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Easy Cloning of Mini-Tn*10* Insertions from the *Bacillus subtilis* Chromosome

MICHEL STEINMETZ* AND ROSELYNE RICHTER

Laboratoire de Génétique des Microorganismes, URA537 du Centre National de La Recherche Scientifique, Institut National de la Recherche Agronomique, and Institut National Agronomique Paris-Grignon, F-78850 Thiverval-Grignon, France

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Delivery vectors for mini-Tn*10* transposons function in *Bacillus subtilis* (M. A. Petit, C. Bruand, L. Janniére, and S. D. Ehrlich, J. Bacteriol. 172:6736–6740, 1990). Using this system, we identified a new gene (*sytA*) whose inactivation affected regulation of genes of sucrose metabolism. For cloning the *sytA*::Tn*10* insertion in *Escherichia coli*, we developed a methodology similar to that commonly used for *B. subtilis* Tn*917* insertions. We constructed a plasmid which can be used to insert (by in vivo recombination) a ColE1 origin linked to a spectinomycin resistance gene (ori-spc element) into mini-Tn*10* transposons inserted into the *B. subtilis* chromosome. DNA extracted from a *sytA*::Tn*10*::ori-spc transformant was cut with restriction enzymes that do not cut into the Tn*10*::ori-spc sequence; plasmids containing the *sytA*::Tn*10* insertion were cloned by self-ligation, followed by transformation of *E. coli*. To obtain the wild-type *sytA* region, one of these plasmids was ligated with an *E. coli-B. subtilis* shuttle vector conferring erythromycin resistance, and the hybrid was used to transform the wild-type *B. subtilis* strain. Erythromycin-resistant transformants, detected as spectinomycin sensitive, resulted from conversion of the insertion mutation by the resident wild-type locus. The shuttle plasmid containing the wild-type locus could then be recovered in *E. coli*.

Many derivatives of natural transposons which increase their manageability and the range of their utilization have been constructed (3). A large collection of derivatives has been constructed from the *Salmonella typhimurium* Tn*10* transposon by Kleckner and collaborators; in delivery vectors for small Tn*10* derivatives (mini-Tn*10*), the transposase gene is located outside the transposon, rendering the insertion very stable (17). Recently, mini-Tn*10* vectors were adapted to the gram-positive bacterium *Bacillus subtilis* (11). A collection of manageable transposons and delivery vectors, functional in *B. subtilis* and other gram-positive bacteria, has been constructed by Youngman and collaborators from the *Streptococcus faecalis* Tn*917* transposon (19, 20). Here we present tools and techniques to render the Tn*10* system both competitive and complementary to the Tn*917*-derived tools in *B. subtilis*. These tools and techniques were tested on the *B. subtilis* sucrose (Suc) system.

Isolation of the *sytA*::Tn*10* mutation. The products of the *B. subtilis sacPA* operon mediate utilization of sucrose. Mutants affected in the *sacT* gene, which encodes an antiterminator protein involved in *sacPA* induction, are impaired for sucrose utilization (6). This defect is not absolute, because *B. subtilis* can also assimilate sucrose through another pathway involving the product of the *sacB* gene whose expression is dependent on SacY (5, 12). SacY is an antiterminator protein structurally related to SacT and able to partially replace it in *sacPA* activation (6, 14). On the other hand, a Δ(*sacT*) Δ(*sacY*) Δ(*sacB*) mutant such as GM1042 is strongly impaired in sucrose utilization. GM1042, a derivative of GM904 (1), carries in its chromosome (inserted into the *amyE* gene and associated to a phleomycin resistance gene) a promoterless *lacZ* gene fused to a promoter-leader region similar to that in *sacPA* (this fusion is sucrose inducible in *sacT*$^+$ strains and repressed in GM1042). Spontaneous Suc$^+$ revertants were selected from GM1042; about 20% of them expressed the *lacZ* fusion (LacZ$^+$ phenotype). Thus, this strain was suitable for identification of *trans*-acting suppressors of *sacT* and *sacY* deletions. To determine whether gene inactivation could result in such Suc$^+$ revertants, GM1042 was transformed with plasmid pHV1248; pHV1248 is a delivery vector for a mini-Tn*10* element conferring chloramphenicol (CAM) resistance (11); Suc$^+$ transposants were screened for. A Suc$^+$ LacZ$^+$ Tn*10* mutant (GM1107) was obtained. The transposon target was called *sytA* for suppressor of deletions of *sacY* and *sacT*.

Cloning of the *sytA*::Tn*10* insertion. To clone the *sytA*::Tn*10* insertion, we adapted a methodology previously developed for *B. subtilis* Tn*917* insertions (19). Strain GM1107 was transformed by plasmid pIC216 (Fig. 1) to insert a ColE1 origin (linked to a spectinomycin [SPC] resistance gene; a 2-kb sequence called ori-spc) into the transposon sequence. DNA extracted from the resulting GM1115 transformant was cut with restriction enzymes (*Eco*RI and *Bgl*II) that did not cut in the Tn*10*::ori-spc sequence. Digested DNA was ligated at a low DNA concentration to favor monomolecular ligation events (19). Both ligations gave SPC-resistant transformants in *E. coli*. Plasmids pIC251 and pIC252, which resulted from restriction by *Eco*RI and *Bgl*II, respectively, were 11 and 17.5 kb long, respectively (Fig. 1).

Cloning of the wild-type *sytA* gene. Plasmids added to *B. subtilis* competent cells are often subject to conversion events when they contain sequences homologous to sequences in the resident genome; this process can remove insertions, point mutations, or deletions, present in the homology region of the incoming DNA; this process is particularly efficient with transforming plasmid DNA extracted from a *recA E. coli* strain (and therefore monomeric) (7). This property was previously used in our laboratory to clone 11 mutant alleles of the *sacY* gene (5). We used this property to clone the wild-type *sytA* locus (Fig. 2). First, plasmid pIC251 was ligated to the *E. coli-B.*

* Corresponding author. Mailing address: Laboratoire de Génétique, INA-PG, 78850 Thiverval-Grignon, France. Phone: (33 1) 30 81 54 47. Fax: (33 1) 30 81 54 57.

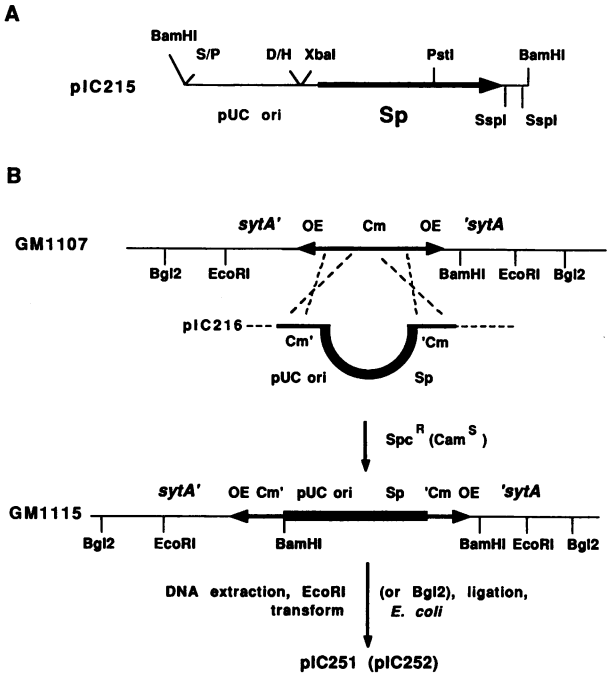

FIG. 1. (A) Plasmid pIC215, shown linearized at its *Bam*HI site, was constructed by ligation of the *Dra*I-*Pvu*II fragment from pUC8 (18), which contains the replication origin (pUC ori), with the *Hin*dII (*Sal*I)-*Sma*I SPC resistance cassette (Sp) from pIC156 (15). This cassette corresponds to a *Bam*HI-*Cla*I fragment of the Tn*554* transposon (10). D/H and S/P, *Dra*I-*Hin*dII and *Sma*I-*Pvu*II ligations, respectively. (B) Cloning of the *sytA*::Tn*10* insertion. The pUC8 origin of replication was inserted into the Tn*10* element in *sytA* by transformation of GM1107 with pIC216 plasmid DNA (pIC216 is a hybrid of the *E. coli-B. subtilis* shuttle plasmid pIC177 [15], cut with *Nco*I into its CAM resistance gene, and pIC215, cut with *Ssp*I); SPC-resistant *B. subtilis* transformants were selected on Luria-Bertani (LB) medium (9) containing SPC (100 mg/liter) and screened for CAM sensitivity on LB plates containing both CAM (5 mg/liter) and SPC. DNA extracted from a CAM-sensitive transformant (GM1115) was digested with *Eco*RI and self-ligated. The ligation mixture was used to transform *E. coli*. SPC-resistant transformants were selected on LB medium containing SPC (200 mg/liter) (most *E. coli* strains, for example, HB101 or DH5α, are efficiently transformed in these conditions; however, some strains, for example, DK1, are spontaneously resistant to SPC). Cm, CAM resistance gene in the mini-Tn*10* element; OE, outside ends of Tn*10*. There are sites for *Bam*HI, *Nde*I, *Pst*I, and *Xba*I within the Tn*10*::ori-spc sequence; this sequence is not cut by *Bgl*II, *Cla*I, *Eco*RI, *Hin*dIII, *Kpn*I, *Sac*I, *Sac*II, or *Sal*I.

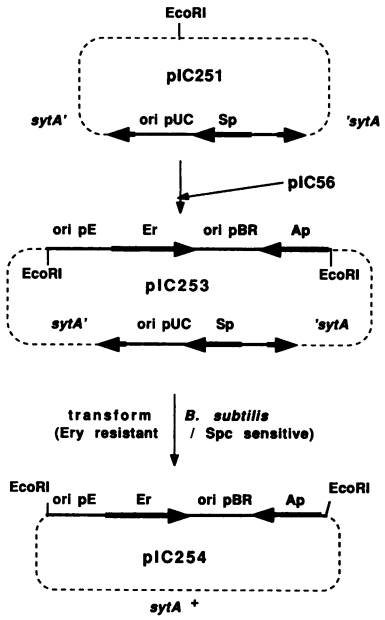

FIG. 2. Cloning of the wild-type *sytA* gene. pIC251 (Fig. 1) and pIC56 were cut with *Eco*RI and ligated together; pIC56 (15) is an *E. coli-B. subtilis* shuttle plasmid conferring resistance to ERY and ampicillin (AMP) in *B. subtilis* and *E. coli*, respectively; the resulting hybrid plasmid, pIC253, was easily selected in *E. coli*, since it conferred resistance to both SPC and AMP. pIC253 DNA was used to transform a *sytA*$^+$ *B. subtilis* strain to ERY resistance; a conversion event, detected as yielding an SPC-sensitive transformant, resulted in pIC254 which contains the wild-type *sytA* gene. Ap, Er, ori pE, and ori pBR, AMP resistance gene, ERY resistance gene, and origins of replication of pE194 and pBR322, respectively.

subtilis shuttle plasmid pIC56 (15), which confers erythromycin (ERY) resistance to *B. subtilis*. The hybrid plasmid (pIC253) was used to transform GM1010 (wild type for *sytA*) to ERY resistance. About 90% of transformants were sensitive to SPC, presumably because of a conversion event. The plasmid from one transformant was isolated and used to transform *E. coli*. Restriction analysis showed that this plasmid (pIC254) was structurally similar to pIC253, but the Tn*10*::ori-spc (3-kb) sequence had been lost (Fig. 2). pIC254 and shorter derivatives complemented the *sytA*::Tn*10* mutation; the restriction map of the *sytA* locus, as well as determination of its position on the *B. subtilis* chromosome map (near the *hut* operon), demonstrated that *sytA* was different from all previously identified sucrose genes (13).

Perspectives. The transposition efficiency of a recent version of Tn*10* delivery vector functioning in *B. subtilis* (8) is similar to that of the most efficient Tn*917* delivery vector, pLTV1 (4). One advantage of the pLTV1 system is the presence of a ColE1 origin within the Tn*917* element. On the other hand, the Tn*917* derivatives are long (about 15 kb for that in pLTV1) and contain many commonly used restriction sites; cloning the entire region containing the inserted transposon on a single plasmid is therefore difficult. In contrast, this was easy for the *sytA*::Tn*10* insertion (Fig. 1); as a consequence, cloning the wild-type *sytA* region was straightforward (Fig. 2). This comparison helps to identify improvements useful for Tn*10* delivery systems adapted to *B. subtilis*. An improved mini-Tn*10* delivery plasmid was recently constructed (16); this vector (pIC333; 7 kb) contains (i) a 2.4-kb mini-Tn*10* containing a ColE1 origin and an SPC resistance gene, (ii) an ERY resistance gene, (iii) a thermosensitive origin of replication for gram-positive hosts (inactive at temperatures higher than 35°C) (8), and (iv) a Tn*10* transposase gene with mutations previously shown to decrease the target specificity of transposition in *E. coli* (2). Experiments in our laboratory and elsewhere showed that pIC333 is an efficient mini-Tn*10* delivery vector in *B. subtilis* (16).

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REFERENCES

1. **Aymerich, S., and M. Steinmetz.** 1992. Specificity determinants and structural features in the RNA target of the bacterial antiterminator proteins of the BglG/SacY family. Proc. Natl. Acad. Sci. USA **89:**10410–10414.
2. **Bender, J., and N. Kleckner.** 1992. IS*10* transposase mutations that specifically alter target site recognition. EMBO J. **11:**741–750.
3. **Berg, C., D. Berg, and E. Groisman.** 1989. Transposable elements and the genetic engineering of bacteria, p. 879–925. *In* D. Berg and M. Howe (ed.), Mobile DNA. American Society for Microbiology, Washington, D.C.
4. **Camilli, A., D. A. Portnoy, and P. Youngman.** 1990. Insertion mutagenesis of *Listeria monocytogenes* with a novel Tn*917* derivative that allows direct cloning of DNA flanking transposon insertions. J. Bacteriol. **172:**3738–3744.
5. **Crutz, A.-M., M. Steinmetz, S. Aymerich, R. Richter, and D. Le Coq.** 1990. Induction of levansucrase in *Bacillus subtilis*: an antitermination mechanism negatively controlled by the phosphotransferase system. J. Bacteriol. **172:**1043–1050.
6. **Debarbouille, M., M. Arnaud, A. Fouet, A. Klier, and G. Rapoport.** 1990. The *sacT* gene regulating the *sacPA* operon in *Bacillus subtilis* shares strong homology with transcriptional antiterminators. J. Bacteriol. **172:**3966–3973.
7. **Iglesias, A., and T. A. Trautner.** 1983. Plasmid transformation in *Bacillus subtilis*: symmetry of gene conversion in transformation with a hybrid plasmid containing chromosomal DNA. Mol. Gen. Genet. **189:**73–76.
8. **Maguin, E., P. Duwat, T. Hege, S. D. Ehrlich, and A. Gruss.** 1992. New thermosensitive plasmid for gram-positive bacteria. J. Bacteriol. **174:**5633–5638.
9. **Miller, J.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
10. **Murphy, E., L. Huwyler, and M. C. de Freire Bastos.** 1985. Transposon Tn*554*: complete nucleotide sequence and isolation of transposition-defective and antibiotic-sensitive mutants. EMBO J. **4:**3357–3365.
11. **Petit, M. A., C. Bruand, L. Jannière, and S. D. Ehrlich.** 1990. Tn*10*-derived transposons active in *Bacillus subtilis*. J. Bacteriol. **172:**6736–6740.
12. **Steinmetz, M.** 1993. Carbohydrate catabolism: enzymes, pathways, and evolution, p. 157–170. *In* A. L. Sonenshein, R. Losick, and J. A. Hoch (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.
13. **Steinmetz, M., V. Azevado, P. Serror, R. Richter, and D. Le Coq.** Unpublished results.
14. **Steinmetz, M., D. Le Coq, and S. Aymerich.** 1989. Induction by sucrose of saccharolytic enzymes in *Bacillus subtilis*: evidence for two partially interchangeable regulatory pathways. J. Bacteriol. **171:**1519–1523.
15. **Steinmetz, M., and R. Richter.** 1994. Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*. Gene, in press.
16. **Steinmetz, M., R. Richter, P. Stragier, and C. Karmazyn-Campelli.** Unpublished results.
17. **Way, J. C., M. A. Davies, D. Morisato, D. E. Roberts, and N. Kleckner.** 1984. New Tn*10* derivatives for transposon mutagenesis and construction of *lacZ* fusions by transposition. Gene **32:**369–379.
18. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–109.
19. **Youngman, P.** 1990. Use of transposons and integrational vectors for mutagenesis and construction of gene fusion in *Bacillus* species, p. 221–266. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biological methods for *Bacillus*. John Wiley and Sons, Chichester, United Kingdom.
20. **Youngman, P.** 1993. Transposons and their applications, p. 585–596. *In* A. L. Sonenshein, R. Losick, and J. A. Hoch (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, D.C.