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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.

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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; Zerrouh, 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 *Sma*I-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 MgCl₂ (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 10⁸ protoplasts/ml with the same buffer.

Table 1
Bacterial strains and plasmids used in this study

Strains or plasmids	Description	Reference
<i>Strains</i>		
<i>B. subtilis</i> UMAF6614	Producer of bacillomycin, fengycin and surfactin	Romero et al. (2005)
<i>B. subtilis</i> UMAF6639	Producer of iturin, fengycin and surfactin	Romero et al. (2005)
<i>B. subtilis</i> 168 $\Delta fenA$	<i>fenA</i> : :Cm; Cm ^R	O. P. Kuipers
<i>E. coli</i> MC1061	Forms multimeric DNA, F ⁺	Wertman et al. (1986)
<i>E. coli</i> XL-1 blue		Stratagene
<i>Plasmids</i>		
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 <i>fenB</i> : :Cm; Ap ^R , Cm ^R (5 kb)	This study
pItu2-2 ^c	pUC18 carrying <i>ituD</i> : :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, 5 µg/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

Table 2
Electrotransformation efficiency of *B. subtilis*

Strains	Volume (μ l)	Replicative plasmids		Integrative plasmids		<i>B. subtilis</i> chromosomal DNA (<i>fenA</i> : :Cm)
		pE194	pGSP12	pFen2-2	pItu2-2	
UMAF6614	60	nd ^a	10 ^b	nd	14	nd
	120	150	160	55	600	55
UMAF6639	60	nd	0	nd	0	nd
	120	60	40	134	36	130

DNA sources and protoplast volumes were varied in electrotransformation experiments. Each value represents the average of three experiments.

^a Not determined.

^b Calculated as number of transformants per microgram of DNA.

regeneration in the different media (data not shown), since the original DM3 medium contains sodium succinate, which is an inhibitor of Km and Spc activities thus hindering the selection of resistant colonies (Jandova and Tichy, 1987), only mannitol or sorbitol were routinely used as osmotic agents.

Transformation was first attempted in 60 μ l volumes, but transformants were only observed for the strain UMAF6614 in a non-reproducible manner (Table 2). However, the use of 120 μ l volumes increased transformation efficiency by one order of magnitude, being associated with a protoplast mortality of 90–99% (data not shown). Considering the importance of the applied voltage with regards to the transformation efficiency, different voltages were tested (data not shown). With higher voltages arcing occurred, resulting in 100% of protoplast mortality, whereas with lower voltages no transformants were obtained.

For replicative plasmids, transformation efficiency was found to be similar regardless of the plasmid size (3 or 12 kb) (Table 2). In order to exclude the possibility that the antibiotic resistant colonies that arose was simply due to acquisition of spontaneous resistance, the antibiotic resistant electrotransformants were characterised by plasmid analysis and plasmids with the expected sizes were recovered (data not shown). However, the differences observed between strains may indicate that transformation efficiency was strain-dependent and optimisation may be required to improve transformation efficiency for a particular strain (Serror et al., 2002).

Because of our interest to carry out site-directed mutagenesis analysis of selected genes of these strains, integrative plasmids and chromosomal DNA from *B. subtilis* 168 showing a fengycin-mutant phenotype were included in this study. The transformation efficiencies obtained were different to those observed for replicative plasmids and also in a strain-dependent manner. As shown in Table 2, the strain UMAF6614 was more susceptible to transformation with replicative plasmids,

whereas UMAF6639 was shown to be more receptive to transformation with integrative plasmids or chromosomal DNA. Molecular analysis of the antibiotic resistant colonies was carried out by PCR. Furthermore, phenotypic analysis for fengycin and iturin or bacillo-mycin production by TLC of those putative integrative mutants revealed the expected suppression phenotype for several electrotransformants (data not shown). These results indicated that integration events had taken place, and, although at relative low frequencies, it was enough to select stable integrants.

In this work we have described a reliable and reproducible electroporation protocol to transform two recalcitrant strains of *B. subtilis*, which is shown in Table 3. The possibility to introduce DNA from different sources is clear evidence that this procedure is suitable for genetic transformation and gene manipulation in undomesticated *Bacillus* strains. To our knowledge, this is the first report describing transformation of Gram-positive bacteria by protoplast electroporation and it opens the field of molecular genetic analysis with

Table 3
Transformation method for undomesticated strains of *B. subtilis* proposed in this study

1. Growth cells in PAB at 37 °C until OD₆₀₀ = 1.7–2.
2. Collect cells and suspend them in SMPP, and obtain protoplasts in presence of lysozyme and mutanolysin after 30 min incubation at 37 °C and 100 rpm. Inspect microscopically.
3. Harvest protoplasts by centrifugation at 5200 $\times$ g and 4 °C for 5 min, wash twice with ice cold washing-electrotransformation buffer, and suspend and adjust to 10⁸ protoplasts/ml with the same solution.
4. Mix 120 μ l of protoplast suspension with 2.5 μ l of DNA (1 μ g), keep on ice for 5 min, and electroporate at 25 μ F, 400 Ω and 0.7 kV. After pulse, add 1 ml of SMMP and incubate at 37 °C and 100 rpm for 12 h.
5. Plate transformation mixture in DM3 (replacing 1 M sodium succinate by 0.5 M mannitol or 0.5 M sorbitol) supplemented with the appropriate antibiotics and incubated at 37 °C for 48 h.

See text for details.

strains recalcitrant to transformation by conventional methods.

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