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Plasmid transformation of competent *Bacillus subtilis* by lysed protoplast DNA

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Transformation of competent *Bacillus subtilis* with DNA obtained from lysed protoplasts (LP transformation) was analyzed using several different plasmid vectors: pC194, pUB110, pCB1 (consisting of pC194 and pBluescript II SK+), and pAC32R2 (consisting of pUB110 derivative and pUC19). LP transformation of *B. subtilis* QB936 with pCB1 was 6500-fold higher than that achieved using conventional transformation with purified DNA. Greater transformation efficiencies were also obtained using pAC32R2. However, transformation frequencies using both protoplast-derived and purified pC194 were very low ($1.4–2.0 \times 10^2$ transformants per μg DNA). Hence, the efficiency of transformation depends on the nucleotide sequence of the donor plasmid. The LP transformation frequency using pC194 obtained from an *add5* mutant was remarkably enhanced (1.6×10^8 transformants per μg DNA), indicating that this unique form of high molecular weight DNA is likely responsible for part of the stimulatory effect. Chromosomal DNA inhibited plasmid transformation using pC194 and pUB110, but had little effect on pCB1 transformation. Conversely, pCB1 DNA did not inhibit transformation with protoplast-derived chromosomal DNA. Competence proteins under the control of transcription factor ComK were likely required for LP plasmid transformation. The DNA concentration-dependence of plasmid transformation was first order and the slope value was one.

[**Key words:** *Bacillus subtilis*; Lysed protoplast; Competent; Plasmid transformation; High efficiency; First order; Specific factor; Nucleotide sequence]

Bacillus subtilis is a gram-positive spore-forming bacterium capable of developing competence, allowing for natural transformation of *B. subtilis* with purified DNA (1–3). Transformation is used for strain manipulation, genetic mapping, and gene cloning (4–6). Interestingly, the covalently closed circular (CCC) monomer form of pC194 DNA is inactive in transformation, but the CCC multimeric form is active (7,8). An attractive working hypothesis on competent transformation with plasmid DNA has been proposed (7–10), which begins with an oligomeric molecule of plasmid DNA binding to the competent cell surface at a DNA receptor site. Double-strand cleavage occurs at two neighboring cleavage sites and attachment of one terminus resulting from each cleavage occurs, with the loss of an intervening segment. Independent entry of the complementary single strands takes place with the same polarity (for example, 3′–5′). Degradation of the non-entering strands accompanies this process, and some DNA region is lost from one or both ends of each entering strand. Complementary strands anneal and circularization occurs as a result of the presence of redundant single-strand termini. Repair to yield a complete CCC monomer takes place. The model accommodates other parameters of plasmid transformation, such as the linear relationship between DNA concentration and number of transformants when using multimeric DNA, the sensitivity of plasmid transformation to restriction endonucleases, and the generality of the requirement for multimeric forms in transformations with *B. subtilis* plasmids.

A large number of DNA-uptake proteins have been identified in *B. subtilis*, and all are under control of the competence transcription factor, ComK (11–17). Most of the DNA-uptake proteins are either membrane-associated or are exported across the membrane and associated with the cell wall (18–24). DNA-uptake proteins can be divided into three classes (25). One group resembles components of the type IV pilus assembly and type 2 secretion systems. ComGC, ComGD, ComGE, ComGG, ComGA, ComGB, ComC, BdbD, and BdbC proteins belong to this class. The second group consists of three proteins, ComEA, ComEC and ComFA, which participate in the transport of DNA across the cell membrane. The final group consists of the cytosolic proteins Smf and YwpH.

A striking feature of plasmid transformation is reported with a DNA form called high-molecular-weight (HMW) multimer (26–28). One of the plasmids capable of producing this form of DNA is pC194, a rolling circle plasmid of *Staphylococcus aureus* (29). The plasmid is mostly present as monomers and dimers when propagated in wild-type *B. subtilis*. However, about 20% of the total plasmid DNA is HMW multimers in cells lacking the major cellular nuclease, AddAB (30–34), which would degrade HMW multimers in wild-type cells (26). Purified AddAB binds at a dsDNA end and functions as both a DNA helicase and nuclease, the combined action of which results in the degradation of both strands of the DNA duplex (26). During the DNA unwinding process, recognition of the properly oriented sequence 5′-AGCGG-3′, the *B. subtilis* chi site, causes attenuation of the AddAB enzyme nuclease activity (34).

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TABLE 1. Bacterial strains and plasmids.

Strain or plasmid	Genotype, phenotype, or plasmid marker	Source or reference
168S	*trpC2 rpsL smo1*	38
QB936	*trpC2 aroG932 leuB8 ald1*	38
1A334	*add5 hisH2 metB5*	BGSC[a]
AYG2	*cysA14 metC3 trpC2 leuB8 argH1 aroG932*	1
KUS1	*add5 leuB trpC ald1*	Tfm (QB936: 1A334DNA, Aro$^+$ UV-s)[b]
KUS10	*com14::Tn917 aroG932 leuB8 trpC2 ald1*	39
KUS11	*comR::Tn917 aroG932 leuB8 trpC2 ald1*	39
KUS12	*comA9 leuB8 trpC2 ald1*	39
KUS13	*comK leuB8 trpC2 ald1*	39
KUS14	*comM leuB8 trpC2 ald1*	39
KUS15	*com31 leu8B trpC2 ald1*	39
HH4	*cysA14 metC3 hisH leuB8 argH1*	39
E. coli		
JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi* Δ(*lac-proAB*) F' [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	53
Plasmids		
pC194	Cm-r	29
pBluescript II SK +	Ap-r	54
pUC19	Ap-r	53
pUB110	Km-r	35
pAC3	Ap-r, Km-r	52
pAC32R2	Ap-r, Km-r	51, The length of the plasmid is 5699 bases
pCB1	Ap-r, Cm-r	39, The length of the plasmid is 5875 bases

UV-s, UV-sensitive. Samples were irradiated with a UV lamp at a distance of 30 cm (GL15 lamp, 254 nm, 5.0 Jm-2 s-1; Toshiba Corporation, Japan) for 10 s and incubated at 37°C overnight.

[a] BGSC, Bacillus Genetic Stock Center.

[b] Tfm, transformation. Tfm (QB936: 1A334DNA, Aro$^+$ UV-s) indicates a Aro$^+$ UV-s transformant of QB936 with native DNA (below 12 ng/mL) of 1A334.

Transformation efficiency with HMW multimer DNA is 40-fold higher than that with oligomer DNA (27).

When *B. subtilis* carrying pUB110 (35) was turned into protoplasts and added into competent culture, transformation frequency with pUB110 was 10,000-fold higher than that of conventional transformation using purified DNA (4). Transformation of competent *B. subtilis* with lysed protoplast chromosomal DNA (LP transformation) also showed 200–1000-fold higher transformation efficiency than that of conventional methods using purified DNA (36–38). Several other studies demonstrated successful transformation of entire genomes into *B. subtilis* by LP transformation, as well as the uptake of double-stranded DNA (dsDNA) fragments of up to 1271 kb (39–41). However, because LP transformation also depends on the ComK transcriptional factor (39), ComEC channel (40), ComFA (40), and other competence genes (*comA9*, *comM*, *com31*, *com14*, and *comR*) (39), the same competence proteins (19) will be employed during the LP transformation.

We have proposed a working hypothesis regarding DNA uptake mechanisms by competent *B. subtilis* during LP transformation as follows (39,41). ComGC is located outside of the cell membrane and is a member of the type IV competence pseudopili (20). Various DNA-binding proteins attached to donor DNA are likely to interact with proteins bound to the type IV pseudopilus region, leading to high-frequency transformation (42). Attachment of the donor DNA complex to the pilus complex leads to retraction of the type IV pili (19,43,44) and transforming DNA is then brought into proximity with the outside region of the ComEC channel (19). ComEA is required for DNA uptake, which is assisted by ATPase ComFA (19,40). The energy to mediate the DNA uptake is likely provided by Brownian motion (40,45–48), not ATP hydrolysis. ComFA functions in a similar manner to the DEAD family of ATP-driven helicases (40,49,50) and Brownian ratchets (40,45). With the assistance of Brownian ratchets and the helicase, transforming DNA is transferred from outside the cell membrane into the cell (40).

In this study we have analyzed the high frequency of plasmid transformation with lysed protoplast DNA and the involvement of AddAB in the transformation. We have also investigated LP transformation using competitor DNA and the requirement of competence proteins for the transformation.

MATERIALS AND METHODS

Bacterial strains and plasmids The bacterial strains and plasmids used in this study are listed in Table 1. pAC32R2 (51,52) is derived from pUC19 (53) and pUB110. pCB1 is derived from pBluescript II SK+ (54) and pC194. These vectors are shuttle plasmids between *Escherichia coli* and *B. subtilis*. pAC32R2 confers ampicillin (Ap-r) and kanamycin-resistance (Km-r) in *E. coli* and Km-r in *B. subtilis*. pCB1 confers Ap-r and chloramphenicol resistance (Cm-r) in *E. coli* and Cm-r in *B. subtilis*.

Media Bacteria were grown in Luria–Bertani (LB) medium (38) or on LB agar supplemented with the appropriate antibiotics. To select resistant *E. coli* cells, Ap (50 μg/mL) was used. Cm (5 μg/mL) and Km (5 μg/mL) were used to select resistant *B. subtilis* cells. Spizizen minimal (SM) medium (38) was used as a basal medium for the preparation of competent cells. For the isolation of auxotrophs or transformants, low SM (LSM) medium (38) was used and supplemented with amino acids when necessary.

Preparation of *B. subtilis* protoplasts Protoplasts were prepared as described by Saito et al. (41) and incubated in SMM buffer (0.5 M sucrose, 0.02 M maleate buffer pH 6.5, 0.02 M $MgCl_2$) containing lysozyme at a final concentration of 250 μg/mL for 30–45 min at 40°C. After centrifugation and resuspension in SMM buffer, the density of the protoplast suspension was measured by absorbance at 660 nm (A_{660}). An A_{660} of 2.88 corresponded to 2.74×10^9 protoplasts/mL. Following dilution with SMM buffer, an aliquot (0.1 mL) of the protoplast suspension was used as the source of donor DNA. When 0.1 mL of the protoplast suspension was added to 0.9 mL of sterilized water, colonies appeared on LB agar plates at a frequency of less than 1×10^{-9} cfu/protoplast. Microscopic observations confirmed that all of the cells examined ($\sim 1 \times 10^5$ cells) were spherical in shape (data not shown).

LP transformation of *B. subtilis* and co-transformation analysis Competent cells were prepared as described by Saito et al. (41). An aliquot (0.1 mL) of a protoplast suspension was added to 1 mL of competent cell culture and incubated at 37°C for 30 min. Cells were then plated onto minimal agar with appropriate nutrients. For plasmid transformation, 1 mL of LB medium was added to each tube containing 1.1 mL of the transformation mixture. The tubes were incubated at 37°C for 90 min and cells were plated onto LB agar medium supplemented with 10 μg/mL Cm. After incubating overnight at 37°C, transformation frequencies were assessed. The co-transformation analysis was carried out as described by Akamatsu and Taguchi (38). After single-colony isolation of transformants, each colony was transferred to a diagnostic agar plate and unselected marker(s) were identified.

Competition experiment Vegetative cells of *B. subtilis* 168S were converted into protoplasts, centrifuged, and suspended in SMM buffer and the protoplast suspension was used as competitor DNA solution in a competition experiment. The transformation mixture contained 1.0 mL of competent cell suspension, 0.05 mL of donor DNA solution in protoplast lysate of *B. subtilis* 168S or 1A334 containing plasmid, and 0.05 mL of competitor DNA in protoplast lysate of *B. subtilis* 168S. After incubating at 37°C for 30 min, the cells were spread onto appropriate selection plates.

DNA manipulation Chromosomal DNA was prepared as described by Akamatsu et al. (55). Preparation of plasmids from *E. coli* and protoplasts of *B. subtilis* was carried out according to the methods described by Birnboim and Doly (56). Manipulation of recombinant DNA was performed by standard techniques (57), using enzymes obtained from Toyobo (Tokyo, Japan).

RESULTS

LP transformation analysis using native plasmid DNA LP transformation of *B. subtilis* QB936 with pCB1, pAC32R2, and pC194 derived from lysed protoplasts (lpDNA) was analyzed and compared to conventional transformation using alkaline lysis-purified DNA (alDNA). The LP transformation efficiency of wild-type strain QB936 with pCB1 lpDNA was 2.8×10^9 transformants per μg DNA (Table 2), a 6500-fold increase compared to conventional transformation using alDNA (4.3×10^5 transformants per μg DNA). Similar results