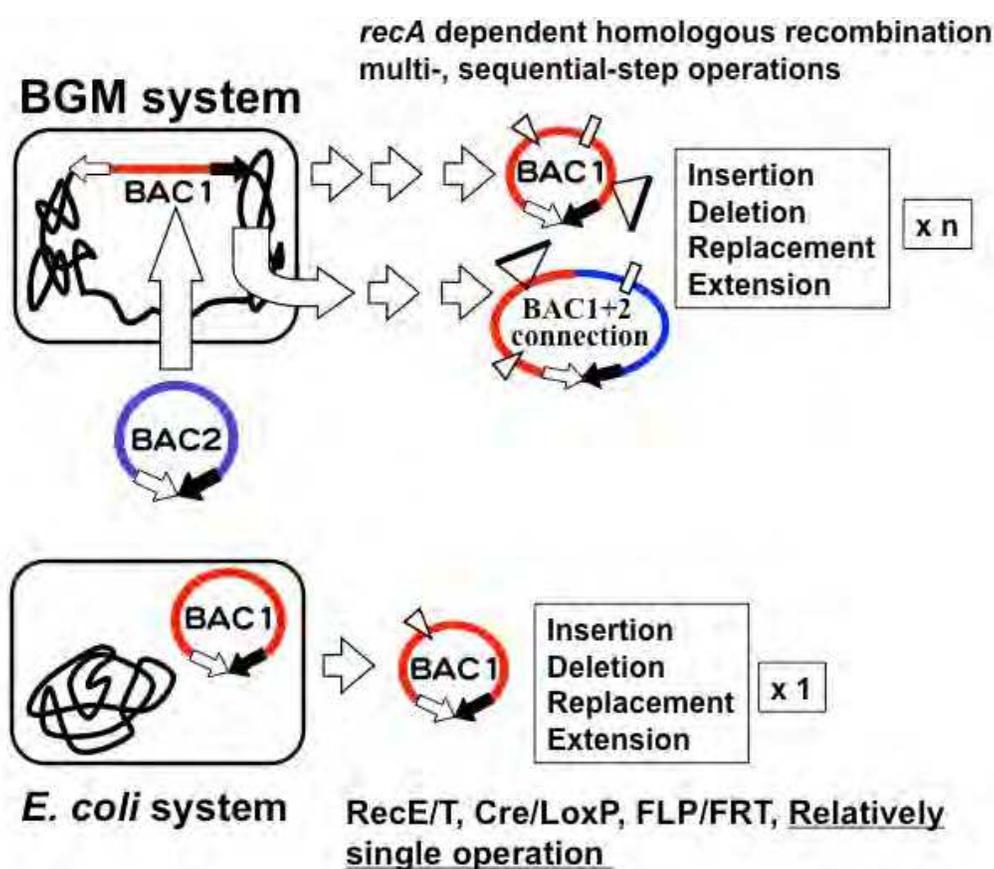

Fig. 2. The *B. subtilis* Genome (BGM) system.
Multiple manipulations of large BAC-DNA fragments are possible in BGM; in the *E. coli* system only single manipulations can be performed. The stability of DNA attributable to the one-copy state of the *B. subtilis* genome assures the maneuverability of *recA*-dependent homologous recombination. Maneuvers can be repeated unlimited times.

The minimal requirements to ensure the stability of BACs before and after engineering operations in *E. coli* are shown in Fig. 2. There are size limitations for the stable handling of BACs in *E. coli*; it has been suggested that the largest clonable size does not exceed 500 kbp. To date not even the existing minimal genome 585-kbp circular one of *Mycoplasma genitalium* has been successfully cloned in *E. coli* (Gibson et al., 2008, 2010).

2. The emerging *B. subtilis* host

B. subtilis has emerged as an appropriate host to supplement the use of BACs from *E. coli* (Itaya, 2009). Our favorite cloning host, *B. subtilis* 168, yet unfamiliar to many researchers, is a Gram-positive firmicute bacterium. It grows as rapidly and in the same media as *E. coli*. Consequently, many protocols used with *E. coli* can be used with *B. subtilis.* However, features inherent in *B. subtilis* facilitate natural transformation, in addition, the bacterium forms endospores that survive for extraordinarily long periods. *E. coli* lacks these features.

2.1 What is *B. subtilis?* - A more detailed explanation

We will place special emphasis on the use of natural transformation in the engineering of BACs (Fig. 1b). The general homologous recombination associated with *B. subtilis* transformation is distinct from the induced recombination adopted by most *E. coli* DNA engineering systems. The *B. subtilis* 168 strain domesticated in the laboratory was isolated as a recipient that facilitates DNA-mediated transformation (Spizizen, 1958). The subsequent elucidation of its detailed molecular mechanisms revealed that competent *B. subtilis* cells can incorporate extracellular DNA into their cytoplasm via the process of natural transformation (Kidane & Graumann, 2005). It is possible to conduct genetic crosses in *B. subtilis* and like *E. coli* K-12, it has been subjected to extensive biochemical and genetic analyses. Although *E. coli* was thought to accept extracellular DNA via a chemical transformation process, this type of transformation was limited to the plasmid delivery method (Mandel & Higa, 1970; Hanahan, 1983). The cloning in and the propagation of BACs in *E. coli* are subsumed under the term DNA delivery. The process of DNA delivery through the cell membrane of *E. coli* involves elaborate physical and chemical treatments of the host (Fig. 1b). In contrast, the transformation system of *B. subtilis* applies to plasmid delivery as well as genomic gene engineering. Competent *B. subtilis* cells positively grab extracellular DNA and pull the fragment(s) into their cytoplasm in single-strand form. The molecular mechanisms, the processing of double-stranded DNA from the cell surface and its conversion into single-strand DNA for entry through the cellular membrane, are carried out in a concerted manner by several proteins encoded by *B. subtilis* genes (Kovács et al., 2009). This process is too complex to be explained here; in short, *B. subtilis* does, while *E. coli* does not possess a set of genes to conduct natural transformation.

As incorporated BACs cannot replicate in *B. subtilis* in plasmid form because there is no replication origin sequence for this host, BACs must enter cellular recombination pathways initiated by the existing *recA* protein (Kidane & Graumann, 2005). If homologous sequences are present in the *B. subtilis* chromosome (BAC* in Fig. 1b), the incoming BAC is integrated into the homologous region. Such homologous recombination-mediated transformation yields the integrated form at high frequency in the *B. subtilis* genome (Fig. 1b). This well-known natural transformation system for the *B. subtilis* 168 strain has been used in genetic research targeting the original genome. Two striking results were the derivation of a 33-fold mutant of *B. subtilis* by repeating the transformation 33 times to introduce mutations at 33 chromosomal loci (Itaya & Tanaka, 1991) and the reduction of the genome to 75% of its original size by the consecutive deletion of genes unaffected by growth (Ara et al., 2007). Fortuitously *B. subtilis* 168 possesses no original/cognate plasmid.

2.2 Why is the *B. subtilis* host advantageous?

A homologous sequence is the sole requirement for extra DNA-engineering in *B. subtilis.* The transformation process shown in Fig. 2 can be repeated and the number of repetitions is practically unlimited. Consequently, due to its ability to repeat transformation, the assembly of large DNA fragments in the *B. subtilis* genome is possible. Typically, if DNA fragments with partial overlaps are prepared, repetitive integration by using the overlapping regions allows the reconstruction of the original DNA in the *B. subtilis* genome (Itaya & Tsuge, 2011). Even before its whole genome sequence determination in 1997, *B. subtilis* became a workhorse for the cloning and manipulations particularly of giant DNAs that cannot be handled by *E. coli.* As *B. subtilis* forms endospores that manifest significant resistance to vacuuming, dryness, and radiation, it has become a reservoir for giant DNA maintained at room temperature. Additional details will be presented in section 3-4.

2.3 Why the *B. subtilis* host - An unprecedented genome vector

Despite conceptual differences between *E. coli* and *B. subtilis* with respect to the cloning of DNA, *B. subtilis* is highly advantageous because it allows the use of small DNAs prepared by routine molecular cloning in *E. coli*. A high workload can be handled by *B. subtilis* via the integration of DNA fragments cloned once in an *E. coli* pBR322 plasmid or BAC. The plasmid transfer from *E. coli* to the *B. subtilis* genome shown in Fig. 1b renders the *B. subtilis* genome a big cloning vector. The transfer of *E. coli*-borne plasmids to the *B. subtilis* genome is advantageous because after the DNA is stably integrated into the *B. subtilis* genome, it becomes part of its genome. A wide range of genetic manipulations is now possible in *B. subtilis* regardless of the origin of the integrated DNA. The number and effectiveness of tools available for genetic manipulations in *B. subtilis* far exceed the tools available in the *E. coli* K-12 system. They include the faithful and stable insertion and deletion of any DNA at designated loci. Another advantage of *B. subtilis* is its extraordinary stability, this eliminates the selection pressure to maintain DNA. This is in sharp contrast with the stringent requirements for the maintenance of plasmids in *E. coli*. During growth, plasmids that replicate independent of the host genome segregate out without selection pressure (Fig. 1b and Fig. 2). Indeed, we have demonstrated that *B. subtilis* stably accommodated DNAs far larger than those covered by *E. coli* BACs. The largest size was up to 3,500 kb (Itaya et al., 2005). We think that the stability of DNA integrated into the *B. subtilis* genome is attributable to the presence of a single-copy genome per bacterial cell. In subsequent sections we focus on the handling of BACs transferred to the *B. subtilis* genome and their recovery. This vector system can handle DNA manifesting sequence variations such as short- (mouse genome) or long repeats (IR), and different GC contents.

3. Type of engineered BACs in the *B. subtilis* genome (BGM) vector

Hereafter, DNA introduced into the *B. subtilis* host will be called guest DNA. Guest DNA cloned in the host becomes integrated into the host genome. The single-copy genome of each *B. subtilis* cell can accommodate chloroplast and mitochondrial guest genomes (Itaya et al., 2008) and the genome of the bacterium *Synechocystis* PCC6803 (Itaya et al., 2005). *B. subtilis* strains developed as hosts for these DNAs are called BGM vectors, an acronym for *Bacillus* GenoMe vector. The wild-type strain *B. subtilis* 168 was the first BGM vector to host one pBR322 sequence (Itaya, 1993). This sequence was successfully introduced at different genomic loci (Itaya et al., 2005). The integrated pBR322, a 4.3 kbp *E. coli* plasmid, served as a

common cloning locus in a manner reminiscent of the integration by homologous recombination illustrated in Fig 1b. Before the creation of the BAC vector, pBR322 and its derivatives were widely used for various gene-cloning experiments as they offered several advantages, e.g. a small size, a medium-sized copy number, and an ability to carry DNA up to 30 kbp. DNA cloned in pBR322 via the *E. coli* molecular cloning system immediately became guest DNA in the pBR322-based BGM. Integration required only two homologous sequences and appropriate selection markers for the bacterium. The DNA flow from *E. coli* BACs to the BGM vector is shown in Fig. 1b; it is similar to the pBR322-based system but requires major modifications.

3.1 Direct transfer of guest BACs

As commercially- or laboratory-prepared BAC clones carried no antibiotic resistance markers for *B. subtilis*, the first BAC-BGM required the pre-installation of a counter-selection system to stimulate the integration process (see Fig. 3). In our initial experiments on the integration of mouse genomic DNA carried by BACs (Kaneko et al., 2003, 2005, 2009; Itaya et al., 2000), we observed no structural disorder during the integration process despite the short repeats generally present in the mouse genome (Itaya et al., 2000; Kaneko et al., 2003, 2009). In addition, the BGM stably carried the 25-kbp-long inverted repeats present in the rice chloroplast genome (Itaya et al., 2008). Consequently, we thought that the BGM could accommodate not only very large-sized DNA but also a wide range of sequence variations from other genomes.
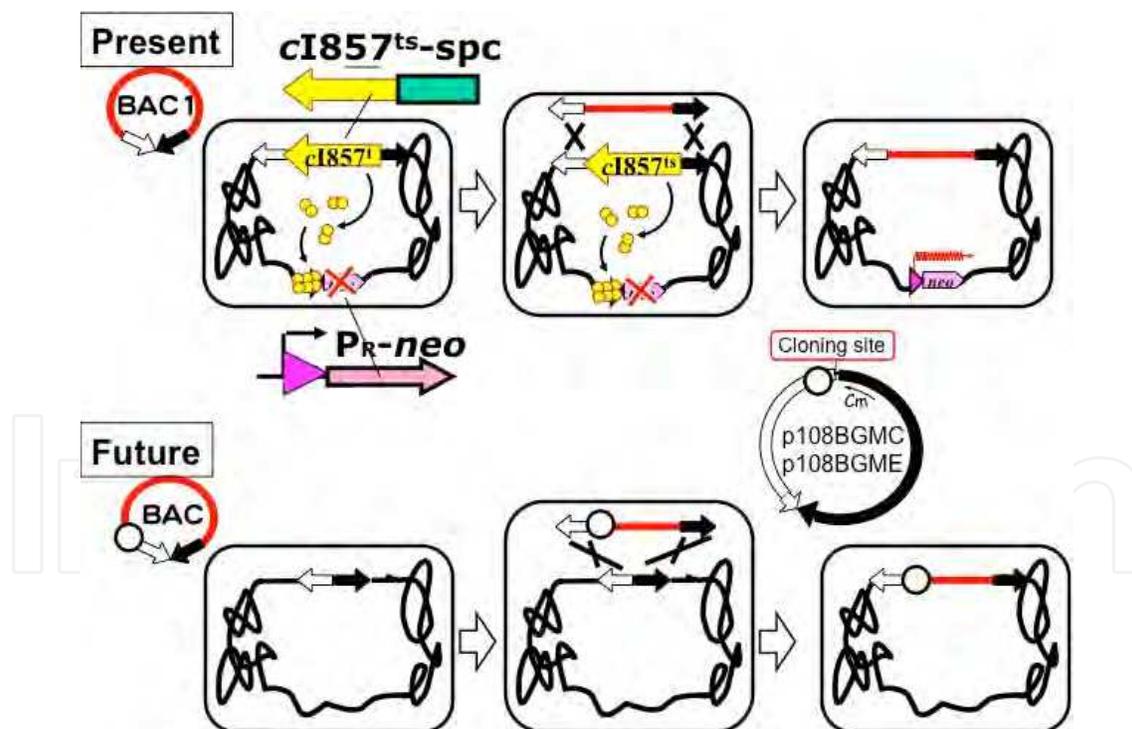

Fig. 3. Cloning of BACs carrying a non-marker for *B. subtilis.*
Top: The present counter selection system is shown. The *c*I repressor gene and the neomycin-resistance gene under the Pr prompter result in the positive selection of marker-less BACs for integration.
Bottom: BAC clones in the new BAC vectors, p108BGMC or p108BGME, carrying an antibiotics marker for *B. subtilis* can be cloned directly in BAC-BGM.

After BAC clones were regularly used both in *E. coli* and *B. subtilis*, the next step was to elaborate the engineering/manipulation of the DNA. Figure 4 illustrates the design and modification of nucleotide sequences inside the guest DNA. Examples are detailed below; various size ranges in section 3-2, connecting two overlapping BAC clones in 3-3, applying genome techniques developed for *B. subtilis* in 3-4, and the unique preservation of designed BACs in BGM for prolonged storage in the absence of special facilities in 3-5.
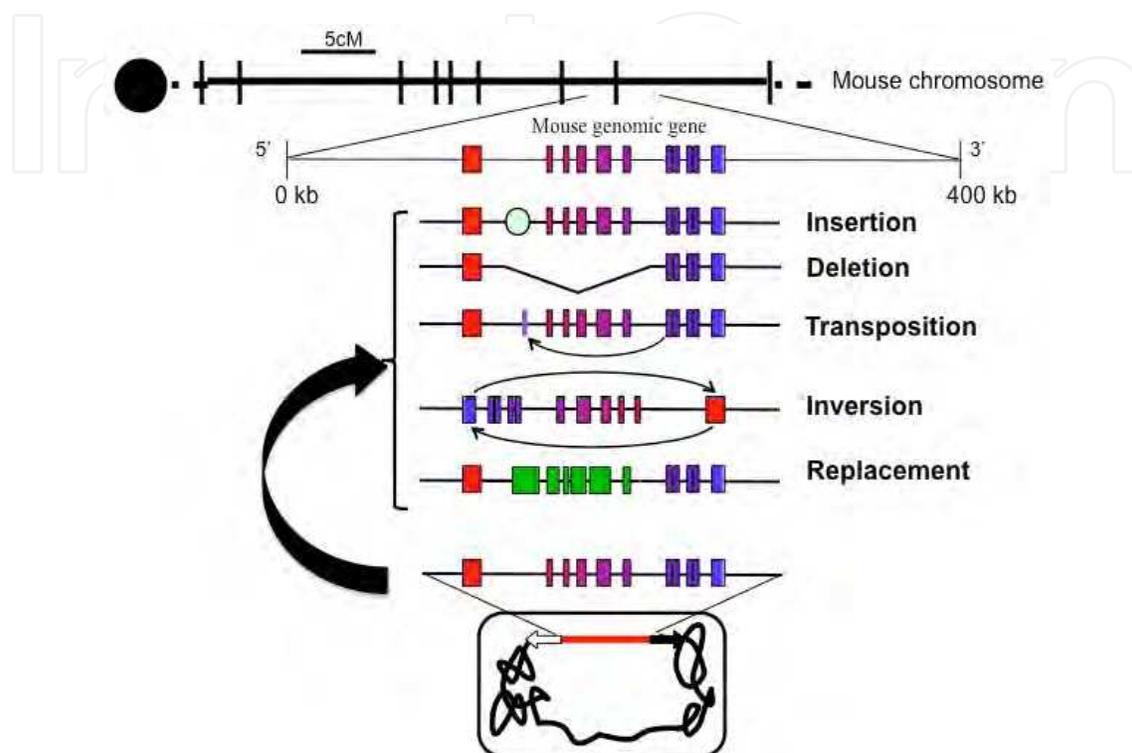

Fig. 4. Manipulation of the guest DNA.
A giant mouse genomic region integrated, for example, into BGM can be re-designed to the indicated structures by relying on the high fidelity of homologous recombination in *B. subtilis*.

3.2 Direct manipulation of guest BACs

As guest DNA replicates as part of the host genome, sequence modifications/conversions targeting regions in the *B. subtilis* genome are possible (Fig. 4). In addition to sequence conversions or small DNA insertions, the formation of systematic deletions was examined for a guest DNA derived from the mouse genome. The protocol to induce deletion shown in Fig. 5a applies a method that is the reverse of the method for integration; it uses two homologous recombinations. Two small DNA segments with deletion endpoints are connected so as to flank appropriate antibiotic resistance markers for *B. subtilis*. The region to be deleted was replaced by a marker gene via two homologous recombinations at the two flanking segments. Kaneko et al. (2003) succeeded in inducing four deletions ranging from 11 to 86 kbp in the mouse *jumonji* (*jmj*) gene locus (Fig. 5b). The preparation of DNA tools by using conventional gene engineering technologies in *E. coli* plasmids and their introduction into competent BGM are now routine. We do not think that such designed and well-controlled deletion formation is possible using standard BAC manipulation kits developed for *E. coli*. Other auxiliary tools, antibiotics resistance genes, and rare-cutting endonucleases, to apply and improve these protocols are presented in Chapter 4 below.
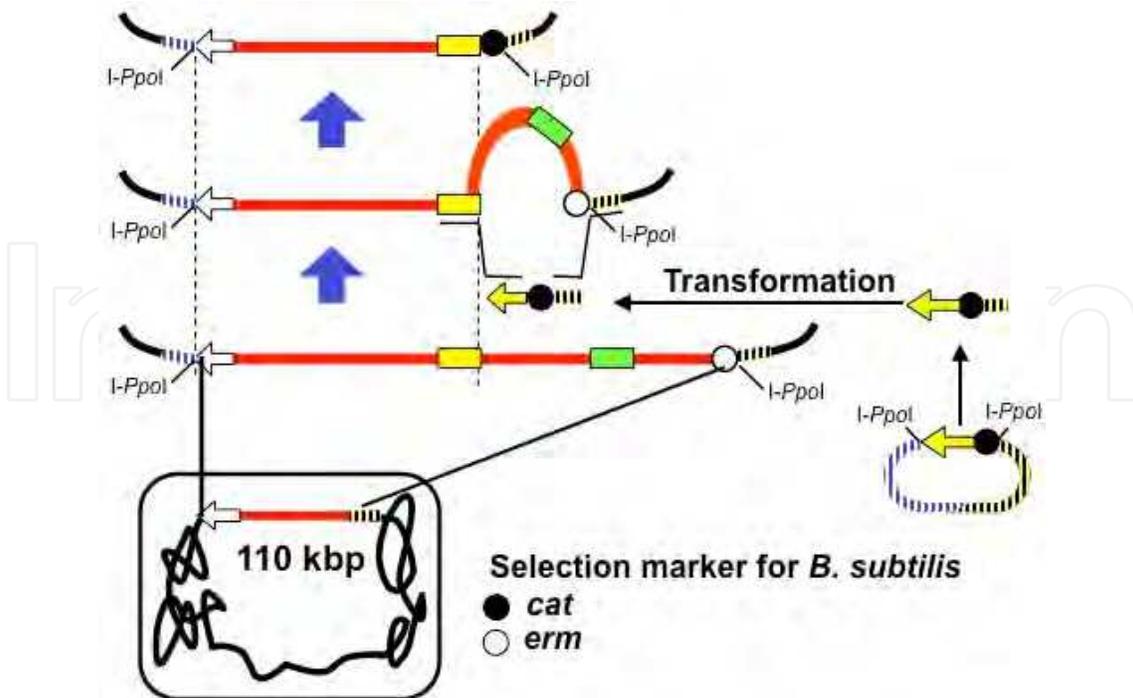
Fig. 5a. Concept underlying the formation of deletions.
Transformation using the optional small DNA fragments and antibiotic resistance markers
for *B. subtilis* produces the designed deletion formation via homologous recombination.
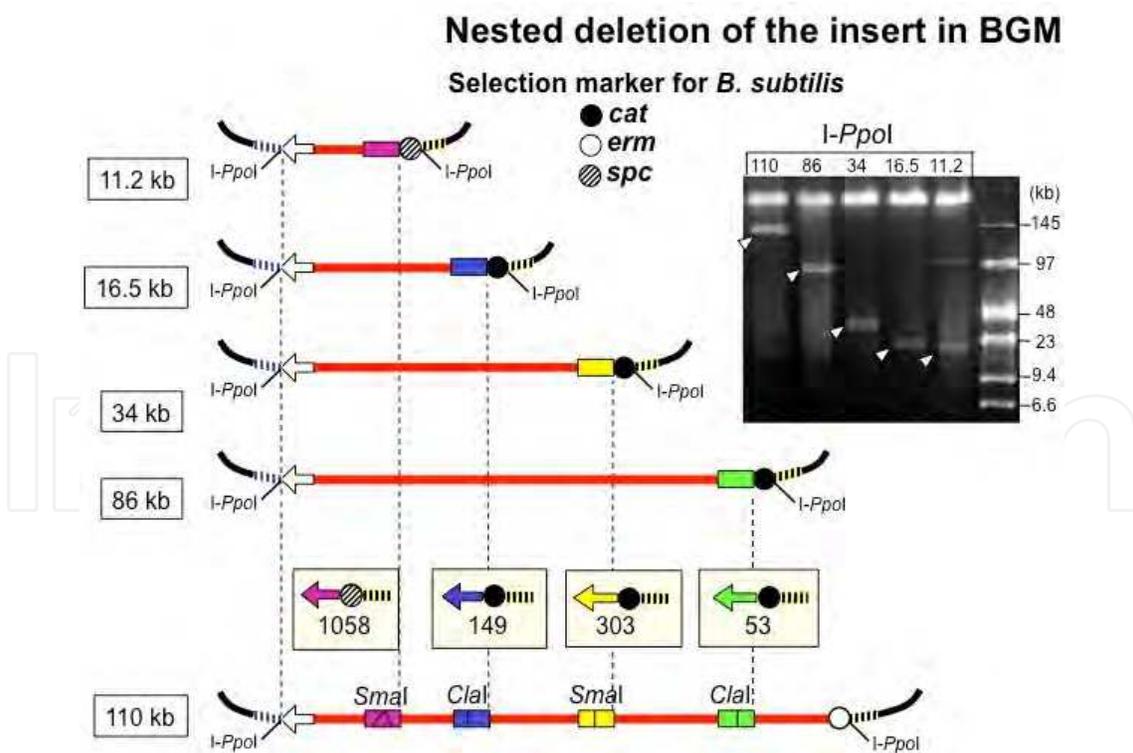

Fig. 5b. Systematic deletion formation from a mouse genomic region (110kb).
The BGM system makes it possible to conduct massive and systematic deletions. The picture
on the right includes I-*Ppo*I fragments resolved by gel electrophoresis (open arrowheads
with the size of the deletion indicated on the left). The BGM vector (4.2 Mb) migrates slowly.

3.3 Connection of two adjacent BACs in BGM

Itaya et al. (2008) documented that the integration of two partially overlapping fragments is possible in BGM. Overlaying the second on the first fragment in BGM resulted in elongation or connection. Each fragment is called a domino. If serial dominos are toppled, or all dominos are connected in BGM, reconstruction of the full-length guest DNA covered by these dominos completes. This concept was first realized by using dominos made in the pBR322-based plasmids, pCISP401(*cat*) and pCISP402(*erm*) in *E. coli*. Up to 31 dominos designed to completely cover the 135-kbp rice chloroplast genome produced a reconstructed full-length guest DNA (Itaya et al., 2008). The use of BACs instead of pBR322 should work in a similar manner. The scenario for connecting two adjacent BACs in a BGM vector is shown in Fig. 6a. However, selection markers present a problem in the preparation of BAC-dominos for immediate use. pBR-dominos are commonly prepared from PCR products of less than a few dozen kbp. However, BACs normally carry 100-kbp DNA fragments that exceed the limit of PCR-mediated amplification. Therefore at present we are forced to use BAC clones, such as commercially-available mouse genomic BAC libraries. However, their BAC vector does not possess *ab initio* selection markers for *B. subtilis*.
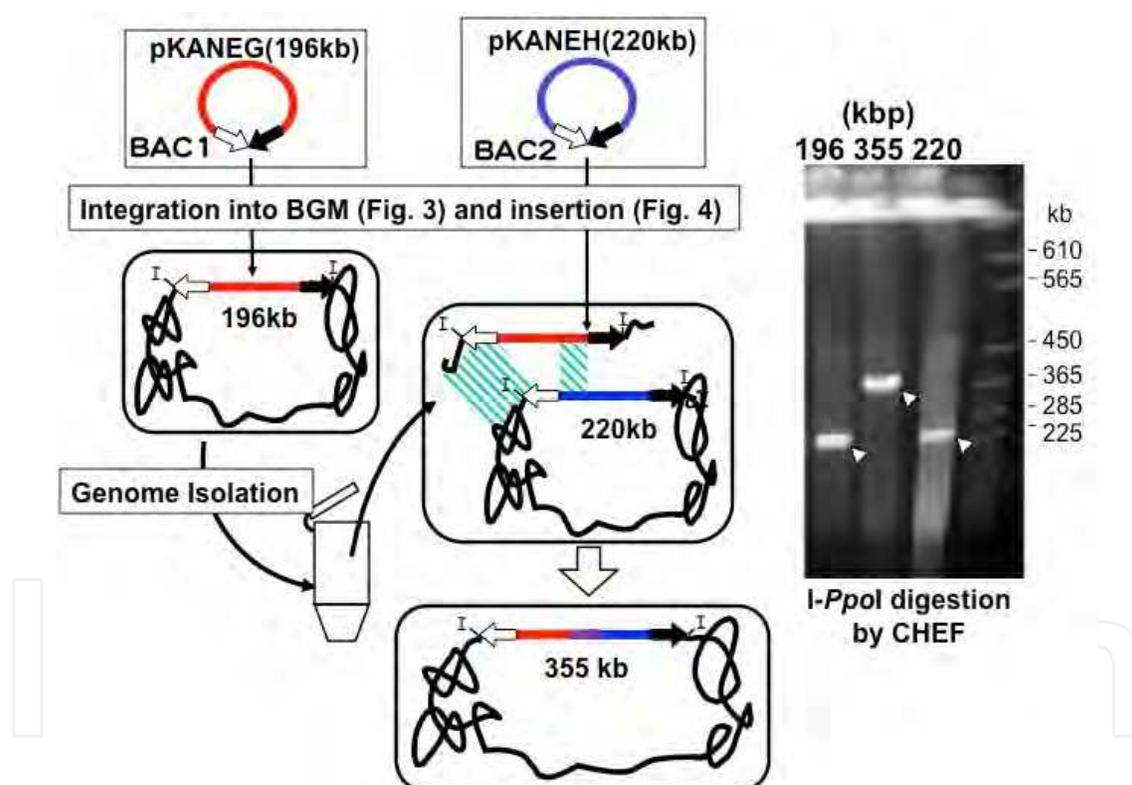

Fig. 6a. Connecting two adjacent BACs in BGM.
Two BAC clones, pKANEG (196 kb) and pKANEH (220 kb), cover the mouse genomic *jmj* region; there is a 60-kb overlap sequence. Each BAC was individually integrated into BGM. Transformation of the BGM carrying the 220-kb BAC2-DNA by using purified genomic DNA from another BGM carrying the 196-kb BAC1-DNA leads to homologous recombination between the 60-kb overlapping region and the sequence shared with the *B. subtilis* genome portion (see the splice boxes). This results in the production of a reconstructed mouse genomic *jmj* region (355kb). BAC insertion after I-*Ppo*I digestion was confirmed by agarose gel electrophoresis (right panel). The I-*Ppo*I recognition sequence is indicated by I.
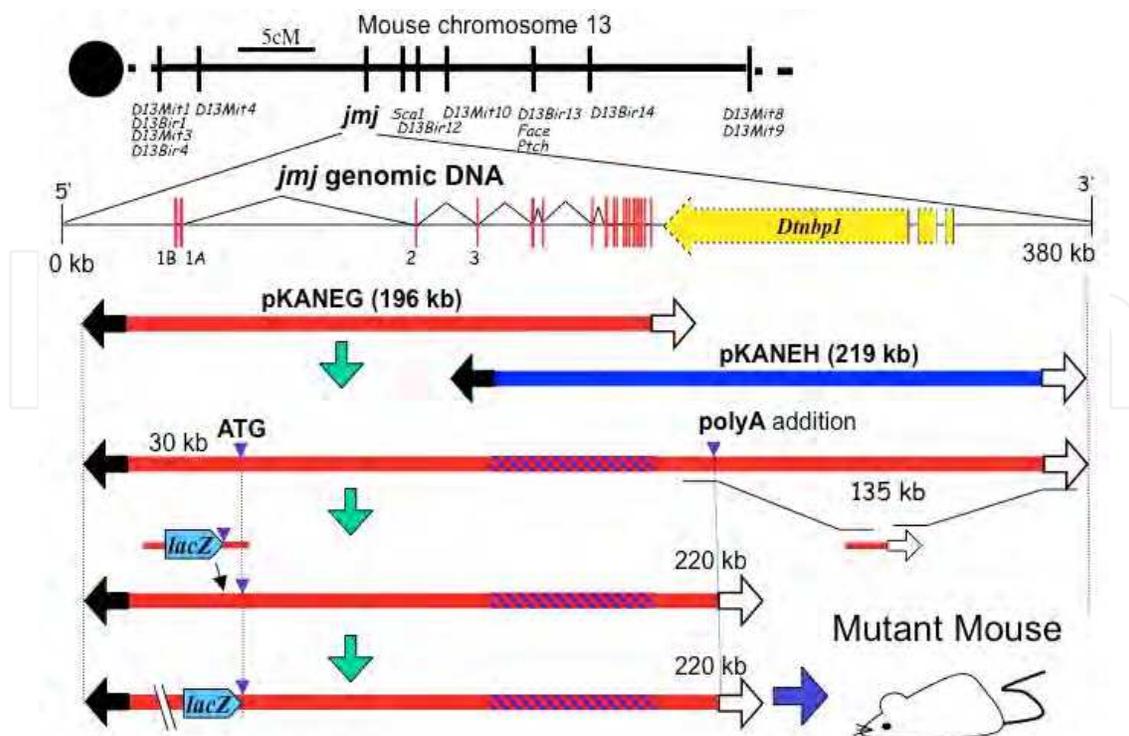
Fig. 6b. Summary of the current achievements made with the mouse a *jmj* gene.

As shown in Fig. 6b, the connection of two sequential guest BACs was first demonstrated for the two BACs covering the mouse gene *jumonji* locus (Kaneko et al., 2009). The two inserts, 196 kbp- and the 220 kbp guests of BGM, shared DNA approximately 60 kbp in size. While the sketch illustrating the connection looks simple, there are difficulties in using marker genes for *B. subtilis*. The combinatorial use of antibiotic markers, already reported by Kaneko et al. (2009) is omitted in Fig. 6a. Instead, the difference from the pBR322-based domino-connection is clearly shown: total genomic DNA isolated from one domino BGM was added to competent cells of the second domino BGM to force double homologous recombination. The transformants obtained by rational antibiotics selection produced a 355 kb-long connected BAC (Kaneko et al., 2009). A summary of our current achievements with the mouse *jmj* gene is presented in Fig. 6b; the most time- and labor-consuming step is the integration of unmarked BACs into the BAC-BGM. Besides these two elaborated experimental works using regular BACs, we have prepared BAC vectors that are designed to accomplish versatile aims in the BGM system. The two new BAC vectors, p108BGMC(*cat*) and p108BGME(*erm*), feature the *B. subtilis* markers shown in Fig. 3. Their presence should facilitate the domino-mediated elongation/reconstruction of BAC-based guest DNA (our unpublished data).

3.4 Implementation of sequence engineering in BACs (inversion)

What can we do with guest DNA? Figure 4 presents a list of possible modifications. Among them, techniques to induce the inversion of guest DNA appears as important as elongation. It is difficult to regulate the orientation of the inserted DNA in BACs. As this difficulty is frequently encountered at the construction of random BAC libraries, tools are needed to invert the insert present in BGM. The method and timing for the induction of large regions of the host *B. subtilis* genome have been described (Toda et al., 1996; Kuroki et al., 2007); they were applied to reverse the orientation of BAC inserts in BGM. Two partially overlapping

fragments [ne] and [eo] derived from a neomycin resistant gene [*neo*] play an essential role; [ne] and [eo] are inserted at the terminus of the insert to be inverted in the BGM. Because these two fragments have an identical region designated [e], homologous recombination here produces two segments, [neo] and [e], that accompany the inversion of the intermediate insert between [ne] and [eo] (Toda et al., 1996). The inversion formation is always associated with the formation of [neo] and can be monitored by resistance to neomycin. This manipulation, theoretically simple but complex in its application, is one of the key technologies for BGM. We have already applied this tool to BACs covering other mouse genomic regions (unpublished findings).

3.5 Long-term storage of guest BACs in *B. subtilis* endospores

The BGM derived from *B. subtilis* is capable of forming spores reminiscent of plant seeds. Spores survive for a long time in unfavorable environments including aridity (Nicholson et al., 2000, 2002; Nicholson & Galeano, 2003; Takahashi et al., 1999; Benardini et al., 2003). *B. subtilis* spores readily start to germinate when moved back into nutrition-rich broth and start to grow immediately. We examined the stability of DNA inside spores (Kaneko et al., 2005) and found that the spores are stable for year at room temperature without requiring special devices. BGM spores may not be of value for use with small DNA that is synthesized and prepared routinely. However, as the size of the guest DNA increases, these spores should become more valuable. BGM spores should provide a low-cost, long-term reservoir for guest DNAs.

4. Heavily engineered BACs for downstream pipelines

Engineered DNA molecules must be delivered to other host systems for diverse applications. The use of BACs for purposes other than sequence determinations, for example, their use in mutagenesis studies on animal models was of limited success (Yang et al., 1997) because complex animal genes range from dozens to hundreds of kbp. They include factors, promoters, 5'untranslated regulatory sequences, introns, exons, splicing- and alternative splicing sites, 3'long terminal repeats, and polyA addition sites (Fig. 6b). Functional accessory sequences contain encoding small RNAs and non-coding RNA, and are regulated by, for example, methylation, histone-binding, and nucleosomes. The study of these complex mammal genes by reverse genetic approaches requires systems that facilitate the delivery of larger DNA than is used for microbial reverse genetics where gene units tend not to exceed kbp orders. For such studies, engineered BACs in BGM must be retrievable. A few methods for retrieving DNA from the *B. subtilis* genome are summarized in Fig. 7, three such methods have been applied to retrieve engineered BACs.

4.1 Fragment isolation using sequence-specific endonucleases

The simplest and most straightforward method involves (i) digestion by special endonucleases and (ii) subsequent isolation/purification of the engineered BAC. The use of two endonucleases facilitates the recognition of extremely infrequent sequences. They are: I-*Ppo*I for the 23-base sequence ATGACTCTCTTAA/GGTAGCCAAA, and I-*Sce*I for the 18-base sequence TAGGGATAA/CAGGGTAAT (Itaya, 2009). Indeed, two I-*Ppo*I recognition sequences are created in the *B. subtilis* genome to cut the BAC part out of the host genome (Figs. 5b and 6a). The linear DNA produced by I-*Ppo*I digestion is readily isolated from agarose gels resolved by pulsed-field gel electrophoresis. The handling of giant DNA longer

than several hundred kbp has been difficult because of its fragility in test tubes. However, its use for microinjection into, for example, fertilized mouse eggs requires pure and undamaged DNA. We implemented and tested various steps to isolate undamaged giant DNA maintained in liquids. Our improved protocol that involves 2 gel electrophoresis runs and collection on a dialysis membrane made it possible to concentrate undamaged giant DNA. The successful purification of up to 220 kb mouse genomic DNA carrying reporter genes facilitated the creation of transgenic mice (manuscript in preparation). Because our method is technically simple it will be of great value in future studies.
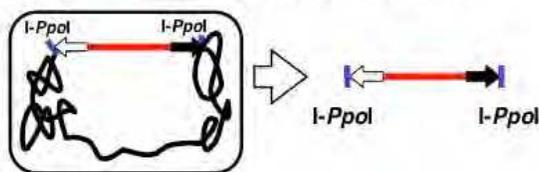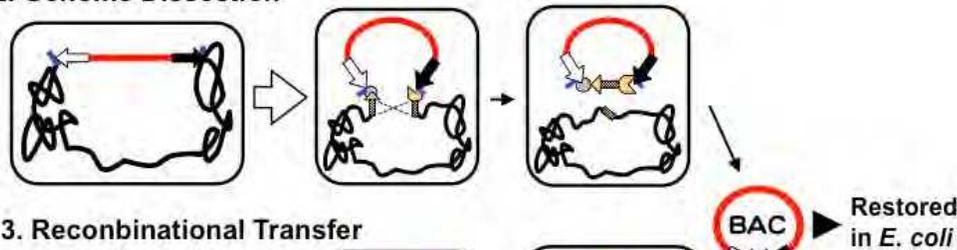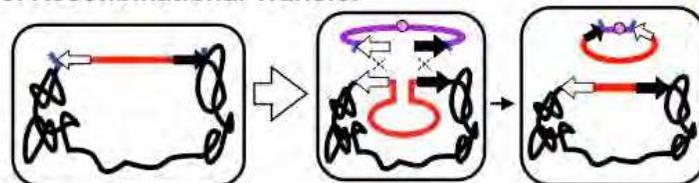

Fig. 7. Three methods to retrieve BAC-DNA from BGM.
1. Fragmentation using endonucleases is simple. Improvements for the isolation step are described in the text.
2. Only one report describing genome dissection has been published (Itaya & Tanaka, 1997). The potential for isolating DNA above 300-kbp is described.
3. For recombinational transfer see the text and refer to Tsuge & Itaya (2000) and Itaya & Tsuge (2011). Although the process appears to be complicated, the BAC insert can be copied in the BGM and pasted into a linear plasmid to complete circularization. This process yields a plasmid carrying the copied DNA segment.

4.2 Genetic dissection of the guest portion from the *B. subtilis* genome

The second method, genome dissection, largely depends on *B. subtilis* genetic systems. Although this method appears complex at first glance because of the molecular apparatus briefly described in Fig. 7, it is in fact simple. Intrachromosomal homologous recombination between the two DNA repeats makes it possible to physically disconnect the BAC segments. As the disconnected DNA was designed to carry a DNA replication origin site (*oriN*) different from *oriC* of the chromosome, it starts replicating autonomously as a plasmid independent from the chromosome. Itaya and Tanaka (1997) reported a 300-kbp DNA fragment that manifested superb genetic stability. Despite the potential to produce circular

DNA larger than 300 kbp by this method, its application has been restricted by the rather complicated procedures involved in its creation (Itaya, M., unpublished observations).

4.3 Retrieval by copying segments of the *B. subtilis* genome

The third method involves a yet more complicated genetic process referred to as *Bacillus* Recombinational Transfer (BReT) (Tsuge & Itaya, 2001; Kaneko et al., 2005). Indeed, BReT relies on homologous recombination in *B. subtilis* and should be as simple as the genetic disconnection procedure described above. Different from the disconnection procedure, the engineered BAC parts from the genome are copied and pasted into the existing plasmid. DNA retrieval from the genome to the plasmid involves a reverse direction of the DNA delivery into a host pBR322 or BAC as illustrated in Fig. 1b. The complete circular form yielded by the BReT pathway, selection with plasmid-linked markers followed by standard extraction of plasmid DNA, resulted in the purification of complete recombinant genomes of lambda (Tsuge & Itaya, 2001), organelle genomes from mitochondria and chloroplast (Itaya et al., 2008; Yonemura et al., 2007), and several BACs (Kaneko et al., 2005). The engineering of organelle genomes, mitochondria, and chloroplasts opens an exciting field because typical organelle genome sizes, ranging from 100-200 kbp for chloroplast, are below the carrying capability of BACs. Although there are currently no reliable technical tools to deliver them back to natural cells, the availability of circular freely engineered mitochondria or chloroplast genomes raises the need for such tools (Gibson et al., 2008, 2010; Itaya et al., 2008; Itaya, 2010).

5. Choice of the two hosts for BAC engineering

The use of BACs addresses different aims and goals in different cellular systems and some disadvantages must still be overcome. One seeming disadvantages is that our *B. subtilis* system is still too new for wide dissemination. Examples of BACs that have been manipulated in BGM systems to date are listed in Table 1. Advantages are that the *B. subtilis* host system is obviously superior to BAC technologies that make use of the *E. coli* system (Fig. 2). We focused on using BACs in our mouse genetics studies; investigations of gene loci other than *jmj* (Fig. 6b) are in progress. Another advantage is that a total of 9 antibiotic resistance gene cassettes are currently available for *B. subtilis* selection: neomycin (*neo*), spectinomycin (*spc*), chloramphenicol (*cat*), tetracycline (*tet*), erythromycin (*erm*), blasticidin S (*bsr*), kanamycin (*kam*), phleomycin (*phl*), and hygromycin (*hyg*). These antibiotic resistance genes will greatly facilitate the development of multipurpose BGM vectors that allow all desired manipulations. Although examples for the practical application of BAC technologies remain limited, we are in the process of producing a BAC-BGM kit that involves a simple protocol. The next generation of BGM should be accompanied by handy manuals so that even non-expert users of *B. subtilis* can perform routine experiments based on a basic understanding of the principles that are the foundation of BGM. The rapid preparation and assembly of fragments in the host is key for simplifying necessary procedures and for shortening the time required for the production of engineered fragments. From this perspective, we think that the BGM system is an invaluable tool for the creation of accurately designed guest DNA that does not rely on existing BAC modification kits in *E. coli*. However, the role of *E. coli* as an initial producer of the BACs prior to BGM will remain unaltered. Initially, our new BAC vectors, p108BGMC(*cat*) and p108BGME(*erm*), can be expected to replace the currently widely-used BAC vectors that lack markers for BGM.

DNA source from	insert[*2] size (kbp)	Accommodation in[*3] BGM by Fig.1b	Retrieval in plasmid as Fig.7	Restored in[*4] *E. coli*
***Arabidopsis thaliana* mtDNA**[†1]				
F1O22	101	**Yes**	**Yes**	**Yes**
F2L14	100	**Yes**	**Yes**	**Yes**
F3A21	100	**Yes**	**Yes**	**Yes**
F3B3	115	**Yes**	**Yes**	**Yes**
F4C19	100	**Yes**	**Yes**	**Yes**
F4I8	100	**Yes**	**Yes**	**Yes**
F4O20	144	**Yes**	**Yes**	**Yes**
F6A21	115	**Yes**	**Yes**	**Yes**
F6A7	105	**Yes**	**Yes**	**Yes**
F7I2	85	**Yes**	**Yes**	**Yes**
F7J2	98.5	**Yes**	**Yes**	**Yes**
F9J20	120	**Yes**	**Yes**	**Yes**
F10J8	90	**Yes**	**Yes**	**Yes**
F10L13	89.5	**Yes**	**Yes**	**Yes**
F10F17	100	**Yes**	**Yes**	**Yes**
F11E12	80	**Yes**	**Yes**	**Yes**
F13E8	90	**Yes**	**Yes**	**Yes**
F13O24	115	**Yes**	**Yes**	**Yes**
Mouse *Jmj* gene (Chromosome 13)				
pKANED[†2]	150	**Yes**	(NT)	(NT)
	(Deletion of pKANED as in Fig.5a and 5b)			
	110	**Yes**	(NT)	(NT)
	86	**Yes**	(NT)	(NT)
	34	**Yes**	**Yes**	**Yes**
	16.5	**Yes**	**Yes**	(NT)
	11.2	**Yes**	**Yes**	**Yes**
pKANEG[†3]	198	**Yes**	(NT)	(NT)
pKANEH[†3]	219	**Yes**	(NT)	(NT)
(Connection: pKANEG +pKANEH in Fig.6a)				
	355	**Yes**	(NT)	(NT)
Mouse *TS* gene DNA (Chromosome 11) [†4]				
p185A21	120	**Yes**	(NT)	(NT)

[*1]BAC clones in literature [*2]Estimated by gel electrophoresis. [*3]Confirmed by Southern blot analysis using original BAC clone as probes. [*4]Used for transformation of *E. coli* strains DH1 or DH10B. Refereed by [†1] Kaneko et al., 2005, [†2] Kaneko et al., 2003 [†3] Kaneko et al., 2008, and [†4] Itaya et al., 2000.

Table 1. BAC clones[*1] handled by BGM systems

6. Future perspectives

Based on available data, we think that the use of *B. subtilis* will facilitate the rapid and efficient engineering of BACs. While both *E. coli* and *B. subtilis* are excellent BAC hosts, several experimental steps require that DNA be manipulated outside these hosts. Steps involved in the delivery of DNA from *E. coli* to BGM and in the isolation of engineered guest DNA render the fragments maintained in liquid vulnerable to damage due to the intrinsic physicochemical nature of these liquids.

Elsewhere we reported that the conventional delivery step may be replaced (Kaneko & Itaya, 2010a, 2010b; Itaya & Kaneko, 2010). We fortuitously found that plasmid DNA remains intact for a while after the induction of lambda phage of *E. coli*. While *E. coli* was lysed and its genome degraded, the co-existing plasmid remained intact and available for transfer to competent *B. subtilis*. The stability of plasmids in the lambda-induced *E. coli* lysate also applied to BACs (Kaneko & Itaya, 2010a, 2010b; Itaya & Kaneko, 2010). Consequently, it is no longer necessary to attempt to prepare undamaged BACs in test tubes.

The techniques involved in the isolation of undamaged DNA from electrophoresis gels have been improved and applied to fragments of DNA measuring up to 220 kbp. In the post-genome-sequencing era, reverse genetics using designed and manipulated BACs and BGM will play an essential role in the construction of various genetic mutants.

7. Concluding remarks

Genome-sequence determination techniques are now applied to a vast range of species. In contrast, techniques to engineer genomes, even of bacteria far smaller than those of higher eukaryotes, remain scarce. Recently, two independent laboratories, one of them ours, demonstrated that whole bacterial genomes can be cloned/manipulated (Itaya et al., 2005). This technological breakthrough has dramatically changed various aspects of genome engineering. However, these cutting-edge technologies are not yet widely applied because they remain labor-intensive with respect to their use in genomes and because the cost of producing correct genomes is high. Besides introducing the reader to the cloning of whole bacterial genomes, this chapter aimed at describing multipurpose systems by which DNA can be routinely engineered. Giant guest DNA cloned in the BGM vector will play a significant role in versatile gene/genome delivery systems. The goal of genome engineering is not only the propagation of microbes but also the engineering of cells addressed by different branches of the life sciences. BAC-based cloning in *E. coli* (Shizuya et al., 1992), one of many DNA cloning technologies, has been revisited in efforts to develop essential cutting-edge tools that can be applied in the BAC-BGM system. The first BAC transfer to- and manipulation in the BGM system was achieved as little as 10 years earlier (Itaya et al., 2000). Subsequent publications demonstrated that once the BAC guest was incorporated into the BGM host, modified guest DNA and the novel homologous recombination activity of *B. subtilis* made this possible due to the amazing structural stability of guest DNA. Furthermore, depending on experimental requirements, the structure of recovered fragments can be circular or linear. Emerging genome synthesis technologies will yield giant DNA equivalent to the size of bacterial genomes (Gibson et al., 2008, 2010; Gibson, 2011). We are entering a new era of novel synthetic biology that relies on the synthesis/construction of designed biomaterials. With the aid of newly designed genes and proteins, *de novo* synthetic

pathways can be expected to produce yet unknown substances and with the aid of giant DNA, many genes can be delivered simultaneously.

While genome synthesis is gradually changing the way of thinking of researchers in the life sciences, the introduction, maintenance, and manipulation of DNA fragments in BACs will continue to shed light on other host- and biological systems. The *de novo* synthesis of sufficiently large DNA in BACs is only a matter of time and it remains to be seen whether current gene engineering systems will continue to be relevant. We will continue to dedicate our careers to working with DNA pieces irrespective of their size and to shedding light on their divergent applicability to biological systems used in research.

8. Acknowledgement

9. References

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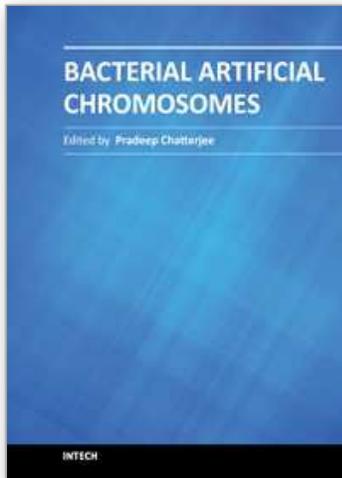
Bacterial Artificial Chromosomes

Edited by Dr Pradeep Chatterjee

This book focuses on the numerous applications of Bacterial Artificial Chromosomes (BACs) in a variety of studies. The topics reviewed range from using BAC libraries as resources for marsupial and monotreme gene mapping and comparative genomic studies, to using BACs as vehicles for maintaining the large infectious DNA genomes of viruses. The large size of the insert DNA in BACs and the ease of engineering mutations in that DNA within the bacterial host, allowed manipulating the BAC-viral DNA of Varicella-Zoster Virus. Other reviews include the maintenance and suitable expression of foreign genes from a Baculovirus genome, including protein complexes, from the BAC-viral DNA and generating vaccines from BAC-viral DNA genomes of Marek's disease virus. Production of multi-purpose BAC clones in the novel Bacillus subtilis host is described, along with chapters that illustrate the use of BAC transgenic animals to address important issues of gene regulation in vertebrates, such as functionally identifying novel cis-acting distal gene regulatory sequences.

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