Control of Synthesis of Wall Teichoic Acid during Balanced Growth of Bacillus subtilis W23

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(Received 23 July 1981; revised 23 September 1981)

Enzymes involved in the synthesis of teichoic acid and its linkage to the wall in Bacillus subtilis W23 were measured in chemostat cultures growing at equilibrium at a dilution rate of 0.2 h⁻¹ in different concentrations of inorganic phosphate. All the enzymes, except teichoic acid glucosyl transferase, which was insensitive to changes in phosphate concentration, were almost undetectable at 0.5 mM-phosphate. At higher phosphate concentrations the changes in activity of the enzymes of linkage unit synthesis were sufficient to account for the changes in the rate of incorporation of teichoic acid into the wall in vivo. Between 3.5 and 4.5 mM-phosphate the amount of teichoic acid synthesized in vivo increased, but no increase in the ability of toluenized bacteria to synthesize teichoic acid could be detected. Allosteric regulation might therefore be important at high phosphate concentrations. Bacteria maintained a constant ATP content and a constant adenylate energy charge during chemostat growth at all phosphate concentrations.

INTRODUCTION

The cell walls of Bacillus subtilis W23 contain β-glucosyl poly(ribitol phosphate) attached to peptidoglycan by a linkage unit of three glycerol phosphate residues and one N-acetylglucosamine 1-phosphate (Coley et al., 1978). CDPglycerol and UDP-N-acetylglucosamine are the precursors of linkage unit, which is built up on a lipid carrier before accepting poly(ribitol phosphate) chains synthesized from CDPribitol (Hancock & Baddiley, 1976; Hancock et al., 1976, 1981; Wyke & Ward, 1977). Little is known of the control of teichoic acid synthesis, but the activity of CDPglycerol pyrophosphorylase, the enzyme responsible for the synthesis of CDPglycerol, and the activity of CDPribitol:teichoic acid phosphoribitol transferase (polymerase) are regulated in B. subtilis W23 (Cheah et al., 1981).

Previous studies of the control of teichoic acid synthesis in Bacillus species have exploited the inhibition that occurs when cultures become phosphate-limited in a chemostat (Ellwood & Tempest, 1972) or starved of phosphate in batch culture. This inhibition is accompanied by an increase in the synthesis of teichuronic acid. Phosphate-starved B. subtilis W23 is unable to synthesize linkage unit and lacks the enzyme responsible for the synthesis of lipid I, the first lipid intermediate in the pathway (Glaser & Loewy, 1979), although it is not known whether this is the only point of inhibition. CDPglycerol pyrophosphorylase is also less active in these bacteria. Either of these changes might be sufficient to prevent synthesis of wall teichoic acid, which is dependent on linkage unit synthesis (Hancock, 1981).

These studies with B. subtilis W23 suffer from the disadvantage that the phosphate-starved bacteria cannot grow and they give little indication of which enzymes are responsible for regulation of teichoic acid synthesis in growing cultures. We have therefore studied the pathway in chemostat cultures in which the bacteria are in balanced growth. In these cultures...
METHODS

Materials. D-[U-14C]Glycerol 1-phosphate, ammonium salt, [U-14C]cytidine triphosphate, ammonium salt, [U-14C]glucose 1-phosphate, potassium salt and potassium borohydride were purchased from Amersham. [1-1H]Ribitol 5-phosphate was prepared by reduction of D-ribose 5-phosphate with potassium borohydride. CDP[1-3H]ribitol was synthesized enzymically from [1-3H]ribitol 5-phosphate and CTP using a crude CDPribitol pyrophosphorylase preparation from Staphylococcus aureus (Shaw, 1962). UDP[U-14C]glucose was prepared enzymically from [U-14C]glucose 1-phosphate and UTP using a commercial preparation of UDPglucose pyrophosphorylase. The preparation of other substrates has been described previously (Hancock 1976). ZnCl₂, 0.061 g; CuCl₂·2H₂O, 0.0153 g; CoCl₂·6H₂O, 0.0215 g; Na₂MoO₄·2H₂O, 0.215 g; K₂SO₄, 4.7 g; MgSO₄·7H₂O, 4.5 g; CaCl₂·6H₂O, 0.39 g; FeCl₃·6H₂O, 0.485 g; MnCl₂·4H₂O, 0.09 g; KCl, 0.061 g; CuCl₂·2H₂O, 0.0153 g; CoCl₂·6H₂O, 0.0215 g; Na₂MoO₄·2H₂O, 0.215 g; K₂SO₄, 4.7 g; 18 litres distilled water; 3% (w/v) sodium dodecyl sulphate. Toluenized cells contained about 25 mg dry wt membrane (1 culture)⁻¹ (unit A₅₄₀)⁻¹. Membranes were prepared by the method of Cheah et al. (1981) or by a modification of the method of Konings et al. (1973) as follows. Bacteria (about 15 g wet wt) were suspended in 100 ml potassium phosphate buffer (0.05 M-phosphate, pH 6.0) at 37 °C and incubated with lysozyme (500 µg ml⁻¹) at 37 °C for 15 min. Then 1.0 m-MgSO₄ was added to give a final concentration of 10 mM and incubation was continued for 30 min. Radioactivity in lipid I was measured after paper chromatography as described previously for other lipid intermediates (Hancock et al., 1981). Lipid I was the only radioactive lipid formed under these conditions and had an Rₚ of 0.86 on paper chromatography.

Culture techniques. The equipment used for continuous culture was an L.H. Engineering fermenter (type LHE 1/1000) of 5 litre capacity. This was inoculated and operated as described previously except that the culture volume was 2 litres (Hussey et al., 1978). The medium had the following composition: (NH₄)₂SO₄, 118.8 g; citric acid·H₂O, 3.78 g; MgCl₂·6H₂O, 4.5 g; CaCl₂·6H₂O, 0.39 g; FeCl₃·6H₂O, 0.485 g; MnCl₂·4H₂O, 0.09 g; ZnCl₂, 0.061 g; CuCl₂·2H₂O, 0.0153 g; CoCl₂·6H₂O, 0.0215 g; Na₂MoO₄·2H₂O, 0.215 g; K₂SO₄, 4.7 g; 18 litres distilled water; 3% (w/v) glucose. Phosphate was added as NaH₂PO₄·2H₂O to give concentrations between 0.5 and 5.0 mM.

The chemostat was not reinoculated each time the phosphate concentration was changed but was allowed to equilibrate for 72 h at each concentration before samples were taken. The dilution rate remained at 0.2 h⁻¹ at all phosphate concentrations. Potassium became limiting at high phosphate concentrations (Anderson, A. J. et al., 1978).

Enzyme determination. CDPglycerol pyrophosphorylase (EC 2.7.7.39), CDPribitol pyrophosphorylase (EC 2.7.7.40) and poly(ribitol phosphate) polymerase were determined as described previously (Cheah et al., 1981). Linkage unit synthesis was measured in toluenized cells as described by Hancock (1981). The results represent glycerol phosphate residues in linkage unit lipid intermediates and linkage unit transferred to the wall in the absence of teichoic acid synthesis. They have been corrected for the formation of phosphatidylglycerol, which is independent of the presence of UDP-N-acetylglucosamine (Hancock et al., 1981). Glucosyltransferase (EC 2.4.1.53) was measured in membrane preparations made as described below. Reaction mixtures contained 0.1 ml of membrane suspension, 10 mM MgSO₄ and 0.2 mM UDP[U-14C]glucose (0.08 µCi, 3.0 kBq) with and without poly(ribitol phosphate) (1.77 µmol P) extracted by alkali (Hughes & Tanner, 1968) from walls of a mutant of B. subtilis W23 which does not glucosylate its teichoic acid, kindly provided by Professor A. R. Archibald. Incubation was carried out at 30 °C for 5 min and incorporation into polymer was measured by paper chromatography as described for the determination of poly(ribitol phosphate) (Cheah et al., 1981). The synthesis of lipid I, the first lipid intermediate in linkage unit synthesis, was measured using membranes isolated as described below. Reaction mixtures contained 0.1 ml of membrane suspension, 75 nmol UDP-N-14Clacetilglucosamine (0.08 µCi, 3.0 kBq) and 2 µmol MgSO₄ in a total volume of 0.12 ml. They were incubated at 30 °C for 30 min, and reaction was then stopped by heating the mixtures to 80 °C for 5 min. Radioactivity in lipid I was measured after paper chromatography as described previously for other lipid intermediates (Hancock et al., 1981). Lipid I was the only radioactive lipid formed under these conditions and had an Rₚ of 0.86 on paper chromatography.

Enzyme preparations. Cells were toluenized for CDPglycerol pyrophosphorylase and CDPribitol pyrophosphorylase determinations as described by Cheah et al. (1981). Toluenization of bacteria for linkage unit investigation was by the method of Hancock (1981). In these toluenized cells at least 80% of the teichoic acid synthesized with linkage unit attached became bound to the cell wall as indicated by insolubility in boiling 4% (w/v) sodium dodecyl sulphate. Toluenedi cells contained about 25 mg dry wt membrane (1 culture)⁻¹ (unit A₅₄₀)⁻¹. Membranes were prepared by the method of Cheah et al. (1981) or by a modification of the method of Konings et al. (1973) as follows. Bacteria (about 15 g wet wt) were suspended in 100 ml potassium phosphate buffer (0.05 m-phosphate, pH 8.0) at 37 °C and incubated with lysozyme (500 µg ml⁻¹) at 37 °C for 15 min. Then 1.0 m-MgSO₄ was added to give a final concentration of 10 mM and incubation was continued for 30 min. Next, 0.3 m-Na₂EDTA was added to give a final concentration of 15 mM and the suspension was incubated for a further 15 min. Finally, 0.05 ml 2-mercaptoproethanol was added and the suspension was centrifuged twice for 5 min at 5000 g and 4 °C to remove unlysed cells (less than 1% of the original cell wet weight). The supernatant was centrifuged at 48000 g (maximum) for 20 min and the membrane pellet was washed once in 100 ml
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0.05 M-phosphate buffer, pH 8.0, containing 5 mM-2-thioethanol. The membranes were suspended in the same buffer at about 25 mg dry wt ml⁻¹ and kept frozen at −70 °C.

Preparation of bacterial cell walls. Samples for the preparation of cell walls were collected from the chemostat overflow, chilled on ice and the cells were then harvested by centrifugation at 15000 g for 5 min at 4 °C. After resuspension in water to give a 50% (wt/wt) suspension, the cells were broken and walls were prepared as previously described except that the trypsin treatment was omitted (Hussey et al., 1978).

Determination of adenine nucleotides. Adenine nucleotides were determined using the luciferin–luciferase enzyme system. Light intensity was measured in a Packard Tricarb liquid scintillation counter (model 3385) set for counting tritium.

Cell extracts were prepared by trichloroacetic acid extraction as follows. Culture samples were removed from the sampling port of the chemostat. A 1 ml portion of each sample was mixed with 1.2 ml 0.51 M-trichloroacetic acid containing 1-25 mM-EDTA at 0 °C. Sampling and mixing were completed within 15 s. After standing at 0 °C for 15 min, insoluble material was removed by centrifugation at 17000 g for 10 min at 4 °C. Trichloroacetic acid was removed by repeated extraction with water-saturated ether and residual ether was removed by bubbling with water-saturated air. Extracts were kept at −20 °C until used. For ATP determination, 100 μl of diluted cell extract was added to 50 μl 0.02 M-Tris/H₂SO₄ buffer, pH 7-7.5, containing 0.18 μmol K₂SO₄ and 0.06 μmol magnesium acetate. For ATP + ADP determination, 100 μl of diluted cell extract was added to 50 μl 0.02 M-Tris/H₂SO₄ buffer, pH 7-7.5, containing 0.18 μmol K₂SO₄, 0.06 μmol magnesium acetate, 0.1 μmol phosphoenolpyruvate and 2 units of pyruvate kinase. A similar mixture with the addition of 2 units of adenylyl kinase was used for determination of total adenylates. The mixtures were incubated for 15 min at 30 °C and the kinases were then inactivated by heating at 100 °C for 30 s. After centrifugation the solutions were cooled to 0 °C. Then 100 μmol from each incubated sample was added to 850 μl 0.02 M-Tris/H₂SO₄ buffer, pH 7-7.5, containing 0.06 pmol magnesium acetate and 50 μl of firefly lantern extract was added. After thorough mixing the sample was loaded into the scintillation counter, and 10 s after the addition of firefly lantern extract counting was started and continued for 30 s. Standard curves were obtained using appropriate nucleotide solutions treated in the same way as the extracts. The count recorded was proportional to the square of ATP content over the range 0-20 pmol. Internal standardization was carried out by determining an additional known amount of appropriate nucleotide added to the incubation mixtures above. All determinations were made in triplicate.

RESULTS

Figure 1 shows the amounts of teichoic acid and teichuronic acid in the walls of bacteria growing at equilibrium with different concentrations of phosphate in the medium. As reported by Anderson, A. J. et al. (1978), the amount of teichoic acid increased with increasing medium phosphate even when the culture density was no longer significantly affected. The chain length of the teichoic acid was constant under these conditions (A. R. Archibald, personal communication). The ability of tolenes billionaire bacteria to synthesize teichoic acid attached to its linkage unit, from exogenous precursors, and incorporate it into their walls roughly paralleled the amount of teichoic acid in the walls up to 3.5 mm-phosphate. The enzymatic activity in tolenes billionaire cells thus appeared to be regulated in the same way as in vivo synthesis of teichoic acid, under the conditions of the assay with saturating concentrations of substrates. At higher phosphate concentrations this was not the case, and additional factors must have affected the regulation in vivo.
Fig. 1. Variation of cell wall anionic polymers and teichoic acid synthetic ability in *B. subtilis* W23 grown under balanced conditions at various medium phosphate concentrations. Bacteria were grown in continuous culture in media containing 3.0 mM-potassium and 0.5-5.0 mM-phosphate at a dilution rate of 0.2 h⁻¹. Linkage unit-dependent poly(ribitol phosphate) synthesis (△) was measured in toluenized cells prepared by the method of Hancock (1981). Cell walls were prepared as described in Methods and examined for phosphorus (◇) and glucuronic acid (■). Culture density (○).

Fig. 2. Variation of the enzymes of polymer and linkage unit synthesis under balanced conditions at various medium phosphate concentrations. Bacteria were grown as described for Fig. 1. Poly(ribitol phosphate) polymerase (△), glucosyl transferase (◇) and synthesis of lipid intermediate I of linkage unit synthesis (○) were measured in membranes prepared by the modified method of Konings et al. (1973). Linkage unit synthesis (■) was measured in toluenized cells (Hancock, 1981).
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Fig. 3. Variation of CDPglycerol pyrophosphorylase, CDPribitol pyrophosphorylase and poly(ribitol phosphate) polymerase activities in \textit{B. subtilis} W23 grown under balanced conditions at various medium phosphate concentrations. Poly(ribitol phosphate) polymerase activity (\(\Delta\)) was measured in membranes prepared by the method of Cheah \textit{et al.} (1981). CDPribitol pyrophosphorylase (\(\bullet\)) and CDPglycerol pyrophosphorylase (\(\square\)) were measured in toluenized cells. Culture density (\(\circ\)).

Activities of individual enzymes of teichoic acid synthesis

In toluenized bacteria, synthesis of teichoic acid from exogenous CDPribitol was dependent on the concomitant synthesis of linkage unit (Hancock, 1981). The overall synthesis shown in Fig. 1 might therefore have been regulated by either the polymerase or the enzymes of linkage unit synthesis. Polymerase, synthesis of lipid intermediate I of linkage unit synthesis, and formation of glycerol phosphate-containing linkage unit intermediates were measured separately (Fig. 2). None of these activities varied with medium phosphate concentration in the same way as overall teichoic acid synthesis. Bacteria grown at 2.5 mM-phosphate contained less than 25\% of their maximum amount of teichoic acid and displayed about 6\% of the maximum capacity for overall teichoic acid synthesis, even though polymerase activity was at its maximum. In contrast, the activity of linkage unit synthesis was considerably higher in bacteria grown at 4.5 mM-phosphate than in those grown at 3.5 mM-phosphate although the overall activity of teichoic acid synthesis had reached its maximum at 3.5 mM-phosphate. It therefore appeared that polymerase was present in excess at intermediate phosphate concentrations and that under these conditions the rate of linkage unit synthesis or the availability of CDPribitol, the substrate for the polymerase, might control the overall rate of teichoic acid synthesis.

The activity of the glucosyl transferase that catalyses the glucosylation of poly(ribitol phosphate) was also examined (Fig. 2). In toluenized bacteria the enzyme glucosylated endogenous poly(ribitol phosphate) (Hancock, 1981) and therefore could not be measured in bacteria that contained only small amounts of \(\varepsilon\)-teichoic acid. Instead, the ability of isolated membranes to glucosylate externally added poly(ribitol phosphate) was measured. The enzyme was very active in bacteria from all concentrations of medium phosphate and therefore was not regulated by phosphate like the other enzymes of the pathway.

Activities of alditol nucleotide pyrophosphorylases

The assays described above were all carried out at saturating substrate concentrations. The actual intracellular concentrations of CDPglycerol and CDPribitol are not known. We
therefore measured the activities of the enzymes catalysing their synthesis, CDPglycerol pyrophosphorylase and CDPribitol pyrophosphorylase, in an experiment similar to that described above. In this case the culture ceased to be phosphate-limited at a lower phosphate concentration and polymerase activity appeared at a correspondingly lower phosphate concentration (Fig. 3). Like the polymerase, CDPribitol pyrophosphorylase was detectable at low phosphate concentrations although it reached its maximum level at a higher phosphate concentration than did the polymerase. On the other hand, CDPglycerol pyrophosphorylase only became detectable at 2 mM-phosphate when the polymerase was already at its maximum level. Thus, synthesis of CDPglycerol, necessary for linkage unit synthesis, appeared to be regulated in a similar manner to the enzymes of linkage unit assembly shown in Fig. 2.

Energy charge

The ability to synthesize alditol nucleotides would depend not only on the activities of the pyrophosphorylases but also on the energy state of the bacteria and hence the availability of nucleoside triphosphates. The intracellular ATP content remained constant in bacteria growing at phosphate concentrations between 0.5 and 4.5 mM, at about 1.28 nmol (ml culture)⁻¹ (unit A₆₀₀)⁻¹. The energy charge was maintained at between 0.81 and 0.88 throughout this range.

DISCUSSION

Our results give some indication of the complexity of the regulatory mechanisms for anionic wall polymer synthesis. With the exception of the teichoic acid glucosyl transferase, all the enzymes of teichoic acid synthesis that we have been able to measure were sensitive to the concentration of inorganic phosphate in the growth medium. They did not, however, undergo coordinate derepression as the phosphate concentration was raised. While all the sensitive enzymes were almost undetectable during severely phosphate-limited growth, polymerase and CDPribitol pyrophosphorylase activities were present in phosphate-limited bacteria growing at higher concentrations of phosphate. The polymerase was close to its maximum activity and the pyrophosphorylase had 60% of maximum activity at the highest phosphate concentration that still limited growth. These enzymes therefore appeared to be present in excess during phosphate limitation since the walls of bacteria grown at 2.5 mM-phosphate, which contained the maximum amount of polymerase, contained less than 25% of the maximum amount of teichoic acid. It is possible that measurement of wall teichoic acid underestimated the synthetic capacity of the bacteria. Recently, de Boer et al. (1981) reported excretion of unlinked teichoic acid from B. subtilis in chemostat cultures. However, in our toluenized cells teichoic acid synthesis was equally dependent on linkage unit synthesis and attachment to wall at low and high phosphate concentrations. At low phosphate concentrations the rate of incorporation of teichoic acid into the wall was limited by the low activities of the enzymes responsible for linkage unit synthesis. These enzymes only appeared in substantial amounts in bacteria grown at phosphate concentrations about 2 mM and thereafter their activities rose with phosphate concentration in a similar manner to the proportion of teichoic acid found in the walls. The quantitative differences in the appearance of lipid I synthesis and linkage unit synthesis suggested that glycerol phosphate incorporation was also regulated. CDPglycerol pyrophosphorylase behaved in a similar way and thus appeared to be regulated as an enzyme of linkage unit synthesis. This is the only role known for the enzyme in B. subtilis W23. Between 3.5 and 4.5 mM-phosphate, the activities of the linkage unit enzymes increased (Fig. 2), but the ability of toluenized bacteria to synthesize teichoic acid did not (Fig. 1). It is possible that the activity of the polymerase became the rate-limiting factor in these potassium-limited bacteria, but the proportion of teichoic acid in the walls increased over this range of phosphate concentration (Fig. 1).

We conclude that, while all the enzymes of teichoic acid synthesis except glucosyl transferase are sensitive to the concentration of phosphate in the medium, regulation of
linkage unit synthesis is sufficient to account for the control of teichoic acid synthesis during phosphate-limited growth. Presumably the regulation is mediated by intracellular inorganic phosphate or by a phosphorylated metabolite. It is not known if the intracellular concentration of inorganic phosphate is directly proportional to the concentration in the culture supernatant in these cultures. At the highest phosphate concentration other factors must play a part, in a manner that is not expressed in toluenized cells. The allosteric regulation of one or more of the enzymes by a low molecular weight compound would fulfill such a role. CDPglycerol pyrophosphorylase is known to be allosterically regulated in *Bacillus licheniformis* (Anderson, R. G. *et al.*, 1973).

Since our measurements of teichoic acid synthesis in toluenized cells were carried out in the absence of peptidoglycan synthesis, the teichoic acid must have become linked to endogenous attachment sites in the wall or membrane (Hancock, 1981). It is possible that these sites became saturated during assay of teichoic acid synthesis in toluenized cells from the culture with the highest concentration of phosphate, thus preventing detection of an increase in the biosynthetic capacity of the bacteria. However, no such saturation has been detected; toluenized bacteria from all cultures synthesized teichoic acid approximately linearly for 30 min.

This work was supported by the Science Research Council. S.-C. Cheah was supported by the Commonwealth Scholarship Commission.

**REFERENCES**


