Transformation of vegetative cells of *Bacillus anthracis* with plasmid DNA

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Methods have been developed for chemical transformation and electro-transformation (electroporation) of vegetative cells of *Bacillus anthracis* with supercoiled plasmid DNA. Chemical transformation was dependent on incubation in Tris/HCl with osmotic support and transformation with plasmid DNA was effected by treatment with polyethylene glycol 3350. Maximum transformation frequencies were 3.8×10^{-5} transformant c.f.u. per viable c.f.u. (1×10^3 c.f.u. per μg DNA). Optimal frequencies were pH dependent and were affected by growth-medium composition. Transformation was not observed with linear or multimeric plasmid DNA. Electro-transformation of *B. anthracis* using high field intensity electroporation was dependent on the composition of both the growth medium and the electroporation buffer. Maximum electro-transformation frequencies were 5.3×10^{-4} c.f.u. per viable c.f.u. (2.6×10^4 c.f.u. per μg DNA). The use of early exponential phase cells was critical to both procedures and the maximum efficiency (c.f.u. per μg DNA) of each system was strain dependent under the conditions described.

Introduction

Bacillus anthracis, the causative agent of anthrax, possesses two known virulence determinants: a tripartite protein toxin and a poly-D-glutamic acid capsule (Smith & Keppie, 1954; Zwartouw & Smith, 1956). Although the structural genes coding for these determinants have been cloned in *Escherichia coli* (Vodkin & Leppla, 1983; Robertson & Leppla, 1986; Makino *et al.*, 1988; Mock *et al.*, 1988) and sequenced (Bragg & Robertson, 1989; Robertson *et al.*, 1988; Todd-Tippetts & Robertson, 1988; Welkos *et al.*, 1988; Makino *et al.*, 1989), a complete understanding of their expression, control and role in pathogenicity will only be achieved when they can be efficiently cloned into their native environment.

Battisti *et al.* (1985) developed a mating system for the transfer of large and small plasmids amongst *B. anthracis*, *B. cereus*, and *B. thuringiensis* based on the *B. thuringiensis* fertility plasmid pX012 and Rufhel *et al.* (1984) reported transduction of small plasmids amongst these species using the *B. cereus* temperate phage CP-51. However, these methods do not easily lend themselves to

cloning experiments, and Koehler & Thorne (1987) subsequently developed the pLS20-mediated transfer of small plasmids between *B. subtilis* and *B. anthracis*. However, the use of intermediate hosts or plasmid mobilization strategies is not ideal. *B. subtilis* is known for its characteristic re-arrangement of cloned material, making it an unpredictable host for certain recombinant DNA molecules (Hardy, 1985), and Green *et al.* (1989) have reported that pX012-mediated plasmid transfer involves a transposable element (Tn4430) which may insert at random into conducted plasmids and recipient strain DNA.

Makino *et al.* (1987) reported transformation of protoplasts of *B. anthracis* with the small plasmid pUB110. Protoplast technology, however, requires development of complex media for both protoplast formation and cell wall regeneration. Reversion to the bacillary form may require extended periods of incubation and the transformation frequency may vary greatly between laboratories (Takahashi *et al.*, 1983; Heierson *et al.*, 1987).

In this paper we report the development of two direct gene transfer systems for *B. anthracis* based on chemical induction of cell competence and on transformation by high field intensity electroporation (electro-transformation) which should enable both direct cloning into *B. anthracis* or transformation with shuttle vector DNA isolated from intermediate cloning hosts.

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Abbreviations: BHI, brain heart infusion broth; CB, competence buffer; EB, electroporation buffer; FPLC, fast protein liquid chromatography.

Table 1. *Strains and plasmids used*

Strain or plasmid	Characteristics*	Source/reference
<i>B. anthracis</i> Sterne 34F ₂	Toxigenic (pX01 ⁺), non-capsulated (pX02 ⁻), avirulent	Sterne (1937)
<i>B. anthracis</i> Ames	Virulent (pX01 ⁺ , pX02 ⁺)	Little & Knudson (1986) Dancer (1981)
<i>B. anthracis</i> Vollum	Virulent (pX01 ⁺ , pX02 ⁺)	
<i>B. subtilis</i> 168 (<i>trpC2</i>)	Tryptophan auxotroph	
pUB110	Kan ^R Neo ^R	Gryczan <i>et al.</i> (1978)
pHV33	pBR322/pC194 shuttle vector; Cap ^R Tet ^R	Primrose & Ehrlich (1981)
pAB124	Tet ^R	Bingham <i>et al.</i> (1980)

* Cap^R, Kan^R, Neo^R, Tet^R resistance to chloramphenicol, kanamycin, neomycin, tetracycline.

Methods

Strains and plasmids. These are listed in Table 1.

Plasmid isolation and manipulation. Plasmid DNA was transformed into protoplasts of *B. subtilis* 168 by the method of Chang & Cohen (1979), extracted by the alkaline-lysis method of Maniatis *et al.* (1982) and purified as the supercoiled form by Superose-12 gel-filtration in 50 mM-Tris/HCl pH 8.0 containing 1 mM-EDTA on a Fast Protein Liquid Chromatography (FPLC) system (Pharmacia). Plasmid pUB110 was linearized by complete digestion with *Bam*HI (BRL) as directed by the supplier and multimers were generated by re-ligation with T4 ligase (BRL) in the presence of polyethylene glycol (Maniatis *et al.*, 1982). Except where indicated, plasmid DNA was supercoiled pUB110 used at 0.5 µg ml⁻¹ for electro-transformation and 1.0 µg ml⁻¹ for chemical transformation. All protocols were developed using the *B. anthracis* Sterne 34F₂ vaccine strain and results were recorded as the mean of three experiments.

Media. LBG contained, per litre, 10 g tryptone, 5 g yeast extract, 10 g NaCl and 1.0 g glucose; the pH was adjusted to 7.0 with concentrated NaOH. For LBG agar Difco Bacto-agar was added at 10.0 g l⁻¹.

LB medium was identical to LBG except that glucose was omitted. M3CG medium contained, per litre, 2.0 g (NH₄)₂SO₄, 0.1 g MgSO₄ · 7H₂O, 6.0 g KH₂PO₄, 14.0 g K₂HPO₄, 1.0 g trisodium citrate, 2.0 g L-glutamic acid, 0.2 g glycine, 0.01 g thiamin.HCl, 0.00025 g MnSO₄ and 5.0 g Difco Casamino acids. The medium was brought to pH 7.0 with concentrated NaOH, autoclaved at 15 p.s.i. for 15 min, allowed to cool, and filter-sterilized FeCl₃ · 6H₂O and glucose were added to final concentrations of 0.04 g l⁻¹ and 0.5 g l⁻¹ respectively.

M3C medium was identical to M3CG except that glucose was omitted.

Brain heart infusion broth (BHI) (Oxoid), used as supplied, contains, per litre, 12.5 g calf brain infusion solids, 5.0 g beef heart infusion solids, 10.0 g proteose peptone, 5.0 g NaCl, 2.0 g glucose, 2.5 g Na₂HPO₄; pH 7.4.

Phosphate-buffered saline (PBS) contained, per litre, 8.0 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, 0.2 g KH₂PO₄; pH 7.3.

LBP comprised equal volumes of 2 × LBG and 1 × PBS.

Competence buffer (CB) consisted of 0.1 M-Tris/HCl (molecular-biology grade, Sigma), 0.5 M-isethionic acid (sodium salt) (Sigma); the pH was adjusted to 7.0 with concentrated NaOH.

Polyethylene glycol 3350 (PEG 3350; Sigma) solution was 40% (w/v) in 1 × PBS.

Electroporation buffer (EB) contained 10% (w/v) sucrose, 15% (v/v) glycerol in 2 mM-potassium phosphate buffer pH 7.8.

Except where otherwise stated all media were sterilized by autoclaving at 15 p.s.i. for 15 min.

Chemical transformation – optimized procedure. A 0.1 ml inoculum from a 16 h LBG culture of *B. anthracis* Sterne 34F₂ vaccine strain was added to 25 ml M3CG in a 250 ml conical flask and incubated with shaking at 37 °C, 100 r.p.m., until the OD₆₀₀ was 0.15–0.2. The culture was then harvested by centrifugation (4000 g, 5 min) and washed twice in 5 ml CB. Cells were resuspended in 12.5 ml CB, pipetted into a 250 ml conical flask and incubated at 37 °C with slow shaking (25 r.p.m.) for 50 min. The cells were again harvested, resuspended in 0.5 ml LBP in a 15 ml screw-cap polypropylene tube and plasmid DNA (1 µg ml⁻¹) was added in a total volume of 0.1 ml LBP. PEG 3350 (40%, w/v) (1.5 ml) was then added and the suspension mixed gently with a wide-bore pipette. Cells were incubated with gentle agitation at 37 °C for 10 min, harvested by centrifugation and the pellet resuspended in 1.5 ml LBG. Incubation was continued at 37 °C, 50 r.p.m., for a further 90 min expression period, after which samples (0.1 ml) were diluted in LBG and plated for antibiotic selection and surface-spread viable count. Competent cell transformation frequency was determined as transformant colony-forming units (c.f.u.) per viable c.f.u.

Electro-transformation – optimized procedure. The electroporation equipment used was a Bio-Rad Gene Pulser apparatus with a Pulse Controller and Capacitance Extender (Bio-Rad). Two *B. anthracis* Sterne 34F₂ vaccine strain colonies from a 16 h LBG agar plate were resuspended in 1.0 ml LBG and held at 37 °C for 10 min. This suspension was used to inoculate a fresh 25 ml volume of LBG in a 250 ml conical flask to give an OD₆₀₀ of 0.05.

The culture was incubated with shaking at 37 °C, 100 r.p.m., until the OD₆₀₀ was 0.18–0.20. Cells were then harvested by centrifugation (4000 g, 5 min) and washed twice in EB. After the second wash the cells were resuspended in 0.4 ml EB, transferred to a 0.4 cm electrode-gap electroporation cuvette (Bio-Rad) and incubated on ice for 10 min; plasmid DNA was then added at 0.5 µg ml⁻¹ in 10 µl EB. A sample (10 µl) was then removed for viable count and the remaining cells were treated with a single pulse at 6.00 kV cm⁻¹, 25 µF, 200 Ω (mean time constant 4.7 ms).

After pulsing, the cells were replaced on ice for 10 min, diluted to 1.0 ml in LBG and samples (100 µl) plated directly onto LBG agar containing appropriate antibiotics. Plates were incubated for 16 h at 35–37 °C. Frequency of electro-transformation was determined as transformant c.f.u. per viable c.f.u.

DNA screening. Screening for transformant plasmid DNA was done by alkaline-SDS lysis as described by Reddy *et al.* (1987). Agarose gel electrophoresis was done at 70 V for 2 h in a 0.7% agarose mini-gel with Tris/borate electrophoresis buffer pH 8.4 using a GNA-100 submarine mini-gel apparatus (Pharmacia). Double-stranded DNA was stained with ethidium bromide as described by Maniatis *et al.* (1982).

Results

Chemical transformation

Successful induction of cell competence and optimal transformation frequency was found to be dependent on several factors. The process was Tris-dependent, requiring a minimum of 40 min incubation, and was optimal within a concentration range of 0.05–0.15 M and a pH range of 6.7–7.5. Incubation of the Sterne 34F₂ vaccine strain in Tris/HCl consistently resulted in considerable cellular damage and low levels of protoplast formation (5–10%) as observed under oil-immersion light microscopy. Low levels of protoplast formation, and cellular damage, were also observed with the Vollum strain but not with the Ames strain. Induction of competence in the non-carbohydrate osmotic support sodium isethionate also enhanced transformation frequencies.

Chemical transformation of competent cells with supercoiled plasmid DNA was dependent on treatment with polyethylene glycol. Under these conditions maximum frequencies were obtained using PEG 3350 at 30% (w/v) final concentration as described above. Transformation frequency fell rapidly beyond early exponential-phase growth (Fig. 1) and saturation was approached at a supercoiled DNA concentration of 5 µg ml⁻¹. Transformation was not observed for linear or multimeric plasmid DNA (data not shown). Of the strains tested, maximum transformation frequencies and efficiencies were obtained with the Ames strain of *B. anthracis* (3.8×10^{-5}) and pUB110 (1.0×10^3 c.f.u. per µg DNA). Screening of transformant colonies for plasmid content indicated DNA bands co-migrating in the region of purified, supercoiled pUB110 DNA.

Electro-transformation

As with chemical transformation of competent *B. anthracis*, optimal electro-transformation was dependent on use of early exponential-phase cells (Fig. 2). The electro-transformation frequency was also dependent on the pH of the electrode buffer and its composition, with optimal frequencies obtained at pH 8.0 (Fig. 3) and in the presence of 15% (v/v) glycerol, which stabilized time constants in the region 4.6–4.8 ms.

Electro-transformation frequencies increased in proportion to supercoiled plasmid DNA concentration and saturation was not observed up to 3.0 µg plasmid DNA ml⁻¹ (Fig. 4). Optimal electro-transformation frequency was associated with a 30% decrease in viable count (data not shown) at a field strength of 6.0 kV cm⁻¹. Under the conditions described here, maximum electro-transformation frequency (5.3×10^{-4}) was obtained with the Ames strain of *B. anthracis* and the *Bacillus* plasmid

Fig. 1. Effect of culture growth stage (OD₆₀₀) on competent cell transformation frequency in *B. anthracis* 34F₂. Cultures (M3CG, 25 ml) were harvested during early to late exponential phase and cell density adjusted to OD₆₀₀ 0.15. Samples (25 ml) were then processed for Tris/HCl-induced competence as described in Methods. Supercoiled pUB110 DNA was added to 1.0 µg ml⁻¹ and transformants selected on LBG agar containing 20 µg neomycin ml⁻¹. Error bars indicate one standard deviation from the mean.

Fig. 2. Variation of electro-transformation frequency in *B. anthracis* 34F₂ with culture growth stage. Cells were harvested from LBG medium during early to late exponential phase and adjusted to give an OD₆₀₀ of 0.18–0.2. Samples (25 ml) were prepared for electroporation as described in the text. Supercoiled pUB110 DNA was used at 0.5 µg ml⁻¹. Error bars indicate one standard deviation from the mean.

pAB124 (2.6×10^4 c.f.u. per µg DNA). As with chemically transformed cells, no electro-transformation was observed using linear or multimeric plasmid DNA. Electro-transformant colonies contained plasmid DNA migrating in the region of purified supercoiled plasmid DNA. Inclusion of glucose at 1.0 g l⁻¹ in LB medium produced a 10-fold increase in electro-transformation frequency. However, when this medium was used for chemical transformation, considerable Tris-induced cell lysis was observed for all test strains and no transfor-

Fig. 3. Effect of electrode buffer pH on electro-transformation of *B. anthracis* 34F₂ with supercoiled pUB110 DNA. Cells from a 25 ml LBG culture were harvested during early exponential-phase growth and prepared for electroporation as described in the text. In these experiments EB was buffered using 5 mM-Tris/HCl over the pH range illustrated. Supercoiled pUB110 DNA was used at 0.5 µg ml⁻¹ and transformant colonies were selected on LBG-agar containing 20 µg neomycin ml⁻¹. Error bars indicate one standard deviation from the mean.

Fig. 4. Variation of electro-transformation frequency in *B. anthracis* 34F₂ with concentration of supercoiled pUB110 DNA. Plasmid was added in a total volume of 10 µl EB as described in the text. Transformant colonies were selected on LBG agar containing 20 µg neomycin ml⁻¹. Error bars indicate one standard deviation from the mean.

transformants were recovered (Table 2). The addition of glucose to M3C medium enhanced the efficiency of both procedures, being most effective for competent cell transformation.

Discussion

Tris-induced competence was first reported in *Bacillus brevis* by Takahashi *et al.* (1983), where it was thought to be associated with removal of outer protein layers of the

Table 2. Effect of medium composition on competent cell and electro-transformation in *B. anthracis* Sterne 34F₂ using pUB110 as donor DNA

Cells were grown in 25 ml volumes of the required medium until the OD₆₀₀ was 0.18–0.2, and then harvested and washed as described in the text. After the second wash the cells were resuspended in 0.4 ml of the same buffer, transferred to a 0.4 cm electrode-gap electroporation cuvette and incubated on ice for 10 min; plasmid DNA was then added at 0.5 µg ml⁻¹. A sample (10 µl) was then removed from each cuvette for viable count and the remaining cells pulsed once at 6.0 kV cm⁻¹, 25 µF, 200 Ω (mean time constant 4.7 ms). After pulsing, the cells were placed on ice for 10 min before dilution in LBG and direct plating on LBG agar plus antibiotics. Plates were incubated for 16 h at 35–37 °C. Frequency of transformation was determined as transformant c.f.u. per viable c.f.u. Results represent the mean of three experiments.

* Limit of detection (transformants per viable cell).

Medium	No. of transformants per viable cell	
	Competent cell transformation	Electro-transformation
LB	6.1 × 10 ⁻⁷	1.4 × 10 ⁻⁶
LBG	<1 × 10 ⁻⁸ *	2.1 × 10 ⁻⁴
M3C	6.1 × 10 ⁻⁷	2.2 × 10 ⁻⁶
M3CG	1.5 × 10 ⁻⁵	7.9 × 10 ⁻⁶
BHI	<1 × 10 ⁻⁸	3.7 × 10 ⁻⁶

bacterial cell wall. However, Heierson *et al.* (1987) reported that this effect could be reproduced in *B. thuringiensis* with a high degree of efficiency and proposed that some other, as yet undescribed, mechanism must be responsible. We have reported here a further extension of this phenomenon for the successful transformation of *B. anthracis* with small plasmids to equivalent or higher efficiencies than previously published (Makino *et al.*, 1987). Electro-transformation procedures have also been employed. Both techniques are simple, rapid in their execution, use only conventional laboratory media and allow recovery of transformants within 16 h. The effect of medium composition and a subsequent pH-dependent step are important for optimal yields using either protocol. The inclusion of glucose at low levels in growth media appeared to induce a structural weakness in the *B. anthracis* cell and this was exploited to optimize DNA uptake. Both techniques exhibited a degree of strain dependence, with the highly virulent Ames strain transforming with the highest frequencies. On the basis of the data presented here, size-selection alone does not appear to be critical for plasmids up to 6.9 kb.

Expression of the *Staphylococcus aureus* pC194 replicon, as part of the shuttle vector pHV33 (Primrose & Ehrlich, 1981), has also been demonstrated. Use of this or related shuttle vectors will facilitate initiation of cloning experiments for cryptic or regulatory anthrax genes in *E. coli* with subsequent transfer back into *B.*

anthracis either directly or via a suitable intermediate *Bacillus* host.

With the current advances in the understanding of anthrax at the molecular level (Leppla *et al.*, 1988; Bartkus & Leppla, 1989; Singh *et al.*, 1989) there is an increasing need for an efficient gene transfer system in *B. anthracis*. The data presented here assume that only one cell per c.f.u. acquires plasmid DNA. However, as *B. anthracis* grows in filaments which can exceed 20 cells in length, this assumption is probably an underestimate of the true potential of these techniques.

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