Conjugational transfer of pLS20 in Bacillus subtilis

Marburg 168 is restricted by the BsuM restriction-modification system. Restriction efficiency was measured using pLS20 derivatives possessing various numbers of XhoI sites, which are known to be recognized by BsuM. An increase in XhoI sites clearly reduced the conjugational efficiency of pLS20 as compared with that of pUB110 plasmid lacking XhoI.

Key words: Bacillus subtilis; conjugational transfer; restriction modification; BsuM

pLS20, a 65-kb conjugational plasmid, was isolated from a Bacillus subtilis (natto) strain and was characterized as transmissible among B. subtilis 168 derivatives.1,2) The conjugation step is so simple that the mixing of donor and recipient cells in Luria-Bertani (LB) media for only 15 min gives thousands of transconjugants per ml. The ability of the pLS20 derivatives to burden extra DNA segments, up to 100 kb and probably more, and be transmitted into another B. subtilis strain,3) was recently reported. These characteristics of pLS20 conjugation can be introduced into DNA engineering. B. subtilis 168 exhibited great potential to rebuild a giant DNA through the assembly of a number of smaller DNA pieces.4,5) In the subsequent transfer of the rebuilt giant DNA to another proper strain of B. subtilis, such as a recA mutant, a normally competence-mediated transformation protocol is applied where a liquid solution of naked DNA is required. However, biochemical isolation of the intact giant DNA becomes harder with increases above 50 kb in size. Therefore, pLS20 is a candidate for a new DNA transporter among B. subtilis strains.

B. subtilis 168 possesses an inherent restriction and modification system, BsuM, which has been characterized as targeting the XhoI sequence (CTCGAG).6) Although B. subtilis strains derived from the BsuM-deficient strain RM125 (ΔSspI AhsΔRM arg leu) are used for giant DNA cloning,4,5) no special attention has been paid to the XhoI sites. The difficulty of delivering DNAs carrying multiple XhoI sites from RM125 to another proficient strain (our unpublished observations) induced us to investigate the effects of the BsuM on pLS20 conjugation.

First, the original unique XhoI site of pLS20 was eradicated by insertion of a chloramphenicol acetyl transferase gene (cat) cassette isolated from pC194 in which the cat gene was controlled by its intrinsic promoter, resulting in pLS20X, as shown in Fig. 1A. The cassette was sandwiched between two SaII sites. Since a cohesive end by SaII is identical to that by XhoI, the two ends can join with one another, but the resulting joint cannot be recut by the enzymes. Thus, pLS20X allowed a systematic investigation to confirm that the new XhoI sites were phased in. Ligation of the two fragments, which had extraordinarily different sizes, pLS20 at 65 kb and the SaII-cat-SaII cassette at 1.0 kb, was efficiently carried out by the OGAB method developed by our group.7) Briefly, ligation in the presence of 15% PEG of the cat prepared from pCISP4015) on SaII digestion with equal moles of pLS20 digested with XhoI generated long linear DNAs consisting of alternative repeats of the two segments in vitro. These linear DNAs processed by competent B. subtilis RM125 generated a circular plasmid, which rendered the transformant resistant to chloramphenicol (Cm). One representative pLS20X among dozens of CmR colonies exhibited the expected structure and normal conjugational activities.

Insertion of the cat cassette into pLS20 in a unique SaII site caused no interference with conjugation.1) Hence, this site was chosen for the systematic addition of XhoI sites. As illustrated in Fig. 1, insertion of a hygromycin phosphotransferase gene (hyg) cassette, in which one XhoI site at one end (Xhyg) or two sites at both ends (XhygX) were introduced, added new XhoI sites to pLS20X. The Xhyg and the XhygX gene cassette were prepared by PCR using pHG108 (obtained from Dr. Y. Koyama) as a template. Since the primers for PCR carried the S10 promoter sequence and XhoI and SaII sites, the DNA fragments, as shown schematically in Fig. 1A, were amplified. These cassettes were used in the replacement of the cat gene in the 4.2-kb EcoRI-EcoRV fragment from pLS20cat,1) which was cloned between the EcoRI and NruI sites of pBR322. Since both EcoRV

Note

Restriction on Conjugational Transfer of pLS20 in Bacillus subtilis 168

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and NruI generate blunt ends, the two ends can join with each other. The resulting E. coli plasmids, illustrated in Fig. 1A, were used to transform RM125 harboring pLS20X. Plasmids were linearized with PvuII prior to transformation to avoid Campbell-type integration. The expected derivatives, pLS20X1 and pLS20X2, as illustrated in Fig. 1B, were obtained from transformants selected with hygromycin (Hg). In a similar way, pLS20X0 without an XhoI site was also constructed. Preparation and transformation of competent B. subtilis were performed as previously described.4,5,7) Tetracycline (Tc, 10 μg/ml), chloramphenicol (Cm, 5 μg/ml), kanamycin (Km, 10 μg/ml), and hygromycin (Hg, 50 μg/ml) were added for B. subtilis selection.

B. subtilis 168 (= 1A1) (hsdRM+ trpC2) was obtained from the Bacillus Genetic Stock Center (Columbus, OH). BEST2125,1) which is resistant to Tc due to a marker gene (tetL) inserted into the proB gene in its genome, was used to provide the 1A1 cells for the recipient. This tetL gene was transferred to RM125 (ΔhsdRM) by genetic transformation, resulting in BEST40569 for the recipient. Conjugational kinetics were carried out by the reported protocol. Briefly, both donor and recipient B. subtilis cultures grown at 37 °C for 15–17 h in LB medium were diluted by 1:20 in 20 ml of pre-warmed LB in a 150 ml-flask and shaken at 120 rpm at 37 °C. Aliquots (0.1 ml) of the donor and recipient cells were mixed together every h and kept for 15 min at 37 °C without shaking. An appropriate volume of the mating mixture was spread on an LB plate supplemented with Tc and Cm, or Tc and Km for transcipient selection. Colonies formed after incubation at 37 °C for 24 h were scored.

Conjugational transfers of three pLS20 derivatives, pLS20X0, X1, and X2, were examined. The conjugation efficiencies in RM125 (ΔhsdRM), in 1A1 (hsdRM+), and from 1A1 to RM125 (BEST40569) were similar among three plasmids, suggesting that the number of XhoI sites has no effect on conjugation frequencies in these combinations of donor and recipient cells (data not shown). In contrast, conjugational transfer from RM125 to 1A1 (BEST2125) clearly reduced the number of transcipients as the number of XhoI sites increased (data not shown). Since the former results suggest that the inherent conjugative abilities of the three pLS20 derivatives are almost the same, the latter result implies that the conjugational transfers of pLS20X1 and X2 are affected by the BsuM restriction system of the recipient.

Fig. 1. Construction of pLS20 Derivatives.
A. Restriction enzyme sites, EcoRI, EcoRV, SalI, PvuII, and XhoI, are designated RI, RV, S, Pv, and X respectively. The original pLS20 possesses single SalI and XhoI sites. The insertion of a cat cassette in the SalI site resulted in pLS20cat in a previous study. E. coli plasmids are surrounded with a dashed box, and the dotted circle represents the pBR322 sequence. (X) indicates an XhoI site associated with the XhygX gene only. The hyg cassettes are shown schematically at the bottom. B. Plasmids pLS20X0, X1, and X2 were derived from pLS20X by insertion of the second antibiotic gene hyg with zero, one, and two XhoI sites respectively. Crossed lines indicate a homologous recombination.
BEST2125. To show more explicitly that the transmission of the pLS20 is restricted by the presence of XhoI sites, the conjugation assay illustrated in Fig. 2A was performed. The pLS20-related conjugation system mobilizes not only pLS20 itself but also the small plasmid pUB110 (4.6 kb), if it coexists in a donor cell. This is because pUB110 possesses a consensus origin of transfer (oriT) region\(^{21}\) of both plasmids traverses through the protein channel connecting donor and recipient. The recipient possesses a genomic tetL marker. B. The number of transciipients selected by two antibiotic markers, Cm and Tc for pLS20 derivatives, and Km and Tc for pUB110, as indicated in panel A. The number of transciipients/ml at 37 °C, pLS20X0 ( ○ ), X1 ( □ ), X2 ( ▴ ), and pUB110 ( △ ) are plotted. Transciipients appeared at an early growth stage, reached a maximum at 2 h of cultivation, and were rapidly lost after 3 h. A typical growth-stage dependent conjugational profile of pLS20 was observed for both pLS20 derivatives and pUB110. The pUB110 profile is represented by that which cohabited with pLS20X0 because the other two were nearly identical in the experiments, done in triplicate. Dotted lines indicate that no transciipient was observed at 4 h. In the inset panel at the upper right, the transciipient number at 2 h is plotted along with the number of XhoI sites. Those for pUB110 are plotted together with pLS20 derivatives.

References


