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Stable Positional Cloning of Long Continuous DNA in the *Bacillus subtilis* Genome Vector

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Direct cloning of a long continuous genome segment in a *Bacillus subtilis* genome vector was demonstrated for the first time. Two small DNA fragments had to be installed in the vector prior to cloning. The DNA between these two fragments was cloned via homologous recombination. The efficiency of cloning was estimated using the 3,573-kb genome of a cyanobacterium, *Synechocystis* sp. PCC 6803. Recombinants were selected using the internal selection system of the *Bacillus* genome vector or with the antibiotic resistance marker in the cyanobacterial genome. Designated genomic segments as large as 77-kb were cloned by means of a single procedure. Cloning efficiency is affected by the molecular weight of the donor DNA and the size of the DNA to be cloned. The method is suitable for direct target cloning of large-sized DNA.

Key words: genome vector, homologous recombination, positional cloning, transformation.

Abbreviation: CHEF, contour-clamped homogeneous electric field.

DNA cloning technology emerged in the late 70s and has led to great successes in DNA manipulation (*1*, *2*). The complete sequences of a number of genomes, of from bacteria to eukaryotes, have unveiled much about genome structure as well as the functions of gene products. Given that all the ORF information has been presented, the handling of a large number of genes as one set becomes important (*3*, *4*). As regards the size of clonable DNA, *Escherichia coli* vectors such as BAC (*5*) and PAC (*6*) can be used for handling DNA larger than 100 kb. The YAC vector in *Saccharomyces cerevisiae* can harbor larger DNA segments, however, it is associated with a high degree of chimera formation and clonal instability (*7*). There are few methods, however, for precise positional cloning for DNA above the limit of PCR-mediated amplification.

A novel *Bacillus subtilis* genome vector was developed to fill this technical gap (*8*, *9*). The cloning principle of the genome vector differs from that of the prevailing plasmid-born vectors. *B. subtilis* develops natural competency by which DNA outside the cell is taken up into the cytoplasm (*10*, *11*). If a homologous sequence is present in the genome, the DNA taken up is integrated into the genome through the *recA*-dependent homologous recombination pathway (*12*, *13*). According to this protocol, two short DNA fragments that flank the target region are required (Fig. 1). These short DNA, or LPSs, standing for *L*anding *P*ad *S*equences (*9*), are prepared in the *E. coli* plasmid pBR322 and installed in the pBR322 sequence of the *B. subtilis* genome (*12*). The intervening DNA segment delineated precisely by the two LPSs is integrated via homologous recombination at both LPSs. The *B. subtilis* strain is called a BGM vector, standing for *Bacillus* GenoMe vector (*9*), and the cloning procedure for the BGM vector itself is equivalent to positional cloning. A positive selection system developed previously facilitates the cloning process (*14*). Two examples have been reported, one includes a 48.5-kb *E. coli* bacteriophage lambda DNA (*8*), and the other mouse genomic DNA ranging 100–140 kb in size (*9*). These cloned segments were stably maintained in the BGM vector regardless of the highly repetitive nature of the mouse genome. Cloning in the BGM vector in the two latter cases, however, resulted in the transfer of DNA previously cloned by means of a different technology (*8*, *9*). Cloning directly from genomic DNA has not been examined.

Direct cloning of the designated region of a cyanobacterium genome was attempted in this study and it was demonstrated that continuous genomic DNA of up to 77 kb could be successfully cloned in the BGM vector. Factors affecting the cloning efficiency were quantitatively measured. This genome vector has high potential to provide properly manipulated DNA through direct cloning of submega-sized DNA.

MATERIALS AND METHODS

1.1. Bacterial Strains and Plasmids—*B. subtilis* 168 *trpC2*, from the *Bacillus* Genetic Stock Center (Ohio, USA), and *E. coli* JA221 are routinely used as hosts for molecular cloning in this laboratory (*15*). Competent *B. subtilis* cells were prepared (*10*) and stored at –70°C in the presence of 20% (v/v) glycerol before use (*13*).

1.2. B. Subtilis Genome Vector—Two DNA sequences characterize the *B. subtilis* genome vector (*9*). They are a 4.3-kb pBR322 sequence inserted in the *Not*I site of the *proB* gene (*12*) and the neomycin resistance gene [*Pr-neo*] integrated into the *Not*I site in the *yvfC-yveP* gene (*14*).

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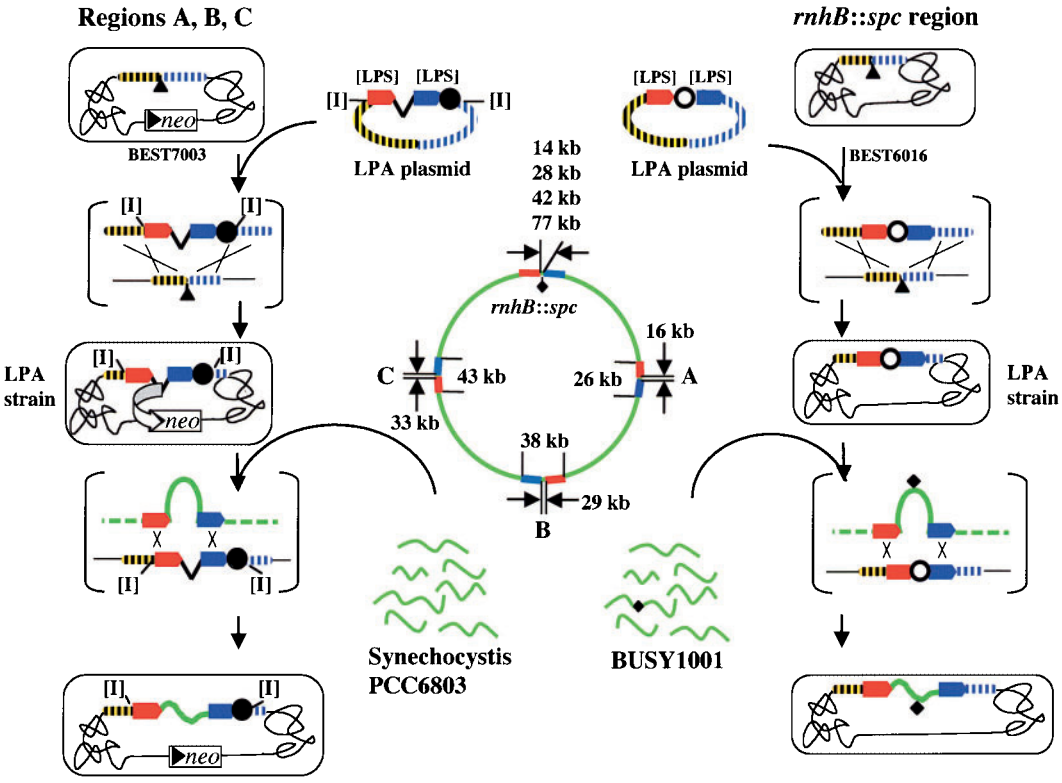

Fig. 1. **Positional cloning of the *Synechocystis* genome in the *B. subtilis* genome (BGM) vector.** BEST6016 and BEST7003 are genome vectors for direct and indirect selection. The structure of the intermediate genome is shown in parentheses. X indicates homologous recombination. The genomic pBR322 sequence is presented in the yellow (amp-half) and blue (tet-half) hatched boxes divided by the cloning site. Antibiotic resistance genes are indicated by closed circles (chloramphenicol), open circles (erythromycin), closed triangles (tetracycline), and closed diamonds (spectinomycin). DNA fragment sizes are not drawn to scale. A twisted arrow indicates suppression of the *P*r-promoter by the CI gene product. [I] for BEST7003 indicates the site for I-*Ppo*I. Screening of the bottom recombinants is described in the text.

The integrated pBR322 is referred to hereafter as the genomic pBR and is divided into two halves, the 2.4 kb amp-half including a β-lactamase gene and the 1.9 kb tet-half including a tetracycline resistance determinant gene. DNA is cloned between these two-halves, as illustrated in Fig. 1. Expression of the *neo* gene is regulated by a *P*r promoter to which the *c*I repressor protein binds, which represses it (*14*).

1.3. Synechocystis Strains—The genome of unicellular cyanobacterium *Synechocystis* sp. strain PCC (Pasteur Culture Collection) 6803 has been sequenced (*16*). This photosynthetic bacterium was chosen because its genome sequence is known (http://www.kazusa.or.jp/cyano/) and it is non-pathogenic. Cyanobacteria are unique organisms that perform oxygenic photosynthesis like chloroplasts of plants. Systematic analysis of gene expression has been carried out with many tools such as DNA microarrays (*17*). BG11 medium (*18*) was used to cultivate *Synechocystis* strains in liquid and on agar plates. The *Synechocystis rnhB* mutant was derived from the wild type strain of PCC 6803. The gene encoding ribonuclease H [EC. 3.1.26.4] chosen in this study is related to another target of our laboratory (*19*). The *Synechocystis rnhB* gene was replaced by the mutated gene *rnhB*::*spc* of pBRSYNrnhBS1 according to the method developed by Sugita and Sugiura (*20*). Colonies formed after 3 days on BG11 plates containing spectinomycin at 20 μg/ml under illumination at 50 μmol photons m^{-2} s^{-2} at 30°C. They were transferred to fresh BG11 medium containing spectinomycin (20 μg/ml) and then incubated for 4 weeks with occasional dilution, allowing them to pass through 11 generations. As the multiplicity of the *Synechocystis* genome is 12 (*21*), the mutant can be validated by the ratio of Southern band intensities of the mutant and wild type alleles. BUSY1001 contained 94.9% mutant *rnhB*::*spc* allele. The incomplete replacement by the mutant allele together with no associated phenotype indicates that the *rnhB* gene may be essential for the strain.

1.4. Preparation of High Molecular Weight Synechocystis Genome DNA—Genomic DNA prepared according to the reported method (*21*, *22*) included little DNA above 50 kb, as shown in Fig. 2A, and gave no recombinants. The extraction protocol in reference 21 was modified to obtain high-molecular weight DNA. A 50-ml *Synechocystis* culture was treated three times by freeze-thawing in dry ice and a water bath before harvesting. The cell pellet was washed with 5 ml of a NaCl/EDTA solution (120 mM/50 mM, pH 8.0), and then suspended in 4 ml of NaCl/EDTA and 1 ml of a saturated sodium iodide (NaI) solution. After incubation at 37°C for 30 min, 5 ml of a 25% sucrose TES solution (100 mM Tris-HCl [pH 7.6], 10 mM NaCl, and 1 mM EDTA) supplemented with 10 mg of lysozyme ml^{-1} was added, followed by incubation for 2 h at 37°C. After incubation for 17 hours with protei-

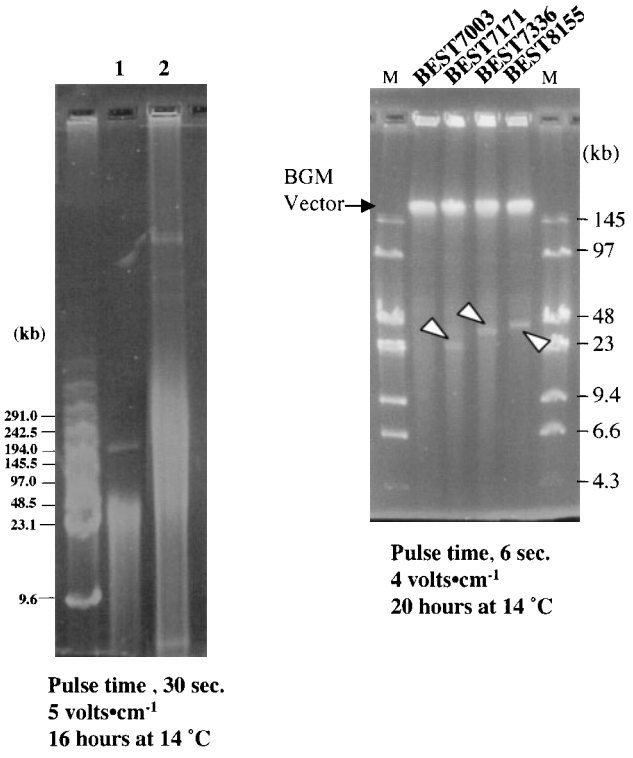

Fig. 2. **A. High molecular weight genomic DNA of *Synechocystis*.** BUSY1001 DNA was prepared by the standard method (lane 1) or by the modified method, as described under materials and methods (lane 2). The discrete bands are plasmids of this bacterium (*16*). Lambda oligomers plus *Hin*dIII digests with their sizes are given on the left. **B. I-*Ppo*I fragments resolved by CHEF.** The *Synechocystis* DNA of 27, 39 and 44 kb was from BEST7171, BEST7336, and BEST8155. These sequences include LPSs and a Cm (1 kb) or Em (1.2 kb) resistance gene. M includes Lambda oligomers plus *Hin*dIII digests, with their sizes on the right.

nase K at 250 μg ml^{-1} and 1.0% sodium dodesyl sulfate (w/v), a 5.75 ml sample was shaken with an equal amount of a phenol/chloroform mixture, followed by centrifugation at 3,500 ×*g* for 15 min. The upper phase, approximately 5 ml, was transferred to a fresh 50 ml tube (Falcon, 2070), and then three times the volume of Ethanol was added to precipitate the genomic DNA. The DNA collected by centrifugation at 3,500 ×*g* for 15 min was rinsed in 10 ml of 70% ethanol. The concentration of the DNA dissolved in 0.2 ml was determined by UV absorption at 260 nm using GeneQuantII (Pharmacia Biotech). This process yielded 5.69 mg/ml for PCC6803 and 3.6 mg/ml for BUSY1001. These DNA preparations included high molecular weight DNA (above 100 kb), as shown in Fig. 2A, and are used throughout this work.

1.5. Preparation of B. Subtilis Genome DNA—Intact unsheared DNA for CHEF gel electrophoretic analysis was prepared in agarose plugs as described previously (*23*). Liquid DNA for conventional Southern analysis was prepared by the method of Saito and Miura (*24*). Agarose gel (1.0% w/v) in TBE solution (45 mM Tris-borate, [pH8.0], 1.0 mM EDTA) was used for CHEF gel electrophoresis at a constant voltage of 3 or 4 V cm^{-1} at 14°C. The pulse and running times are specified in the legends to the figures. Agarose gel (1.0% v/v) in a TAE solution (50 mM Tris-acetate [pH 8.0] and 1.0 mM EDTA) was used for conventional gel electrophoresis at room temperature. The DNA in the gels after electrophoresis was stained with an ethidium bromide solution (60 μg/ml) for 15 min, followed by photography. The Southern hybridization experiment was performed according to the protocol for a DNA labeling and detection kit (Roche, USA)

RESULTS

2.1. Cloning with an Indirect Selection Marker—As *Synechocystis* has no appropriate selection markers, the use of the internal selection system of the BGM vector (*14*) allows cloning of any genomic loci. Cloning from three genomic loci, the 16-kb region A (0882–0899), the 29-kb region B (1696–1725), and the 33-kb region C (2558–2591), is illustrated in Fig. 1.

The LPS plasmids and the plasmids combined with the two LPSs for each region were prepared in *E. coli* as listed in Table 1. The latter plasmid is referred to as an LPA plasmid, standing for LPS Array plasmid. *B. subtilis* strains having LPA in the genomic pBR were constructed separately aiming at different target regions; BEST7166 for region A, BEST7332 for region B, and BEST8135 for region C (Table 1). For region A, 271 colonies were selected using neomycin at 3 μg/ml after the transformation of BEST7166 with PCC6803 genome DNA. Nine clones sensitive to spectinomycin at 50 μg/ml were subjected to colony PCR screening. The internal 4,650-bp of the 16 kb was amplified from two clones. Both carry the same 27–kb DNA generated by I-*Ppo*I-digestion. The I-*Ppo*I fragment produced from BEST7171 was resolved by pulsed-field gel electrophoresis (Fig. 2B). The primer set used to amplify the internal 4,650-bp segment of 16 kb was 5′-GTGGCAGAGTCGGTATTGGCTC-3′ (0898890F) and 5′-CCGGGTATTTATGGATCCCTAACC-3′ (0903540R).

Similarly, the 29-kb segment of region B was cloned in BEST7336. Only this strain was isolated from 15 neomycin-resistant and spectinomycin-sensitive candidates *via* colony PCR screening using 5′-GGGATCAACTACAGTGCCCGG-3′ (1724984F) and 5′-TGTGGATCCTTGGATTTCATCAGG-3′ (1729678R) to detect the internal 4,694-bp segment. Cloning of the 33-kb region C resulted in only one strain, BEST8155, on screening for the 55 neomycin-resistant and spectinomycin-sensitive clones by colony PCR screening. The primer set for the internal 726-bp segment of 33 kb was 5′-CAATTCCCTCAGTCCCGACG-3′ (2591470F) and 5′-GGTCCTGGGCGTTAAAGGC-3′ (2592196R). The I-*Ppo*I segments from these strains were confirmed, as shown in Fig. 2B. Southern analysis of *Bam*HI and *Hin*dIII digests using *Synechocystis* genomic DNA as a probe verified that these cloned DNA were identical with those predicted from the sequences in the database (data not shown). These results indicated that target cloning with an internal positive selection system proceeds through the mechanism illustrated in Fig. 1.

2.2. Cloning with a Direct Selection Marker—Despite the effectiveness of the internal marker system, the cloning of DNA segments longer than 50 kb was unsuccessful (data not shown). The number of true recombinants seemed underestimated due to the large number of background neomycin for resistant colonies for a poorly

Table 1. **Bacterial strains and plasmids for indirect selection.**

Bacteria	Genotypes	Sources or references	
Synechocystis sp. PCC6803		(Institut Pasteur, France)	
BUSY1001	*rnhB*::*spc*	pBRSYNrnhBS1 × PCC6803	
Bacillus subtilis (1A1)	*trpC2*	(BGSC, Ohio, USA)	
RM125	*arg leu*	(*9*)	
BEST6016	*proB*::pBRTc	(*13*)	
BEST7003	*proB*::pBRTc, *yah*::pr-*neo*	this study	
BEST4110	*leuB*::*cat*	this study	
LPA strains and recombinants			
BEST7166	*proB*::pC[0878/0903]	CmR, SpcR, NmS	pC[0878/0903] × BEST7003
BEST7171	*proB*::pC[26 kb]	NmR, SpcS, CmR	PCC6803 × BEST7166
BEST7332	*proB*::pC[1691/1729]	CmR, SpcR, NmS	pC[1691/1729] × BEST7003
BEST7336	*proB*::pC[38 kb]	NmR, SpcS, CmR	PCC6803 × BEST7166
BEST8135	*proB*::pE[2553/2596]	EmR, SpcR, NmS	pE[2553/2596] × BEST7003
BEST8155	*proB*::pE[43 kb]	NmR, SpcS, EmR	PCC6803 × BEST8135
Plasmids	Construction or features	References	
pBRSYNrnhB	*rnhB* (PCC6803)	gene amplified cloned in pBR322	
pBRSYNrnhBS1	*rnhB*::*spc*	1.3kb/*Sma*I into pBRSYNrnhB/*Aat*I	
pBMAP105TT	*leuB*::*tet*	(*13*)	
LPS clones isolated from PCC6803 and derivatives			
LPS	Plasmid	Segment size (kb)	Region or vector
[0878]	pCR[0878–0882]¶	4.67	878,001 to 882,669
[0903]	pCR[0899–0903]¶	4.53	899,015 to 903,540
[1691]	pCR[1691–1696]¶	4.74	1,691,653 to 1,696,392
[1729]	pCR[1725–1729]¶	4.54	1,725,139 to 1,729,678
[2553]	pCR[2553–2558]¶	4.91	2,553,209 to 2,558,117
[2596]	pCR[2591–2596]¶	4.96	2,591,742 to 2,596,704
pC0878	pCR[0878–0882]/*Bam*HI		pCISP310B/*Bam*HI†
pC1691	pCR[1691–1696]/*Bam*HI		pCISP310B/*Bam*HI†
pE2553	pCR[2553–2558]/BamHI		pCISP311B/*Bam*HI†
LPA plasmids for integration into BEST7003			
pC[0878/0903]	pCR[0899–0903]/*Eco*RI	pC0878/*Eco*RI	
pC[1691/1729]	pCR[1725–1729]/*Eco*RI	pC1691/*Eco*RI	
pE[2553/2596]	pCR[2591–2596]/*Eco*RI	pE2553/*Eco*RI	

Cm, chloramphenicol; Sp, spectinomycin; Nm, neomycin; Em, erythromycin; R, resistance; S, sensitive. ¶Cloned in pCR-XL-TOPO vector. †pCISP310B and pCISP311B are reported in Ref. *9*.

understood reason (*9*, *14*). a direct selection method was used for quantitative measurements of size-dependent efficiency. A *Synechocystis* derivative, strain BUSY1001, that has a spectinomycin resistance gene in the *rnhB* gene was constructed. As the spectinomycin resistance gene functions for selection of *B. subtilis*, only recombinants containing the resistance gene are selected directly.

Four *B. subtilis* strains for cloning of *rnhB*::*spc* segments of various sizes, from 14 kb to 77 kb, were constructed. Initially, the number of spectinomycin-resistant colonies varied among the experiments but the number became reproducible when the outgrowth period after DNA uptake was extended from 1 hour to 2 hours. It is likely that appropriate expression from the spectinomycin resistance gene requires prolonged incubation. This condition was employed throughout this study.

Recombinants selected using spectinomycin at 50 μg/ml were all sensitive to erythromycin at 5 μg/ml. Six representative clones for 14-kb, 4 for 28-kb, 4 for 42-kb, and 3 for 77-kb were analyzed by Southern hybridization using *Synechocystis* genome DNA as a probe. As shown in Fig. 3, the numbers and sizes of the *Not*I and *Bgl*II Southern bands were consistent with those predicted from the sequence information. The lack of unexpected bands indicated high structural stability of the cloned segment in the BGM vector. No structural alteration of the *B. subtilis* genome part was detected on *Sfi*I and *Not*I fragment analysis (data not shown). These results demonstrated that all colonies selected with spectinomycin integrated the *rnhB*::*spc* segment replacing the *erm* gene between the two LPSs, as shown in Fig. 1.

2.3. Size-Dependent Efficiency of Cloning—Experiments were performed in triplicate with two BUSY1001 DNA concentrations, 1.80 and 6.05 μg/ml. The average number of spectinomycin- resistant transformants was plotted with standard deviations (Fig. 4). The number decreased as the size of the clone increased from 14 kb to 77 kb at both DNA concentrations. Saturation by *Synechocystis* DNA was not clear under the present conditions. This is consistent with the observation that the frequency of segment transfer between *B. subtilis* genomes

Table 2. **Bacterial strains and plasmids for direct selection.**

Bacteria	Genotypes	Antibiotic markers	Sources or references
BEST7004	proB::p[LPS1/Em/LPS2]	EmR	p[LPS1/Em/LPS2] × BEST6016
BEST7016	proB::pBR[16.5 kb (89554–115223)]	SpR, EmS	BUSY1001 × BEST7016
BEST7012	proB::p[LPS1/Em/LPS3]	EmR	p[LPS1/Em/LPS3] × BEST6016
BEST7017	proB::pBR[42kb (75564–115223)]	SpR, EmS	BUSY1001 × BEST7016
BEST7008	proB::p[LPS1/Em/LPS4]	EmR	p[LPS1/Em/LPS4] × BEST6016
BEST7018	proB::pBR[50 kb (61054–115223)]	SpR, EmS	BUSY1001 × BEST7008
BEST7015	proB::p[LPS1/Em/LPS5]	EmR	p[LPS1/Em/LPS5] × BEST6016
BEST7019	proB::pBR[90 kb (25603–115223)]	SpR, EmS	BUSY1001 × BEST7015
BEST7021	proB::pBR[90 kb (25603–115223)]	*leuB*::*tet*, SpS	see the text and Fig. 5
LPS clones isolated from PCC6803 and derivatives			
Plasmid	Segment size (kb)	Region	Vector
[LPS1] pSYNrnhB2′	7.48	107755–115223	*Hin*dIII/pBR322
[LPS2] pCRNHBOT	4.18	89554–93735	pT7BlueT
[LPS3] pCRNHB-101	4.17	75564–79734	*Bam*HI/pBR322
[LPS4] pSYN3-TO	5.01	61054–66059	pCR-XL-TOPO
[LPS5] pCRNHB-203	5.11	25603–30713	*Eco*RI/pBRcI′.
LPA plasmids	Donor	Vector	
p[LPS1/LPS2]	pSYNrnhB2′/*Bam*HI	pCRNHBOT/*Bam*HI	
p[LPS1/LPS3]	pSYNrnhB2′/*Bam*HI	pCRNHB-101/*Bam*HI	
p[LPS1/LPS4]	pSYNrnhB2′/*Bam*HI	pSYN3-TO/*Bam*HI	
p[LPS1/LPS5]	pSYNrnhB2′/*Bam*HI	pCRNHB-203/*Bam*HI	
LPA plasmids for integration into BEST6016[§]			
p[LPS1/Em/LPS2]	p[LPS1/LPS2]/*Eco*RV		
p[LPS1/Em/LPS3]	p[LPS1/LPS3]/*Eco*RV		
p[LPS1/Em/LPS4]	p[LPS1/LPS4]/*Eco*RV		
p[LPS1/Em/LPS5]	p[LPS1/LPS5]/*Nhe*I-T4DNApolymerase		

[†]http://www.kazusa.or.jp/cyano/. [§]The *erm* cassette prepared from pBEST701 (*9*) with *Sma*I was inserted into the site indicated between the two LPSs. A 1.3-kb fragment containing the *rnhB* gene was obtained by PCR-mediated amplification from genomic DNA of BUSY101 using primers *rnhB*, 5′-CCTAATCAAAACGGAGTACG-3′ and 5′-AATTGCCATTGAAGTGGCGG-3′. The primer sets used for amplification were: [LPS5] pCRNHB-203: SYNrnhB-20F 5′-GGGACCAAGTACAACAACTC-3′ and SYNrnhB-20R 5′-GGGGGAGGAAATTATCAGCG-3′; [LPS4] pSYN3-TO: SYN3F 5′-GCCGAATTCCGGGCTGGATCCCCT-3′ and SYN3R 5′-TATGGATCCTAACAACGACTTCCCC-3′; [LPS3] pCRNHB-101: SYNrnhB1F 5′-GGAGGAAGTCTGTTGCTCGG-3′ and SYNrnhB1R 5′-GTTGGGGTTTACAGGCGGTG-3′; [LPS2]: CRNHBOF 5′-TCAGGATCCGCCACCTTTTGACCGTTG-3′ and CRNHBOR 5′-TCAGGATCCCGGTCCAGAAGCATGG-3′; [LPS1]: SYNrnhB2F 5′-GGGGTTAAACGAATGACAGCGG-3′ and SYNrnhB2R 5′-AGGGACTGGAGGGGACAATG-3′.

appeared to be inversely proportional to the segment size (*13*). The degrees of competency of the four parental strains determined by a standard method (*13*) did not differ significantly, as indicated in Fig. 4. The cloning efficiency of cyanobacterial fragments was normalized by comparison with the degree of competency: 3.1% for 14 kb, 0.85% for 28 kb, 0.42% for 42 kb, and 0.09% for 77 kb. The rate-determining step may be the integration process, as discussed previously (*13*).

2.4. Cloned Segment as Part of the BGM Vector—These recombinants showed no apparent reduction in the rate of growth, as measured in antibiotic-free LB medium at three temperatures, 25, 35, and 45°C (data not shown). Although the expression profiles of genes in the cloned region were not investigated, the cloned *Synechocystis* segment likely replicates as part of the *B. subtilis* genome. This suggests that cloned *Synechocystis* DNA functions similarly with respect to transformation. The 77 kb *Synechocystis*–originated DNA of BEST7019 was examined for transformation of *rnhB*::*spc* of BUSY1001. As shown in Fig. 5, BEST7021 was derived from BEST7019 through conversion of *rnhB*::*spc* to *rnhB*. This conversion was carried out by gene-directed mutagenesis using *leuB*::*tet* as a catalyst gene (*25*). Transformation of BEST7021 with the BUSY1001(*rnhB*::*spc*) DNA gave a substantial number of spectinomycin-resistant colonies. The number, 2.83×10^2, *i.e.* 43.3% of the relevant transfer of *leuB*::*tet*→*leuB*::*cat*, 6.55×10^2, by BEST4110, indicated that the 90-kb cyano segment and other loci in the *B. subtilis* genome are not discriminatory in terms of genetic transformation. The slightly lower frequency may be accounted for by the limited length for homologous recombination, only 10 kb, as indicated in Fig. 5.

DISCUSSION

The present results strongly indicate that the target DNA of the *B. subtilis* genome vector basically can be of any kind. The preparation of two flanking segments prior to BGM construction is unavoidable but allows for the positional cloning of large DNA beyond the limit of PCR technology. Cloning in the *B. subtilis* genome vector has several advantages compared with cloning in plasmids. The DNA integrated in the genome exhibited high genetic stability and did not segregate even in antibiotic-free LB-medium. It was proven that the cloned DNA becomes indistinguishable from the rest of the *B. subtilis* genome with respect to genetic transformation. This finding raised the possibility of unlimited manipulation against the cloned segment in the BGM vector (*15*, *26*).

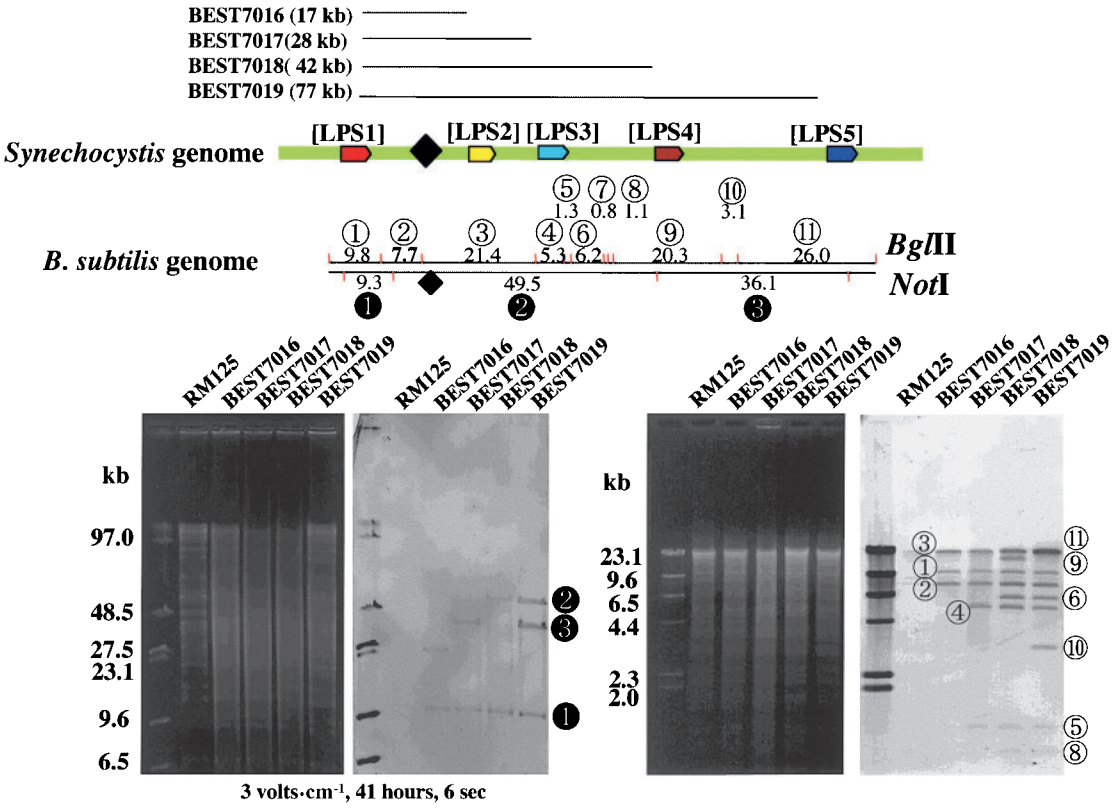

Fig. 3. **The cloned cyanobacterial segment in the *B. subtilis* genome vector.** Genomic DNA of the indicated strains digested with *Not*I (left) or *Bgl*II (right) was run. The running conditions for CHEF are shown. The probe was prepared using BUSY1001 genomic DNA after complete digestion with *Hin*dIII. The Southern band indicated by the circled number corresponds to the *Synechocystis* genomic *Not*I and *Bgl*II restriction map of this region. The end fragments were altered due to the cloning in the BGM vector. Fragment 7 of BglII is too small to be seen under these conditions.

Precise positional cloning depends on effective double homologous recombination. The cloning process starts with the incorporation of DNA by competent cells. According to the proposed mechanism (*11*), the double-stranded DNA is converted to single-stranded DNA by a competent complex formed in the cell wall. About 20–30 kb fragments enter the competent *B. subtilis* cells effec-

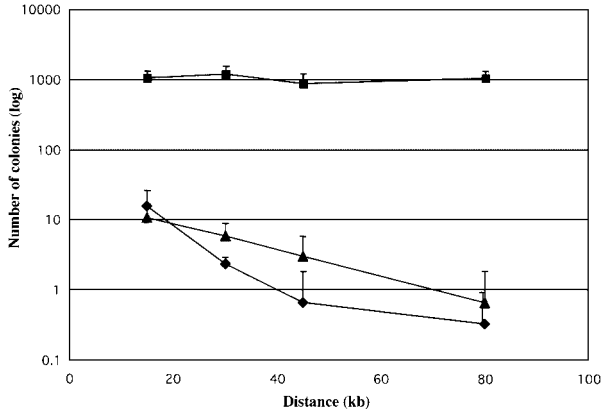

Fig. 4. **Size-dependent efficiency of cloning.** The number of spectinomycin-resistant transformants is plotted against the size of the cloned segment. Two concentrations of *Synechocystis* genomic DNA, 1.80 μg/ml (closed diamonds) and 6.05 μg/ml (closed triangles), were used. The degrees of competency of the four strains measured as the number of tetracycline-resistant transformants DNA (closed box) were nearly identical. Standard deviation (SD) is indicated by vertical bars.

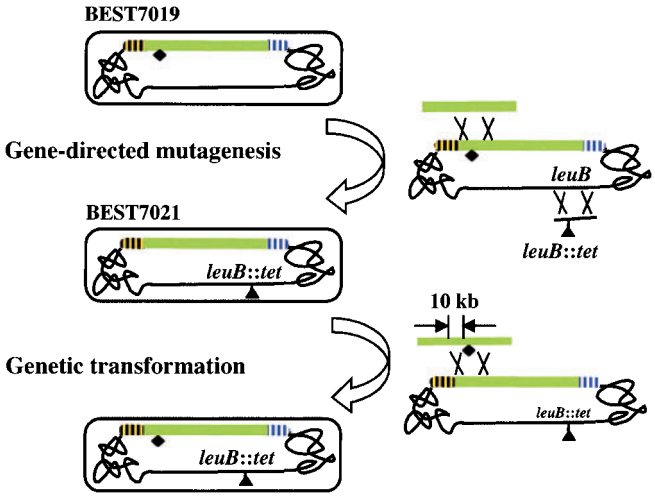

Fig. 5. **Genetic transformation within the new genome region. X indicates homologous recombination.** Cognate genomic transformation was measured using BEST4110 DNA. Other symbols are the same as in Fig. 1. BEST7021 supplies only 10 kb of *Synechocystis* DNA of the left end instead of the sequence longer than 70 kb of the right.

tively during transformation (*27*), and a single-stranded DNA of approximately 7 kb is found in the cells (*11*). The actual size of the DNA incorporated has been controversial. In our previous study, it was demonstrated that continuous DNA of longer than 50 kb actually enters a competent cell and recombines with the genome (*13*). This size was nearly doubled in the present study, leaving the argument unresolved. The efficiency of integration apparently decreased as the size of the DNA to be cloned increased at all concentrations. Although LPS was empirically employed as 5 to 10% of the target DNA length (*9*), no significant difference in cloning efficiency between the smallest [LPS3] (4.17 kb) and largest [LPS5] (5.11 kb) was observed. Above all, high molecular weight donor DNA is critical for effective cloning as well as for determination of the maximum DNA size entering a competent cell.

The internal selection system facilitates regional-specific cloning from not only bacterial but also eukaryote genomes, as long as high molecular weight DNA is prepared. In addition to the present *Synechocystis* genome, whose G+C content is 45% (*16*), which is close to that (43%) of *B. subtilis* (*28*), cloning of DNA with a higher or lower G+C content is underway to exploit the use of the BGM vector. It is likely that larger DNA could be harbored if the internal selection marker system is used repeatedly (Itaya and Fujita, unpublished experiment). Manipulation of the cloned DNA with a combination of efficient recovery tools (*15*, *27*) makes the BGM system prominent.

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