doi:10.1016/j.jmb.2005.04.041 J. Mol. Biol. (2005) 349, 1036–1044

JMB

Available online at www.sciencedirect.com

SCIENCE DIRECT®

ELSEVIER

DNA Shuttling Between Plasmid Vectors and a Genome Vector: Systematic Conversion and Preservation of DNA Libraries Using the *Bacillus subtilis* Genome (BGM) Vector

Shinya Kaneko, Manami Akioka, Kenji Tsuge and Mitsuhiro Itaya*

Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya Machida, Tokyo 194-8511 Japan

The combined use of the contemporary vector systems, the bacterial artificial chromosome (BAC) vector and the *Bacillus subtilis* genome (BGM) vector, makes possible the handling of giant-length DNA (above 100 kb). Our newly constructed BGM vector efficiently integrated DNA prepared in the BAC vector. A BAC library comprised of 18 independent clones prepared from mitochondrial DNA (mtDNA) of *Arabidopsis thaliana* was converted to a parallel BGM library using the new BGM vector. The effectiveness of the combined use of the vector systems was confirmed by the stable recovery of all 18 DNAs as BAC clones from the respective BGM clones. We show that DNA in BGM was stably preserved at room temperature after spore formation of the host *B. subtilis*. Rapid and stable shuttling between *Escherichia coli* and the *B. subtilis* host, combined with spore-mediated DNA storage, may facilitate the long-term and low-cost preservation and the transportation of DNA resources.

Keywords: genome vector; recombination; transformation; recombinational transfer; spore formation

**Corresponding author*

Introduction

The two reliable cloning vehicles for large-sized DNA (>100 kb), the bacterial artificial chromosome (BAC)[1–3] and the *Bacillus subtilis* genome (BGM) vector, were developed with different cloning concepts. BAC expanded the utility of the F′ replicon (*oriS*) of *Escherichia coli* and made it possible to replicate as low as a single-copy state per cell and to clone lengths as long as 350 kb.[1,4,5] Although the preparation of large intact DNA was a prerequisite, random DNA library construction required a relatively simple protocol and protocols for the construction of BAC libraries were readily available.[6–8] Technical developments such as the GET-rec system that employs site-specific recombination of host *E. coli*[9–12] proved the BAC cloning system invaluable for the manipulation of large DNAs, despite the inherent genetic instability, because its episomal replication mode is similar to other plasmid vectors.

Cloning in the BGM vector involves different principles. DNA is actively taken up due to competency developed by the host *B. subtilis*.[13–17] The target DNA in the cytoplasm is guided to integrate at the cloning site where two short guide-sequences are introduced. These guide-sequences, termed landing pad sequences (LPSs), are normally constructed in *E. coli* pBR322 plasmid. These LPSs are pre-installed in the genomic pBR322 sequence (GpBR) of the BGM vector by homologous recombination.[16–19] We applied the stable cloning described here (Figure 1) and in our previous reports[16–18,20] to DNA from different species and used DNA of a size comparable to that of BAC clones (100–200 kb) in target cloning. The cloned DNA segments in the BGM vector were highly stable both structurally and genetically; this reflects

Abbreviations used: BAC, bacterial artificial chromosome; bp, base-pair(s); kb, kilo bases; Mb, mega bases; *cat*, chloramphenicol acetyl transferase gene; *spc*, spectinomycin resistance gene; *c*I, *c*I repressor gene; *tet*, tetracycline resistance determinant gene; *km*, kanamycin resistance gene; CHEF, contour-clamped homogeneous electric field gel electrophoresis; mtDNA, mitochondrial DNA; ccc, covalently closed circular; LPS, landing pad sequence; GpBR, the genomic pBR322 sequence.

E-mail address of the corresponding author: ita@libra.ls.m-kagaku.co.jp

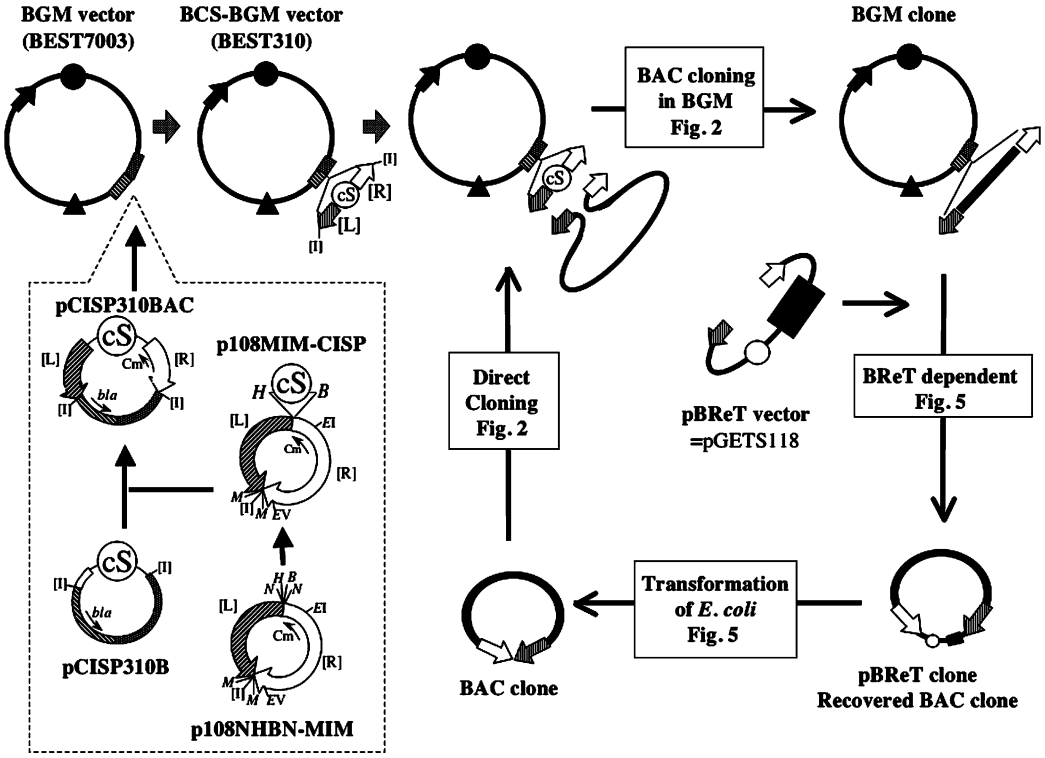

Figure 1. Structure of the standard BGM vector (BEST7003) and derivatives used in this study. Filled circles and triangles represent *oriC* and *terC* of *B. subtilis*, respectively. The two boxes in the genome indicate the genomic pBR322 (GpBR); dots and stripes correspond to *tet*-half and *amp*-half. The genomic BAC-vector sequence of BEST310 is represented by open and striped arrows that correspond to [L] and [R], respectively. Details of [L] and [R] and the construction of BEST310 are contained in the area surrounded by dashes (see also Materials and Methods). The *cat* gene (Cm) for *E. coli* selection and *oriS* are included in [R]. The circled cS indicates the positive selection cassette containing the *c*I repressor- and spectinomycin-resistance gene. Pr-neo (Lambda Pr promoter fused to the neomycin resistance gene) for the positive selection (filled arrow) is positioned at 3516–3522 kb of the *B. subtilis* genome.[16] *H*, *B*, *N*, and *E*V and [I] represent recognition sequences for HindIII, BamHI, NotI, EcoRV, and I-PpoI. The shuttling of BAC clones between *E. coli* and the new BGM vector (BEST310) is shown on the right. Details of each step in the cycle are described in the legends to Figures 2 and 5. BreT, *Bacillus* recombinational transfer mechanism using linearized pBReT vector.[16,22,45]

the stability of the host genome.[21] Taking advantage of the great structural stability of its DNA, a characteristic of the BGM vector, and the competency of *B. subtilis*, we successfully performed several kinds of manipulation of cloned DNA.[16,18] We processed recovered DNA segments after such manipulations by the reverse order of integration, availing ourselves of the intrinsic property of the *B. subtilis* recombinational transfer (BReT) mechanism.[16,22] This system, inherent to BGM, resulted in the production of a complete copy of the cloned segment as a covalently closed circular (ccc) plasmid (pBReT plasmid) in *B. subtilis*. As the plasmid that we constructed can shuttle between *E. coli* and *B. subtilis*, plasmid DNA can be prepared from either host. Because different principles underlie cloning in BAC and BGM, they cannot be used together. Therefore, BAC has primarily been used for random DNA library construction and GET-rec, and BGM for target cloning and manipulation. A more useful means of handling DNA larger than 100 kb requires a system that allows for rapid and flexible shuttling between BAC and BGM.

An important, sometimes overlooked feature of the BGM vector is the ability of *B. subtilis* to form endospores. Sporulation of *B. subtilis* is triggered when a vegetatively growing cell depleted of nutrients goes into the dormant state where it shows no detectable metabolism and a higher degree of resistance to inactivation by various physical insults. Despite their metabolic inactivity, continuous monitoring for changes in the nutritional status in their environment results in a rapid response and the spores germinate and resume vegetative growth.[23] We postulated that if cloned DNA in the genome of *B. subtilis* were maintained undamaged in dormant state, the BGM vector could serve as a long-term storage system at room temperature without special equipment. Here we present our novel shuttling system between BGM and BAC. It permits the precise and delicate manipulation of 100 kb DNA. We demonstrate the effectiveness of our system by converting a BAC library to a BGM library in which DNA was stably preserved in spores.

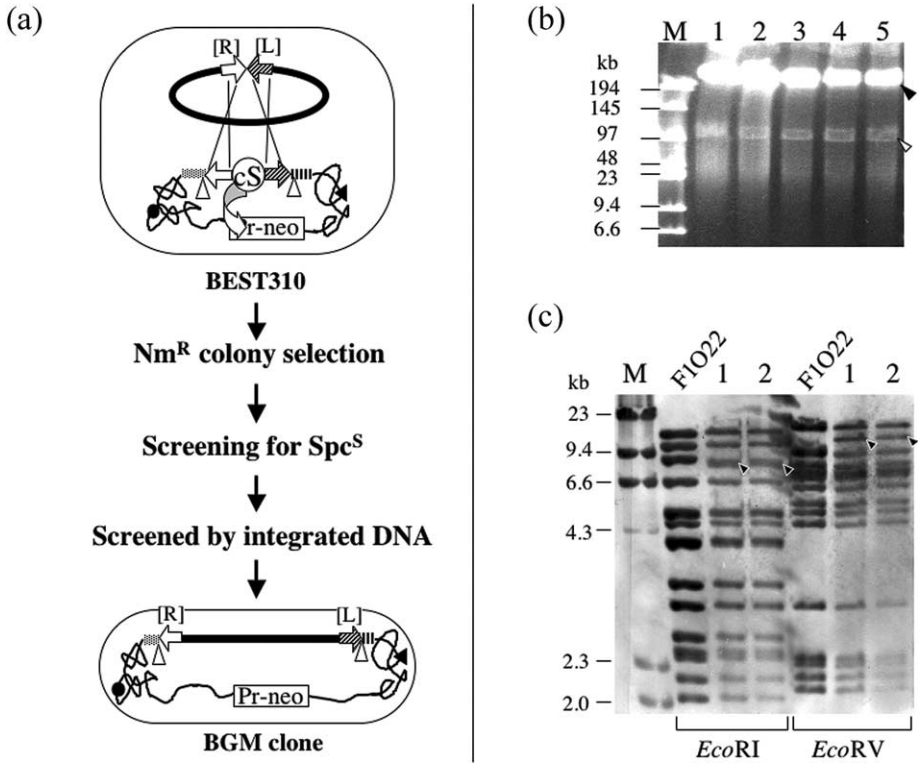

Figure 2. One-step cloning in BEST310. (a) Replacement of the *c*I-*spc* cassette (circled cS) that confers repression of the Pr promoter of the neomycin resistance gene (Pr-*neo*) with the insert in the BAC clone results in expression of neomycin resistance (Nm^R). Several hundred fmol of each purified BAC clone were used for each transformation. Screening for spectinomycin-sensitive (Spc^S) colonies avoided false-positives associated with spontaneous *c*I mutation.[16,19] Nm^R and Spc^S clones were subjected to fragment analysis by Southern hybridization after I-PpoI digestion. Two I-PpoI recognition sequences are identified with open triangles inserted between each BAC sequence ([L] and [R]) and the pBR322 sequence (dotted and striped boxes)(see Figure 1). (b) An example of screening for I-PpoI digestion in the cloning of F1O22. All five screened Nm^R, Spc^S clones contained two I-PpoI fragments that corresponded to the cloned, approximately 100 kb BAC-DNA (open arrowhead) and the approximately 4.2 Mb BGM vector (filled arrowhead). The running conditions were: 4 V cm^{-1}, 12 seconds pulse time, 17 hours running time at 14 °C. (c) Genomic DNA of BEST6487 and BEST 6488 (lanes 1 and 2 in (b)), and purified F1O22 digested with the indicated restriction enzymes were hybridized with F1O22 as a probe. Both BGM recombinants contained signal fragments identical with those of F1O22 except for the fragments indicated with filled arrowheads. The additional bands were from a BAC vector sequence pre-installed in linearized form in the genome of BEST310. Running conditions: 3 V cm^{-1}, 18 seconds pulse time, 18 hours running time at 14 °C.

Results

Construction of a new BGM vector for rapid cloning of BAC inserts

Elsewhere we have reported the cloning of BAC inserts in the standard BGM vector[16–19] (strain BEST7003 in Figure 1) that carries the GpBR sequence for the cloning locus in the *B. subtilis* genome. As strain BEST7003 was not suitable for direct cloning of the insert in the BAC vector, we constructed a new BGM vector, strain BEST310, from BEST7003. It has two divided guide-sequences as the BAC vector region (BAC-[R] and BAC-[L]) in the GpBR sequence (Figure 1). BAC clones taken up by BEST310 were directly integrated *via* double crossings-over with the genomic BAC-[R] and BAC-[L] (3.5 kb each) during transformation as described in Figure 1. Selection of BGM recombinants uses an internal positive selection system because no appropriate marker for *B. subtilis* is carried by BAC (Figure 2(a)).[16,19] Briefly, the *c*I gene product, a repression protein of bacteriophage lambda for *E. coli* expressed from the *c*I-*spc* cassette (circled cS in Figure 1) inserted between BAC-[R] and BAC-[L], binds to the Pr-promoter of the neomycin resistance gene (Pr-neo) of BEST310 and represses transcript from the Pr-neo gene. The concomitant loss of the *c*I gene upon replacement by the BAC insert confers to BGM host resistance to neomycin *via* the induced expression of the Pr-*neo* gene. For cloning of the BAC insert in BEST310 we used a BAC clone, pBACCm, that carries the 1.1 kb chloramphenicol acetyl transferase (*cat*) gene for *B. subtilis*, as a model. BEST310 recombinants that integrated the *cat* sequence were selected either directly by chloramphenicol or indirectly by neomycin (data not shown).

Conversion of BAC library to BGM library

We used 18 BAC clones (provided by the Kazusa

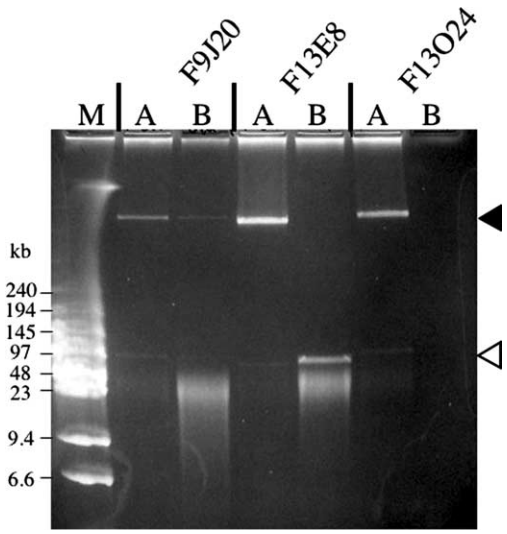

Figure 3. Intact unshared DNA is required for high-efficiency BGM (BEST310) cloning. Comparison of the indicated BAC-DNA used in separate transformation experiments. A, Many recombinants were obtained; B, almost no recombinants. DNA in lane B that lost intact ccc is indicated by filled arrowheads; degraded DNA by open arrowheads. Lane M, concatemer of lambda DNA used as a size marker. Running conditions: 4 V cm^{-1}, 30 seconds pulse time, 22 hours running time at 14 °C.

DNA Research Institute, Chiba, Japan) to test the feasibility of cloning real BAC clones in BEST310. The 18 BAC clones were selected from 113 clones that carried mtDNA of *Arabidopsis thaliana* in the IGF *Arabidopsis* BAC library prepared for the *A. thaliana* genome project.[24–26] The size of the inserts, estimated by contour-clamped homogeneous electric field gel electrophoresis (CHEF) after NotI digestion, is shown in Table 2.

These BAC clones were cloned in BEST310 as described in the legend to Figure 2. Isolated BGM clones for the 18 BAC clones are listed in Table 2. As cloning efficiency varied from experiment to experiment, we carefully investigated cases with extremely low cloning efficiency. We found that the quality of BAC used for transformation drastically affected the number of BGM recombinants (Figure 3). High-efficiency cloning requires highly purified and unshared BAC-DNA. We checked the converted BGM library consisting of 18 recombinants for stability, preservation and recovery.

Stability of cloned BAC in BGM

The structure of all BAC-DNAs cloned in the BGM vector was stably maintained. The structure of F1O22 (101 kb) is provided as a typical example (Figure 2(b) and (c)). It should be noted that stability

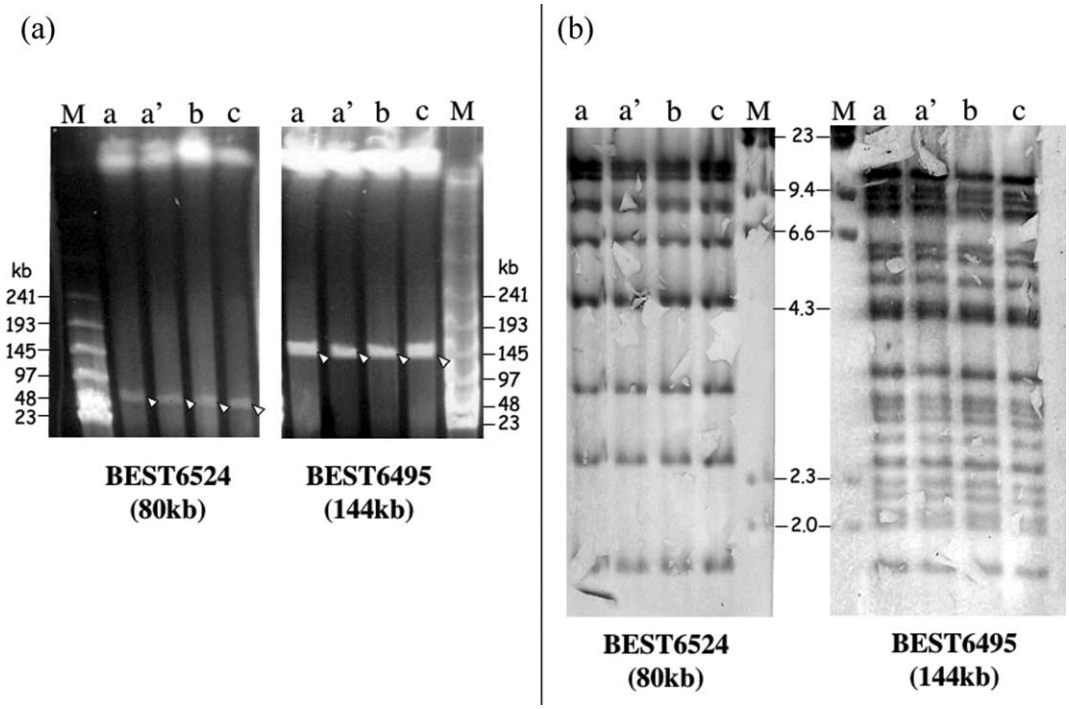

Figure 4. Unaltered structure of cloned DNA in germinated spores derived from BGM clones BEST6524 (F11E12) and BEST6495 (F4O20). The letters above each lane correspond as follows; a a′, isolated from heat-treated spores; b, isolated from spores after one month on Schaeffer's plate; c, from frozen stock. Analysis was by (a) CHEF resolution after I-PpoI digestion and (b) Southern blot hybridization with each original BAC clone as a probe. (a) I-PpoI fragments of approximately 80 kb for all BEST6524 strains and 144 kb for all BEST6495 strains remained constant as indicated by open arrowheads. Running condition: 4 V cm^{-1}, 30 seconds pulse time, 20 hours running time at 14 °C. (b) The structure of each digest of genomic DNA from BEST6524 and BEST6495 was identical with the BAC structure. Running condition: 3 V cm^{-1}, 18 seconds pulse time, 17 hours running time at 14 °C.

was unaltered whether or not antibiotics were used during culture preparation (data not shown). The cultivation of BGM does not require antibiotics except for recombinant screening; this is consistent with our other reports.[16–18]

The apparent high stability was further assessed by checking sporulation. All clones in the BGM library formed spores when cultivated in Schaeffer's sporulation liquid medium; the sporulation rate ranged from 11 to 77%, nearly equivalent to that of standard *B. subtilis* 168 *trpC2* (~50%). Sporulation was confirmed by germinated colonies after heat treatment (80 °C, 15 minutes). Two colonies, a and a′ from strains BEST6524 and BEST6495 in Figure 4(a) and (b), yielded I-*Ppo*I fragments and Southern signals identical with those of the original isolates. These results confirmed that the cloned BAC-DNAs of F11E12 (80 kb) and F4O20 (144 kb) remained unaltered throughout the sporulation and germination process.

The structural and genetic stability of the cloned BAC-DNAs in sporulated BGM was examined in regards to their possible prolonged preservation on plates. Colonies formed on Schaeffer's sporulation agar plates were monitored by looking for the appearance of the characteristic brown pigmentation of sporulating colonies after one month incubation at 30 °C. Germinated colony b from BEST6524 and BEST6495 in Figure 4(a) and (b) manifested BAC structure F11E12, F4O20, identical with that of the initial recombinant. Furthermore, the similar germinated colony isolated from the same plates after six months, completely dried up, gave identical results with b of Figure 4 (data not shown). Heat treatment process (80 °C, 15 minutes) in the isolation from the six months plate also concluded with the same result. Excellent stability was also confirmed in other BGM clones, for example, BEST6487 and BEST6489 carrying F1O22 (101 kb) and F3A21 (100 kb), respectively (data not shown). Our results indicate that the BGM vector is suitable for the stable and long-term preservation of cloned DNA at room temperature after sporulation. Studies are underway in our laboratory to examine the effect(s) of longer storage on the present BGM library.

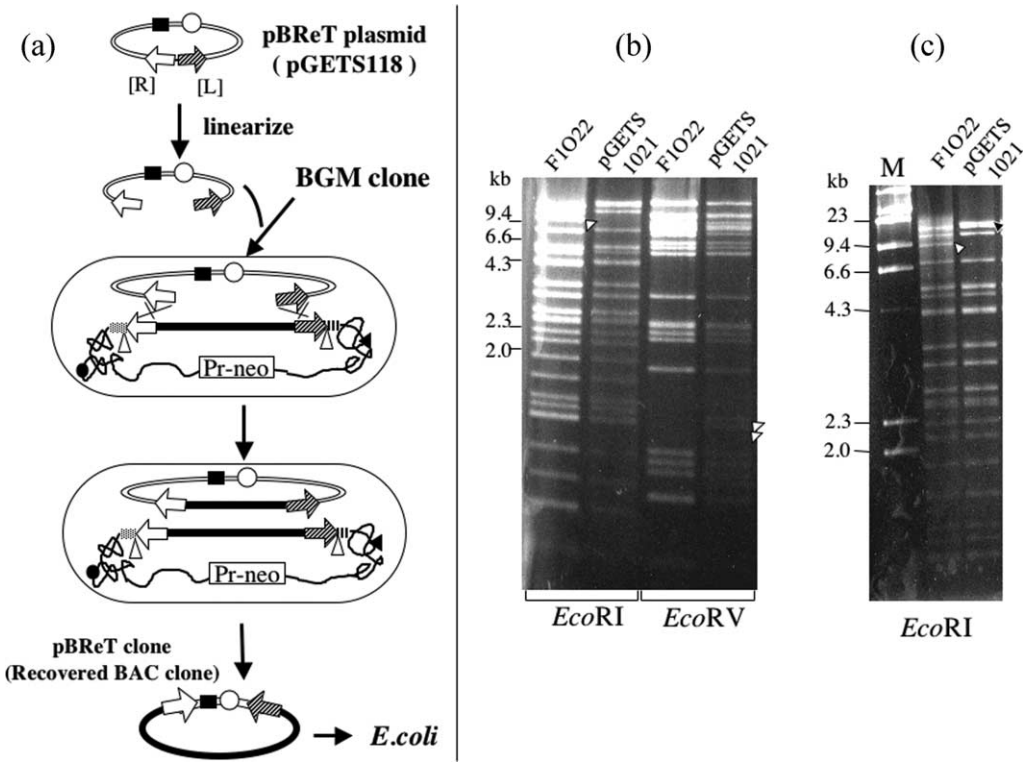

Figure 5. Recovery of BAC clone from BGM and shuttle-back to *E. coli*. (a) Linearized pGETS118 taken up by competent BGM copied the cloned BAC-DNA by the BReT mechanism we described elsewhere.[16,22] BAC vector sequences [L] and [R] provided LPSs for recombination. The ccc form of the pGETS118 recombinant plasmid renders the host tetracycline-resistant by the tetracycline determinant gene for *B. subtilis* indicated by the filled box. The open circle in the pBReT plasmid represents the replicon for *B. subtilis*. Filled circles and triangles in the genome represent *oriC* and *terC* for the initiation of DNA replication and the termination of *B. subtilis*. (b) The purified original BAC clone (F1O22) and pGETS1021 (recovered BAC clone) were digested with EcoRI and EcoRV and run on CHEF. Running condition: 3 V cm^{-1}, 18 seconds pulse time, 9.5 hours running time at 14 °C. The BAC vector fragment (pBeloBAC-Kan, open arrowheads) converted to pGETS118 vector. (c) Comparison of EcoRI fragments of pGETS1021 isolated from *E. coli* and fragments of F1O22 (original BAC clone). Running condition: 2.67 V cm^{-1}, 18 seconds pulse time, 20 hours running time at 14 °C. The original BAC vector (open arrowhead) converted to the pGETS118 vector of pGETS1021 (filled arrowhead).

Recovery of BAC-DNA from BGM

The cloned BAC-DNA replicates as part of the *B. subtilis* genome in BGM. As we consider recovery of this BAC-DNA in ccc form highly advantageous, we constructed a pBReT vector, pGETS118, carrying the BAC vector sequence ([L] and [R]) and *B. subtilis* replicon (*repA*) and used it to recover BAC-DNAs from the BEST310-derived BGM library. The recovery process is based on the recombinational transfer principle[22] and is described in the legend to Figure 5(a). Briefly, the linearized pGETS118, incorporated by the competent BGM clones during transformation, copies the intervening BAC segment from the genome by homologous recombination with the two flanking regions [L] and [R]. As a result, the linearized pGETS118 is re-circularized and established as a "pBReT clone" plasmid. Recombinants that harbor the pBReT clone can be selected by tetracycline. Recombinational transfer-mediated recovery was applied to the 18 BGM clones; tetracycline-resistant colonies were obtained from all 18 clones. Each corresponding pBReT clone ($n=18$) was isolated and analysis by restriction enzyme digestion revealed that its structure was identical with the original BAC in *E. coli*. Figure 5(b) shows a representative pGETS1021 with the original BAC clone F1O22. The other 17 pBReT clones are listed in Table 2.

We postulated that these pBReT clones should be able to replicate in *E. coli* as BAC replicons. Transformation of *E. coli* by electroporation using these pBReT clones resulted in the expected transformations (Table 2). The copy numbers in *E. coli* remained unaltered from the original BAC clone (data not shown). A representative pGETS1021 is presented in Figure 5(c); there was no structural alteration through the transfer in the cyclic manner shown in Figure 1. Therefore, the efficient shuttling of DNA between BAC and BGM was demonstrated.

Discussion

To examine the proposed system for shuttling between BAC and BGM we used a BGM vector (BEST310) and a DNA library of the mtDNA of *A. thaliana*. Our complete transfer results are instructive. An LPS length of 3.5 kb made possible the integration of 80–144 kb. We determined the proper LPS length empirically as approximately 10% of the target region;[17,18] while it can be shortened, efficient cloning was accelerated by the use of damage-free BAC-DNA. Our complete success in shuttling provides opportunities for performing not only various kinds of manipulations,[16,18] but may make possible the long-term, stable preservation of DNA in spores at room temperature. We are working to improve the efficiency of each step to make possible high-throughput shuttling between BAC and BGM libraries.

At present, the long-term storage of DNA in *E. coli* is expensive in terms of labor and instrumentation. Bacterial recombinants are currently preserved by freezing[27–29] and techniques for the long-term preservation of bacterial cultures have been devised,[27,30–33] including stab culture[34] and lyophilization.[35,36] However, viability may decrease with prolonged storage.[37,38] It was found that cryopreservation with glycerol even appeared to diminish viability,[29] although this method is widely used. As demonstrated in Figure 4, the BAC-DNA in the genome of *B. subtilis* was structurally unaltered through spore formation and germination. We attribute this stability not only to the spore coats and the relative impermeability of the spore core, but also to the saturation of spore DNA with α/β-type small, acid-soluble proteins (SASP) and the repair of macromolecular damage during spore germination and outgrowth.[23] The resistance of spores to physical and chemical insults such as wet and dry heat, UV and gamma radiation, extreme desiccation (including vacuum), and oxidizing agents[23] leads us to suggest that DNA stored in spores can be safely transported even under otherwise suboptimal environmental conditions.

Based on the data presented here we propose that the stable mtDNA BGM library of *A. thaliana*[39,40] may be used for the reconstruction of a mitochondrial genome of *A. thaliana* in the BGM vector.

Table 1. Bacterial strains used in this study

Bacterial strain	Genotype or insert	Antibiotic selection[a]	Reference or source
E. coli			
JA221	F$^-$ *hsdR hsdM*$^+$ *trp leu lacY recA1*		46
DH10B	F$^-$ *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) ϕ80d*lacZ*ΔM15Δ*lac*X74 *deo*R *rec*A1 *end*A1 *ara*D139 Δ(*ara, leu*)7697 *gal*U *gal*Kλ$^-$ *rps*L *nup*G		This study
DH1	F$^-$ *end*A1, *gyr*A96, hsdR17($r_k^-m_k^+$), *rec*A1, *rel*A1, *sup*E44, *thi*−1		This study
B. subtilis			
1A1 (=168*trpC2*)	*trpC2*		BGSC[b]
RM125	*leuB8 arg-15* ΔSPβ		41
BEST7003[c]	RM125 plus *proB*::pBRTc Pr-*neo*	Nm, Tc	16
BEST310	*proB*::pBR[BAC]	Spc	This study

[a] Tc, tetracycline resistance; Nm, neomycin resistance; Spc, spectinomycin resistance.
[b] *Bacillus* Genetic Stock Center (Ohio State University, Columbus, OH).
[c] Pr-*neo* gene (Lambda Pr promoter fused to the neomycin resistance gene) is inserted between NotI sites of *yvfC* and *yveP*.

Table 2. BGM strains with BAC clones and recovered BAC clones

Original BAC clone[a]	Insert size (kb)[b]	*B. subtilis* representative[c]	PBReT clone[d]	*B. subtilis* representative	*E. coli* representative
F1O22	101	BEST6487	PGETS1021	BEST6508	MEC5704
F2L14	100	BEST6491	pGETS1024	BEST6560	MEC5724
F3A21	100	BEST6489	pGETS1023	BEST6559	MEC5723
F3B3	115	BEST6492	pGETS1027	BEST6572	MEC5736
F4C19	100	BEST6500	pGETS1026	BEST6563	MEC5730
F4I8	100	BEST6496	pGETS1025	BEST6574	MEC5738
F4O20	144	BEST6495	pGETS1037	BEST6592	MEC5753
F6A21	115	BEST6498	pGETS1028	BEST6575	MEC5752
F6A7	105	BEST6501	pGETS1032	BEST6576	MEC5739
F7I2	85	BEST6509	pGETS1029	BEST6566	MEC5733
F7J2	98.5	BEST6517	pGETS1042	BEST6593	MEC5757
F9J20	120	BEST6596	pGETS1043	BEST6602	MEC5765
F10J8	90	BEST6521	pGETS1030	BEST6567	MEC5734
F10L13	89.5	BEST6511	pGETS1034	BEST6583	MEC5744
F10F17	100	BEST6540	pGETS1035	BEST6584	MEC5745
F11E12	80	BEST6524	pGETS1036	BEST6585	MEC5746
F13E8	90	BEST6598	pGETS1044	BEST6603	MEC5766
F13O24	115	BEST6600	pGETS1045	BEST6604	MEC5767

[a] Transformant of DH10B by original BAC provided by Drs Kaneko and Tabata.
[b] Estimated by CHEF with NotI digestion.
[c] Confirmed by Southern blot analysis using original BAC clone as probe.
[d] Used for transformation of DH1. Strain DH10B was transformed by pGETS1037, pGETS1028 and pGETS1042.

Materials and Methods

Bacterial strains

The bacterial strains used in this study are listed in Tables 1 and 2. *B. subtilis* strains, BEST7003, and BEST310, were used as the host strains of the BGM vector which has no Bsu restriction–modification system.[16,41]

Bacterial culture and transformation

E. coli and *B. subtilis* were grown at 37 °C unless otherwise specified. Each bacterial transformation was carried out according to our previously described method.[42] Selection by antibiotics for both *E. coli* and *B. subtilis* was under reported conditions.[18] *B. subtilis* genomic DNA in liquid or in agarose plugs was prepared from each culture using Luria-Bertani (LB) medium without antibiotics as described.[42]

Bacterial plasmids

All BAC clones used for integration into the BGM vector (BEST310 strain) and all pBReT clones recovered by the *B. subtilis* BReT mechanism are listed in Table 2. Details of the BReT mechanism are presented in Figure 1. The 18 BAC clones were selected from the IGF *Arabidopsis* BAC library that contained mtDNA of *A. thaliana* originally prepared by Mozo *et al*.[25] They were stocked at the Kazusa DNA Research Institute and kindly provided by T. Kaneko and S. Tabata. Other bacterial plasmids used in this study are listed in Table 3. The pCISP310BAC vector carrying the *c*I-*spc* cassette and the BAC vector part were constructed from pCISP310B[18] as described in Figure 1. p108NHBN-MIM was derived from pBAC108L by consecutive insertion of two linkers, NotI-HindIII-BamHI-NotI (5′-GCGGCCGCAAGCTTGGATCCGCGG CCGC-3′) and MluI-I-PpoI-MluI (5′-ACGCGTCTCTCTT AAGGTAGCACGCGT-3′). Insertion of the *c*I cassette consisting of the *c*I857 gene and the spectinomycin-resistance gene (*spc*) in the HindIII and BamHI sites of p108NHBN-MIM resulted in p108MIM-CISP. After digestion with I-PpoI, p108MIM-CISP was ligated at the I-PpoI site of the pBR322-based pCISP310B. The resultant chimeric plasmid pCISP310BAC replicated in *E. coli* with a moderate copy number; it probably used the pCISP310B- rather than the BAC replicon.

pGETS118 was constructed as a new pBReT vector for the BReT system by replacing the pBR322 part of pBR322-based pGETS109[22] with a BAC vector. pGETS118 replicates according to the F′ replicon (*oriS*) property in *E. coli*

Table 3. *E. coli* plasmids constructed in this study

Plasmids	Description[a]	Reference or source
pCISP310B	Ap^R, Sp^R, Cm^R, BS^R	19
pBAC108L	BAC vector (6.8 kb), Cm^R	19
p108NHBN-MIM	BAC with two linkers, Cm^R	This study
p108MIM-CISP	[*c*I], Cm^R, Sp^R	This study
LPS Array plasmid		
pCISP310BAC	pCISP310B ligated with p108MIM-CISP	This study
pBReT vector		
pGETS109	Derived from pGETS103. Tc^R	16
pGETS118	Derived from pGETS109. Tc^R	This study

[a] Ap^R, ampicillin resistance; Sp^R, spectinomycin resistance; Cm^R, chloramphenicol resistance; BS^R, blasticidin S resistance; Em^R, erythromycin resistance; Tc^R, tetracycline resistance; [*c*I], *c*I cassette.

and yields as low as one copy per cell. Linearized pGETS118 by NotI was used for BReT as depicted in Figure 1. Recovered pBReT clones in *B. subtilis* were purified as described elsewhere.[22] Type II restriction enzymes and T4 DNA ligase were from Toyobo (Tokyo, Japan), NotI was from TaKaRa (Kyoto, Japan) and I-PpoI from Promega (Madison, WI, USA).

B. subtilis spore formation

Sporulation is induced when the source of a readily metabolized form of carbon, nitrogen, or (more rarely) phosphorus is depleted in the environment. This nutrient limitation occurs naturally when cells are allowed to exhaust nutrients in their solid or liquid growth medium. Schaeffer's is the most commonly used sporulation medium; it can be used for spore formation in solid (agar) or liquid substrates.[43,44] The conditions for sporulation and germination have been published elsewhere.[44] We used Schaeffer's sporulation agar plates for prolonged preservation at room temperature. For *B. subtilis* sporulation, BGM strains and the standard strain, 168*trp*C2, grown in Schaeffer's liquid sporulation medium (37 °C, 24 hours) and diluted appropriately in 1.5 ml tubes, were incubated in a water bath (80 °C, 15 minutes) and then spread onto LB agar plates for germination. The sporulation rate was calculated from the ratio of the number of colonies per ml with and without 80 °C heat treatment. No spores formed when these strains were cultivated in LB medium.

Acknowledgements

We thank Drs T. Kaneko and S. Tabata of the KAZUSA DNA Institute for supplying the BAC clones and Dr Yanagawa of KEIO University for helpful discussions and continuous encouragement.

References

1. Shizuya, H., Birren, B., Kim, U. J., Mancino, V., Slepak, T., Tachiiri, Y. *et al*. (1992). Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F− factor-based vector. *Proc. Natl Acad. Sci. USA*, **89**, 8794–8797.
2. Kim, U. J., Birren, B. W., Slepak, T., Mancino, V., Boysen, C., Kang, H. L. *et al*. (1996). Construction and characterization of a human bacterial artificial chromosome library. *Genomics*, **34**, 213–218.
3. Frengen, E., Weichenhan, D., Zhao, B., Osoegawa, K., Geel, M. V., Jong, P. J. *et al*. (1999). A modular, positive selection bacterial artificial chromosome vector with multiple cloning sites. *Genomics*, **58**, 250–253.
4. Nordström, K. & Austin, S. J. (1989). Mechanisms that contribute to the stable segregation of plasmids. In *Annual Review of Genetics* (Campbell, A., ed.), vol. 23, pp. 37–69, Annual Reviews Inc., Palo Alto, CA, USA.
5. Hosoda, F., Nishimura, S., Uchida, H. & Ohku, M. (1990). An F factor based cloning system for large DNA fragments. *Nucl. Acids Res.* **18**, 3863–3869.
6. Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G. *et al*. (2001). The sequence of the human genome. *Science*, **291**, 1304–1351.
7. International Human Genome Sequencing Consortium. Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J. *et al*. (2001). Initial sequencing and analysis of the human genome. *Nature*, **409**, 860–921.
8. Mouse Genome Sequencing Consortium. Waterston, R. H., Lindblad-Toh, K., Birney, E., Rogers, J., Abril, J. F., Agarwal, P. *et al*. (2002). Initial sequencing and comparative analysis of the mouse genome. *Nature*, **420**, 520–562.
9. Yang, X. W., Model, P. & Heintz, N. (1997). Homologous recombination based modification in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. *Nature Biotechnol.* **15**, 859–865.
10. Narayanan, K., Williamson, R., Zhang, Y., Stewart, A. F. & Ioannou, P. A. (1999). Efficient and precise engineering of a 200 kb beta-globin human/bacterial artificial chromosome in *E. coli* DH10B using an inducible homologous recombination system. *Gene Ther.* **6**, 442–447.
11. Mikhail, N., Robert, W. & Panayiotis, A. I. (2000). Insertion of disease-causing mutation in BACs by homologous recombination in *Escherichia coli*. *Nucl. Acids Res.* **28**, e79.
12. Michael, O., Mikhail, N., Jim, V., Faten, Z., Robert, W. & Panayiotis, A. I. (2000). Engineering EGFP reporter constructs into a 200 kb human β-globin BAC clone using GET recombination. *Nucl. Acids Res.* **28**, e84.
13. Spizizen, J. (1958). Transformation of biochemically deficient strains of *Bacillus subtilis* by deoxyribonuclease. *Proc. Natl Acad Sci. USA*, **44**, 1072–1078.
14. Dubnau, D. (1999). DNA uptake in bacteria. *Ann. Rev. Microbiol.* **53**, 217–244.
15. Itaya, M. (1999). Genetic transfer of large DNA inserts to designated loci of the *Bacillus subtilis* 168 genome. *J. Bacteriol.* **181**, 1045–1048.
16. Kaneko, S., Tsuge, K. & Takeuchi, T. (2003). Conversion of sub-megasized DNA to desired structures using a novel *Bacillus subtilis* genome vector. *Nucl. Acids Res.* **31**, e112.
17. Itaya, M., Fujita, K., Ikeuchi, M., Koizumi, M. & Tsuge, K. (2003). Stable positional cloning of long continuous DNA in the *Bacillus subtilis* genome vector. *J. Biochem.* **134**, 513–519.
18. Itaya, M., Nagata, T., Shiroishi, T., Fujita, K. & Tsuge, K. (2000). Efficient cloning and engineering of giant DNAs in a novel *Bacillus subtilis* genome vector. *J. Biochem.* **128**, 869–875.
19. Itaya, M. (1999). Effective cloning of unmarked DNA fragments in the *Bacillus subtilis* 168 genome. *Biosci. Biotechnol. Biochem.* **63**, 602–604.
20. Itaya, M. (1995). Toward a bacterial genome technology: integration of the *Escherichia coli* prophage lambda genome into the *Bacillus subtilis* 168 chromosome. *Mol. Gen. Genet.* **248**, 9–16.
21. Itaya, M. (1993). Integration of repeated sequences (pBR322) in the *Bacillus subtilis* 168 chromosome without affecting the genome structure. *Mol. Gen. Genet.* **241**, 287–297.
22. Tsuge, K. & Itaya, M. (2001). Recombinational transfer of 100-kilobase genomic DNA to plasmid in *Bacillus subtilis* 168. *J. Bacteriol.* **183**, 5453–5458.
23. Nicholson, W. L., Munakata, N., Horneck, G., Melosh, H. J. & Setlow, P. (2000). Resistance of *Bacillus* endospores to extreme terrestrial and extraterrestrial environments. *Microbiol. Mol. Biol. Rev.* **64**, 548–572.
24. Mozo, T., Fisher, S., Meier-Ewert, S., Lehrach, H. &

Altmann, T. (1998). Use of the IGF library for physical mapping of the *Arabidopsis thaliana* genome. *Plant J.* **16**, 377–384.
25. Mozo, T., Fisher, S., Shizuya, H. & Altmann, T. (1998). Construction and characterization of the IGF *Arabidopsis* BAC library. *Mol. Gen. Genet.* **258**, 562–570.
26. The *Arabidopsis* Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature*, **408**, 796–815.
27. Howard, D. H. (1956). The preservation of bacteria by freezing in glycerol broth. *J. Bacteriol.* **71**, 625.
28. Kocka, F. E. & Bretz, H. W. (1969). Survival of *Escherichia coli* after storage in various frozen menstrua. *Antonie van Leeuwenhoek*, **35**, 65–76.
29. Sidyakina, T. M. & Golimbet, V. E. (1991). Viability and genetic stability of the bacterium *Escherichia coli* HB101 with the recombinant plasmid during preservation by various methods. *Cryobiology*, **28**, 251–254.
30. Salt, D. T., Arret, B. & Wilner, J. (1973). Low temperature maintenance of test organism suspensions for antibiotic assays. *J. Pharm. Sci.* **62**, 2040–2043.
31. Grivell, A. R., Ganesan, A. K. & Hanawalt, P. C. (1972). Preservation of *Escherichia coli* on silica gel. *Microb. Genet. Bull.* **34**, 15.
32. Devay, J. E. & Schnathorst, W. C. (1963). Single-cell isolation and preservation of bacterial cultures. *Nature*, **199**, 775–777.
33. Dietz, A. (1975). Nitrogen preservation of stock cultures of unicellular and filamentous microorganisms. In *Round Table Conference on the Cryogenic Preservation of Cell Cultures* (Rinfret, A. P. & LaSalle, B., eds), National Academic of Science, Washington, DC.
34. Antheunisse, J. (1972). Preservation of microorganisms. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **38**, 617–622.
35. Antheunisse, J. (1973). Viability of lyophilized microorganisms after storage. *Antonie van Leeuwenhoek J. Microbiol. Serol.* **39**, 243–248.
36. Lapage, S. P., Shelton, J. E., Mitchell, T. G. & Mackenzie, A. R. (1970). Culture collections and the preservation of bacteria. *Methods in Microbiology* (Norris, J. R. & Ribbons D. W., eds), vol. 3a, pp. 135–228, Academic Press, New York.
37. Kropinski, A. M. (1975). Stability of bacterial mutants in saline. *Appl. Microbiol.* **29**, 448–450.
38. Miyamoto-Shinohara, Y., Imaizumi, T., Sukenobe, J., Murakami, S., Kawamura, S. & Komatsu, Y. (2000). Survival rate of microbes after freeze-drying and long-term storage. *Cryobiology*, **41**, 251–255.
39. Klein, M., Eckert-Ossenkopp, U., Schmiedeberg, I., Brandt, P., Unseld, M., Brennicke, A. *et al*. (1994). Physical mapping of the mitochondrial genome of *Arabidopsis thaliana* by cosmid and YAC clones. *Plant J.* **6**, 447–455.
40. Unseld, M., Marienfeld, J. R., Brandt, P. & Brennicke, A. (1997). The mitochondrial genome of *Arabidopsis thaliana* contains 57 genes in 366,924 nucleotides. *Nature Genet.* **15**, 57–61.
41. Itaya, M. & Tanaka, T. (1997). Predicted and unsuspected alterations of the genome structure of genetically defined *Bacillus subtilis* 168 strains. *Biosci. Biotechnol. Biochem.* **61**, 56–64.
42. Itaya, M. & Tanaka, T. (1991). Complete physical map of the *Bacillus subtilis* 168 chromosome constructed by a gene-directed mutagenesis method. *J. Mol. Biol.* **220**, 631–648.
43. Schaeffer, P., Millet, J. & Aubert, J. P. (1965). Catabolic repression of bacterial sporulation. *Proc. Natl Acad. Sci. USA*, **54**, 704–711.
44. Nicholson, W. & Setlow, P. (1990). Sporulation, germination and outgrowth. In *Molecular Biological Methods for Bacillus.* (Harwood, C. R. & Cutting, S. M., eds), John Wiley and Sons, Chichester, England.
45. Tomita, S., Tsuge, K., Kikuchi, Y. & Itaya, M. (2004). Targeted isolation of a designated region of the *Bacillus subtilis* genome by recombinational transfer. *Appl. Environ. Microbiol.* **70**, 2508–2513.
46. Itaya, M. (1990). Isolation and characterization of a second RNase H (RNase HII) of *Escherichia coli* K-12 encoded by the *rnhB* gene. *Proc. Natl Acad. Sci. USA*, **87**, 8587–8591.

Edited by J. Karn

(Received 22 February 2005; received in revised form 11 April 2005; accepted 19 April 2005)