Protein Phosphorylation in *Bacillus thuringiensis* during Growth and δ -Endotoxin Production

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At least 14 phosphopolypeptides in which the phosphate groups were present as mono-esters were detected by pulse labelling of *Bacillus thuringiensis* subsp. *kurstaki* HD-1-Dipel with [32 P]orthophosphate at different stages of growth and differentiation. Marked changes in the profile of phosphopolypeptides were observed primarily during the late exponential phase of growth. Several phosphopolypeptides co-purified with the endotoxin crystal of this subspecies and the phosphoamino acid residue of the most abundant (M_r 25 000) phosphopolypeptide was identified as phosphothreonine. Comparison of the phosphopolypeptides in endotoxin crystals from several subspecies suggested that M_r 25 000 species might be a common component.

INTRODUCTION

In recent years there have been reports describing protein phosphorylation in a diverse range of prokaryotes (see Turner & Mann, 1986) indicating that this form of post-translational covalent modification is widespread among prokaryotes. In the case of both *Myxococcus xanthus* (Komano *et al.*, 1982) and *Rhodospirillum rubrum* (Turner & Mann, 1986) clear changes in the pattern of protein phosphorylation have been correlated with the processes of differentiation exhibited by these organisms. In some species the phosphorylation of specific proteins has been characterized and related to the control of their activities. Thus the acetate-dependent attenuation of isocitrate dehydrogenase activity in *Escherichia coli* is mediated by phosphorylation (Nimmo, 1984) and in *Clostridium sphenoides* the active form of citrate lyase ligase has been shown to be phosphorylated (Antranikian *et al.*, 1985). Furthermore, in *Streptococcus pyogenes* the HPr component of the phosphotransferase system is phosphorylated at both a serine and a histidine residue (Reizer *et al.*, 1985).

In this paper we describe studies aimed to establish whether protein phosphorylation occurs in the Gram-positive spore-forming bacterium *Bacillus thuringiensis* and whether the pattern of phosphorylation reflects transitions in the growth state of the organism. *B. thuringiensis* is of interest primarily because of the formation of an insecticidal crystal protein inclusion, the δ -endotoxin, in parallel with the process of sporulation (Bulla *et al.*, 1980). The protoxin component of the crystal protein inclusion has been reported to be covalently modified by glycosylation (Bulla *et al.*, 1980) and Holmes & Monro (1969) reported a phosphate component in the purified crystals. Consequently, it was also of interest to establish whether any protein components of the crystal were subject to phosphorylation.

METHODS

Materials. O-Phospho-L-serine, O-phospho-L-tyrosine, O-phospho-DL-threonine, DNAase and RNAase were obtained from Sigma. Acrylamide and PAGE blue 83, obtained from BDH, were of Electran grade. All other gel components were obtained from Bio-Rad and were of electrophoresis purity. Protein standards for M_r estimations were obtained from Pharmacia. Acid-free, carrier-free [32 P]orthophosphate (5000 Ci mmol $^{-1}$; 185 TBq mmol $^{-1}$) was obtained from Amersham. HPTLC cellulose plates (10 $\times$ 10 cm) were obtained from BDH.

Organisms and culture conditions. *B. thuringiensis* subsp. *kurstaki* HD-1 and HD-1-Dipel, subsp. *thuringiensis* and subsp. *israelensis* were supplied by Microbial Resources. All strains were grown in the nutrient broth of Singer &

Rogoff (1968), or on solid medium composed of nutrient broth containing 1.5% (w/v) Bacto-agar. Growth in liquid medium was monitored spectrophotometrically at 650 nm. Starter cultures (10 ml) in 50 ml Erlenmeyer flasks were inoculated with a colony of *B. thuringiensis* from an overnight plate. All cultures were grown at 30 °C and 150 r.p.m. in a shaking water-bath.

Continuous and pulse labelling. Cultures to be labelled with [32 P]orthophosphate were grown in Singer and Rogoff nutrient broth lacking the phosphate component. When a starter culture had grown to an OD_{650} of 1 it was used to inoculate (1:100) fresh medium. For continuous labelling 10 ml cultures were grown in 50 ml Erlenmeyer flasks into late exponential phase when 1 mCi (37 MBq) [32 P]orthophosphate was added to the culture. These cultures were then allowed to grow until they had fully sporulated and lysed. For pulse labelling the cells were grown as above in 50 ml medium in a 250 ml Erlenmeyer flask. At appropriate time intervals samples were removed from the culture and dispensed into prewarmed Universal bottles containing 0.25 mCi (9.25 MBq) [32 P]orthophosphate and incubated at 30 °C for 30 min. The sample volumes taken were such that a constant amount of cellular material in terms of OD_{650} was obtained [sample volume (ml) = $2.5/OD_{650}$].

Crystal purification. The continuously labelled culture was harvested by centrifuging at 6000 *g* for 10 min, washed twice in ice-cold distilled water and resuspended in a final volume of 0.5 ml ice-cold distilled water. This was then sonicated five times for 20 s at an amplitude of 30 μ m with cooling on ice. The crystals were then harvested and resuspended in 0.5 ml 20 mM-Tris/HCl, pH 7.5, 10 mM-MgCl₂ containing DNAase and RNAase at 40 μ g ml⁻¹ and 80 μ g ml⁻¹ respectively and incubated at 30 °C for 1 h. The crystal preparation was then loaded onto a 12 ml 70–85% (w/v) sucrose gradient and centrifuged at 40000 *g*, 4 °C overnight. The crystal band was harvested by puncturing the tube and removing with a syringe, diluted with 20 ml ice-cold distilled water and the crystals collected by centrifugation. The crystals were then resuspended in 2 ml ice-cold distilled water and sonicated twice for 30 s at an amplitude of 30 μ m with cooling on ice. The crystals were again collected by centrifugation, resuspended in 0.5 ml ice-cold distilled water and then subjected to a second cycle of centrifugation on a 12 ml 31–62% (w/v) sodium diatrizoate gradient (Sharpe *et al.*, 1975) under the same conditions used for the sucrose gradient. The crystal band was harvested and washed as above and resuspended in a final volume of 1 ml ice-cold distilled water. Crystals were solubilized by adding an equal volume of 2 $\times$ sample buffer: 100 mM-Tris/HCl pH 7.5, 2% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 2 mM-EDTA, 20% (w/v) glycerol and 0.005% (w/v) bromophenol blue. Samples were incubated at 100 °C for 5 min for complete solubilization; 100 μ l was then loaded per gel lane and any material not used immediately was stored at -20 °C.

Pulse labelled sample preparation. Cells from pulse labelled samples were harvested by centrifugation at 6000 *g* for 10 min, resuspended in 40 μ l ice-cold distilled water, solubilized as above, and then any insoluble material was removed by centrifuging at 10000 *g* for 10 min. The supernatant was removed and stored as above. The entire sample after the addition of 1 μ l RNAase (20 mg ml⁻¹) was loaded into a single lane of the gel.

Electrophoresis and autoradiography. Samples were analysed on 10–30 or 4–30% (w/v) acrylamide-SDS exponential gradient gels and protein bands were visualized by staining with PAGE blue 83 and destaining with several changes of 10% (v/v) propan-2-ol, 10% (v/v) acetic acid. Gels on which crystal proteins had been analysed were then dried and autoradiographed. Gels on which pulse labelled proteins were analysed were destained and treated with 16% (w/v) trichloroacetic acid (TCA) at 95 °C by the method of Manai & Cozzzone (1982) before drying and autoradiography. Autoradiographs were allowed to develop for 1–7 d at -70 °C.

Preparation and analysis of phosphoamino acids. The phosphopolypeptide band of interest was cut from the gel and electroeluted using a Biotrap (Schleicher and Schuell). The eluate was chilled on ice and the phosphopolypeptide precipitated by the addition of 50% (w/v) ice-cold TCA to a final concentration of 20% (w/v). The sample was kept on ice for 10 min and the precipitate was collected by centrifugation in an Eppendorf microcentrifuge. The pellet was washed twice with diethyl ether, resuspended in 200 μ l 6 M-HCl and then autoclaved at 10 p.s.i. for 90 min. The hydrolysate was dried under nitrogen and redissolved in 3.5 μ l water; 1.5 μ l of solutions containing each of the authentic phosphoamino acids at a concentration of 10 mg ml⁻¹ was added to the hydrolysate. The phosphoamino acids were then resolved by two dimensional TLC as described by Mann & Turner (1988).

RESULTS

In order to establish whether phosphopolypeptides were detectable, and if so whether their profile was determined by the stage of growth or sporulation, samples were withdrawn from a culture of *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel in Singer and Rogoff broth at different stages of growth (Fig. 1) and pulse labelled with [32 P]orthophosphate. The cell extracts, representing a constant amount of cell material, were analysed by SDS-PAGE and autoradiography (Fig. 2, tracks 1–9). The 32 P-labelled bands detected represent phosphate esters since the gel had been treated with hot TCA which destroys amidophosphate and

Fig. 1. Growth of *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel in low phosphate Singer and Rogoff nutrient broth monitored spectrophotometrically at 650 nm. Times of removal of samples for continuous and pulse labelling with [32 P]orthophosphate are indicated by arrows.

Fig. 2. Autoradiograph of 32 P-labelled polypeptides in samples of *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel resolved by electrophoresis through a 10–30% (w/v) SDS-polyacrylamide gel and subsequently treated with hot TCA. Tracks 1–9 (exposure time 2 d) represent pulse labelled samples taken at different stages of growth as indicated in Fig. 1. Track 10 represents a sample continuously labelled from late exponential phase. M_r values of the major phosphopolypeptides are shown on the right and the positions of the M_r standards are indicated on the left.

Fig. 3. Analysis of the phosphopolypeptides in purified δ -endotoxin crystals of *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel by electrophoresis through a 4–30% (w/v) SDS-polyacrylamide gel. Track A shows the polypeptides stained with PAGE blue 83; track B is the corresponding autoradiograph (exposure time 7 d). M_r values of the major phosphopolypeptides are shown on the right and the positions of the M_r standards are indicated on the left.

acylphosphate linkages. At least fourteen phosphopolypeptides were detected, several of which exhibited a pronounced growth-state-dependent pattern. In the early stages of exponential growth (Fig. 2, tracks 1–4) at least seven phosphopolypeptide bands were clearly detected, with abundant species of M_r 77 000, 18 000, 12 500 and 10 000. Longer autoradiographic exposures revealed additional minor species (data not shown). In addition there was a particularly heavily phosphorylated polypeptide(s) of M_r 8000. A characteristic pattern of biphasic growth was observed (Fig. 1) when *B. thuringiensis* was grown in the nutrient broth of Singer and Rogoff lacking phosphate and this was correlated with particular changes in the pattern of phosphopolypeptides. During the reduction in growth rate (Fig. 2, tracks 2 and 3) there was a considerable reduction in abundance of the M_r 18 000 phosphopolypeptide. As the culture growth rate began to increase again the M_r 18 000 band returned to its former intensity and then declined again as the culture reached late exponential phase. A similar pattern of modulation was observed for the M_r 10 000 phosphopolypeptide; however, the M_r 12 500 species exhibited the inverse pattern of modulation.

A significant alteration in the profile of phosphopolypeptides was detected from mid to late exponential phase (Fig 2, tracks 5–7) with a major species of M_r 52 000 becoming considerably

Fig. 4. Identification of phosphoamino acids by two dimensional TLC. Material from the gel band corresponding to the p25 phosphopolypeptide of the endotoxin crystal preparation was electroeluted and analysed for its phosphoamino acid residues (as described in Methods). A, Ninhydrin-stained TLC plate; B, corresponding autoradiograph (exposure time 7 d). The positions of the phosphoamino acid standards phosphoserine (P-Ser), phosphothreonine (P-Thr) and phosphotyrosine (P-Tyr) are indicated.

more abundant and virtually all the species characteristic of early exponential phase disappearing, with the exception of the extremely heavily phosphorylated band(s) of around M_r 8000. There were no further significant changes in Fig. 2, track 9, which corresponds to an early point in sporulation with no mature spores or crystals detectable microscopically.

A 1.1 ml sample was also withdrawn from the same culture during late exponential phase and continuously labelled with [^{32}P]orthophosphate until the majority of the cells in the culture had sporulated, produced δ -endotoxin crystals and lysed. The δ -endotoxin crystals, spores and unlysed cells present in this sample were separated from the bulk of the cellular debris by centrifugation and analysed by SDS-PAGE (Fig. 2, track 10). Although some phosphopolypeptides as judged by their M_r may be common to both the pulse labelled vegetative cells and the continuously labelled crude spore and crystal preparation it is clear that several phosphopolypeptides are unique to this preparation, including a particularly abundant species of M_r 25 000.

In order to establish whether any of the phosphopolypeptides were genuinely components of the δ -endotoxin crystal a culture of *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel was continuously labelled with [^{32}P]orthophosphate until virtually all the cells had sporulated and lysed. The crystals were then purified by two cycles of density gradient centrifugation and after solubilization analysed by SDS-PAGE and autoradiography (Fig. 3). The pattern of phosphopolypeptides associated with the purified δ -endotoxin crystals (Fig. 3) is somewhat simplified compared to that of the partially purified preparation (Fig. 2, track 10) though the abundant M_r 25 000 polypeptide is still present; it is presumably a minor component of the crystals since it could not be detected by staining with PAGE blue 83. By comparison with authentic unlabelled stained bands the major M_r 155 000 protoxin is apparently also phosphorylated, though very weakly. The nature of the phosphorylated amino residue in the M_r 25 000 polypeptide was identified as phosphothreonine by two dimensional thin layer chromatography (Fig. 4).

Crystals labelled with ^{32}P were prepared in a similar fashion from *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel, subsp. *thuringiensis*, subsp. *kurstaki* HD-1 and subsp. *israelensis* in order to establish whether the phosphopolypeptide profile was characteristic of particular types of

Fig. 5. Analysis of ^{32}P -labelled polypeptides in purified endotoxin crystal preparations from different subspecies of *B. thuringiensis*. Track 1, subsp. *kurstaki* HD-1-Dipel; 2, subsp. *kurstaki* HD-1; 3, subsp. *thuringiensis*; 4, subsp. *israelensis*. The position of the M_r 25000/21000 phosphopolypeptide doublet is shown on the right and the positions of the M_r standards are indicated on the left.

δ -endotoxin crystal. The respective crystal preparations were solubilized and analysed by SDS-PAGE and autoradiography (Fig. 5). It is clear that an abundant phosphopolypeptide of approximate M_r 25000, detected initially in the δ -endotoxin crystals of subsp. *kurstaki* HD-1-Dipel, was present in the crystals of all four subspecies examined. The nature of the phosphorylated residue in the M_r 25000 phosphopolypeptide of the crystals of subsp. *israelensis* was again found to be phosphothreonine (data not shown). Although the M_r 25000 species was consistently present in δ -endotoxin crystal preparations the species of M_r 21000 and about 12000 (Fig. 5) were variably present and probably do not represent genuine components of the crystal.

DISCUSSION

At least 14 phosphopolypeptides were detected in *B. thuringiensis* subsp. *kurstaki* HD-1-Dipel by SDS-PAGE in one dimension. In the pulse labelling experiments described here the hot acid treatment of the gel before drying and autoradiography ensured that all the ^{32}P -labelled bands represented ester-linked phosphopolypeptides as opposed to amidophosphates or acylphosphates which are unstable under these conditions. However, two dimensional analyses of the pattern of phosphopolypeptides in *Escherichia coli* have revealed at least 128 phosphopolypeptides (Cortay *et al.*, 1986) and it would consequently be reasonable to assume that the number of

phosphorylated species detected in *B. thuringiensis* is a considerable underestimate. Several of the phosphopolypeptides exhibited a growth-stage-dependent pattern of appearance. Firstly, the abundance of the M_r 18000, 12500 and 10000 phosphopolypeptides was modulated in response to the changing growth rate both early and late in the growth of the culture. The late exponential phase of growth was characterized by radical changes in the abundance of several phosphopolypeptides including the appearance of a species of M_r 52000 and the disappearance of species of M_r 18000, 12500 and 10000. Although there was no observable change in the growth rate over this period this clearly represents a transition phase. These experiments were not intended to enable the identification of any specific proteins subject to phosphorylation. However, the HPr component of the phosphotransferase system in *Bacillus subtilis* has an M_r of 7500 (Kalbitzer *et al.*, 1982) and the corresponding protein in *Streptococcus pyogenes* is phosphorylated at a serine residue (Deutscher & Saier, 1983). Consequently the abundant phosphopolypeptide(s) of M_r 8000 observed in *B. thuringiensis* growing on glucose may represent the HPr protein of this species.

Analysis of the phosphopolypeptides co-purifying with the δ -endotoxin crystal revealed at least five species; one of these clearly corresponds in M_r to the P1 protoxin. However, one of the most abundant phosphopolypeptides was a species of M_r 25000. Phosphorylated species of the same size were detected in the crystals of four distinct subspecies of *B. thuringiensis* suggesting that this polypeptide might be a universal component of the endotoxin crystals. The phosphorylated residue was found to be threonine in the polypeptide from *B. thuringiensis* subspecies *kurstaki* HD-1-Dipel and *israelensis*.

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