Targeted Isolation of a Designated Region of the *Bacillus subtilis* Genome by Recombinational Transfer

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A method for positional cloning of the *Bacillus subtilis* genome was developed. The method requires a set of two small DNA fragments that flank the region to be copied. A 38-kb segment that carries genes *ppsABCDE* encoding five enzymes for antibiotic plipastatin synthesis and another genome locus as large as 100 kb including one essential gene were examined for positional cloning. The positional cloning vector for *ppsABCDE* was constructed using a *B. subtilis* low-copy-number plasmid that faithfully copied the precise length of the 38-kb DNA in vivo via the recombinational transfer system of this bacterium. Structure of the copied DNA was confirmed by restriction enzyme analyses. Furthermore, the unaltered structure of the 38-kb DNA was demonstrated by complementation of a *ppsABCDE* deletion mutant.

Results of comparative genomic studies for a number of sequenced bacterial genomes indicate ubiquitous traits for DNA transfer between bacterial genomes (3, 4, 14). We are interested in horizontal DNA transfer, particularly by the process of integration into and elimination of large DNA segments in the genome. *Bacillus subtilis* has historically been used for this kind of investigation due to the ability to develop competency (1, 5, 17). It has been demonstrated that heterologous DNA segments of 50 to 100 kb, larger than previously considered, are taken up by competent *B. subtilis* and integrated into the genome (6, 7, 8, 11).

In contrast to these studies of the integration process, investigation of the reverse process has been limited to two experimental approaches (9, 19). One approach includes dissection of part of the *B. subtilis* genome to physically separate a 300-kb genomic segment that becomes an independent replicon or a 300-kb subgenome (9, 10). The other approach involves the copy and transfer of the cloned heterologous DNAs to an independent replicon by the *Bacillus* recombinational transfer mechanism (BReT) (19). The mechanism of BReT-mediated transfer is indicated in Fig. 1. It requires the two sequences called landing pad sequences (LPSs), where homologous recombination occurs during *B. subtilis* transformation. Intervening DNA between the two LPSs is copied and transferred to the BReT plasmid. Initially, the BReT system was developed to recover the heterologous DNAs cloned into the two halves of pBR322 sequences of the *B. subtilis* genome vector (19). The pBReT plasmid pGETS103 has the two halves of pBR322 sequences that are used as the two LPSs to designate the cloned DNA (19). It was demonstrated that pGETS103 stably maintains DNA segments of up to 100 kb due to its θ -type replicative form for *B. subtilis* (2, 19).

As the BReT depends on the intrinsic homologous recom-

ination pathways of *B. subtilis*, it should be applied for isolation of the internal segment of the *B. subtilis* genome by designing the appropriate LPSs. However, the attempt using pGETS103 was unsuccessful probably due to the plasmid's high copy number, nine on average (our unpublished observation). Therefore, pGETS109 that replicates with a copy number as low as one was derived from pGETS103 by the insertion of a *rho*-independent terminator in front of the *repA* gene, which put *repA* expression under the control of the *Pspac-lacI* repressor (20). The tetracycline resistance marker gene of pGETS109 was converted into the chloramphenicol resistance gene of pGETS113 that was used throughout this study for construction of pBReT vectors (Fig. 1).

A region carrying the genes with known function was first examined. *B. subtilis* Marburg strain produces a fungicidal lipopeptide, plipastatin, the peptide moiety of which is synthesized in a nonribosomal manner by the five peptide synthetases encoded by *ppsA*, *ppsB*, *ppsC*, *ppsD*, and *ppsE* (18). The five genes residing in the 38.2-kb-long *pps* operon (18) are *ppsA* (7.7 kb), *ppsB* (7.7 kb), *ppsC* (7.7 kb), *ppsD* (10.8 kb), and *ppsE* (3.9 kb). Cloning of this large DNA segment is not reported. Sequence information was downloaded from <http://www.Pasteur.fr/Bio/SubtiList.html> (13). Two LPSs, one of 1.9 kb (designated [LPS-P]) and one of 2.0 kb (designated [LPS-S]), were prepared by PCR-mediated amplification using primer sets listed in Table 1. Insertion of [LPS-P] and [LPS-S] into pGETS113 in the correct order and orientation was done as indicated in Fig. 1 and Table 1. The pBReT vector, pGETS113-ppsAE, was obtained.

PCR products were cloned using *Escherichia coli* TOP10 [F^- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) ϕ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *deoR* *araD139* Δ (*ara-leu*)7697 *galU* *galK* *rpsL*(Str^r) *endA1* *nupG*] supplied by Invitrogen Inc. (Carlsbad, Calif.). Subcloning to construct pBReT vectors was done by using *E. coli* JA221 [F^- *hsdR* *hsdM*⁺ *trp* *leu* *lacY* *recA1*], and JM109 [F^- *endA1* *recA1* *gyrA96* *thi* *hsdR17*(r_K⁻ m_K⁺) *relA1* *supE44* Δ (*lac-proAB*)(F' *traD36* *proAB* *lacI*^q *lacZ* Δ M15)] was routinely used as a host for molecular cloning. Luria-Bertani broth was used

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FIG. 1. Transfer of genomic segments into plasmids by recombinational transfer. Target segments of the genome are shadowed. The pBREt vector comprised pGETS113 (bold lines) and LPSs (parts of the open arrow). Recombinational transfer proceeds by homologous recombination between LPSs of the genome and the pBREt vector. The pBREt plasmid replicates as a single copy in the recombinant. The two genome loci are enlarged. A dark rhomb indicates the location of *menA*. Construction of the pBREt vector for the *pps* operon is described in Table 1. pBREt vectors for the other genome locus are represented by that for the 24.6-kb segment. The pBR322 sequence of pGETS1203 drawn by two lines is eliminated upon linearization.

for all bacteria at 37°C. Tetracycline (10 µg/ml) and ampicillin (50 µg/ml) were added for JA221 or JM109 selection. Manipulation of the plasmid in *E. coli* was done according to the established method (16). A large amount of plasmid was purified with a CsCl gradient formed by ultracentrifugation in the presence of ethidium bromide (16). Restriction enzymes were obtained from Toyobo (Tokyo, Japan). Blunting of DNA fragments and ligation were done using the TaKaRa blunting kit and TaKaRa DNA ligation kit (Takara Shuzo, Kyoto, Japan).

B. subtilis 168 *trpC2* (1A1) was from the *Bacillus* Genetic Stock Center (Columbus, Ohio). The competent 1A1 cell was prepared by the two-step culture method developed by Anagnostopoulos and Spizizen (1) with modifications as described previously (8). [LPS-P] and [LPS-S] of pGETS113-ppsAE were physically separated by complete NotI digestion prior to *B. subtilis* transformation. Only plasmid that copied the intervening 38.2-kb DNA by the BREt mechanism was established as a circular plasmid and rendered *B. subtilis* resistant to chloramphenicol (Fig. 1). One microgram of pGETS113-ppsAE yielded 143 chloramphenicol-resistant colonies selected at the

5- μ g/ml concentration. Plasmids prepared from four colonies by the miniprep method were identical in their SfiI fragments, and a representative plasmid was referred to as pGETS113-ppsABCDE. The structure of the 38.2-kb DNA segment plus [LPS-P] and [LPS-S] in pGETS113-ppsABCDE was checked using 11 restriction enzymes, BglII, EcoRI, EcoRV, HindIII, KpnI, SacI, SalI, SmaI, PstI, PvuII, and NotI (data not shown). The number and sizes of the fragments yielded by restriction enzyme digestion were all identical to those predicted using nucleotides of the *pps* locus (1,959,404 to 1,997,591) (13). Examples of HindIII and BglII digests are presented in Fig. 2A. To examine the gene function of the copied segment, the pGETS113-ppsABCDE plasmid was used to transform BUSY8519, a mutant that lacked the *pps* operon. Plipastatin production was fully restored in the chloramphenicol-resistant transformant as shown in Fig. 2B. These results proved that the five *pps* genes of pGETS113-ppsABCDE are functional and that no structural alteration was introduced during the BREt process, regardless of the high level of repetition in sequences in the *pps* operon (18) such as the 2.8-kb

TABLE 1. Plasmids for recombinational transfer

Plasmid	Description	Size (kb) of clone or cloned insert	Primers ^b	Positions cloned
LPS primary clones^a				
pCRppsA	[LPS-P]	1.9	ppsAF-SfiI, ppsAR-NotI	
pCRppsE	[LPS-S]	2.0	ppsEF-NotI, ppsER-SfiI	
pDAS101	[LPS-A]	2.6	AF, AR	
pDAS102	[LPS-B(100.3)]	1.8	B100.3-F, B100.3-R	
pDAS148	[LPS-B(24.6)]	1.9	B-24.6F, B24.6-R	
pDAS149	[LPS-B(46.6)]	2.2	B-46.6F, B46.6-R	
pDAS145	[LPS-B(58.8)]	1.8	B-58.8F, B58.8-R	
pDAS146	[LPS-B(88.7)]	2.1	B-88.7F, B88.7-R	
Assembled LPS primary clones				
pGETS113-ppsE	[LPS-S]/HindIII in pGETS113			
pDAS110	[LPS-A]/EcoRI and MunI inserted into the EcoRI site of pBR322			
pDAS152 ^c	[LPS-A] and [LPS-B (24.6)] inserted into pBR			
pDAS153 ^c	[LPS-A] and [LPS-B (46.6)] inserted into pBR			
pDAS154 ^c	[LPS-A] and [LPS-B (58.8)] inserted into pBR			
pDAS166 ^c	[LPS-A] and [LPS-B (88.7)] inserted into pBR			
pDAS117 ^c	[LPS-A] and [LPS-B (100.3)] inserted into pBR			
pBRet vectors				
pGETS113-ppsAE ^d	[LPS-P] and [LPS-S] in pGETS113			
pGETS1203 ^e	pDAS152 inserted into pGETS113			
pGETS1204 ^e	pDAS153 inserted into pGETS113			
pGETS1205 ^e	pDAS154 inserted into pGETS113			
pGETS1207 ^e	pDAS166 inserted into pGETS113			
pGETS1202 ^e	pDAS117 inserted into pGETS113			
pBRet plasmids				
pGETS113-ppsABCDE		38.2		1,959,404–1,997,591
pGETS1208		24.6		3,932,990–3,952,542
pGETS1209		46.6		3,932,990–3,979,557
pGETS1210		58.8		3,932,990–3,991,791
pGETS1212		88.7		3,932,990–4,021,664
pGETS1201		100.3		3,932,990–4,033,322

^a PCR-amplified fragments cloned in pCR-XL-TOPO by TA cloning. TOP10 transformants were selected by using kanamycin.

^b The primer sequences were as follows: ppsAF-SfiI, 5'-CGGCCGATGTGGCCCGGGAGACCTGTTTTCAGAG-3'; ppsAR-NotI, 5'-GCGGCCGCGTTCA GACAGGACACCGACG-3'; ppsEF-NotI, 5'-AAGCTTGGCCGCGCTCAGGATCAACAGGCCG-3'; ppsER-SfiI, 5'-AAGCTTGGCCAGACGCGCCATA CAAAAAGAAAGACGACAGAGAATGGC-3'; AF, 5'-GAATTCGGGTGTATACATGTGACGTGTGCGC-3'; AR, 5'-CAATTGGCGGCCGCGACGCTG GAAATCGTGCC-3'; B100.3-F, 5'-CAATTGGCGGCCGCGGTTCTGTGATCCCCATCAGCG-3'; B100.3-R, 5'-GAATTCGCCGTTACCTCTCCCTAAAGG-3'; B24.6-F, 5'-CAATTGGCGGCCGCGCTTAGGGCCGTTTTCG-3'; B24.6-R, 5'-GAATTCGAATCACTTCGCGTCACTCCC-3'; B46.6-F, 5'-CAATTGGCGGCC CGCCACAGCTGTGACCTACCCG-3'; B46.6-R, 5'-GAATTCACATTGGCACTGGTTGTAC-3'; B58.8-F, 5'-CAATTGGCGGCCGCGAATCATGGCTGC CTGAGAGC-3'; B58.8-R, 5'-GAATTCGTGGAAGGACAAGTGTCAACG-3'; B88.7-R, 5'-GAATTCGCAACGCGCGCGAAC-3'; and B88.7-F, 5'-CAATTG GCGGCCGCTCACCCAGTGGGCTTTTCAC-3'. EcoRI, MunI, HindIII, EagI, and NotI sites are underlined.

^c [LPS-B] digested by EcoRI was cloned into the EcoRI and MunI sites of pDAS110.

^d The [LPS-P]/EagI fragment of pCRppsA was ligated into the NotI site of pGETS113-ppsE.

^e A 7.5-kb BamHI-EcoRI fragment (blunted) of pGETS113 was cloned into the EcoRI sites of the indicated pDAS clones.

segments of *ppsA* and *ppsC* (97% identity in nucleotide sequences), the 1.8-kb segments (97% identity) of *ppsD* and *ppsA*, and the 1.7-kb segments (97% identity) of *ppsC* and *ppsD*.

As pGETS113 carries a ColEI origin of replication and is able to replicate in *E. coli*, the 50-kb pGETS113-ppsABCDE was introduced into JA221 by CaCl₂-mediated transformation with a frequency of 1.2×10^2 CFU/ μ g (15). Frequencies of transformation of JA221 and JM109 by electroporation were 6.8×10^4 and 2.6×10^2 CFU/ μ g, respectively. These transfor-

mants showed no apparent growth reduction. The plasmids isolated from these *E. coli* transformants were identical to pGETS113-ppsABCDE (Fig. 2A). This is the first example of the positional cloning of a 50-kb fragment in *E. coli* via *B. subtilis*.

To prove applicability to other internal DNAs, positional cloning of a 100-kb segment from another genome locus, 3,932,990 to 4,033,322 (13), was examined. First, cloning of the 24.6-kb region including an essential gene, *menA* (3,949,750 to 3,950,682), encoding 14-dihydroxy-2-naphthoate octaprenyl-

FIG. 2. (A) pBReT plasmids containing the *pps* operon. pGETS113-ppsAE (lane 1) and pGETS113-ppsABCDE prepared from *B. subtilis* (lane 2) and from *E. coli* JM109 (lane 3) were digested by the indicated restriction enzyme. Lambda/HindIII marker fragments were run in lane M with their sizes indicated on the left. Fragments shown by open arrowheads came from the internal region of the pBReT vector pGETS113-ppsAE. The number and sizes of the other fragments were identical to those predicted from a sequence data file constructed with nucleotide sequences of pGETS113 and of the *pps* locus (1,959,404 to 1,997,591) (13). (B) Complementation of plipastatin production by the *pps* operon of the pBReT plasmid. *B. subtilis* strain BEST8628 (plipastatin producer) and BUSY8519 (plipastatin nonproducer) are our laboratory stock. Samples were prepared according to the method described in reference 18 from the culture in 40 ml of ACS medium in 200-ml Erlenmeyer flasks at 30°C and 120 strokes/min. Three-day cultures were used for BEST8628 and BUSY8519, and four-day cultures were used for plasmid transformants. The indicated high-performance liquid chromatography (HPLC) peaks represent plipastatin stereoisomers within lipid and peptide moieties (18) and surfactins. Conditions for reverse-phase HPLC were the same as those described in reference 18.

transferase (12) was attempted using the pBReT vector pGETS1203. pGETS1203 was constructed by insertion of [LPS-A] and [LPS-B(24.6)] into pGETS113 as indicated in Fig. 1 and Table 1. Transformation of 1A1 by pGETS1203 (1 μ g) linearized with NotI yielded 10 chloramphenicol-resistant colonies. The plasmids isolated from these 10 recombinants had identical structures with respect to digestion by EcoRI, BamHI, and BglIII. The cloned 24.6-kb segment of the representative pGETS1208 was confirmed using restriction endonucleases EcoRV, KpnI, SacI, SalI, SfiI, SmaI, PstI, PvuII, and

NotI (data not shown except for PvuII in Fig. 3A). To examine positional cloning of a larger portion of this region, four pBReT vectors that designate 46.6, 58.8, 88.7, and 100.3 kb of this genome locus were separately constructed as described in Table 1. Transformation experiments with strain 1A1 were done in triplicate using 1 μ g of pBReT vectors linearized with NotI. The average numbers of chloramphenicol-resistant transformants were as follows: 11 ± 0.6 with pGETS1203, 28 ± 3.5 with pGETS1204, 5 ± 1.2 with pGETS1205, 8 ± 2.1 with pGETS1207, and 11 ± 1.2 with pGETS1202. All the plasmids

Pulse time 6 sec.
3 volts $\cdot$ cm $^{-1}$
9 hours at 14 $^{\circ}$ C

Probe: pGETS1201 plus lambda DNA

Pulse time 6 sec.
3 volts $\cdot$ cm $^{-1}$
9 hours at 14 $^{\circ}$ C

Probe: pGETS1201 plus lambda DNA

FIG. 3. (A) Positionally cloned inserts from pBReT plasmids. The indicated pBReT plasmids (cloned sizes [in kilobases] are in parentheses) were digested by PvuII and separated by contour-clamped homogeneous electric field (CHEF) gel electrophoresis. Running conditions for CHEF electrophoresis are given at the bottom. Lambda/HindIII marker fragments were run in lane M with their sizes indicated on the right. Left panel, gel stained with ethidium bromide. Right panel, Southern bands obtained using a mixture of probes made by pGETS1201 (100.3 kb) and lambda DNA digested with HindIII. The use of mixed probes established in reference 7 made lambda markers visible for direct comparison. The lambda/HindIII marker bands are seen in lane M. The PvuII fragments of the smaller pBReT plasmids are all hybridized by the longest insert (100.3 kb) of pGETS1201. Open arrowheads indicate two fragments containing the pGETS113 part divided by the internal PvuII site. (B) Identification of the positionally cloned region. DNA of 1A1 digested by the indicated restriction enzymes was separated by CHEF electrophoresis. Running conditions for CHEF are given at the bottom. Lambda/HindIII marker fragments were run in lane M with their sizes indicated on the right. Southern bands using the same mixed probe pGETS1201 (100.3 kb) and lambda DNA are consistent with those deduced from the sequence data (13).

isolated from each pBrET transformant had identical structures (data not shown). Insert structures of the representative pBrET plasmids pGETS1209 (46.6 kb), pGETS1210 (58.8 kb), pGETS1212 (88.7 kb), and pGETS1201 (100.3 kb) were confirmed using several restriction endonucleases (data not shown except for PvuII in Fig. 3A). Figure 3B indicates that the 100.3-kb segment cloned in pGETS1201 detected no alteration of Southern signals of genome digests by PstI, PvuII, EcoRI, BglII, and SacI. Transfer of these positionally cloned segments to *E. coli* remained to be examined because of the lack of a pBR322 sequence in these pBrET plasmids. It was established by adopting a low-copy-number pBrET vector that the BrET mechanism also functioned for positional cloning of the internal DNA of the *B. subtilis* genome. The nearly equal numbers of chloramphenicol-resistant transformants indicate that the BrET proceeds at equal rates regardless of the DNA length to be copied. As discussed previously, the rate-determining step for the BrET is not the uptake-replication process but the process of formation of simultaneous homologous pairs (19). This conclusion is consistent with the similar degrees of efficiency of the cloning of the internal 100.3-kb in this study and the previous cloning of the 90.6-kb heterologous DNA originating from the *Cyanobacterium* genome into the *B. subtilis* genome (19). The successful positional cloning of internal DNA facilitates precise cloning of DNA fragments such as lysogenic phages that could not be obtained with other cloning systems.

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