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Note

Transformation of undomesticated strains of *Bacillus subtilis* by protoplast electroporation

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Abstract

A rapid method combining the use of protoplasts and electroporation was developed to transform recalcitrant wild strains of *Bacillus subtilis*. The method described here allows transformation with both replicative and integrative plasmids, as well as with chromosomal DNA, and provides a valuable tool for molecular genetic analysis of interesting *Bacillus* strains, which are hard to transform by conventional methods.

Keywords: *Bacillus* spp.; Electrotransformation; Genetic manipulation; Gram-positive bacteria

Bacillus subtilis is an ubiquitous bacterium showing interesting features for industrial and agronomical activities. Its genetic manipulation is an essential tool to get insight into the molecular basis involved in the different phenotypes and is required for strain improvement. Natural competence is the ability of bacteria to take up exogenous DNA and incorporate it into the genome. This phenomenon has allowed gene cloning, mutant generation and gene mapping in *B. subtilis* (Anagnostopoulos and Spizizen, 1961; Kunst and Rapoport, 1995). The low competence or unnaturally competence showed for several *Bacillus* strains led to development of other strategies involving phage transduction (Yasbin and Young, 1974), protoplast fusion (Chang and Cohen, 1979) and, finally, the highly versatile electrotransformation method. A variety of electroporation protocols have been reported which were focussed to improve the transformation efficiencies using intact cells or in combination with cell wall wakening agents, modifying the composition of washing and electroporation buffers, altering the electrical pulse or varying the nature of the DNA used to transform (Ohse et al., 1995; Xue et al., 1999; Ito and Nagane, 2001). However, most of these strategies have been developed using reference or culture collection strains, well adapted to laboratory conditions, and do not always work for undomesticated strains recalcitrant to transformation. Consequently, such strains are often abandoned in favour of related or amenable ones (Yao et al., 2003).

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In our laboratory, two bacterial strains UMAF6614 and UMAF6639 have been isolated, identified as *B. subtilis* according to biochemical and physiological tests as well as 16S-rDNA sequence, and proved to be very interesting as biological control agents of microbial plant diseases (Romero et al., 2004; Zeriouh, 2005). In order to understand the molecular bases of their biological control capabilities, transformation is essential and has become a bottleneck. Several attempts, involving natural competence methods, protoplast fusion and several electroporation protocols, failed to transform these strains. Electroporation of protoplasts is a transformation strategy which has been successfully applied to yeasts, fungi and plants (Nickoloff, 1995). Thus, the aim of this study was to develop a reproducible transformation method for *B. subtilis* based on the electroporation of protoplasts, which could be potentially used with other *Bacillus* strains refractory to transformation by conventional methods.

In order to analyse the role of the DNA nature in the transformation efficiency, we have tested self-replicating plasmids of different sizes, integrative plasmids and *B. subtilis* chromosomal DNA (Table 1). For the construction of integrative plasmids, DNA fragments of the genes *fenB* (fengycin production) and *ituD* (iturin and bacillomycin production) were obtained by PCR with specific primers and cloned into the SmaI-digested pUC18 plasmid, and the resulting plasmids were subsequently linearized with suitable enzymes and ligated to appropriate antibiotic resistance cassettes (Guérot-Fleury et al., 1995).

Protoplasting was carried out following a modification of a method previously described (Chang and Cohen, 1979). Briefly, cells were grown in 20 ml of Penassay broth (PAB) at 37 °C until the onset of the stationary phase of growth (OD_{600} = 1.7–2). Subsequently, cells were collected by centrifugation, suspended in 10 ml of SMPP medium (0.3% bovine serum albumin, 5% 2 M sucrose, 25% 4× PAB, 50% 2× SMM), composition of 2× SMM being 1 M sucrose, 0.04 M maleic acid and 0.04 $MgCl_2$ (pH 6.5), and protoplasts were obtained after incubation at 37 °C on a rotary shaker at 100 rpm for 30 min in presence of lysozyme (10 mg/ml) and mutanolysin (75 U/ml) (Serror et al., 2002). The presence of protoplasts was verified by phase contrast microscopy. Protoplasts were then carefully harvested by centrifugation at 5200 ×*g* and 4 °C for 5 min, washed twice with ice cold washing-electrotransformation buffer (SMMP medium without PAB), and finally suspended in this solution. The protoplasts were counted microscopically in order to adjust the final concentration to 10^8 protoplasts/ml with the same buffer.

Table 1
Bacterial strains and plasmids used in this study

Strains or plasmids	Description	Reference
Strains		
B. subtilis UMAF6614	Producer of bacillomycin, fengycin and surfactin	Romero et al. (2005)
B. subtilis UMAF6639	Producer of iturin, fengycin and surfactin	Romero et al. (2005)
B. subtilis 168 Δ*fenA*	*fenA*: :Cm; Cm^R	O. P. Kuipers
E. coli MC1061	Forms multimeric DNA, F^+	Wertman et al. (1986)
E. coli XL-1 blue		Stratagene
Plasmids		
pE194	Em^R (3 kb)	BGSC [a]
pGSP12	Derivative of pHP12; Em^R (12 kb)	Van Sinderen et al. (1995)
pFen2-2 [b]	pUC18 carrying *fenB*: :Cm; Ap^R, Cm^R (5 kb)	This study
pItu2-2 [c]	pUC18 carrying *ituD*: :Spc; Ap^R, Spc^R (4 kb)	This study

[a] *Bacillus* Genetic Stock Center.
[b] Integrative plasmids containing *fenB* gene sequences from *B. subtilis*, respectively.
[c] Integrative plasmids containing *ituD* gene sequences from *B. subtilis*, respectively.

For the electroporation trials, volumes of 60 or 120 μl of protoplast suspensions were mixed with 2.5 μl of DNA (0.3–1 μg) and kept on ice for at least 5 min. The mixture was then transferred to a pre-chilled electroporation cuvette (0.2 cm electrode gap) and exposed to a single electrical pulse in a Gene Pulser Xcell System (Bio-Rad Laboratories, USA) set at 25 μF, 400 Ω and 0.7 kV. Immediately after the pulse delivery, 1 ml of recovering medium (SMMP) was added to the cuvette, and the mixture was transferred to a 2 ml tube and incubated at 37 °C and 100 rpm for 12 h. After incubation, the mixture was spread on DM3 agar plates, the protoplast regenerating selective medium (Chang and Cohen, 1979). In order to optimize the protoplast regeneration ratio, the following osmotic agents were studied: 1 M sodium succinate used in the original DM3 medium, 0.25 M sucrose (Cue et al., 1997), 0.5 M mannitol (Bourne and Dancer, 1986) and 0.5 M sorbitol (Jandova and Tichy, 1987). When required, the following antibiotics were added to the culture media at different concentrations depending on the osmotic agent: chloramphenicol (Cm, 5μg/ml), erythromycin (Em, 5 μg/ml), kanamycin (Km, 10 μg/ml or 800 μg/ml) and spectinomycin (Spc, 100 or 500 μg/ml). After spreading, plates were incubated at 37 °C for 48 h and the transformants were counted. Despite the fact that no differences were obtained regarding protoplast