

SHORT COMMUNICATION

A Procedure for Isolating High Quality DNA from Spores of *Bacillus subtilis* 168

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A method of isolating DNA from spores of *Bacillus subtilis* is described. The DNA has approximately the same efficiency in genetic transformation as DNA isolated from vegetative cells and gives a restriction pattern similar to that from vegetative DNA.

INTRODUCTION

There are many indications, in the literature, of the difficulty in releasing DNA from spores reproducibly in high yield and retaining good activity in transformation (Takahashi, 1968; Sakakibara *et al.*, 1970; Gillin & Ganesan, 1975; Callister & Wake, 1974; Dunn *et al.*, 1978). An improved isolation procedure would be of value in marker frequency analysis (Callister & Wake, 1974). Moreover, it may facilitate investigations of the special chromosomal configuration thought to be present in spores (Baillie *et al.*, 1974) and of chromosome replication during commitment to sporulation (Dunn *et al.*, 1978).

Mechanical methods of releasing spore DNA, such as that of Yoshikawa & Sueoka (1963) in which spores are ground in liquid nitrogen, have been used but are only suitable on a large scale and require considerable finesse in order to obtain a good product. DNA has also been extracted, using conventional methods, from spores germinated in the presence of inhibitors of DNA synthesis (Ephrati-Elizur & Borenstein, 1971). Disruption of the spore coat by chemical means has been the most widely used approach to releasing DNA. Even so, the yields of DNA obtained using these methods are low and not reproducible, but the source of the problem has been difficult to identify. The rationale of these methods has been to reduce the disulphide bridges found in the keratin-like outer coat, usually at extreme pH values in the presence of protein-solubilizing agents such as urea and sodium dodecyl sulphate. Fitz-James (1971) and Aronson & Fitz-James (1976) have shown that alkali-soluble proteins are extracted from the inner spore coat without dissolving the outer layers. This allows lysozyme to penetrate to and to solubilize the peptidoglycan layer causing the spore to become phase-dark.

The refractory nature of the outer layer of the spore coat of *Bacillus subtilis* undoubtedly prevents complete extraction of DNA (Fitz-James, 1971; Aronson & Fitz-James, 1976). The possibility of covalent cross-links between separate proteins has been considered as the cause of this insolubility by analogy with cross-linked proteins found in multicellular organisms (Aronson & Fitz-James, 1976; Pandey & Aronson, 1979). Sakakibara *et al.* (1970) have reported that older spores are more difficult to lyse than younger spores using their procedure; this may reflect the development of cross-links substantially later than the appearance of refractility. Milhaud & Balassa (1973) claim that maximum heat resistance of spores is attained very late in sporogenesis. A number of unusual compounds have been

found in hydrolysates of spore proteins which may indicate interchain covalent cross-links. Aspartyl-*ε*-lysine has been found in spores of *Bacillus sphaericus* (Tipper & Gauthier, 1972) and dityrosine has been found in *B. subtilis* (Pandey & Aronson, 1979). Goldman & Tipper (1978) have estimated that there is no more than 1 mol dityrosine per 3.6×10^5 g spore coat, which suggests that extensive inter-protein cross-linking by this mechanism is unlikely. It may prove that the refractory proteins of the spore coat are uniquely insoluble in aqueous solvents.

Extreme extraction conditions may generate covalent cross-links or may modify proteins so that they cannot be solubilized. In the presence of urea a variety of groups in amino acids may be cyanylated (Stark *et al.*, 1960). Cysteine can be converted to lanthionine at very high pH (du Vigneaud *et al.*, 1941) or dehydroalanine above pH 10 which then reacts with *ε*-amino groups of lysine (Aronson & Fitz-James, 1976). The amount of dityrosine in spore proteins can apparently be increased by performic acid treatment (Pandey & Aronson, 1979). By investigating conditions for spore coat disruption where undesirable effects are minimized, a useful system of spore extraction has been devised.

METHODS

Preparation of spores. Spores of a number of *B. subtilis* 168 strains [168 Trp⁻; 168 Trp⁻ Thy⁻; BD280 (ϕ 105) (D. Dubnau); ED53 (J. Neuhard); RB1030 (ϕ SPO2) (R. Buxton); RB1124 (Su³⁺) (R. Buxton); RUB2300 (ϕ 3T) (F. Young)] were obtained using the resuspension method of Sterlini & Mandelstam (1969). Spores were routinely harvested after 20 h unless otherwise stated. They were purified after treatment with lysozyme and sodium dodecyl sulphate (SDS) by centrifugation (50000 *g* for 20 min) through a layer of Urografin [2 ml of 45% (w/v) in distilled water]. The spores were washed in distilled water and their purity was checked by phase microscopy. Preparations contaminated with bacterial cells or debris were repurified. All preparations used were completely free of bacterial contamination.

Extraction of DNA from spores. Pure spore preparations in distilled water were resuspended at a concentration of 2×10^9 ml⁻¹ in freshly prepared solution A [urea (BDH Aristar grade), 8 M; Tris, 1 M; SDS, 1% (w/v); ammonium acetate, 0.1 M; dithioerythritol (DTE), 0.1 M; final pH 9.8]. Extraction was carried out at 30 °C for 16 h in a sealed tube after which the spores were sedimented (16000 *g* for 5 min), washed at room temperature successively with solution B [sodium chloride, 1 M; DTE, 0.01 M] and solution C [Tris/HCl buffer, 0.1 M, pH 7.3; Tween 80, 0.25% (v/v); EDTA, 0.01 M; DTE, 0.01 M] and then resuspended in solution C containing 0.5 M-sucrose. Lysozyme was added (1 mg ml⁻¹) and the spores were incubated at 50 °C for 1 h. The spores were centrifuged and resuspended in 1 ml of solution C and were then frozen and thawed five times. At this stage the spores appeared swollen and phase-light. Sodium sarcosinate (1%, w/v, final concentration) and *Streptomyces griseus* protease (Sigma, type V, 200 µg ml⁻¹) were added and incubation was continued for a further hour during which time the lysate became clear and viscous. The lysate was then centrifuged at 15000 *g* for 5 min and the sediment was resuspended in 8 M-guanidinium chloride (Cox, 1968) and allowed to stand for 30 min at room temperature. This mixture was then centrifuged for 5 min at 16000 *g* and the supernatant was pooled with the first extract after dialysis. The sediment usually contained less than 5% of the original DNA and was discarded. Tris (pH 8.0) and EDTA were added to the pooled supernatants to give 0.2 and 0.1 M, respectively, and the sample was extracted with phenol (equilibrated with 0.1 M-Tris) at 50 °C for 30 min. Phenol was extracted from the aqueous phase with ether. Sodium acetate was added to give 0.3 M followed by 2 vol. ice-cold absolute ethanol. After leaving at least 2 h at -20 °C, DNA was sedimented at 31000 *g* for 15 min. The precipitate was dissolved in the minimum volume of $0.1 \times$ SSC (SSC: 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0) and reprecipitated with ethanol. Samples were dialysed extensively against $0.1 \times$ SSC.

Transformation. Strain SL35 (Piggot & de Lencastre, 1978) was used as a recipient. Competent cells were obtained by the method of Wilson & Bott (1968). DNA was used at 1 µg ml⁻¹ in all crosses.

RESULTS AND DISCUSSION

The procedure described allows recovery of pure DNA in overall yields greater than 80% while the spore residue retains less than 5% of the total DNA. The method has been used with seven strains (see Methods). In DNA-mediated transformation, 2×10^5 to 5×10^5 transformants per 10^8 recipient cells have been routinely obtained when selecting for

purA16⁺, *argC4⁺* and *metB5⁺* transformants using spore DNA from 168 Trp⁻ and 168 Trp-Thy⁻. Similar frequencies for *metB5⁺* transformants were obtained using vegetative cell DNA. Owing to the higher origin to terminus ratio in vegetative DNA, markers closer to the origin are transformed at up to twofold greater frequencies. At no point in this procedure are the spores or DNA exposed to extreme conditions of pH which may damage DNA. Nucleases are probably inactive throughout the extraction procedure as EDTA is present. EDTA has no effect on the lysis procedure but the frequency of transformants per μg DNA is substantially higher when it is present.

The conditions used to disrupt the spore coat are not greatly different from those used previously by a number of authors to extract spore coat proteins and to render spores sensitive to lysozyme (Fitz-James, 1971; Aronson & Fitz-James, 1976; Pandey & Aronson, 1979). However, to extract sufficient coat protein so that detergents release DNA after lysozyme treatment, the conditions described above should be followed precisely. Using the method of Fitz-James (1971), spores become phase-dark during lysozyme treatment but do not become reproducibly sensitive to detergents. Probably the most important conditions for successful spore lysis are that SDS should be removed completely before treatment with lysozyme and the thiol groups should remain reduced during the washing and lysozyme treatment steps. A wash in 1 M-sodium chloride containing DTE followed by the buffer used for lysozyme treatment is sufficient to achieve this.

The deterioration of urea solutions during the extraction procedure is an important factor affecting the efficiency of extraction. Urea and ammonium cyanate are in equilibrium in alkaline solution so that cyanate ions accumulate in urea solutions after preparation. Cyanate ions react with a variety of groups in proteins (Stark *et al.*, 1960) and may affect their extractability. Ammonium acetate was included in the extraction medium (solution A) to force the equilibrium away from cyanate accumulation.

Spores prepared as described above are, with rare exceptions, fully susceptible to the lysis procedure. However, several batches of spores from strains which routinely lyse easily gave low yields of DNA. These spores were abnormally pigmented. Although older spores were more pigmented, I have been unable to demonstrate any reduced susceptibility to the lysis procedure in spores harvested up to 43 h after resuspension.

After spore lysis the DNA released is very active in transformation, but may not be digestible with restriction endonucleases even after phenol treatment. However, if the lysate is digested with *Streptomyces griseus* protease, extracted with guanidinium chloride (as described by Cox, 1968) and then phenol, the final product after alcohol precipitation is susceptible to *EcoRI* and *BamHI* and gives a limit digest restriction pattern similar to that from vegetative DNA. No attempt has been made to establish if there are minor differences.

The mechanism by which spore lysis is achieved is not fully understood. Gould & Hitchins (1963) and many others have shown that treatment with reducing agents at extreme pH values allows spores to become phase-dark. However, I have been unable to release DNA quantitatively or even in moderate yield from spores treated in this way. Using the procedure described, lysozyme affects spores much more drastically so that they appear phase-light and swollen. Indeed, it appears essential to reach this state in order to extract DNA successfully. The processes involved in spore germination may contribute to the disruption of the spore coat in this procedure and they may be inactivated in acid or alkaline conditions.

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