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 *NdeI*, *SfiI*, and *Ascl* PFGE restriction profiles. It was confirmed by sequencing that one of the insertions, generating an A derivative, 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 ultrarrare *I-ScI* 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 enterotoxin and/or a heat-stable emetic toxin, causing diarrhea and poisoning as well as opportunistic infections. Certain strains of this bacterium, *B. cereus*, 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 enterotoxin and/or a heat-stable emetic toxin, causing diarrhea and poisoning as well as opportunistic infections. Certain strains of this bacterium, *B. cereus*, 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 enterotoxin and/or a heat-stable emetic toxin, causing diarrhea and poisoning as well as opportunistic infections. Certain strains of this bacterium, *B. cereus*, 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 enterotoxin and/or a heat-stable emetic toxin, causing diarrhea and poisoning as well as opportunistic infections. Certain strains of this bacterium, *B. cereus*, 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).
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 plates. Preliminary assays were successfully performed in *E. coli* and plasmids used in this study are listed in Table 1. Constructions of plasmids pGIC055 and pGIC057 are described below.

**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* Pmif promoter (32) in a pNEB193 (New England Biolabs, Inc.) derivative. A kanamycin-resistant (Kan) mini-IS231A delimited by KpnⅠ restriction sites was isolated from pGIG010 (23a) and cloned downstream of the modified transposase gene. In this mini-IS, the Kan gene is associated with the rare-cutting polynucleter (RCP) 1.4 (28). A fragment including both the transposase gene and the mini-IS was then cloned into the pGIC13 gram-positive/gram-negative shuttle vector (24a). This pGIC13 is a derivative of pH1030 (24), fused to the *E. coli* vector pGIC2 (33) which bears the erythromycin resistance (Ery') gene from pGI4010 (18). The resulting plasmid pGIC052 contains the Pmif-TnpA-mini-IS Kan structure. To provide the mini-IS with uracilase restriction sites, the Kan gene was swapped for the spectinomycin resistance (Sp') gene combined to the RCP 2 of pGI300 (28) to give pGIC054. An internal fragment, limited to the Sp' gene was then exchanged with the Kan gene of pGI010 to get pGIC056. This plasmid thus harbors the uracilase restriction sites of RCP 2. Plasmids pGIC054 and pGIC056 were then deleted of a 2.9-kb *Aat*II fragment containing their gram-negative replicons 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 minipreparation (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 EcoRI (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). Labelling, 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 and mini-IS Sp' were prepared by PCR amplification from *E. coli* plasmids bearing the mini-IS, to avoid background labelling 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 Kolot et al. (20), using a CHEF-DR II (Bio-Rad) apparatus. The electrophoresis buffer was 0.5× TBE (45 mM Tris, 45 mM boric acid, 1 mM EDTA [pH 8]) and switch times ranging from 5 to 120 s were used. Total DNA were digested with octanucleotide-recognizing endonucleases NotI, SfiⅠ, and Asci (New England Biolabs) for 8 h, and with the omega-nuclease I-SceI (Boehringer Mannheim) for 1 h, as specified by the manufacturers. Sizes of the fragments were estimated by using lambda DNA concatamers (size range, 48.5 to 1,018.5 kb) and yeast chromosomes (225 to 1,900 kb) markers (New England Biolabs).

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**Table 1. Bacterial strains and plasmids used**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Description*</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td><em>Bacillus cereus</em> ATCC 14579</td>
<td>Type strain</td>
<td>38</td>
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<tr>
<td><em>Bacillus subtilis</em> CU267</td>
<td>Host strain for pGI plasmids and first transposition assays</td>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pGIC052</td>
<td>oriⅠ, oriⅡ, EryⅠ, mini-IS231A KanⅠ, Pmif-TnpA, RCP 1.4, 1 kb</td>
<td>This study</td>
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<td>pGIC054</td>
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<td>This study</td>
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<tr>
<td>pGIC055</td>
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<td>This study</td>
</tr>
<tr>
<td>pGIC056</td>
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<td>pGIC057</td>
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<td>This study</td>
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<td>oriⅠ, Amp’, 2.9 kb</td>
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<td>pH1T030</td>
<td>B. thuringiensis plasmid, thermosensitive replicon</td>
<td>24</td>
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<tr>
<td>pGI13</td>
<td>Fusion of pGIC2 and pH1T030; oriⅠ, oriⅡ, Amp’, EryⅠ, 6.8 kb</td>
<td>24a</td>
</tr>
<tr>
<td>pGI010</td>
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</tr>
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<td>oriⅠ, Amp’, 2.7 kb</td>
<td>New England Biolabs</td>
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<td>pBluescript SK</td>
<td>oriⅠ, Amp’, cloning vector, 3 kb</td>
<td>Stratagene</td>
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<td>pGI102</td>
<td>pBluescript with an EcoRI fragment containing the mini-IS</td>
<td>This study</td>
</tr>
<tr>
<td>pGI105</td>
<td>pBluescript with an EcoRI fragment containing the mini-IS</td>
<td>This study</td>
</tr>
</tbody>
</table>

* oriⅠ, replicative origin for gram-positive host; oriⅡ, replicative origin for gram-negative host; Amp’, EryⅠ, KanⅠ, and Sp’, resistance genes to ampicillin, erythromycin, kanamycin, and spectinomycin, respectively; Pmif-TnpA, IS231A transposase gene expressed under control of the Pmif promoter.
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 minipreparation as described above. After restriction with EcoRI, DNA fragments were cloned in the EcoRI 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, Kanr) and pGIC105 (ade insertion, Spri). 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 IRr and IRl (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, 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 Spri Erys or Kanr Erys candidates (Table 2) per generation. In the conditions used, the transposition frequency in B. subtilis approached $3 \times 10^{-6}$ for pGIC055 and $10^{-4}$ in the case of pGIC057. Moreover, the segregational stabilities of the donor plasmid (proportion of Erys candidates) 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 Eryr, 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 Kanr cells, appearing at a frequency of $10^{-7}$, were still Eryr. In these conditions, the relative transposition frequency reached the proportion of $8 \times 10^{-6}$ Kanr Eyr 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 Kanr 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 NotI, SfiI, and AscI and analyzed by PFGE. Since the mini-IS231A elements carry these rare restriction sites, their

![Diagram](https://via.placeholder.com/150)
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 *AseI* 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 *AseI*, 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 candidates 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, *AseI* 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 Kanr 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.

<table>
<thead>
<tr>
<th>Transposition test*</th>
<th>% of auxotrophs</th>
<th>Auxotrophies identifiedb</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st round (pGIC057)</td>
<td>&lt;0.03</td>
<td>0</td>
</tr>
<tr>
<td>2nd round (pGHC055)</td>
<td>0.6</td>
<td>1 Ura&lt;sup&gt;−&lt;/sup&gt;, 1 Gua&lt;sup&gt;−&lt;/sup&gt;, 5 Ade&lt;sup&gt;−&lt;/sup&gt;, 2 Met&lt;sup&gt;−&lt;/sup&gt;, 2 His&lt;sup&gt;−&lt;/sup&gt;, 2 Cys&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* In each case, 3,000 clones were tested.

b Phenotype abbreviations: Ura, uracil; Gua, guanine; Ade, adenine; Met, methionine; His, histidine; Cys, cysteine.
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 TACCCGCA - 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') in B. cereus ATCC 14579 containing the Kan' 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' Sp' 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' 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' 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).

Several auxotrophs as well as an aphenotypic insertion mutant were further characterized by restriction and PFGE. A total of five 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' into the largest NotI fragment (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).

Ascl 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).
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 SpI 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 IRp 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 $3 \times 10^{-5}$ to $1 \times 10^{-4}$ event per generation. With these transposition levels, each experiment can give rise to $10^7$ to $10^9$ 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 NotI and AccI 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 (SpI) 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 IS1831 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 auxotrophs 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 auxotrophs 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 auxotrophs 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 \textit{E. coli} \cite{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 \cite{28}.

In its current conformation, this system appears to be a powerful tool for insertional mutagenesis of \textit{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 \textit{B. subtilis} has been entirely determined \cite{21}, it would be of particular interest to focus on other remarkable \textit{Bacillus} species, most particularly those relevant for the industry (\textit{B. amyloliquefaciens}, \textit{B. licheniformis}, and \textit{B. stearothermophilus}) or the numerous opportunistic and pathogenic bacteria displaying positive (\textit{B. thuringiensis}, \textit{B. sphaericus} and \textit{B. popilliae}) or negative (\textit{B. cereus} and \textit{B. anthracis}) effects on animals and/or humans.

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