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Essential involvement of the *Bacillus subtilis* ABC transporter, EcsB, in genetic transformation of purified DNA but not native DNA from protoplast lysates

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Involvement of the *Bacillus subtilis* ABC transporter EcsB in genetic transformation with native DNA from protoplast lysate (LP transformation) was investigated using an *ecsB* deletion mutant constructed by fusion polymerase chain reaction. In these experiments, the non-transformability phenotype of the *ecsB* mutant was reversed and high numbers of transformants generated (1.5×10^5/μg DNA). The relative efficiency of transformation (RET) of *ecsB* to wild type (1.2×10^{-2}) was a thousand times higher using native chromosomal DNA than the RET obtained from purified DNA ($<8.6\times10^{-6}$). Similar transformation efficiencies were observed using native plasmid DNA. These results rule out a primary role for EcsB as a competence gene regulator. DNA-binding proteins attached to native DNA are not present in purified DNA preparations, and it is possible that such proteins could account for the transformability of the *ecsB* mutant. Because EcsB may play a role in protein(s) export, we tested exogenous proteins to identify functional replacements. We found that bovine serum albumin (fraction V) partially suppressed the phenotype of the *ecsB* mutation, leading to transformability with purified DNA. Linkage analysis of the *ecsB* mutant by LP co-transformation produced a higher co-transformation ratio (42% and 20%) at a distance of 34 kb and 121 kb in the *ecsB* mutant, compared to the wild-type strain, AYG2 (30.5% and 12.3%). The stimulatory linkage effect observed could be derived from a regulating gene involved in homologous recombination.

[**Key words:** *Bacillus subtilis*; EcsB; DNA uptake; Competent transformation; ATP-binding cassette (ABC) transporter; Type IV pili; Competence pseudopili; Retraction; Brownian motion; Brownian ratchet]

ATP-binding cassette (ABC) transporters, otherwise known as traffic ATPases (1,2), are unidirectional permeases found in both prokaryotic and eukaryotic cells. In bacteria, they function as importers and exporters of various substances, including proteins, amino acids, sugars, ions or drugs. Two hydrophobic and two hydrophilic components are common to all ABC transporters. The hydrophobic components comprise active transporters or channel formers, while the hydrophilic components have ATP binding sites; these components form a membrane-bound complex (1,2). In bacteria, the components are usually separate proteins encoded by a single operon that exist as homo- or heterodimers in the membrane (3–7). Bacterial uptake systems also contain a ligand-binding constituent found in the periplasm (8) or as a membrane-bound lipoprotein (9), in gram-negative and gram-positive bacteria, respectively. ATP hydrolysis by ATPases provides the energy for ligand translocation (5,10,11) and a large number of ABC transporters regulate gene expression where the transported solute serves as the signaling molecule. A good example of this is seen in the regulation of genetic competence and sporulation in *Bacillus subtilis*, where studies have shown that solute binding to its receptor forms part of a signal transduction system (12,13). ABC transporters can also mediate signal transduction across membranes by solute binding alone, a process possibly mediated by a conformational change in the transporter itself (14–16).

The *ecs* (*e*ffect on exoproteins, defect in *c*ompetence and *s*porulation) is a three-cistron operon of *B. subtilis* that encodes proteins that are similar to the ATPase (EcsA) and hydrophobic components (EcsB) of ABC transporters (17). A strong processing defect of a secreted α-amylase precursor and other exoproteins has been identified in the *ecsA26* mutant. The same mutant exhibited diminished spore formation and a lack of transformation competence. Consequently, it is assumed that Ecs plays a regulatory role in the synthesis and/or secretion of extracellular enzymes, as well as in genetic competence and spore formation (16,18).

Successful transformation of DNA is subject to the following; (i) DNA binding to the cell surface, (ii) DNA transport through the cell membrane and (iii) DNA incorporation into the recipient cell by homologous recombination (19). Many of the proteins required for transformation of competent *B. subtilis* have been identified (19–26). Some of these localize at the poles of the competent cells. This localization is functionally significant, because both DNA binding and transport occurs at these sites (27). All transformation-

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related proteins are regulated at the transcriptional level and depend on the ComK transcriptional factor for their synthesis. Seven proteins encoded by the *comG* operon, as well as ComC, BdbC and BdbD, are required to form a macromolecular structure located outside the cell membrane, called the competence pseudopilus (28). This structure is probably required for DNA binding to the cell surface. ComEA, an integral membrane protein, binds DNA at the cell surface using its helix-turn-helix motifs, and plays a role in membrane transport (29,30). ComEC, another integral membrane protein, appears to form a water-filled transport channel (31), while ComFA, (which resides on the inner face of the cell membrane), resembles members of the DEAD family of ATP-driven helicase (32). ComFA possibly functions as a Brownian ratchet, which is required for rectified Brownian motion from outside to inside the cell membrane (33).

Some studies have shown that transformation of competent *B. subtilis* with native DNA obtained from protoplast lysate (LP transformation) can achieve 200- to 1000-fold higher transformation efficiencies than conventional transformation methods using purified DNA (33–36). However, since LP transformation also depends on the ComK transcriptional factor (37), it appears likely that similar proteins may play a role in both conventional and LP transformation. Our experiments have recently demonstrated the transformability of the *comFA*, *comEA*, *comGC*, *comGG*, *and comEC* deletion mutants using LP transformation (33) (Takeno, M., Taguchi, H., and Akamatsu, T., unpublished data). We have also shown that whole genomic DNA (4215 kb) can be taken up into *B. subtilis* competent cells using the LP transformation method (38). In these experiments, a continuous length of DNA exceeding 1271 kb was taken up by the cells (38). Subsequently, co-transformation analysis revealed that ComFA controlled the DNA uptake velocity (33), while ComGC and ComEA controlled the DNA binding efficiency of this process. From this we conclude that LP transformation may be a suitable method for analyzing mutants that are difficult to transform.

A construction method for producing deletion mutants using fusion PCR has recently been developed that can generate a non-homologous sequence in the flanking region of a disrupted gene (39). Since reverse recombination will not occur in such deletion mutants, the method is suitable for analyzing mutants with very low transformation efficiencies.

Although the DNA uptake mechanism for LP transformation of competent *B. subtilis* is not fully understood, we propose that DNA-binding proteins attached to donor DNA molecules interact with DNA receptor proteins, resulting in a high-frequency of transformation (38). When DNA is taken up into competent *B. subtilis*, it is a long continuous double-stranded fragment (>1300 kb) (40). It is most likely that the energy required for DNA uptake into the cell is not derived from ATP hydrolysis of ComFA, but from Brownian motion (33,41,42). Because a rectified Brownian motion is required for DNA uptake (mediated by Brownian ratchets) (33,41–44), ComFA probably functions as both a DEAD family ATP-driven helicase (33,45–47) and a Brownian ratchet (33,42).

In this study, we describe the functional analysis of EcsB by both LP transformation and co-transformation analysis, using disruptants constructed by fusion PCR. Our results are the first to demonstrate the involvement of exogenous protein(s) in transformation, strongly supporting our hypothesis for the mechanism of DNA binding uptake during transformation of competent *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and oligonucleotides The bacterial strains and plasmids used in this study are listed in Table 1(36,37,48–50). *B. subtilis* AYG2 (*cysA14 metC3 trpC2 leuB8 argH1 aroG932*) was used as a parental strain for constructing the *ecsB* mutant. Primers (Genenet, Fukuoka, Japan) used in this study and listed in Table 2 were designed using Primer 3 software (http://frodo.wi.mit.edu/primer3/). *B. subtilis* gene sequences were obtained from the *B. subtilis* genome database (BSORF) (http://bacillus.genome.jp/).

TABLE 1. *Bacillus subtilis* strains and plasmids used in this study.

Bacterial strain	Genotype or phenotype	Reference, source or derivation
Bacterial strain		
Bacillus subtilis		
AYG2	*cysA14 metC3 trpC2 leuB8 argH1 aroG932*	37
168S	*trpC2 rpsL smo1*	36
AC820	*hisH rpsL smo1*	48
MG87	AYG2 *ecsB::spc*	tfm2[a] (AYG2: *ecsB::spc* DNA, Sp-r)
Plasmids		
pAC32R2	*bla kan*	49
pDG1727	*spc*	Sekiguchi[b], 50

[a] tfm2; Transformation; tfm2 (AYG2: *ecsB::spc* DNA, Sp-r) indicates a Sp-r transformant of AYG2 containing the amplified *ecsB::spc* DNA.
[b] Interdisciplinary Graduate School of Science and Technology, Shinshu University, Nagano, Japan.

Media Bacteria were grown in Luria-Bertani (LB) medium (51) or LB agar, supplemented with antibiotics where appropriate. To select resistant *B. subtilis*, spectinomycin (Sp: 75 μg/ml) and kanamycin (Km: 5 μg/ml) were used. Spizizen minimal (SM) medium (52) was used for the preparation of competent cells. Low Spizizen minimal medium (51), supplemented with amino acids where necessary, was used for the isolation of auxotrophs or transformants.

Preparation of *B. subtilis* protoplasts Protoplasts were prepared as described by Saito et al. (38) and incubated in SMM buffer (0.5 M sucrose; 0.02 M maleate buffer pH 6.5; 0.02 M $MgCl_2$) containing lysozyme (final concentration 250 μg/ml) for 15–45 min at 40°C. Following centrifugation, the density of protoplast solution resuspended in SMM buffer was measured by absorbance at 660 nm (A_{660}). An A_{660} of 2.88 corresponded to 2.74×10^9 protoplasts/ml. After dilution with SMM buffer, a 0.1 ml aliquot of 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 with a frequency of approximately 1×10^{-9} (number of osmotically resistant colonies per protoplast). Microscopic observation showed that all of the cells examined (about 1×10^5 cells) were spherical (data not shown).

LP transformation and co-transformation analysis Competent cells were prepared as described by Saito et al. (40). A 0.1 ml aliquot of protoplast suspension was added to 1 ml of competent cell culture and incubated at 37°C for 30 min. Cells were plated onto minimal agar containing appropriate nutrients. For plasmid transformation, 1 ml of LB medium was added to tubes containing 1.1 ml of transformation mixture and incubated at 37°C for 90 min. Cells were plated onto LB agar supplemented with 10 μg/ml chloramphenicol (Cm), incubated overnight at 37°C, and observed for transformants. The co-transformation analysis was done as described by Akamatsu and Taguchi (51). After single-colony isolation of transformants, each colony was transferred to a diagnostic agar plate and unselected marker(s) identified.

LP transformation of *B. subtilis* with DNA and exogenous proteins DNA solutions and competent cell cultures containing 1 mg/ml final concentration of Bovine Serum Albumin fraction V (BSA) were incubated for 15 min at 4°C and 30 min at 37°C, respectively. A 0.1 ml aliquot of the DNA suspension was added to 1 ml of the competent cell culture and incubated at 37°C for 30 min. The cells were then plated onto minimal agar with appropriate nutrients.

DNA manipulation techniques Chromosomal DNA was prepared as described by Akamatsu et al. (53). Preparation of plasmids from *Escherichia coli* and from protoplasts of *B. subtilis* was done according to the procedure of Birnboim and Doly (54). Manipulation of recombinant DNA was performed using standard techniques as

TABLE 2. Synthetic primers for PCR amplification.

Number	Name	Primer sequence (5′ to 3′)
1	ecsB-top-F	cgaagaattgacgctgatgg
2	ecsB-top-Rv-spc	atgtattcaaatatatcctcctcaccatattattcatggccagcg
3	ecsB-bottom-F-spc	ggaaatattcattctaattggtaatcagaaagcacggaaagctgtgaac
4	ecsB-bottom-Rv	gacttacggccatcatcact
5	spc-F	gtgaggaggatatatttgaatacat
6	spc-Rv	tctgattaccaattagaatgaatatttcc
7	cysE-a-f	aagagactgcaattcagcgt
8	cysE-a-c	tgaaatggtgggtacatcct

The primer shares approximately 20 bp homology with the chromosomal or the *spc* marker, and around 30 bp homology with the *spc* marker (underlined).