Conjugational Transfer Kinetics of pLS20 between *Bacillus subtilis* in Liquid Medium

Mitsuhiro Itaya, Nagayoshi Sakaya, Satoko Matsunaga, Kyoko Fujita, and Shinya Kaneko

Mitsubishi Kagaku Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194-8511, Japan

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**Note**

pLS20-mediated conjugational transfer between *Bacillus subtilis* was investigated not on conventional solid media but in liquid culture. Detailed conjugational kinetics revealed that pLS20 transmission occurred at a limited cellular growth stage of both donor and recipient. Mutation of the recipient recA did not significantly interfere with the conjugational transfer process.

**Key words:** *Bacillus subtilis*; conjugational transfer; pLS20

*Bacillus subtilis* 168 is a gram-positive endospore-forming bacterium and is generally regarded as a safe organism. Recombinant gene cloning and engineering by use of both plasmids and genome has been successful due to the ability of *B. subtilis* 168 to develop natural competency.1,2) One of applications is the use of *B. subtilis* 168 as the initial host for cloning1,2) and the subsequent delivery of the engineered DNA to other hosts of interest.4,5) But, *Bacillus* strains other than *B. subtilis* 168 are normally unable to develop competence. Hence gene delivery to these *Bacillus* species requires alternative methods such as genetic manipulation to convert them to competent developers,6) the use of protoplasts, electroporation transfer,7) or conjugational transfer.7) The first method is elegant but limited to strains of researchers’ interest. As regards the two physicochemical-based protocols, the former method is laborious and difficult in our hand to reproduce, and the latter, though technically more simple, is limited to small DNA. Conjugation for interspecies plasmid transfer drew our attention to exploit a novel DNA delivery system. A low copy conjugative *Bacillus* plasmid pLS207–9) can mobilize not only small plasmid pUB110 but also pLS20 itself,7) but the properties underlying the conjugation process between *B. subtilis* remain to be investigated as compared with other conjugation systems.10–12) Transmission of as large as 65 kb pLS20, yet smaller than the DNA size currently handled in our *B. subtilis* cloning and manipulation system,2–5) implies the potential to exploit far larger transferable sizes. Here we present the first report on detailed kinetics for pLS20-mediated conjugational transfer.

pLS20 was purified from strain IFO33358,9) and a physical map with several type II restriction enzymes was constructed (data not shown). Though pLS20, approximately 65-kb, is considered stable in *B. subtilis* 168,7) a tightly associated antibiotic marker was needed for the detailed kinetics of conjugational transfer. A unique enzyme *Sal*I site was chosen for marker gene insertion. The determined sequence of the region including the *Sal*I site indicated that mutations in this site, located between two putative ORFs (data not shown), probably cause little interference with conjugation. A chloramphenicol acetyl transferase (*cat*) gene cassette prepared from pBRCm13) on *Sal*I digestion was mixed with equimolar pLS20 digested with the same enzyme, ligated, and used to transform *B. subtilis* RM125 (*hsdR hsdM leu arg*) according to the method described previously.14) Luria-Bertani broth was used for all bacteria at 37°C, including *Escherichia coli* JA221 (F*− hsdR hsdM trp leu lacY recA1) for molecular cloning at 37°C. Type II restriction enzymes and T4 DNA ligase were purchased from Toyobo (Tokyo). Colonies selected on an LB plate supplemented with chloramphenicol (Cm, 5 μg/ml) were subjected to plasmid isolation for screening. Three out of 12 plasmids examined were identical to the expected structure, and one representative was designated pLS20cat. An LB culture of BEST40401, an RM125 strain carrying the pLS20cat, made after nine generations in the absence of Cm, was spread on the LB plate after appropriate dilutions. The high genetic stability of pLS20cat in BEST40401 was demonstrated by the appearance of no segregants out of 1,100 colonies examined (< 0.1%).

Conjugational transfer experiments on pLS20 between *B. subtilis* have been carried out on solid surface media like other gram-positive bacteria to sustain mating pair formation.7,10) This was due to the apparent lack of pili-like structure of *E. coli*12) with few exceptions, referred to by Andrup and Anderson.15) Because *B. subtilis* 168 does not form inherent aggregation during growth in liquid media, we expected to

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1) To whom correspondence should be addressed. Fax: +81-42-724-6254; E-mail: mita2001@sfc.keio.ac.jp
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observe the conjugational transfer kinetics of pLS20 using BEST40401 as donor and BEST2125 (trpC2 proB::pBRTc)\textsuperscript{16} or BEST8630 (trpC2 recA362::tet; our laboratory stock) as recipient. These recipients can be selected by tetracycline (Tc) at 10 \( \mu \)g/ml due to a resistance gene (tetL) in the proB or recA. B. subtilis cultures grown in LB medium (BEST2125 or BEST8630) or LB medium containing Cm (BEST40401) at 37°C for 15–17 h were diluted by 1:20 in 20 ml pre-warmed LB in a 100 ml flask and shaken at 120 rpm at 37°C. Amounts (0.1 ml) of donor and recipient cells were mixed together at the indicated times and kept for 15 min at 37°C. An appropriate volume of mating mixture was spread on the LB plate supplemented with Cm and Tc and incubated at 37°C for 24 h to select transcipients. Transfer of pLS20\textit{cat} was confirmed by plasmid extraction of six randomly selected transcipients. All transcipients tested had pLS20\textit{cat}. Growth profiles of donor and recipient measured by OD\textit{600} are plotted in Fig. 1a. A typical growth stage-dependent conjugational profile of pLS20\textit{cat} is shown in Fig. 1b. Transcipient appeared at an early growth stage, reached a maximum at 2 h cultivation, and was rapidly lost after 3 h. Mating time was fixed to 15 min, during which induction of the \textit{cat} gene expression appears sufficient, because a prolonged mating time above 30 min can result in plausible second mating by the same donor or a newly generated donor (our unpublished observation).\textsuperscript{12,15} The number of transcipients decreased in proportional to the decrease in donor cells as shown in Fig. 1b. The number of viable donor and recipient cells at the 2 h was scored by counting colonies formed on the LB plate. A transfer frequency \( 4.01 \times 10^{-4} \) / donor / 15 min at 37°C was calculated, one order of magnitude higher than the reported \( 1.3 \times 10^{-5} \), which was measured on solid media.\textsuperscript{7} This might account for the difference in mating carried out in liquid from that on a solid surface.

In experiments where donor culture started 1 h ahead (+1) or behind (−1) with respect to the recipient, the conjugation profiles shifted, apparently linked to the donor growth stages, as shown in Fig. 1c. Similar profiles were observed by sliding the recipient start time, as shown in Fig. 1d, indicating that the growth stage of the recipient also affects mating efficiency. Conjugational kinetics was carried out using the \textit{recA} strain BEST8630 as a recipient. Apparent reduction of the maximum transcipient number to approximately one-tenth of that of the \textit{recA} proficient recipient was interpreted as reduced viability of the \textit{recA} mutant, yielding a transfer frequency of \( 9.25 \times 10^{-5} \) / donor / 15 min at 2 h. The slightly broadened mating profile against time shown in Fig. 1b might reflect the altered recipient stage of the \textit{recA} mutant.

The present results revealed new properties of pLS20-mediated conjugational transfer in liquid mating and offer a standard protocol for mating. pLS20 was shown to transfer small plasmid vectors, categorized as rolling circle replication (RCM) plasmids, including pUB110, widely used in \textit{B. subtilis} cloning.\textsuperscript{1,17} But the stability of these RCR plasmids in \textit{B. subtilis} 168 largely evidenced was not significantly high. The stability as proven in this study of pLS20, categorized as theta replicating plasmid,\textsuperscript{18} can be used to exploit pLS20-based vectors as a stable and readily transmissible delivery vehicle of DNAs cloned and manipulated in \textit{B. subtilis}, especially for large and heterologous inserts.\textsuperscript{2,3,13}
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References


