Glycine-induced cryotransformation of plasmids into *Bacillus anthracis*

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Different cloning vectors (pC194, pBC16, pUB110, pBD10, pBD8, pAM β 1) and *Bacillus anthracis* plasmid pX02 were introduced into *B. anthracis* by a transformation method. To induce an artificial competence state for uptake of isolated plasmid DNA, the cultures were treated with glycine, to reduce cross-linking of peptidoglycan, followed by freezing and thawing. The procedure is extremely rapid and relatively efficient (maximum transformation efficiency about 10^3 c.f.u. per μ g DNA) and allows different cloning vectors with molecular masses ranging from 1.8 to 17.7 MDa to be introduced into *B. anthracis*.

Introduction

Bacillus anthracis, the causative agent of anthrax, is pathogenic to domestic animals and humans. The virulence of this organism is associated with two separate plasmids, pX01 (110 MDa) and pX02 (60 MDa), which code for a trimeric toxin (Tox) and the production of a poly-D-glutamic capsule (Cap), respectively. The genes coding for toxin and capsule synthesis have been cloned in *Escherichia coli* and *Bacillus subtilis* (Vodkin & Leppla, 1983; Robertson & Leppla, 1986; Ivins & Welkos, 1986; Tippets & Robertson, 1988; Makino *et al.*, 1988). However, the expression and toxicity of the cloned genes in a homologous system have not yet been examined because of the lack of a method for reintroducing them into *B. anthracis*.

Different experimental approaches have been used to introduce plasmids into *B. anthracis*. Ruhfel *et al.* (1984) and Stepanov *et al.* (1989), respectively, demonstrated the utility of transducing phages CP51 and CP54ant in transferring plasmids among *B. anthracis*, *Bacillus thuringiensis* and *Bacillus cereus*. Thorne's group showed the conjugative-like mobilization of large (pX01 and pX02) and small (pBC16) plasmids mediated by *B. thuringiensis* fertility plasmids of the Cry⁺ family (Thorne, 1985; Battisti *et al.*, 1985; Reddy *et al.*, 1987). The involvement of Tn4430, located on one of these plasmids, pX012, in mobilization of pX01 and pX02 was demonstrated (Green *et al.*, 1989). This group also used the cryptic plasmid pLS20 to transfer the small cloning

vector plasmids pBC16 and pUB110 among a wide variety of *Bacillus* species including *B. subtilis* and *B. anthracis* (Koehler & Thorne, 1987).

The anthrax bacillus is difficult to transform. To overcome this problem, *N*-acetylmuramidase-induced protoplast transformation (Makino *et al.*, 1987) and an electroporation method (Bartkus & Leppla, 1989) have recently been developed. It was also shown by Heierson *et al.* (1987) that *B. thuringiensis*, which is taxonomically very close to *B. anthracis*, could be transformed by plasmid DNA with a high degree of efficiency using the Tris-alkaline procedure first described for *Bacillus brevis* by Takahashi *et al.* (1983).

In this paper we report a two-step procedure for the introduction of cloning vectors into *B. anthracis*.

Methods

Strains, phages and plasmids. These are listed in Table 1. *B. anthracis* strain Sterne 34F2 was used as the recipient unless otherwise indicated.

Media and growth conditions. Brain heart infusion (BHI) broth and solidified agar medium (Difco) were routinely used unless otherwise noted.

Recipient strains were grown overnight without shaking in 250 ml Erlenmeyer flasks containing 25 ml BHI broth at 37 °C.

Transformation procedure. To 25 ml of an overnight culture of the recipient in BHI broth, glycine (Serva) and MgCl₂ (Merck) were added to final concentrations of 5% (w/v) and 0.05 M, respectively, and incubation was continued with gentle shaking at 37 °C for 2.5 h. Then the cells were sedimented in a bench-top centrifuge (Sigma 202MK)

Table 1. Bacterial strains, phage and plasmids

Phenotype		Reference or source
Strains		
<i>B. anthracis</i>		
Sterne 34F2	Tox ⁺ (pX01 ⁺) Cap ⁻ (pX02 ⁻)	Culture collection of the All-Union Antiplague Research Institute 'Microbe'
KM33	Tox ⁻ (pX01 ⁻) Cap ⁻ (pX02 ⁻)	
KM34	Tox ⁻ (pX01 ⁻) Cap ⁺ (pX02 ⁺)	
KM35	Tox ⁺ (pX01 ⁺) Cap ⁻ (pX02 ⁻)	
Phage		
CP54		Thorne (1978)
Plasmids		
pC194 (1.8 MDa)	Cm ^r	Ehrlich (1977)
pBC16 (2.8 MDa)	Tc ^r	Bernhard <i>et al.</i> (1978)
pUB110 (2.8 MDa)	Km ^r	Gryczan <i>et al.</i> (1978)
pBD10 (4.4 MDa)	Cm ^r Em ^r	Bacillus Genetic Stock Center (Ohio State University)
pBD8 (6.0 MDa)	Km ^r Cm ^r	Clewell <i>et al.</i> (1974) This laboratory
pAMβ1 (17.7 MDa)	MLS ^r	
pX02 (60.0 MDa)	Cap ⁺	

and resuspended to a final density of $1-2 \times 10^9$ viable cells ml⁻¹ in a solution containing, unless otherwise specified, 0.3% (w/v) Bacto-peptone (Spofa), 5% (w/v) glycine, 10% (w/v) polyethylene glycol 6000 (PEG 6000; Serva) and 0.1 M-MgCl₂. Samples (150 μ l) were transferred to glass tubes and combined with 50 μ l DNA in 1 mM-Tris/HCl buffer (pH 7.4). The samples (total volume 200 μ l each) were frozen by immersion in liquid nitrogen for 3 min unless otherwise noted, then thawed at temperatures described in the text (commonly at 37 °C). Finally the cells were transferred to 1 ml BHI broth, incubated at 37 °C with gentle shaking for 2 h, then plated on selective solid media and incubated at 37 °C for 24–36 h.

Viability was estimated by plating dilutions in 0.85% (w/v) NaCl on non-selective medium after freezing and thawing.

The sterility of isolated DNA was checked by plating the initial solution on BHI agar plates followed by incubation at 37 °C for 48 h. The specificity of transformation was verified by addition to the freezing mixture of DNAase (Amersham) to a final concentration of 5 μ g ml⁻¹.

Selection of transformants was performed on BHI agar plates supplemented with one of the following antibiotics: chloramphenicol (20 μ g ml⁻¹), tetracycline (10 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹) and erythromycin (10 μ g ml⁻¹). Cap⁺ (pX02⁺) transformants were selected by treatment with phage CP54 according to Green *et al.* (1985).

The main parameters for cryotransformation were determined with supercoiled DNA of plasmid pBD10. The other plasmids used were pC194, pBC16, pBD8, pUB110, pAM β 1 and pX02, prepared in caesium chloride gradients according to the method of Mozharov (1982).

Results

The various parameters of the transformation protocol were tested in turn.

Effect of glycine

Glycine concentrations from 1 to 10% were tested, with incubation for 2.5 h. Incubation with glycine at concentrations between 3 and 6% gave the maximum yield of

transformants (Table 2a). The concentrations of glycine used did not lead to protoplast formation or sensitivity to osmotic shock.

Effect of divalent cations

Dityatkin *et al.* (1985) reported a dependence of the transformation frequency of *E. coli* on the concentration of divalent cations in the freezing mixture. A significant effect of Mg²⁺ on the yield of *B. anthracis* transformants was observed (Table 2b). The optimum concentration of Mg²⁺ was 0.1–0.15 M. Transformation of *B. anthracis* was also achieved by freeze-thawing in the presence of CaCl₂, although this was rather less efficient than transformation in the presence of MgCl₂ (Table 2b).

Effect of PEG 6000

An approximately 20-fold increase in the yield of transformants was observed when 10% (w/v) PEG was added to the freezing mixture. The other concentrations of PEG used were less effective (Table 2c).

Effect of freezing medium

Distilled water, LB, 2YT and Chottinger broth, and 0.3–1.0% solutions of Bacto-peptone were used, all other conditions being equal. The maximum yield of transformants was observed when distilled water or any concentration of Bacto-peptone were used. (Table 2d).

Freezing-thawing conditions

Freezing at –196 °C (liquid nitrogen for 3 min) and –12 °C (deep freeze for 25 min) were compared. No

Table 2. *Effect of altering parameters of freeze-thaw transformation*

For details of individual treatments see Results. All results are means of at least five experiments; in no case did the replicates vary by more than twofold.

Treatment	Transformation efficiency [c.f.u. ($\mu\text{g DNA}$) ⁻¹]	Treatment	Transformation efficiency [c.f.u. ($\mu\text{g DNA}$) ⁻¹]
(a) <i>Pregrowth with glycine:</i>		(e) <i>Freeze-thaw conditions:</i>	
1%	1.2×10^1	Frozen in liquid N ₂ (-196°C)	3.5×10^2
3%	3.5×10^2	Deep freeze (-12°C)	3.3×10^2
6%	3.4×10^2	Thawing at 42°C	1.1×10^1
10%	0	Thawing at 37°C	3.4×10^2
(b) <i>Divalent cations in freezing mixture:</i>		Thawing at 30°C	1.9×10^2
MgCl ₂ , 0.01 M	1.1×10^1	Thawing at 20°C	1.2×10^1
MgCl ₂ , 0.05 M	1.2×10^2	(f) <i>Time of DNA addition:</i>	
MgCl ₂ , 0.1 M	3.4×10^2	Joint freezing of cells with DNA	3.4×10^2
MgCl ₂ , 0.15 M	3.5×10^2	Addition of DNA 5 s after beginning thawing	1.1×10^1
MgCl ₂ , 0.2 M	1.6×10^2	Addition of DNA 15 s after beginning thawing	0
CaCl ₂ , 0.05 M	5.9×10^1	Addition of DNAase 15 s after beginning thawing	3.5×10^2
CaCl ₂ , 0.1 M	6.8×10^1	(g) <i>Plasmids used as a source of DNA:</i>	
CaCl ₂ , 0.15 M	1.3×10^1	pC194 (1.8 MDa)	3.4×10^2
(c) <i>PEG 6000 in freezing mixture:</i>		pBC16 (2.8 MDa)	3.3×10^2
5%	2.9×10^2	pUB110 (2.8 MDa)	1.0×10^3
10%	3.5×10^2	pBD10 (4.4 MDa)	3.5×10^2
15%	2.5×10^2	pBD8 (6.0 MDa)	3.4×10^2
None	1.8×10^1	pAM β 1 (17.7 MDa)	0.9×10^1
(d) <i>Medium for freezing mixture:</i>		pX02 (60.0 MDa)	0
LB broth	4.1×10^1	pX02 (thawing at 30°C)	3.0×10^{-1}
2YT broth	4.1×10^1	(h) <i>Effect of endogenous plasmids:</i>	
Chottinger broth	6.6×10^1	<i>B. anthracis</i> Sterne 34F2 (pX01 ⁺ pX02 ⁻)	2.8×10^2
Bacto-peptone, 1%	2.3×10^2	<i>B. anthracis</i> KM33 (pX01 ⁻ pX02 ⁻)	1.1×10^2
Bacto-peptone, 0.6%	2.3×10^2	<i>B. anthracis</i> KM34 (pX01 ⁻ pX02 ⁺)	2.9×10^1
Bacto-peptone, 0.3%	2.3×10^2	<i>B. anthracis</i> KM35 (pX01 ⁺ pX02 ⁻)	2.5×10^2
Distilled water	2.1×10^2		

significant effect of freezing temperature was found (Table 2e).

When the thawing temperature was varied from 42 to 30°C , the maximum yield of transformants occurred at 37°C (Table 2e). The only exception was transformation of *B. anthracis* with the homologous high-molecular-mass plasmid pX02, which occurred only when the thawing temperature was reduced to 30°C (Table 2g).

Duration of the competence state

The cells were frozen in the absence of plasmid DNA, and DNA was added at various times after initiation of thawing at 37°C . The induced competence state of the recipient cells was rapidly lost during thawing: the susceptibility of the bacteria to exogenous DNA was completely lost within 15 s. The maximum yield of transformants was observed only if the recipient cells were frozen in the presence of DNA. The addition of

DNAase to the incubation mixture after 15 s incubation at 37°C did not result in a reduction of transformation efficiency, confirming the transient nature of the competence state (Table 2f).

Effect of plasmid size

Merrick *et al.* (1987) reported a reduction of cryotransformation with increasing plasmid size in both *E. coli* and *Klebsiella pneumoniae*. An increase of plasmid molecular mass from 1.8 to 6.0 MDa had no significant effect on freeze-thaw transformation frequency in *B. anthracis*. However, the larger plasmids of 17.7 and 60.0 MDa transformed with significantly lower efficiency (Table 2g).

Additional proof of the introduction of plasmids into *B. anthracis* cells was obtained in transformation experiments performed with plasmid DNA reisolated from transformants (data not shown).

Effect of endogenous plasmids

The isogenic derivatives of virulent strain 81/1 (KM33, KM34, KM35) were used in these experiments. The carriage of plasmid pX01 did not affect the frequency of transformation, but the yield of transformants decreased approximately tenfold when strain KM34, harbouring plasmid pX02, was used as recipient (Table 2h).

Discussion

Since Dityatkin *et al.* (1972) first used the procedure of freeze-thawing for transformation of *E. coli* and *Proteus vulgaris*, cryotransformation has been shown to be useful for induction of DNA uptake by *Agrobacterium tumefaciens* (Holsters *et al.*, 1978), *Bordetella pertussis* (Weiss & Falkow, 1982), *Yersinia pestis* (Kokoushkhin, 1982), *K. pneumoniae* and *Proteus mirabilis* (Merrick *et al.*, 1987), and *B. cereus* (Loginova & Dityatkin, 1988).

We have now shown that cryotransformation can be used to induce plasmid DNA uptake in *B. anthracis*. Because of its rapidity and simplicity, cryotransformation is more attractive than the recently described method of Makino *et al.* (1987), involving the transformation in *N*-acetylmuramidase-induced protoplasts.

DNA uptake by *B. anthracis* in the conditions described by Dityatkin *et al.* (1972) for *E. coli* and *P. vulgaris* (i.e. a low $MgCl_2$ concentration – 10.0 mM – and the absence of treatment with glycine and PEG) was not observed in our experiments (data not shown).

Hammes *et al.* (1973) showed that incorporation of glycine into cell wall peptidoglycan reduces the degree of cross-linking. We therefore examined the effect of glycine treatment on induction of competence in *B. anthracis* prior to freeze-thawing. Incubation of a growing culture for 2.5 h with glycine at a concentration of 3–6% was necessary to obtain transformants.

Other components known to enhance transformation efficiency were also tested. An increase of DNA uptake was observed when Mg^{2+} (0.1–0.15 M) was added to the freezing mixture. Transformation in the presence of Ca^{2+} was considerably less efficient. The addition of PEG 6000 to the freezing mixture to a final concentration of 10% was essential for efficient DNA uptake.

In agreement with the results of Dityatkin *et al.* (1985) with *E. coli* HB101, one of the features of *B. anthracis* as a recipient of the cryotransformation system was its short-term receptibility to DNA. However, an increase of transformation frequency of *B. anthracis* with decreasing temperature of freezing was not demonstrated. The lack of a significant effect of freezing temperature on transformation efficiency is in agreement with results reported by Merrick *et al.* (1987) for *K. pneumoniae*.

Dityatkin *et al.* (1985) showed that deviation of the thawing temperature in either direction from the optimum (42 °C) progressively reduced the maximum yield of *E. coli* transformants. Unlike *E. coli*, the maximum efficiency of transformation for *B. anthracis* was observed at 37 °C. The effect of thawing temperature was particularly noticeable when transformation was performed with homologous DNA of high molecular mass, e.g. plasmid pX02. It was necessary to decrease the temperature of thawing from 37 to 30 °C to obtain Cap^+ transformants.

Thus our cryotransformation procedure may be a convenient tool for examination of cloned virulence-associated genes in a homologous system, for elucidation of their roles in pathogenesis of *B. anthracis*.

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