

Integrated Physical and Genetic Mapping of *Bacillus cereus* and Other Gram-Positive Bacteria Based on IS231A Transposition Vectors

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The genome structure of *Bacillus cereus* is relatively complex, its DNA being modulated between a size-varying chromosome and large plasmids. To study the genetic organization of the *B. cereus* type strain ATCC 14579, thermosensitive transposition vectors were designed on the basis of IS231A-derived cassettes containing uncommon restriction sites. A highly preferred insertion site for IS231A was detected in the chromosome by Southern blotting and pulsed-field gel electrophoresis (PFGE) analyses of independent insertion mutants. However, once this insertional hot spot was occupied, secondary IS231A insertions occurred randomly, as demonstrated by isolation of independent *B. cereus* auxotrophs at a frequency of approximately 0.6%. The hot-spot site, as well as several auxotrophic mutations, were mapped by using *Not*I, *Sfi*I, and *Asc*I PFGE restriction profiles. It was confirmed by sequencing that one of the insertions, generating an Ade[−] phenotype, had disrupted a gene of the purine synthesis pathway. These results showed that combined PFGE and sequencing analyses of mini-IS231A insertions enable the construction of integrated physical and genetic maps of *B. cereus* type strain. Moreover, the presence of the ultrarare *I-Sce*I restriction site in the mini-IS231A allowed the isolation, in double-insertion mutants, of contiguous and nonoverlapping large chromosomal fragments, convenient for direct sequencing. The system detailed in this report is therefore a powerful tool for comparative genetic studies among members of the *B. cereus* group (i.e., *B. cereus*, *B. thuringiensis*, *B. mycoides*, and *B. anthracis*) and could also be applied to more distantly related gram-positive bacteria.

Bacillus cereus, a ubiquitous gram-positive spore-forming soil bacterium, is the causative agent of different types of food poisoning as well as opportunistic infections. Certain strains of this bacterium are capable of producing a heat-labile enterotoxin and/or a heat-stable emetic toxin, causing diarrheal and emetic syndromes, respectively (14). Due to the production of other toxins, *B. cereus* has been associated with septicemia, endophthalmitis, endocarditis, kidney and urinary tracts infections or wound infections, and recently bacteremia and pneumonia (11, 31, 40).

B. cereus belongs to the relatively large group named *B. cereus sensu lato*, which includes animal and human pathogens (*B. cereus* and *B. anthracis*), bacteria with rhizoid growth (*B. mycoides*), and bacteria that produce insecticidal endotoxins widely used as biological control agents (*B. thuringiensis*). Several studies, including comparative analyses of 16S rRNA sequences, have indicated that these bacteria may be considered subspecies of the single generic *B. cereus* species (1, 35). However, more recent analyses suggested that although *B. cereus* and *B. thuringiensis* are effectively the closest taxa to *B. anthracis*, *B. mycoides* is apparently slightly more distant (19).

At the genetic level, only a limited number of genes of *B. cereus* have been characterized. However, the development of the pulsed-field gel electrophoresis technique (PFGE) (37) facilitated the construction of physical maps of several *B. cereus* strains (6–8, 20). Comparison and analysis of these maps showed that the *B. cereus* genome is complex and apparently flexible. Indeed, the size of the chromosome was shown to vary between 2.4 and 6.3 Mb. Furthermore, the *B. cereus* genome displays a particular structure consisting of one constant entity

harboring essential genes and another, less stable region, which could be the subject of rearrangements such as insertions, deletions, and inversions (8). Moreover, it was also found that in the case of the *B. cereus* displaying the smaller chromosome, the pyruvate dehydrogenase gene was carried by one stable extrachromosomal element, indicating that housekeeping genes may exist outside the main chromosome (8).

Substantial progress in understanding the genomic organization and plasticity of this organism and related species requires the development of new genetic tools. Until now, transposable elements have not been exploited in *B. cereus* strains for molecular genetics. This could be due to the lack of suitable transposon delivery systems and/or convenient transposition methods. Some of these limitations could be overcome by using the insertion sequence IS231A. This 1,656-bp element, isolated from *B. thuringiensis*, is delineated by 20-bp inverted repeats (IRs) and encodes a single transposase of 478 amino acids (30). So far, 10 other iso-IS231 elements have been isolated and characterized (9, 27). The 11 IS231 elements can be grouped into two size classes: the 1,650-bp size group includes nine of them (IS231A to -H and -Y), whereas the two others (IS231V and -W) are about 1,950 bp long. Analysis of their distribution among a representative number of strains from the *B. cereus* group revealed that they are largely distributed within these bacteria. However, no obvious association has been established between any iso-IS231 element and a specific strain (22).

Transposition of IS231A was demonstrated in both gram-positive and gram-negative bacteria (27). Moreover, recent study of its mechanism of transposition showed that this insertion sequence transposes exclusively via a simple cut-and-paste pathway (23). Mutagenesis assays, based on this transposable element, have been developed to study its insertional distribution in the chromosome of *Escherichia coli*. These systems are based on suicide transposition with vectors unable to maintain

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description ^a	Reference or source
Strains		
<i>Bacillus cereus</i> ATCC 14579	Type strain	38
<i>Bacillus subtilis</i> CU267	Host strain for pGI plasmids and first transposition assays	13
Plasmids		
pGIC052	ori ⁺ , ori ⁻ , Ery ^r , mini-IS231A Kan ^r , P _{deg} -TnpA, RCP 1.4, 10 kb	This study
pGIC054	ori ⁺ , ori ⁻ , Ery ^r , mini-IS231A Sp ^r , P _{deg} -TnpA, RCP 2, 9.7 kb	This study
pGIC055	ori ⁺ , Ery ^r , mini-IS231A Sp ^r , P _{deg} -TnpA, RCP 2, 6.8 kb	This study
pGIC056	ori ⁺ , ori ⁻ , Ery ^r , mini-IS231A Kan ^r , P _{deg} -TnpA, RCP 2, 10 kb	This study
pGIC057	ori ⁺ , Ery ^r , mini-IS231A Kan ^r , P _{deg} -TnpA, RCP 2, 7.1 kb	This study
pGC2	ori ⁻ , Amp ^r , 2.9 kb	33
pHT1030	<i>B. thuringiensis</i> plasmid, thermosensitive replicon	24
pGC13	Fusion of pGC2 and pHT1030; ori ⁺ , ori ⁻ , Amp ^r , Ery ^r , 6.8 kb	24a
pGIG010	ori ⁻ , Cm ^r , mini-IS231A Kan ^r , RCP 1.4, 4.4 kb	23a
pGI300	ori ⁺ , suicide plasmid with Tn10dSp, RCP 2, 6.6 kb	28
pGI4010	ori ⁻ , Amp ^r , Ery ^r , 3.7 kb	18
pNEB193	ori ⁻ , Amp ^r , 2.7 kb	New England Biolabs
pBluescript SK	ori ⁻ , Amp ^r , cloning vector, 3 kb	Stratagene
pGIC102	pBluescript with an <i>Eco</i> RI fragment containing the mini-IS Kan ^r inserted into the hot spot, 10.3 kb	This study
pGIC105	pBluescript with an <i>Eco</i> RI fragment containing the mini-IS Sp ^r inserted into the <i>ade</i> insertion site, 6.2 kb	This study

^a ori⁺, replicative origin for gram-positive host; ori⁻, replicative origin for gram-negative host; Amp^r, Ery^r, Kan^r, and Sp^r, resistance genes to ampicillin, erythromycin, kanamycin, and spectinomycin, respectively; P_{deg}-TnpA, IS231A transposase gene expressed under control of the P_{deg} promoter.

in the bacterium because of their inappropriate replicative origin. However, such a procedure requires high transformation efficiency of the bacterial host and is thereby not easily applicable to *Bacillus* strains which are poorly transformable. Other strategies are thus required to carry out this type of analysis in gram-positive bacteria.

The system described in this study, designed to deliver mini-IS231A into the chromosome of *Bacillus*, relies on the temperature sensitivity of the replicative origin of the carrier vectors. Preliminary assays were successfully performed in *B. subtilis* and allowed the isolation of thousands of insertion mutants in one single experiment. The system has then been applied to the *B. cereus* type strain ATCC 14579 (38), in which a highly preferred chromosomal site for IS231A insertion was identified. However, it was demonstrated that once this hot spot is occupied, efficient mutagenesis of the bacterium by secondary insertions is observed. The generation of large chromosomal DNA fragments, available for sequencing without cloning, is also described.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and transformation. The bacterial strains and plasmids used in this study are listed in Table 1. Constructions of plasmids pGIC055 and pGIC057 are described below. *Bacillus* cells were grown in liquid or solid Luria-Bertani (LB) medium (36) or 2× LB supplemented, when required, with the antibiotics spectinomycin (400 µg/ml), kanamycin (25 µg/ml), and erythromycin (25 µg/ml). Auxotrophs were isolated by parallel replica plating on LB and M9 minimal media (36). Auxotrophies were identified by using a crossed-pool plates assay (10). Preparation of competent *B. subtilis* and *B. cereus* cells and electroporation were performed as described previously for *B. thuringiensis* (25). All electroporations were done with a Gene Pulser II (Bio-Rad) apparatus.

Construction of pGIC055 and pGIC057. Plasmids pGIC055 and pGIC057 (Fig. 1) only differ by their mini-IS resistance genes. Their construction required the following steps. The IS231A transposase coding sequence, starting at the ATG codon, was fused to a PCR fragment containing the *B. subtilis* P_{deg} promoter (32) in a pNEB193 (New England Biolabs, Inc.) derivative. A kanamycin-resistant (Kan^r) mini-IS231A delineated by *Kpn*I restriction sites was isolated from pGIG010 (23a) and cloned downstream of the modified transposase gene. In this mini-IS, the Kan^r gene is associated with the rare-cutting polylinker (RCP) 1.4 (28). A fragment including both the transposase gene and the mini-IS was then cloned into the pGC13 gram-positive/gram-negative shuttle vector (24a). This pGC13 is a derivative of pHT1030 (24), fused to the *E. coli* vector

pGC2 (33) which bears the erythromycin resistance (Ery^r) gene from pGI4010 (18). The resulting plasmid pGIC052 contains the P_{deg}-TnpA-mini-IS Kan^r structure. To provide the mini-IS with ultrarare restriction sites, the Kan^r gene was swapped for the spectinomycin resistance (Sp^r) gene combined to the RCP 2 of pGI300 (28) to give pGIC054. An internal fragment, limited to the Sp^r gene was then exchanged with the Kan^r gene of pGIG010 to obtain pGIC056. This plasmid thus harbors the ultrarare restriction sites of RCP 2. Plasmids pGIC054 and pGIC056 were then deleted of a 2.9-kb *Aat*II fragment containing their gram-negative replicon to give the gram-positive versions pGIC055 and pGIC057, respectively.

Transposition assays. Single colonies of *B. subtilis* CU267 (13) or *B. cereus* ATCC 14579 containing pGIC055 or pGIC057 were inoculated into 10 ml of LB medium and grown for 8 h at 28°C, diluted 100-fold, and grown in the same conditions for an additional 8 h, in order to allow transposition of the mini-IS231A to occur. The cultures (10 ml of LB inoculated with 100 µl of the previous culture) were then shifted to 52°C for three cycles of 4 h each (±20 generations) for *B. subtilis* CU267 or to 46°C for a combination of 3 8-h overnight cycles and 13 4-h daytime cycles for *B. cereus* ATCC 14579 (±120 generations). From the final culture, different dilutions were plated on LB medium to determine the total number of cells, on LB containing kanamycin (for pGIC057) or spectinomycin (for pGIC055) to detect transposition events, and on erythromycin-containing plates to estimate the number of cells that did not lose the donor plasmid. The plates were incubated at the temperature corresponding to that of the liquid culture.

Southern blotting and hybridization. Total DNA of auxotroph mutants was isolated by miniprep (2): 100 µl of an overnight preculture (10 ml of 2× LB, 28°C) was used to inoculate a 10-ml LB culture incubated for 4 h at 37°C, from which total DNA was extracted. After a 3-h restriction with *Eco*RI (New England Biolabs), the DNA samples were migrated in a 0.8% agarose gel and transferred on a nylon membrane (Hybond-N; Amersham Life Sciences) according to standard transfer protocols (36). Labeling, hybridization, and detection were done according to the protocols for the Dig High Prime Starter Kit II (Boehringer Mannheim). The probes corresponding to the mini-IS Kan^r and mini-IS Sp^r were prepared by PCR amplification from *E. coli* plasmids bearing the mini-IS, to avoid background labeling of *Bacillus* sequences. Amplification of the fragment corresponding to the mini-IS requires only a single primer corresponding to the IS231A IRs (22). After PCR, the DNA was purified from a 0.8% agarose gel with a QIAEX II gel extraction kit (Qiagen) and digoxigenin labeled.

PFGE. Preparation of intact genomic DNA in agarose plugs was performed as described by Kolstø et al. (20), using a CHEF-DR II (Bio-Rad) apparatus. The electrophoresis buffer was 0.5× TBE (45 mM Tris, 45 mM borate, 1 mM EDTA [pH 8]) and switch times ranking from 5 to 120 s were used. Total DNA were digested with octanucleotide-recognizing endonucleases *Nor*I, *Sfi*I, and *Asc*I (New England Biolabs) for 8 h, and with the omega-nuclease *I-Sce*I (Boehringer Mannheim) for 1 h, as specified by the manufacturers. Sizes of the fragments were estimated by using lambda DNA concatemers (size range, 48.5 to 1,018.5 kb) and yeast chromosomes (225 to 1,900 kb) markers (New England Biolabs).

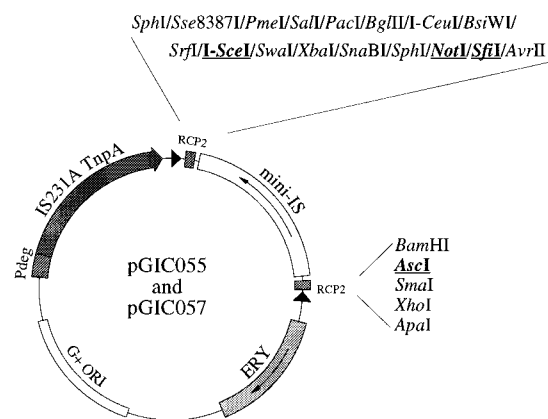


FIG. 1. IS231A-based transposition vectors pGIC055 and pGIC057. ERY, Ery^r gene; G+ ORI, replicative origin for gram-positive host; P_{deg}, strong gram-positive promoter; TnpA, transposase; RCP, rare-cutting polylinker; black triangles represent the IRs of IS231A. The mini-IS resistance gene is Sp^r (pGIC055) or Kan^r (pGIC057).

Cloning and sequencing of the regions flanking the hot spot and *ade* insertion sites. Total DNA from a hot-spot and one of the *ade* auxotroph mutants was isolated by miniprep as described above. After restriction with *Eco*RI, DNA fragments were cloned in the *Eco*RI site of pBluescript SK (Stratagene, La Jolla, Calif.) and electroporated into *E. coli*. Candidates for the cloning of the mini-IS231A in the hot spot site were isolated by using kanamycin as the selectable marker, while screening for the *ade* insertion was performed on spectinomycin. The recombinant plasmids were named pGIC102 (hot spot, Kan^r) and pGIC105 (*ade* insertion, Sp^r). Sequencing was done according to the automated sequencing method based on the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction kit. The primers used corresponded to mini-IS231A internal sequences close to IR_L and IR_R (IR left and IR right, respectively, by reference to the orientation of IS231A transposase gene inside the element) and directed outward the mini-IS.

RESULTS

Transposition from thermosensitive delivery vectors in *B. subtilis* and *B. cereus* strains. Plasmids pGIC055 and pGIC057 (Fig. 1) represent two versions of the same system whose characteristics are a gram-positive thermosensitive replicon from pHT1030 vector (24); the IS231A transposase gene, under the control of P_{deg}, a strong gram-positive promoter (32), located outside the IS231A IRs in order to prevent secondary insertions; a mini-IS231A-containing selectable resistance marker (Kan^r [pGIC057] or Sp^r [pGIC055]), and an RCP (RCP 2 [28]) inside the IRs. Since this transposition system is

based on a gram-positive thermosensitive replicon, the maximum growth temperature of the strains to be used was determined. It was shown that *B. cereus* ATCC 14579 does not sustain temperatures higher than 46°C, while *B. subtilis* CU267 is still able to grow at 52°C.

Initial transposition experiments were performed in *B. subtilis*. Transposition of the mini-IS231A from plasmids pGIC055 or pGIC057 was achieved at 28°C. The donor plasmid was then eliminated by shifting the temperature to 52°C for about 20 generations (three cycles of 4 h). The insertion mutants were selected on spectinomycin or kanamycin and checked for sensitivity to erythromycin. Estimation of the IS231A transposition frequency under these conditions is particularly difficult to assess because of the complexity of the parameters involved (e.g., determination of the exact number of cell generations, including growth on plate, and variation in the plasmid copy number). This frequency can, however, be approximated by the proportion of Sp^r Ery^s or Kan^r Ery^s candidates (Table 2) per generation. In the conditions used, the transposition frequency in *B. subtilis* approached 3×10^{-6} for pGIC055 and 10^{-4} in the case of pGIC057. Moreover, the segregational stabilities of the donor plasmid (proportion of Ery^r colonies) were shown to be close to 10^{-6} and 10^{-5} for pGIC055 and pGIC057, respectively. These results were consistent with those obtained for pHT1030-derived plasmids in *B. subtilis* 168 (24).

Based on these encouraging results, the strategy was adapted for *B. cereus* ATCC 14579. The stability of pGIC057 was first tested during several cycles of cultures of 4 and 8 h at 46°C (Table 2). After approximately 20 generations, 10% of total CFU were Ery^r, indicating that cells carrying the plasmid were still present in the population. However, a satisfactory level of plasmid loss was obtained by increasing the number of culture steps (Table 2). After about 120 generations, the segregational stability of pGIC057 was less than 10^{-7} and none of the Kan^r cells, appearing at a frequency of 10^{-3} , were still Ery^r. In these conditions, the relative transposition frequency reached the proportion of 8×10^{-6} Kan^r Ery^s CFU per generation.

IS231A inserts into a chromosomal hot-spot site of the *B. cereus* type strain. To test whether the mini-IS231A insertions occurred randomly in the chromosome of *B. cereus*, auxotrophic mutations were searched for. A total of 3,000 randomly selected Kan^r colonies were tested for growth on minimal medium. Surprisingly, no auxotrophs were isolated (Table 3). Total DNA from several insertion mutants was then digested with *Not*I, *Sfi*I, and *Asc*I and analyzed by PFGE. Since the mini-IS231A elements carry these rare restriction sites, their

TABLE 2. Transposition in *B. subtilis* CU267 and *B. cereus* ATCC 14579

Species (plasmid)	Incubation for plasmid loss ^a	Estimated no. of generations	Total CFU (10 ⁷ /ml)	Kan ^r or Sp ^r CFU ^b (10 ⁴ /ml)	Kan ^r or Sp ^r cell ratio	Plasmid stability ^c	
						Ery ^r CFU/ml	Ery ^r cell ratio
<i>B. subtilis</i> (pGIC057)	3 × 4 h	20	1.9	3.8	2×10^{-3}	4×10^2	2×10^{-5}
<i>B. subtilis</i> (pGIC055)	3 × 4 h	20	3.4	0.2	6×10^{-5}	1×10^2	3×10^{-6}
<i>B. cereus</i> (pGIC057)	3 × 4 h	20	1.9	570	3×10^{-1}	1.8×10^6	1×10^{-1}
	1 × 8 h + 6 × 4 h	50	4.8	5.8	1.2×10^{-3}	4×10^4	8×10^{-2}
	2 × 8 h + 10 × 4 h	90	5.2	4.1	8×10^{-2}	1×10^2	2×10^{-6}
	3 × 8 h + 13 × 4 h	120	5.6	5.7	1×10^{-3}	$<10^{-7}$	$<10^{-7}$
<i>B. cereus</i> (pGIC055)	3 × 8 h + 13 × 4 h	120	9.8	15	1.5×10^{-3}	$<10^{-7}$	$<10^{-7}$

^a Nonpermissive incubation temperatures were 52°C for *B. subtilis* and 46°C for *B. cereus*. 3 × 4 h represents three cycles of 4 h each.

^b Kan^r or Sp^r CFU include both the insertion mutants and the cells that did not lose the delivery vector pGIC057 or pGIC055.

^c Estimated from the ratio Ery^r CFU/total CFU.

TABLE 3. Auxotroph mutants generated in *B. cereus* by IS231A transposition

Transposition test ^a	% of auxotrophs	Auxotrophies identified ^b
1st round (pGIC057)	<0.03	0
2nd round (pGIC055)	0.6	1 Ura ⁻ , 1 Gua ⁻ , 5 Ade ⁻ , 2 Met ⁻ , 2 His ⁻ , 2 Cys ⁻

^a In each case, 3,000 clones were tested.^b Phenotype abbreviations: Ura, uracil; Gua, guanine; Ade, adenine; Met, methionine; His, histidine; Cys, cysteine.

introduction into the *B. cereus* chromosome results in the modification of the corresponding restriction patterns. The different candidates were shown to display similar *NotI*, *SfiI*, and *AscI* electrophoretic profiles (data not shown), suggesting that these clones could emanate either from independent transposition events into a hot spot or from the emergence of an early transposition event. To unravel this issue, several mutants obtained from new independent experiments were analyzed by PFGE after restriction with *NotI* and *AscI*, and results similar to those mentioned above were observed. The restriction profiles obtained were compared to the physical map of *B. cereus* ATCC 14579 established by Carlson et al. (7). In all the can-

didates analyzed, the largest *NotI* restriction fragment of 1,370 kb (N1, according to Carlson et al. [7]) was split into two fragments of 1,210 and 160 kb (Fig. 2A, lane HS). As expected, the large fragment (1,210 kb) did not enter the gel under the conditions used. In addition, *AscI* restriction of these insertion mutants generated two fragments of 2,940 kb (not apparent) and 550 kb from the large A1 chromosomal segment of 3,490 kb (Fig. 2B). Additional Southern hybridization analysis of eight candidates, originating from four independent transposition assays, confirmed identical mini-IS231A insertions for seven of these eight candidates (data not shown). All of these findings clearly indicated that the chromosome of *B. cereus* exhibits a hot-spot insertion site for IS231A. Based on the different restriction profiles, this hot spot was located on the physical map of *B. cereus* type strain at a distance of about 800 kb (67°) from the *dnaA* locus (Fig. 3).

The mini-IS Kan^r introduced in the hot spot and its flanking sequences were cloned from one of the insertion mutant as an *EcoRI* fragment (the mini-IS is devoid of this restriction site), in the pBluescript SK vector, to give pGIC102. Sequencing of the DNA flanking the mini-IS revealed that this site corresponds to the left IR of another mobile element, Tn4430, whose extremities were previously shown to be the preferred targets for IS231A transposition (16). Insertion of IS231A is known to introduce short direct repeats in the target site,

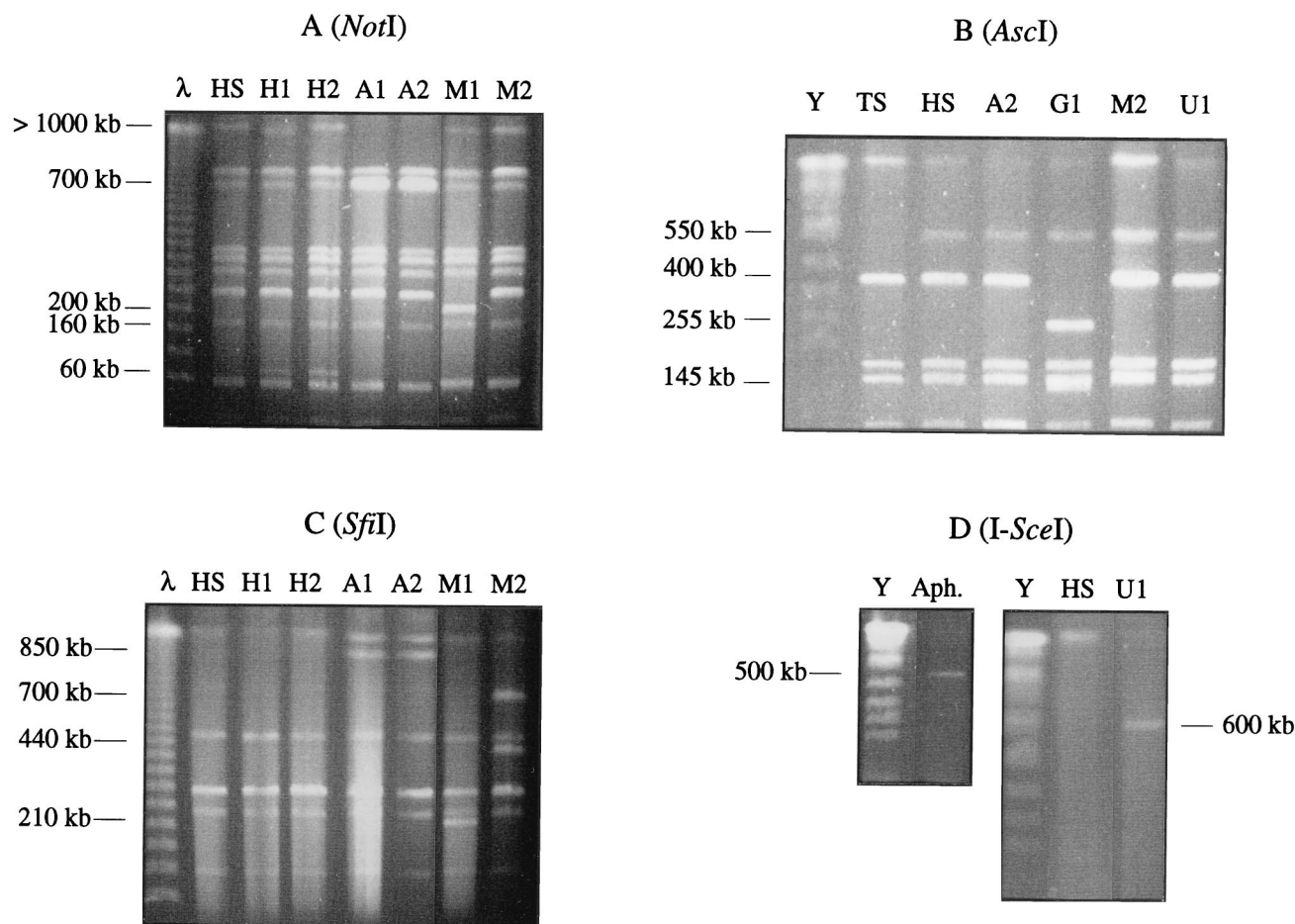


FIG. 2. PFGE profiles of total DNA from *B. cereus* strains restricted by *NotI* (A), *AscI* (B), *SfiI* (C), and *I-SceI* (D). Y, yeast chromosome marker; λ, lambda ladder marker (New England Biolabs); HS, hot-spot candidate with one single mini-IS Kan^r insertion; TS, *B. cereus* ATCC 14579 type strain; A1 and A2, *ade* mutants; G1, *gua* mutant; H1 and H2, *his* mutants; M1 and M2, *met* mutants; U1, *ura* mutant; Aph., aphentotypic double-insertion mutant.

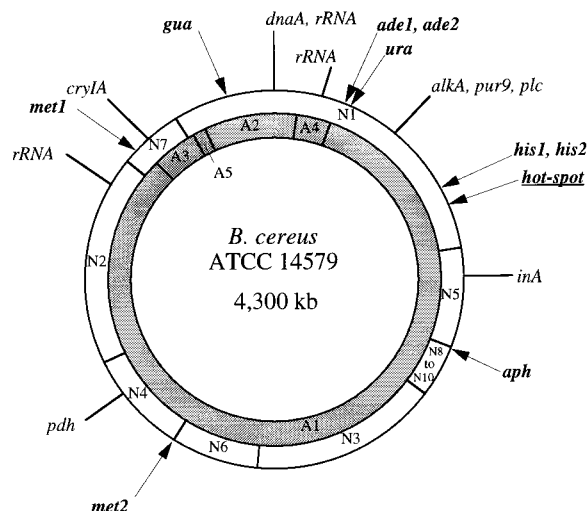


FIG. 3. *B. cereus* chromosomal map. Based on the position of the *dnaA* gene and by comparison of the locations of other genes with those of *B. subtilis*, the restriction map recently published by Carlson et al. (7) has been reoriented to place the potential replicative origin site at the 0°/360° point. Several relevant loci have been retained; their positions were arbitrarily set in the middle of the chromosomal fragment to which they hybridize (7). *plc*, phospholipase C; *inA*, inhibitor A; *pdh*, pyruvate dehydrogenase. Nx and Ax correspond to *NotI* and *AscI* restriction fragments, respectively. The hot-spot insertion site as well as eight other insertions generating auxotrophies are indicated. Aph represents an aphentotypic secondary transposition event.

owing to the staggered nicks introduced in the two strands of the DNA by the transposase, followed by gap repair by the host machinery. As shown in Fig. 4A, the 11-bp sequence 5'-GGG TACCGCCA-3' was duplicated in the case of the hot-spot insertion. However, the Tn4430 sequence present at this site corresponded to a 42-bp vestigial element. None of its flanking sequences showed any obvious homology to other sequences from current databases.

Selection of auxotrophic mutants and characterization by Southern blotting and PFGE analyses. Assuming that the hot spot is unique, one would expect that once it is occupied, additional transposition events will generate random insertions. A second round of transposition was thus carried out with pGIC055 (*Sp^r*) in *B. cereus* ATCC 14579 containing the *Kan^r* insertion into the hot spot. From three separate experiments, using independent electroporated candidates, mini-IS231A insertions that caused auxotrophic mutations were isolated. From a total of 3,000 *Kan^r* *Sp^r* clones, 19 (0.6%) were unable to grow on minimal medium. Among these insertions, a total of 13 defined auxotrophies were identified (Table 3): one uracil (designated U1), one guanine (G1), five adenines (A1, A2, A3, A4, and A5), two methionines (M1 and M2), two histidines (H1 and H2), and two cysteines (C1 and C2).

To confirm that the *Sp^r* insertions occurred randomly, chromosomal DNA isolated from the auxotrophs (with the exception of A5 and C2) was digested by *EcoRI*, separated in an agarose gel, transferred to a nylon membrane, and hybridized with a mini-IS231A *Sp^r* probe (Fig. 5). Since the mini-IS bears no *EcoRI* site, one single fragment is expected to hybridize with the probe, with variable size according to the insertion site. In fact, the labeled fragments were all different except for the four *ade* mutants (A1, A2, A3, and A4), suggesting that these might be identical. In contrast, the mutants displaying the *Met⁻* phenotype, M1 and M2, appeared to be mutated at different positions, as did the *his* mutants H1 and H2 (Fig. 5).

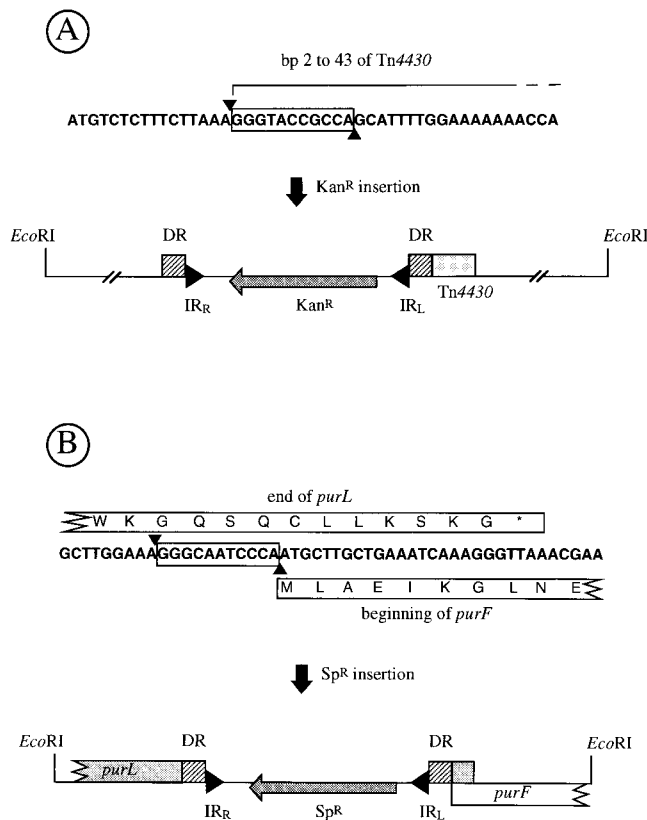


FIG. 4. IS231A target DNA sequence of the hot-spot insertion site (A) and *Ade⁻* insertion site (B) and representation of their respective *EcoRI* fragments cloned in pGIC102 (A) and pGIC105 (B). The 11 bp boxed on the sequence represent the duplicated target site (DR). *IR_L* and *IR_R* refer to IR left and right, respectively, by reference to the transposase gene orientation in IS231A; small black arrows indicate the cleavage generated by the transposase on each strand of the DNA. The size of the *EcoRI* fragment cloned in pGIC102 (A) is 7.3 kb, including 1.9 kb for the mini-IS *Kan^r*. This fragment is 3.2 kb in the case of pGIC105 (B), from which 1.6 kb pertains to the mini-IS *Sp^r*. As for *B. subtilis*, the putative *B. cereus* *purL* and *purF* genes share a 25-bp overlap.

Several auxotrophs as well as an aphentotypic insertion mutant were further characterized by restriction and PFGE. A total of nine insertions plus the hot spot were positioned on the physical map of *B. cereus* by integration of the results obtained with the different enzymes (Fig. 3). Examples of the PFGE profiles obtained are given in Fig. 2. In the case of the *his* mutants H1 and H2, insertion of the mini-IS231A *Sp^r* into the largest *NotI* fragment ($\pm 1,210$ kb) generated fragments of 60 and 1,150 kb (Fig. 2A). For the adenine auxotrophs (A1 and A2), insertion occurred in the middle of this segment, resulting in the emergence of a double band of about 700 kb. Similarly, the M1 (*Met⁻*) auxotroph lost the 250-kb N7 fragment which was split into a 200- and a 50-kb (doublet on the gel) fragment (Fig. 2A).

AscI restriction generated fewer fragments, but in the case of the G1 (guanine) mutant, the mini-IS231A insertion resulted in the loss of one wild-type 400-kb fragment and the appearance of two new fragments of 145 and 255 kb (Fig. 2B). The *SfiI* profiles obtained in the case of the A1, A2, M1, and M2 mutants were also very informative: the largest *SfiI* segment gave rise to a large fragment plus a signal at 850 kb for A1 and A2, and a large fragment and a 210-kb signal for M1, and the S2 *SfiI* segment was divided in fragments of 700 and 440 kb in the case of M2 (Fig. 2C).

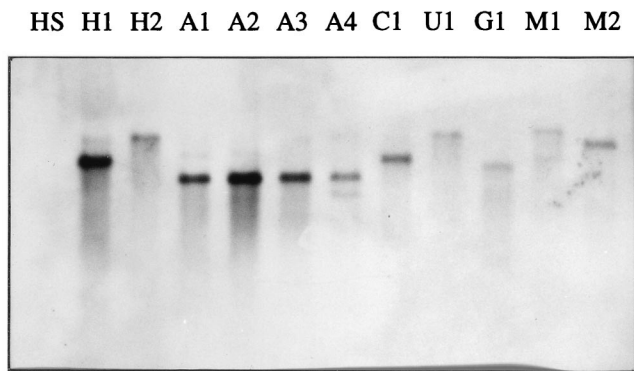


FIG. 5. Hybridization of the total DNA of *B. cereus* auxotrophs restricted by *EcoRI* with a probe corresponding to the *Sp^r* gene. HS, hot-spot candidate with one single mini-IS *Kan^r* insertion (negative control); A1, A2, A3, and A4, *ade* mutants; C1, *cys* mutant; G1, *gua* mutant; H1 and H2, *his* mutants; M1 and M2, *met* mutants; U1, *ura* mutant.

Sequencing of the insertion site from one of the *ade* auxotrophs. To ensure that the observed mutations were actually due to the insertion of the mini-IS231A into a gene of the corresponding pathway, one of the insertions was characterized in detail. The mini-IS231A *Sp^r* insertion responsible of an *Ade⁻* phenotype was cloned in pBluescript SK to give pGIC105. Its flanking sequences were determined by using the same approach as used for the hot-spot insertion site. By comparison with sequences from the databases, the site in which the IS had inserted was shown to have strong similarity with genes of the *pur* operon of *B. subtilis* (Fig. 4B). The sequence flanking the left IR of IS231A is closely related (77% identity over 350 bp [data not shown]) to the *B. subtilis purF* gene, whereas the sequence adjacent to IR_R corresponds to the end of the *purL* gene (67% identity over 350 bp). In fact, the mini-IS231A has inserted just upstream of the ATG codon of the putative *purF* gene, in a region where *purL* and *purF* overlap over a few base pairs (12).

Chromosomal DNA fragments from *B. cereus* generated by *I-SceI* restriction. *I-SceI*, an endonuclease or omega-nuclease encoded by a group I intron of the *Saccharomyces cerevisiae* mitochondrial 21S rRNA, recognizes an 18-bp sequence shown to be absent from most prokaryotic and eukaryotic genomes (39). This *I-SceI* site has been introduced in the mini-IS of plasmids pGIC055 and pGIC057 and is thus comobilized with the mini-IS231A at each transposition event. Consequently, the successive introduction of two mini-IS231A elements, carrying two different antibiotic markers, into the chromosome is expected to yield an *I-SceI* chromosomal segment delimited by these two mini-IS. To illustrate this, the genomes of U1 auxotroph and an aphenotypic double-insertion mutant were digested with endonuclease *I-SceI* and analyzed, together with the hot spot candidate, by PFGE (Fig. 2D). Linearization of the chromosome of the hot-spot candidate gave rise to a large signal visible in the upper part of the gel. The uracil auxotroph displayed a band of apparent size of 600 kb, and the aphenotypic mutant exhibited a band of about 500 kb (Fig. 2D), as expected from mapping of the insertions (Fig. 3).

DISCUSSION

This report describes two versions of the same plasmid, pGIC055 and pGIC057, and their application for integrated physical and genetic analysis of the *B. cereus* type strain. The system is based on the mobile sequence IS231A and the PHT1030 thermo-

sensitive replicon, both isolated from *B. thuringiensis*. The method consists in growing plasmid-bearing *Bacillus* at permissive temperature to allow transposition events to occur and then increasing the temperature for 20 to 120 generations to eliminate the delivery vector. In these tests, it was shown that the mini-IS231A transposed into the *B. subtilis* and *B. cereus* chromosome at frequencies ranging from 3×10^{-6} to 1×10^{-4} event per generation. With these transposition levels, each experiment can give rise to 10^3 to 10^5 independent insertions. The resulting clone banks of chromosomal inserts are suitable for screening of genetic loci of interest, such as genes coding for virulence factors or particular metabolic pathways.

Analysis of several independent candidates generated from a single round of transposition showed that IS231A has one highly preferred target site in *B. cereus* chromosome. This hot spot was localized by *NorI* and *AscI* restriction profile analysis on the *B. cereus* map. Moreover, nucleotide sequence determination of this singular insertion site indicated that it corresponded to the left IR of Tn4430, well known to be a preferred target for IS231A in its natural host *B. thuringiensis* (29) and in *E. coli* (17). It is noteworthy that previous hybridization analyses of *B. cereus* ATCC 14579 with a Tn4430 probe did not detect this transposon, neither on the chromosome nor on any extrachromosomal elements (5). However, the fact that this Tn4430 is vestigial (42 bp) explains this lack of detection.

To test whether IS231A would display a random insertion distribution in the *B. cereus* chromosome once its preferred site is occupied, pGIC055 (*Sp^r*) was introduced in a hot-spot candidate to perform a second round of transposition. With a transposition rate close to that previously observed, this second assay led to the recovery of insertional auxotrophic mutations with a frequency of 0.6%. This result is similar to those obtained with Tn10 derivatives in *B. subtilis* (34) and slightly higher than those obtained with transposon Tn611 in *Mycobacterium smegmatis* (0.1 to 0.4% [15]) or with IS31831 in coryneform bacteria (0.2% [41]). Furthermore, with the exception of four adenine mutants, Southern analysis of 11 auxotrophs, belonging to six different types, showed different secondary insertion sites for each auxotroph, including the two *his* and the two *met* mutants.

By analogy to *B. subtilis*, the replicative origin of *B. cereus* chromosome is thought to be very close to the *dnaA* gene. Comparison of *dnaA* and other gene positions on both chromosomes suggests that the published map of *B. cereus* (7) can be aligned to that of *B. subtilis* by a simple rotation of a few degrees to the left. Using the PFGE technique, we mapped the IS231A hot spot, eight insertions generating auxotrophies, and one aphenotypic insertion on the *B. cereus* chromosome. The secondary insertions were scattered on the molecule, confirming that once the hot spot is occupied, subsequent insertions occurred randomly in the chromosome.

PFGE data led to the mapping of the two *His⁻* inserts at the same locus, although their hybridization patterns were clearly different. This can easily be explained by the fact that the two insertions occurred in different sites of the same gene or operon. Indeed, in *B. subtilis*, most of the *his* genes are grouped in a large cluster of more than 6 kb (21). Also, two *Ade⁻* insertions were mapped in a fragment previously shown to hybridize with a *pur* probe (7). These sites, however, do not converge on Fig. 3, due to the fact that by convention, the *pur9* locus is positioned in the middle of the fragment to which it hybridized. However, by analogy to the *B. subtilis pur* genes, most of which are also assembled into a cluster (21), it is most likely that the *pur9* and *ade* mutants from *B. cereus* reside in the same locus.

The *B. cereus* genome has no *I-SceI* restriction site; thus, this report demonstrated that the introduction of *I-SceI* sequences, together with two successive mini-IS231A insertions, allowed

the recovery of chromosomal segments suitable for genome sequencing as has been recently shown for *E. coli* (3, 4, 26). This procedure avoids the difficulties associated with conventional techniques of genomic cloning. Moreover, it also allows the recovery of large nonoverlapping fragments generated from successive rounds of transposition, using the different markers located on the mini-IS (28).

In its current conformation, this system appears to be a powerful tool for insertional mutagenesis of *B. cereus* strains but also of all gram-positive bacteria able to grow at temperatures above 45°C, where it can rapidly generate integrated physical and genetic maps. Now that the genome sequence of the reference microorganism *B. subtilis* has been entirely determined (21), it would be of particular interest to focus on other remarkable *Bacillus* species, most particularly those relevant for the industry (*B. amyloliquefaciens*, *B. licheniformis*, and *B. stearothermophilus*) or the numerous opportunistic and pathogenic bacteria displaying positive (*B. thuringiensis*, *B. sphaericus* and *B. popilliae*) or negative (*B. cereus* and *B. anthracis*) effects on animals and/or humans.

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