Teichoic acid and lipid metabolism during sporulation of *Bacillus megaterium* KM

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The biochemistry of teichoic acid and lipid metabolism has been studied during sporulation of *Bacillus megaterium* KM. Measurements of cell-wall and membrane teichoic acid have shown that net synthesis of these polymers ceases at the onset of sporulation. Pulse-labelling studies show that the period of asymmetric septation and forespore engulfment is marked by an initiation of turnover of membrane teichoic acid but not of wall teichoic acid. This is reflected in the presence of inner-membrane teichoic acid and the virtual absence of wall teichoic acid in dormant spores. The total amount of lipid phosphorus in the sporulating cell increases by 70% as a result of asymmetric septation and subsequent engulfment of the forespore. The phosphorus requirement for this synthesis is derived from a pool formed during exponential growth, which is not exchangeable with extracellular P_i during sporulation. These results suggest that during sporulation a proportion of the glycerol 3-phosphate produced by preferential degradation of membrane teichoic acid formed during exponential growth is used for phospholipid synthesis during sporulation.

In response to nutrient limitation, certain bacilli undergo a well-defined series of morphological changes that result in the formation of dormant endospores (Ellar, 1978). These changes in vegetative cell function offer a unique system in which to study prokaryotic cellular differentiation (Freese, 1972).

During sporulation a number of important membrane modifications have been observed including synthesis of new proteins (Hogarth *et al.*, 1977a) and new carotenoid pigments (Konciewicz *et al.*, 1977), qualitative and quantitative changes in membrane lipids (Ellar & Posgate, 1974), alterations in lipid/protein ratio (Konciewicz *et al.*, 1977), progressive reduction in electron-transport capacity (Wilkinson & Ellar, 1975), changes in the activity of tricarboxylic acid-cycle enzymes (Ohné, 1975; Andreoli *et al.*, 1977) and initiation of Ca^{2+} uptake (Hogarth & Ellar, 1978). In addition the early phase of sporulation involves a unique modified cell division (Hitchins & Slepecky, 1969) in which an asymmetrically positioned membrane septum proliferates to enclose the forespore compartment. The mechanisms con-

trolling the location and proliferation of this septum have not been established (Hitchins, 1978). Equally important to our knowledge of the control of sporulation is an understanding of the role played by the double membrane that separates the two compartments of the sporulating cell. The two membranes that comprise this barrier have been shown to have reversed polarity with respect to both oxidative and transport capacity (Wilkinson *et al.*, 1975) and some assessment of their integrity, metabolic potential and transport capacity is crucial to establish the degree of co-ordination between forespore and mother-cell compartments. Biochemical characterization of these membranes may help to elucidate the relationships between their structure and function. In the present paper an analysis of teichoic acid metabolism has been carried out and related to changes in phosphorus incorporation into phospholipid during sporulation.

Experimental

Materials

[2- ^{14}C]Acetic acid (2.04 GBq/mmol), [2- 3H]glycerol (18.5 GBq/mmol) and [^{32}P]P_i (carrier-free) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

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Methods

Cultivation of the organism. *Bacillus megaterium* KM was induced to sporulate synchronously as described by Ellar & Posgate (1974) by using the modified growth medium of Stewart *et al.* (1981). The concentration of potassium phosphate in this medium was decreased to 4 mM except where stated.

Extraction of teichoic acids. Wall and membrane teichoic acids were extracted from vegetative and sporulating cells by a modified method of Rogers & Taylor (1978). Cell-culture samples (2.0 ml) labelled with radioactive glycerol were mixed with an equal volume of ice-cold 10% (w/v) trichloroacetic acid. After incubation for 10 min at 0°C, the cells were recovered by centrifugation (4500 g for 8 min at 4°C) and washed with 5 ml of ice-cold water. The pellets were freeze-dried, extracted with 1.5 ml of methanol at 65°C for 5 min and, after cooling to room temperature, 4.5 ml of chloroform was added. The suspension was shaken at frequent intervals for 1 h, the residue recovered by centrifugation (4500 g for 4 min at 20°C) and re-extracted by the same method. The pelleted residues were then dried *in vacuo*, resuspended in 50 mM-NaH₂PO₄ buffer containing 50 mM-NaCl and 100 µg of lysozyme/ml. After incubation at 37°C for 2.5 h the lysozyme-solubilized fractions containing wall teichoic acid were recovered by centrifugation (4500 g for 4 min at 20°C) and counted for radioactivity. The insoluble residue was washed with 5 ml of 50 mM-NaH₂PO₄ containing 50 mM-MgCl₂ and resuspended in the same buffer containing 10 µg of deoxyribonuclease/ml and 1 µg of ribonuclease/ml. After incubation for 30 min at 37°C, the residue recovered by centrifugation (4500 g for 4 min at 20°C) was washed twice with 5 ml of 50 mM-NaH₂PO₄ buffer containing 50 mM-MgCl₂ and resuspended in 2.0 ml of water. This fraction containing membrane teichoic acid was also counted for radioactivity.

Extraction of teichoic acids from dormant-spore fractions. Spores were broken and inner-membrane and integument fractions were prepared as described previously (Hogarth *et al.*, 1977b). Freeze-dried integuments (200 mg) and membranes (100 mg) were extracted twice with methanol (0.1 ml/mg) and chloroform as described above. The residues were extracted twice with 15 ml of water at 100°C for 30 min and the extracts containing lipoteichoic acid (Coley *et al.*, 1975) combined and freeze-dried. The residues were washed twice with 15 ml of water, recovered by centrifugation (4500 g for 4 min at 20°C) and extracted with 15 ml of 0.1 M-NaOH for 1 h at 100°C. After neutralization with HCl, the suspensions were clarified by centrifugation, and the supernatants containing wall teichoic acid (Coley *et al.*, 1975) were freeze-dried.

Lipid extraction and purification. Up to 100 mg (dry weight) of vegetative or sporulating cells was

pelleted in a 10 × 1.5 cm glass centrifuge tube (6000 g for 5 min at 4°C) and resuspended in 0.8 ml of ice-cold 50 mM-Tris/HCl buffer, pH 7.5. After the addition of 4.1 g of glass beads (0.10–0.11 mm diam.), the tube was sealed and the contents were vortex-mixed for 4 × 30 s bursts with intermittent cooling in ice. This procedure broke >95% of the vegetative cells or spores. The suspension of beads and cells was resuspended in 10 ml of propan-2-ol and heated at 100°C for 15 min. After cooling to room temperature, the suspension was added to 100 ml of chloroform/methanol (4:1, v/v) and incubated at room temperature for 2 h. The insoluble residue was removed by filtration and re-extracted overnight in 100 ml of chloroform/methanol (2:1, v/v). The combined extracts were dried by rotary evaporation and purified by passage through a Sephadex G-25 column (1 cm × 14 cm) equilibrated with water-saturated chloroform/methanol (19:1, v/v) as described by Rouser *et al.* (1967).

Preliminary characterization of teichoic acid. Crude teichoic acid extracted with boiling water was initially characterized by chromatography of acid and intestinal phosphatase hydrolytic products (Archibald, 1972).

Analytical methods. The ATP pools of sporulating cells were extracted with boiling propan-1-ol and determined as described by Scott & Ellar (1978). The total phosphorus content of purified lipid samples was measured as described by Marinetti (1962). Radioactivity was determined by mixing aqueous samples with 10 vol. of scintillation fluid containing 7 g of 2,5-diphenyloxazole and 0.35 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene in 1 litre of toluene/Triton X-100 (2:1, v/v) followed by scintillation counting. Lipid samples were dried from chloroform and counted for radioactivity in scintillant containing 4 g of 2,5-bis-(5'-*t*-butylbenzoxazol-2'-yl)thiophen in 1 litre of toluene.

Results

Growth and sporulation

The characteristics of growth and sporulation of *B. megaterium* KM are shown in Fig. 1. In this system sporulation was synchronous as defined by Ellar & Posgate (1974) with each sporulation Stage taking 1–1.5 h. The end of increase in cell numbers is defined as *t*₀. Towards the end of exponential growth the cell ATP pool decreased and then subsequently rose at *t*_{0.5} to a level 2.5 times that of exponentially growing vegetative cells, which was maintained throughout sporulation (Fig. 1). This rise in the cell ATP pool coincides with the onset of sporulation and in this system provides a useful biochemical marker for this event. Similar changes in the cell

Fig. 1. Growth and sporulation of *B. megaterium* in CCY medium

B. megaterium KM was grown in modified CCY medium as described by Stewart *et al.*, (1981) but containing 4 mM-potassium phosphate. t_0 is the time at which the increase in cell numbers ends. Sporulation stages are designated by Roman numerals. ●, A_{600} ; △, cell count; □, ATP.

ATP pool have been described during sporulation of *B. subtilis* (Hutchinson & Hanson, 1974).

Wall and membrane teichoic acid metabolism during sporulation

Previous studies had reported the absence of wall teichoic acid in bacterial spores (Chin *et al.*, 1968). To examine the fate of vegetative cell wall and membrane teichoic acids during sporulation, cells were grown and steady-state-labelled in medium containing [^3H]glycerol. The contents of both membrane and wall teichoic acid remained almost constant up to t_5 (Fig. 2), agreeing with a previous report by Boylen & Ensign (1968). These results show that there is no significant net hydrolysis of wall or membrane teichoic acid until at least Stage IV of sporulation. Sporulating cells were also pulse-labelled with [^3H]glycerol to measure the rate of turnover of cell teichoic acids. The results of this experiment (Fig. 3) demonstrate that wall teichoic acid synthesis decreases rapidly at the end of exponential growth. The radioactivity incorporated into membrane teichoic acid also began to fall at t_0

but subsequently rose during the period of forespore septation and engulfment. These results therefore suggested that membrane teichoic acid is synthesized during forespore engulfment and some or all of the newly synthesized polymer may be located in the forespore membrane(s) and therefore subsequently in the spore membrane(s).

Extraction of spore teichoic acids

Spores were grown in medium containing [^3H]glycerol, fractionated by published procedures and the teichoic acids were extracted. The spore membranes were prepared in the presence of 10 mM-MgCl₂ under which conditions membrane teichoic acid is not released (Shockman & Slade, 1964). The distribution of glycerol in spore fractions is shown in Table 1. An amount of glycerol equal to 65% of that contained in the spore inner-membrane lipid was extracted from lipid-extracted inner membranes by boiling water. This extraction would solubilize membrane teichoic acid with slight nucleic acid and protein contamination (Coley *et al.*, 1972). Since the membranes were washed before extraction, the

Fig. 2. Incorporation of [^3H]glycerol into wall and membrane teichoic acids during growth and sporulation. Cells of *B. megaterium* KM were grown in 125 ml of CCY medium containing 4 mM-potassium phosphate and [^3H]glycerol (13.6 kBq/ μmol ; 6.6 mM). The culture was incubated at 30°C and shaken at 200 rev./min in a 250 ml Erlenmeyer flask. At intervals 2 ml samples were added to an equal volume of ice-cold 10% (w/v) trichloroacetic acid and after 10 min the cells were recovered by centrifugation (6000 g for 4 min at 4°C). The radioactivity incorporated into wall and membrane teichoic acids was determined as described in the text. ●, A_{600} ; □, radioactivity incorporated into wall teichoic acid; △, radioactivity incorporated into membrane teichoic acid.

Table 1. Distribution of [^3H]glycerol in spore fractions. Spores of *B. megaterium* KM grown in CCY medium containing 4 mM-potassium phosphate and [^3H]glycerol (35.8 kBq/mmol; 6.6 mM) were fractionated as shown in the Table and described in the text.

Fraction	Glycerol (nmol/mg of spores)
Inner-membrane lipid	15
Inner-membrane water extract	10
Inner-membrane NaOH extract	4.8
Integument lipid	1.3
Integument water extract	1.8
Integument NaOH extract	4.4

degree of nucleic acid contamination would be minimized. The presence of substantial amounts of teichoic acid in the spore inner membrane is consis-

tent with the synthesis of membrane teichoic acid previously observed during Stages II and III of sporulation. The amount of membrane teichoic acid extractable from the integuments is <20% of that extractable from the inner membranes. Since extraction of walls with 0.1 M-NaOH solubilizes wall teichoic acid (Archibald, 1972), the data shown in Table 1 suggest that there may be small amounts of wall teichoic acid associated with spore inner membrane and integuments.

To demonstrate more conclusively the presence of spore inner-membrane teichoic acid, the products of acid and alkaline phosphatase hydrolysis of boiling-water-extractable material from lipid-free vegetative and spore inner membranes were separated by paper chromatography. By comparison with authentic standards, both glycerol and glycerol phosphate could be demonstrated in these hydrolysates. It must

Fig. 3. Pulse label of [^3H]glycerol into wall and membrane teichoic acids during growth and sporulation. Culture samples (2.0 ml) of *B. megaterium* cells grown in CCY medium as described for Fig. 1 were pulse labelled at 30°C with [^3H]glycerol (206 kBq/ μmol ; 6.6 mM). After 15 min the radioactivity incorporated into wall and membrane teichoic acids was determined as described in the text. ●, A_{600} ; □, radioactivity incorporated into wall teichoic acid; △, radioactivity incorporated into membrane teichoic acid.

be noted, however, that no estimate has yet been made of the extent to which the membrane teichoic acid of this organism is substituted with fatty acids, glycolipids or phospholipids. Therefore the possibility that not all the ^3H that is incorporated is contained in the glycerol phosphate chain should be kept in mind when interpreting these experiments.

Lipid metabolism during sporulation

Typical results of a [^{32}P]P_i pulse-label experiment into phospholipid during sporulation are shown in Fig. 4. The rate of incorporation of phosphate into phospholipid fell rapidly at the end of exponential growth. During the period of formation of the forespore membranes, when the total amount of lipid phosphorus per cell rose by a factor of 1.7, the specific radioactivity of the phospholipids remained

at <2% of that seen at the end of exponential growth. These results suggest that external phosphate is not used as the source of phosphorus for the synthesis of phospholipid headgroups at this time and suggest the utilization of a pool formed during exponential growth for this synthesis. To test this possibility, vegetative cells were steady-state-labelled with [^{32}P]P_i until the end of exponential growth and then chased by the addition of a 40-fold excess of unlabelled P_i (Fig. 5). During the chase period the specific radioactivity of the phospholipids fell with a half-life of 2.5 h. At the time of synthesis of the forespore membranes, there was, however, no decrease in the half-life of the specific radioactivity of the phospholipid. Thus the results of this experiment confirmed those of the pulse-label experiment.

Fig. 4. $[^{32}\text{P}]\text{P}_i$ pulse label of *B. megaterium* KM phospholipid during growth and sporulation

Culture samples (40 ml) of *B. megaterium* KM growing in CCY medium as described in Fig. 1 were transferred to 250 ml Erlenmeyer flasks, which were incubated at 30°C and shaken at 120 oscillations/min in a reciprocating water bath. After a 15 min pulse with $[^{32}\text{P}]\text{P}_i$ (2.6 kBq/µmol; 4 mM) incorporation was terminated by the addition of 40 ml of ice-cold 40 mM-potassium phosphate buffer, pH 7.0. The cells were recovered by centrifugation (8000 g for 1 min at 4°C), broken by vortex-mixing and the lipid was extracted and purified as described above. ●, A_{600} ; △, lipid phosphorus; □, radioactivity incorporated into lipid phosphorus; ▲, radioactivity/µg of lipid phosphorus.

When sporulating cultures were pulse-labelled with $[^{14}\text{C}]\text{acetate}$ and the specific radioactivity of the purified phospholipids was determined, results similar to those reported by Pitel & Gilvarg (1971) were obtained (Fig. 6). The decline of acetate incorporation into phospholipid at the end of exponential growth was followed by a burst of incorporation that coincided with formation of the forespore membrane. The decrease in specific radioactivity of the phospholipids during exponential growth may arise due to the accumulation of acetate in the growth medium (Nakata & Halvorson, 1960). During sporulation poly- β -hydroxybutyrate synthesized during exponential growth is catabolized to produce acetate, which is then incorporated into spore components (Slepecky & Law, 1961; Nakata, 1966). This production of acetate may explain the 1.5-fold decrease in incorporation of acetate into phospholipid during engulfment.

Discussion

The absence of wall teichoic acids from spores of *B. subtilis* and *B. licheniformis* (Chin *et al.*, 1968; Boylen & Ensign, 1968) has been previously reported. The results reported in the present paper have demonstrated the presence of membrane teichoic acid associated with the forespore and dormant-spore inner membranes. Membrane teichoic acids are present in all Gram-positive bacteria that have been examined. They have less diversity in their structure than wall teichoic acids and their presence is less dependent on growth conditions than wall teichoic acids (Duckworth, 1977). Indeed Ellwood & Tempest (1972) have shown that under conditions of phosphate limitation, when synthesis of wall teichoic acid ceases, membrane teichoic acids continue to be produced. Thus membrane teichoic acids may be essential for membrane function, although their

Fig. 5. $[^{32}\text{P}]\text{P}_i$ steady-state chase of *B. megaterium* KM phospholipid

B. megaterium KM cells were grown in CCY medium containing radioactive phosphate (37MBq/mmol; 1 mM). At t_0 sufficient 4 M-potassium phosphate buffer, pH 7.0, was added to make the phosphate concentration 40 mM. Culture samples (40 ml) were added to 40 ml of ice-cold 40 mM-potassium phosphate buffer, pH 7.0, and the cells were harvested and lipid extracted as described in the text. ●, A_{600} ; Δ , lipid phosphorus; $\square$, radioactivity/ μg of lipid phosphorus.

actual role has not been fully established (Lambert *et al.*, 1977). This may explain why the spore can apparently dispense with wall teichoic acid but retain its membrane teichoic acid. There is evidence that glycerol-containing teichoic acids, the type common to all membrane and some wall teichoic acids, are important in the inhibition of autolysis of peptidoglycan (Holtje & Tomasz, 1975; Cleveland *et al.*, 1976). The membrane teichoic acid present in the spore may have a similar role, particularly during germination, when the cortex peptidoglycan is rapidly degraded to leave the germ-cell wall surrounding the spore protoplast (Hsieh & Vary,

1975). The lack of wall teichoic acid in spores would be consistent with the absence of teichoic acid-synthesizing enzymes in dormant spores (Chin *et al.*, 1968). These enzymes were shown to be synthesized during germination (Chin *et al.*, 1968) and subsequently wall teichoic acid is co-ordinately synthesized with peptidoglycan from the time of outgrowth of the germinating spore (Boylan & Ensign, 1968).

A transient increase of external P_i incorporation into phospholipid has been described during fore-spore septation (Bureau & Mazliak, 1968). In that study a phosphate-limiting medium was used for

Fig. 6. [^{14}C]Acetate pulse label of *B. megaterium* phospholipid during growth and sporulation. *B. megaterium* KM cells growing in CCY medium containing 4 mM-potassium phosphate were pulse labelled as described for Fig. 4 except that the radioactive phosphorus was replaced with 9.25 kBq of [2- ^{14}C]acetate/ml. ●, A_{600} ; △, lipid phosphorus; □, radioactivity incorporated into lipid; ▲, radioactivity/ μg of lipid phosphorus.

sporulation in which cells would be expected to be depleted of phosphate pools when sporulation was induced. Under such conditions teichoic acids would be at least partially replaced by teichuronic acids (Ellwood & Tempest, 1972). In the present study, the phosphate concentration in the medium was at least 10 times the growth-limiting concentration and therefore cell phosphate pools would not be depleted during sporulation.

Turnover of membrane teichoic acid during forespore septation may explain the inability to label the phospholipids synthesized during this process with P_i . Wall and membrane teichoic acids account for 10% and 1.5% respectively of the vegetative cell dry weight (Hay *et al.*, 1963; Lambert *et al.*, 1977) and contain 6% phosphorus by weight (Grant, 1979). The total cell lipid phosphorus content increases by $0.5 \mu\text{g}/\text{mg}$ of cells during Stages II and III of sporulation. Thus net hydrolysis or preferential turnover of a proportion of wall or membrane teichoic acid formed during vegetative growth could provide sufficient phosphorus for this synthesis. The observed membrane teichoic acid turnover during

sporulation is in agreement with the induction of polyglycerol phosphate teichoic acid hydrolase during sporulation (Wise *et al.*, 1972). Kessler & Shockman (1979) have also reported that deacylation of membrane teichoic acid with subsequent release of the deacylated residue from the plasma membrane occurs in *Streptococcus faecium* starved of valine. If a similar deacylation occurs during the transition from vegetative cell to spore, it could make an important contribution to turnover and precursor pools. These results demonstrate that different pathways leading to phospholipid synthesis operate in the sporulating cell.

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