Comparison of Methods for Protoplast Formation in *Bacillus thuringiensis*

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In preparation of *Bacillus thuringiensis* protoplasts or cell-free lysates, hydrolysis of cell walls was due to endogenous autolytic activity and was largely independent of lysozyme. Purified cell walls of *B. thuringiensis* were resistant to lysozyme, lysostaphin, chitinase, trypsin and *Bacillus subtilis* proteinase, but were hydrolysed by *B. thuringiensis* autolytic enzymes or an *N*-acetylmuramidase (mutanolysin) from *Streptomyces globisporus*. Mutanolysin was effective in preparation of protoplasts or lysates of *B. thuringiensis*, and, unlike lysozyme, did not interfere with electrophoretic analysis of DNA.

INTRODUCTION

Bacillus thuringiensis has been of increasing interest because it produces insecticidal toxins during growth and sporulation, but physiological and genetic studies are hampered by lack of efficient and versatile techniques for genetic manipulation in this organism (Martin & Dean, 1980*b*). Although transformation of *B. thuringiensis* protoplasts has been reported (Azizbekyan *et al.*, 1982; Martin & Dean, 1980*a*; Miteva *et al.*, 1981), the efficiency of transformation was very low, possibly due to incomplete removal of the cell walls by lysozyme (Alikhanian *et al.*, 1981; Martin *et al.*, 1981). Many researchers have alluded to difficulties in the use of lysozyme for hydrolysis of *B. thuringiensis* cell walls (Miteva *et al.*, 1981; Gonzalez & Carlton, 1982; Kingan & Ensign, 1968*a*; Lereclus *et al.*, 1982). Martin *et al.* (1981) reported that growth of *B. thuringiensis* in L-broth without glucose resulted in greater lysozyme sensitivity than growth in L-broth with glucose. High concentrations of lysozyme have also been used in an effort to obtain efficient cell wall hydrolysis; however, excess lysozyme may lead to undesirable effects such as interference with cell wall regeneration due to strong binding of lysozyme to the protoplast membrane (Harrington & Baddiley, 1983) or proteolytic activity associated with lysozyme in extracts (Oliver & Stadtman, 1983). Experiments were initiated in this laboratory to modify existing methods, or to develop new methods for preparation of *B. thuringiensis* protoplasts.

METHODS

Bacterial strains. *B. thuringiensis* strains KBt4 (var. *kurstaki*, a derivative of HD-1) and HD522 (var. *israelensis*) were used throughout this study. Cultures were routinely grown in L-broth as described previously (Temeyer, 1984).

Preparation of crude autolytic enzyme. The procedure of Kingan & Ensign (1968*b*) was modified by growth of the cells to late exponential phase in tryptic soy broth (Difco) containing 0.1% yeast extract, followed by synchronous sporulation in mineral salts medium (DCE) without glutamate (Temeyer, 1984). Sporulated cultures were collected by centrifugation when 1% of the spores had been released from the sporangia. The pellet was resuspended in 1/25 culture volume of 20 mM-potassium phosphate, pH 8.0, containing MgCl₂, MnCl₂ and CaCl₂ (each 1 mM), plus 0.1 mM-CoCl₂. Toluene was added to 2.5% (v/v) after 2 h at 28 °C with shaking, and the loosely capped slurry was allowed to stand at 22 °C overnight. The autolysate was centrifuged, rinsed with a minimal volume of distilled water, and the supernatants were pooled. One-tenth volume 1 M-potassium phosphate, pH 7.5, was added and the crude autolysate was fractionated by precipitation with solid ammonium sulphate at constant pH while stirring at 0 °C. Material which precipitated at 30–70% saturation (Di Jeso, 1968) was dialysed against 10 mM-Tris/HCl, pH 8.0, freeze-dried, and stored in 50% (v/v) glycerol at –20 °C. Hydrolytic activity of the crude autolytic enzyme preparation was stable for at least one year.

Preparation of *B. thuringiensis* cell walls. Purified cell walls were prepared from frozen, spore-free vegetative cells by sonication in distilled water until more than 99.9% of the cells had been broken as indicated by phase-contrast microscopy. The disrupted cell suspension was centrifuged (23 500 *g*) to pellet particulate matter, resuspended in distilled water, and boiled for 30 min to inactivate lytic enzymes. The suspension was cooled to 37 °C and incubated for 30 min with RNAase A and DNAase I (100 µg ml⁻¹ each) followed by 30 min with 100 µg trypsin ml⁻¹. The cell walls were collected, resuspended, and boiled for 1–5 min in 1% (w/v) sodium dodecyl sulphate (SDS). Particulate matter was pelleted (4800 *g*) and rinsed a total of nine times, first with distilled water (2 ×), then 1 M-NaCl (2 ×), followed by 1 mM-Tris/HCl + 1 mM-Na₂EDTA, pH 7.5 (2 ×), and finally with distilled water (3 ×). A concentrated suspension was freeze-dried, yielding a spongy white material. A water suspension of 1 mg ml⁻¹ gave a Klett reading of 123 (blue, no. 42 filter).

Assay of lytic activity. A colorimetric assay of *B. thuringiensis* cell wall hydrolysis was used to compare relative activity of hydrolytic enzymes. Purified cell walls were dyed with Procion red MX5B as described by Forsberg & Rogers (1971). Cell wall hydrolysis was accompanied by release of non-sedimentable dye. Dyed cell walls of *B. thuringiensis* were incubated with various lytic enzymes at 23 °C with agitation. The suspensions were diluted and centrifuged at 5 °C for 15–30 min (11 000 *g*) and relative hydrolysis was determined as absorbance of the supernatant at 530 nm.

Electrophoretic analysis. Preparations of plasmid DNA (purified by isopycnic centrifugation) containing lysozyme, mutanolysin or autolytic enzyme were incubated at 37 °C for 30 min to test for nuclease activity or the formation of intermolecular complexes interfering with electrophoretic analysis of DNA. The DNA samples were electrophoretically separated on 0.7% agarose gels in Tris/borate buffer (89 mM-Trizma base, 89 mM-boric acid, 2.5 mM-Na₂EDTA, pH 8.3), and visualized by fluorescence of the ethidium-bromide-stained gels under UV (300 nm) illumination.

Enzymes and chemicals. *Streptomyces globisporus* N-acetylmuramidase (mutanolysin) was obtained from Sigma, or from Miles Laboratories. *Bacillus subtilis* proteinase and *Serratia marcescens* chitinase were obtained from US Biochemical Corp. Egg white lysozyme (grade I), lysostaphin, RNAase A, DNAase I, trypsin and D-cycloserine were obtained from Sigma. Procion red MX5B was kindly donated by Dr Richard Bryant of Imperial Chemical Industries. All other materials were of the highest grade available commercially.

RESULTS AND DISCUSSION

Lysozyme sensitivity of *B. thuringiensis* was monitored by measurement of the reduction in turbidity of the cell suspension, clarity of the suspension after addition of SDS to 2%, release of plasmid DNA as monitored by electrophoresis in agarose gels, or conversion to spherical form (spheroplasts) in the presence of 0.25 M-sucrose. Removal of the cell wall with lysozyme was highly dependent on the condition and treatment of the cells during growth and harvest. The presence of glucose during growth in L-broth was not a significant factor in apparent sensitivity to lysozyme. Cells from rapidly growing cultures (with or without glucose) appeared to be much more sensitive to lysozyme than slower growing cultures or those grown in other media, such as tryptic soy broth (Difco) containing 0.1% yeast extract (Difco). Harvest treatments such as washing the cells in Na₂EDTA or repeated washes in distilled water reduced apparent sensitivity of the cells to lysozyme. Freezing the cells further reduced apparent sensitivity to lysozyme. Frozen cells resuspended in L-broth did not recover maximal apparent sensitivity to lysozyme for at least two doublings of the culture turbidity. Apparent sensitivity to lysozyme was maximal when rapidly growing cells from early exponential phase (100–150 Kletts units using a no. 66, red filter) were collected and resuspended in lysozyme without rinsing. These results suggested the involvement of a labile factor associated with the cells during rapid growth, which conferred apparent, rather than actual, sensitivity to lysozyme. Subsequent experiments gave equivalent lysis and release of plasmid DNA with or without lysozyme addition, indicating that intrinsic autolytic factors, rather than lysozyme, were primarily responsible for removal of the cell wall.

Autolytic formation of *B. thuringiensis* protoplasts (autoplasts) was observed by phase-contrast microscopy. Autoplasts were formed by extrusion through a small hole in the encasing cell wall. Rate and efficiency of autoplast formation was dependent upon the same factors as identified for apparent sensitivity of the cells to lysozyme. Autoplast formation was optimal at 37 °C in 50 mM-Tris/HCl, pH 7.5, containing 10 mM-MgCl₂, 1 mM-CaCl₂, 1 mM-MnCl₂, 0.1 mM-CoCl₂, 1 mM-dithiothreitol, bovine serum albumin (1 mg ml⁻¹), and 350–500 mM-

sucrose. Both the rate and efficiency of autoplast formation could be increased by addition of an exogenous preparation of *B. thuringiensis* autolytic enzymes; however, protoplasts prepared in this way exhibited altered refractive index, changing from phase-dark to phase-transparent during subsequent incubation. Use of exogenous autolytic enzymes for preparation of *B. thuringiensis* protoplasts would require additional purification of the autolytic enzymes to remove membrane damaging factors. Autoplasts prepared without addition of exogenous enzyme were stable in phase character for several days.

B. thuringiensis cell walls were purified to remove endogenous lytic factors so that hydrolytic activity of various enzymes could be compared. Initial assays attempted to measure hydrolytic activity turbidimetrically (no. 42, blue filter) using cell wall suspensions, but addition of lysozyme (1–10 mg ml⁻¹) or potassium phosphate (0.1 M) resulted in aggregation and precipitation of the cell walls. For this reason, purified cell walls dyed with Procion red MX5B were used. Release of dye corresponded to decreased size of the cell wall pellet following centrifugation. This method was used for comparative studies but was not standardized for quantification of absolute enzyme activity. Only the *N*-acetylmuramidase from *S. globisporus* (mutanolysin) and the autolytic enzyme preparation from *B. thuringiensis* were effective in hydrolysing the purified cell walls in either the presence or absence of the dye, while lysozyme, lysostaphin, chitinase, trypsin and *B. subtilis* proteinase were completely ineffective. In addition, lysozyme inhibited release of dye by both the *S. globisporus* enzyme and the autolytic enzyme preparation.

Optimal conditions for hydrolytic release of dye by mutanolysin appeared to be at 37 °C with pH 7.9–8.6. Dibasic cations were inhibitory to mutanolysin activity in the order Mn²⁺ and Co²⁺ (most inhibitory) followed by Ca²⁺ then Mg²⁺. Since at least 10 mM-MgCl₂ was required to stabilize *B. thuringiensis* autoplasts, various buffers were tested to determine if Mg²⁺ exhibits different levels of inhibition in different buffers. In general, 10 mM-MgCl₂ resulted in 50% inhibition of mutanolysin activity in different buffer systems, including several different salts of Tris. The exceptions to this general observation occurred with buffers containing phosphate or citrate (0–30% inhibition), which can sequester Mg²⁺ either by formation of a poorly soluble salt or by chelation.

Lysozyme-resistant species of cariogenic *Streptococcus* have been shown to be sensitive to mutanolysin (Calandra & Cole, 1980; Siegel *et al.*, 1981). Other Gram-positive bacteria also show different sensitivities to lysozyme or mutanolysin (Yokogawa *et al.*, 1975; Zipperle *et al.*, 1984). The basis for the different actions of lysozyme and mutanolysin on whole cells or purified cell walls may relate to the presence of accessory structures such as teichoic or teichuronic acids, or protein components of the cell surface and cell wall, rather than to any intrinsic differences in the peptidoglycan structure (Knox *et al.*, 1979; Nesbitt *et al.*, 1980). Other species exhibit *O*-acetylation of glycan chains (Brumfitt *et al.*, 1958) and incomplete *N*-acetylation of amino sugars (Araki *et al.*, 1972) as the basis for lysozyme resistance.

Since resistance to lysozyme hydrolysis may result from modification of the peptidoglycan or from the presence of accessory cell wall components which limit accessibility of the peptidoglycan, *B. thuringiensis* cells were converted to spheroplasts by growth in L-broth containing 2 mM-D-cycloserine and 0.1–0.25 M-sucrose. Resuspension of the spheroplasts in fresh medium without cycloserine resulted in rapid conversion to bacillary form and recovery of resistance to lysis induced by osmotic shock. Addition of lysozyme to the spheroplasts prevented both the subsequent return to bacillary form and recovery of resistance to osmotic shock.

Lysozyme and mutanolysin lacked nuclease activity; however, lysozyme formed complexes with the plasmid DNA and prevented electrophoretic migration into the gel, necessitating its removal by phenol extraction prior to electrophoretic analysis. Mutanolysin did not interfere with normal electrophoretic migration of the DNA.

Lysozyme was thus not only ineffective in hydrolysing cell walls of *B. thuringiensis*, but may be detrimental to a variety of subsequent biological or biochemical manipulations. Three alternatives to the use of lysozyme are presented. (1) Intrinsic autolytic activity may be utilized to produce cell lysates or protoplasts, but this action was relatively slow. (2) Addition of autolytic enzymes of *B. thuringiensis* (free of proteases and lipases) appeared to be possible and effective.

(3) Mutanolysin was effective in hydrolysing cell walls of *B. thuringiensis*. Use of mutanolysin at 1 mg ml⁻¹ in 10–50 mM-Tris/HCl, pH 8.0, resulted in rapid lysis of *B. thuringiensis* cells, or conversion to protoplasts in 10–50 mM-potassium phosphate, pH 8.0, with 10–20 mM-MgCl₂ and 0.3 M-sucrose. Additional stability was obtained by addition of MnCl₂ and CaCl₂ (each to 1 mM) and bovine serum albumin (to 1 mg ml⁻¹). Although the optimal temperature was 37–45 °C, similar results were achieved using 10 mg mutanolysin ml⁻¹ at 5 °C.

Mention of a commercial product does not constitute recommendation for use by the Agricultural Research Service, US Department of Agriculture.

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