CONTROL OF TEICHURONIC ACID SYNTHESIS IN *BACILLUS LICHENIFORMIS* ATCC 9945

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1. Introduction

Biosynthesis, and more specifically, regulatory control of the anionic polymers teichoic and teichuronic acid, in bacterial cell walls has provided an area of continued interest. The scheme whereby regulation could be achieved through the intermediates of one pathway repressing the synthesis of enzymes of the other [1] was questioned when glycerol containing intermediates, unique to teichoic acid, was reported not to repress the synthesis of teichuronic acid [2]. That such intermediates could modulate enzyme activity was, however, conceded.

A chemostat investigation of the teichoic acid-synthesising enzymes in *Bacillus licheniformis* presented evidence for the rapid inactivation of CDP glycerol pyrophosphorylase during changeover from non-phosphate to phosphate limited growth [3]. The mechanism of inactivation was not investigated, but was shown to be independent of new protein synthesis. In [4] cessation of teichoic acid synthesis in phosphate limited cells of *Bacillus subtilis* was reported not to be effected through a loss of CDP glycerol pyrophosphorylase activity. And, because new protein synthesis was required, an involvement of one of the teichuronic acid-synthesising enzymes in teichoic acid disappearance was tentatively assumed [5]. These observations are indicative of a difference in the polymer control mechanism in the two organisms. Here, we describe the behaviour of the teichuronic acid-synthesising enzymes in *B. licheniformis* during transitions from phosphate and non-phosphate limited growth and provide an insight into the mode of polymer control.

2. Materials and methods

2.1. *Culture techniques*

Procedures for the growth of *Bacillus licheniformis* ATCC 9945 in batch and continuous cultures have been described [3].

2.2. *Cell wall preparation and analyses*

Cells were disrupted by sonication and walls prepared as in [6]. Glucuronic acid was determined by the method in [7] and phosphate as in [8].

2.3. *Enzyme determination*

Alkaline phosphatase, UDP glucose dehydrogenase and UDP *N*-acetylglucosamine 4-epimerase were determined on lysozyme-treated cells. Chemostat samples (2 ml) were incubated with 0.2 ml Tris–HCl buffer (50 mM, pH 8.0) containing DTT (5 mM), lysozyme (1 mg/ml), DNase (50 μg/ml) and RNase (50 μg/ml) at 37°C for 30 min. Batch grown cells (10 ml) were treated with 0.1 ml of the above mixture.

Alkaline phosphatase was measured as in [3] with a 10 min incubation period.

Determination of UDP glucose dehydrogenase was based on the method in [9]. Mixtures contained 4 mM UDP glucose, 0.09 μCi UDP–[U-^{14}C]glucose (303 μCi/μmol), 4 mM NAD, 4 mM magnesium acetate and 40 μl enzyme preparation in 80 μl total vol. Mixtures were incubated for 15 min at 37°C. UDP glucose dehydrogenase activity was located in the cytoplasmic fraction.

A radiochemical method for the determination of UDP *N*-acetylglucosamine 4-epimerase was developed from the colorimetric method in [10]. Reaction mixtures contained 1 mM UDP *N*-acetylglucosamine,

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0.2 μCi UDP*N*-[*acetyl*-^{14}C]acetylglucosamine (59 μCi/μmol), 2.5 mM $MgCl_2$, 25 mM glycine (pH 8.6) and 5 μl enzyme preparation in 60 μl total vol. After incubation at 37°C for 10 min the reaction was terminated by adding 15 μl 0.5 M HCl and heating at 100°C for 15 min. Inactivated reaction mixtures were applied to borate-treated (0.2 M $Na_2B_4O_7$, pH 8.0) chromatography paper (Whatman no. 1) and the acetylated amino sugars separated by development in *n*-butanol: pyridine:H_2O (6:4:3, by vol.).

Teichuronic acid synthetase was determined using toluenised cells. Chemostat samples (2.5 ml), in 0.5 ml Tris–HCl buffer (50 mM, pH 8.0) containing 20 mM magnesium acetate and 5 mM DTT were mixed with 25 μl toluene for 30 min at 4°C, centrifuged, washed and resuspended in the same buffer. Batch grown cells (10 ml) were treated with 0.1 ml buffer and 10 μl toluene for 30 min at 20°C. Incubation mixtures contained 0.4 mM UDP glucuronic acid, 0.1 μCi UDP-[U-^{14}C]glucuronic acid (307 mCi/mmol), 0.4 mM UDP*N*-acetylglucosamine, 20 mM magnesium acetate, 5 mM DTT, 50 mM Tris–HCl buffer (pH 8.0) and 40 μl enzyme preparation in 0.1 ml total vol. After incubation for 60 min at 20°C the reaction was terminated by heating at 100°C for 2 min. Polymer was separated on Whatman no. 1 chromatography paper in 0.5 M ammonium acetate (pH 3.8):ethanol (5:2, v/v) and located on the origin. Polymer was identified after acid hydrolysis (4 M HCl for 4 h at 100°C) and comparison of the hydrolysis products with those from authentic teichuronic acid. Sugars were located with silver nitrate [11].

One unit of enzyme activity is defined as that required to produce either 1 nmol (UDP glucose dehydrogenase and teichuronic acid synthetase) or 1 μmol (alkaline phosphatase) of enzyme product under standard assay conditions. Protein was determined with the Coomassie blue reagent [12].

2.4. *Radiochemical detection*

Radioactive material from paper chromatograms was counted in a scintillation counter (Packard Tricarb, model 3255) in liquid scintillant containing 4 g 2,5-diphenyloxazole and 0.1 g 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene/l toluene.

2.5. *Determination of component washout in the chemostat*

The theoretical curve for washout was calculated from the formula $X_t\ X_0 = e^{-Dt}$, where X_0 and X_t are the component concentrations initially and after time t, and D is the dilution rate.

2.6. *Materials*

UDP–D-[U-^{14}C] glucuronic acid and [^{14}C]glucose-1-phosphate were purchased from The Radiochemical Centre (Amersham, Bucks). UDP*N*-[*acetyl*-^{14}C]acetylglucosamine was prepared as in [13]. UDP–D-[^{14}C]-glucose was prepared enzymically from [^{14}C]glucose-1-phosphate. [^{14}C]Glucose-1-phosphate (48.4 μCi; 3 μCi/μmol) and UTP (13 mg) in 4 ml Tris–HCl buffer (50 mM, pH 8.0) containing magnesium acetate (2 mM) were incubated with UDP glucose pyrophosphorylase/inorganic pyrophosphatase from yeast (Sigma Chemical Co., St Louis MO) for 3 h at 25°C. The product was purified by chromatography on Whatman 3 MM paper in ethanol:1 M ammonium acetate, pH 7.5 (5:2, v/v).

3. Results and discussion

Adaptation of *B. licheniformis* to phosphate limiting conditions, following growth on medium rich in phosphate, is accompanied by variations in the anionic polymer content of the wall. These involve the loss of teichoic acid and an increase in teichuronic

Table 1
Steady state[a] levels of teichuronic acid synthesising enzymes in *B. licheniformis*

Enzyme	Spec. act. (nmol . min^{-1} . mg protein^{-1})	
	Glucose-limited	PO_4^{3-}-limited
UDP–Glucose dehydrogenase	5.0	10.6
UDP–*N*-acetylglucosamine 4-epimerase	28.1	21.9
Teichuronic acid synthetase	1.8×10^{-2}	4.1×10^{-2}

[a] Cells were grown at a dilution rate of 0.2 h^{-1}

acid [3]. The biosynthesis of teichuronic acid, a polymer of *N*-acetylglucosamine and glucuronic acid [14], requires UDP-*N*-acetylglucosamine 4-epimerase and UDP-glucose dehydrogenase as well as the polymerising activity of teichuronic acid synthetase. Examination of the levels of these 3 enzymes in cells derived from glucose and phosphate-limited chemostat cultures revealed significant variations in the activities of the dehydrogenase and synthetase only (table 1). In an attempt to elucidate the mechanism(s) involved in the regulation of anionic polymer biosynthesis, the adaptation process has been studied in transient state cultures and the results are now described.

During the change from glucose to phosphate limitation, the earliest discernable biochemical change consistently occurred between 2–3 h, when the level of teichuronic acid synthetase fell to <50% of its initial steady state value (fig.1). The converse effect on teichoic acid biosynthesis has been reported in [3]. At this stage in the chemostat, the culture has been released from glucose limitation by the incoming phosphate-poor medium, but has not yet become phosphate limited because of residual phosphate from the initial medium remaining in the culture vessel [3]. The nature of the limitation at this stage is not known, but might be oxygen supply. Minimal activity coincides with maximal activity of poly(glucosylglycerol phosphate) synthetase. As reported in [3] teichuronic acid (as expressed by glucuronic acid) in the wall also decreased drastically, again to <50% the initial level, within the 2 h period commencing 2 h after medium changeover. UDP–glucose dehydrogenase was not subject to these dramatic variations during the early stages of adaptation. Both teichuronic acid synthesising enzymes started to increase 3 h after the medium change attaining steady levels after 7 h; CDP–glycerol pyrophosphorylase and poly(glucosylglycerol phosphate) synthetase activities began to decline at 3 h while by 7 h CDP–glycerol pyrophosphorylase activity was completely lost [3].

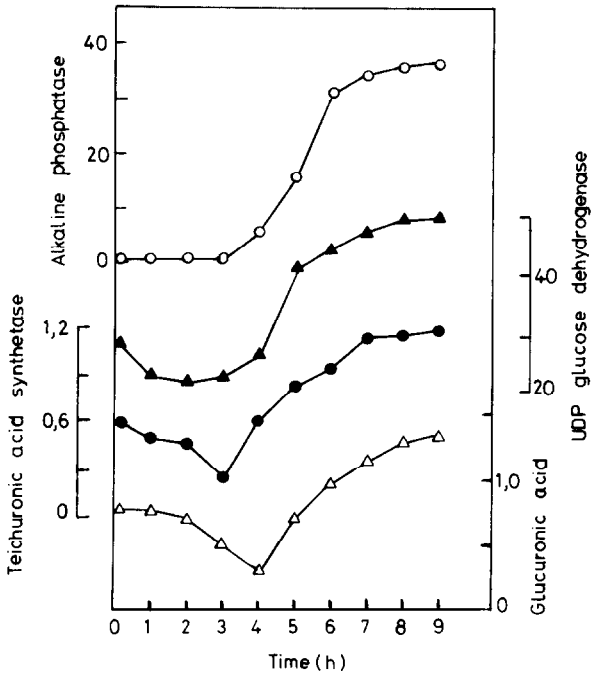


Fig.1. Changes in wall glucuronic acid and teichuronic acid-synthesising enzymes during transition from glucose- to phosphate-limited growth. Cells were grown at a dilution rate of 0.2 h^{-1} and collected for 30 min periods for wall analysis: (△) glucuronic acid; (●) teichuronic acid synthetase; (▲) UDP glucose dehydrogenase; (○) alkaline phosphatase. Enzymes quoted as units/mg cell dry wt; glucuronic acid as μmol/mg wall dry wt.

When glucose-limited cells were taken from the chemostat and grown in batch culture on a phosphate-poor medium, similar changes to those occurring after medium switch-over in the chemostat were observed (fig.2) but in this case the transient release from glucose and phosphate limitation was much less marked due to the low concentration of phosphate in the new medium. The bacteria responded to phosphate starvation within 30 min of transfer. These changes were prevented by chloramphenicol.

The biochemical changes accompanying the transition from phosphate to glucose limitation are shown in fig.3. Comparison of fig.1 and 3 suggests that the transition from phosphate-limited growth is less complex in terms of membrane and wall reorganisation than that to phosphate-limited growth. Within 3 h of the change from phosphate to glucose limiting medium 90% of teichuronic acid synthetase activity was lost. Over the same period teichuronic acid decreased by 75% while the level of teichoic acid (as expressed by phosphorus) increased correspondingly. The decline in synthetase activity exceeded the theoretical washout rate. Synthetase activity fell rapidly, also, upon transfer of phosphate-limited cells to batch culture on medium rich in phosphate and this occurred even in the presence of chloramphenicol (fig.4).

A requirement for de novo protein synthesis for the onset of teichoic acid synthesis in phosphate-rich cells of *B. licheniformis* ATCC 9945 was demonstrated

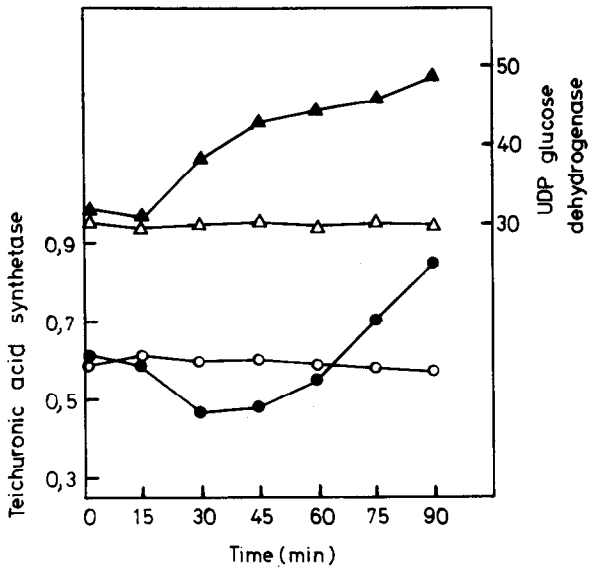
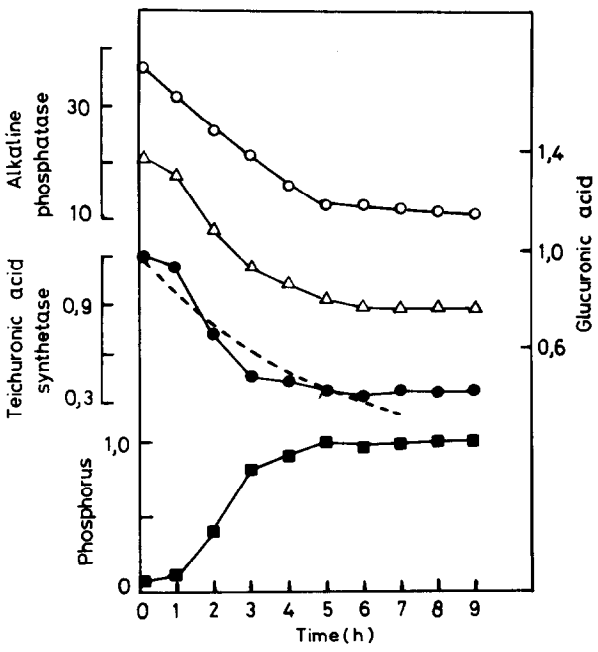


Fig.2. Changes in teichuronic acid synthesising enzymes following transfer of glucose-limited cells to phosphate-poor medium; the effect of chloramphenicol. Chemostat culture (25 ml), harvested by centrifugation and resuspended in prewarmed, low phosphate medium was transferred to 250 ml of the same medium. Culture growth continued in batch. Chloramphenicol (50 μg/ml) was added to medium prior to cell transfer. (●,○) Teichuronic acid synthetase; (▲,△) UDP glucose dehydrogenase; (closed symbols) without chloramphenicol; (open symbols) with chloramphenicol. Enzymes quoted as units/mg cell dry wt.



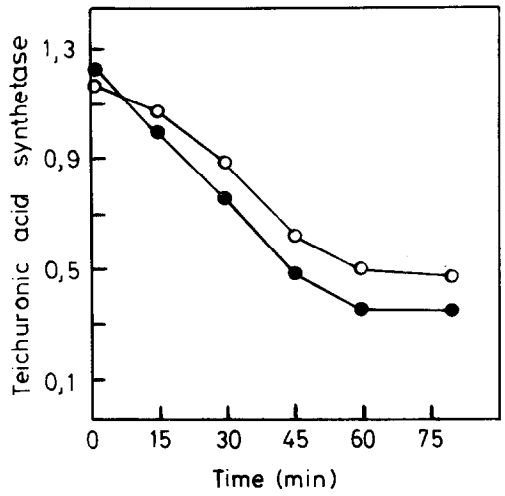


Fig.4. Change in teichuronic acid synthetase activity following transfer of phosphate-limited cells to phosphate-rich medium; the effect of chloramphenicol. Sampling, with the omission of the centrifugation step and other procedures as in fig.2; (closed symbols) without chloramphenicol; (open symbols) with chloramphenicol. Enzyme activity quoted as units/mg cell dry wt.

in [3]. The disappearance of teichoic acid in phosphate-limited cells, however, as manifest in CDP–glycerol pyrophosphorylase levels was found to be independent of new protein synthesis. The results presented herein, in conjunction with these earlier observations, show de novo protein synthesis to be essential for an increase in the enzymic potential associated with one anionic polymer but not the simultaneous reduction in that of the other, thus rendering any intimate relationship between the corresponding biosynthetic pathways unlikely. Moreover, the rapid decline in the activities of the soluble enzyme CDP–glycerol pyrophosphorylase and of the membrane-bound synthetases that catalyse the polymerisation of teichoic acid and teichuronic acid points to a degradative mechanism and has been intimated [3] the intracellular P_i levels, which vary considerably with changing phosphate supply may be important. We envisage a

Fig.3. Changes in wall phosphorus, glucuronic acid and teichuronic acid synthetase activity during transition from phosphate to glucose limited growth. Dilution rate and sampling as in fig.1. (■) Phosphorus; (– – –) theoretical washout rate; other symbols as in fig.1. Enzymes quoted as units/mg cell dry wt; wall components as μmol/mg wall dry wt.

conformational change in the polymer synthesising enzymes occurring in response to altered phosphate levels, thus rendering them susceptible to proteolytic degradation. Such a mechanism would be dependent upon the availability of pre-existent proteinase. Intracellular proteinase with broad specificity is known to be present in *B. licheniformis* [15] but its precise physiological role has not been determined. It is of interest, however, that very little of the potential activity is expressed in vivo and that denatured over native protein is the preferred substrate. Proteinases have already been implicated in the control of fungal cell wall biosynthesis [16–18].

Finally, in the light of the above considerations and because of the ubiquitous presence of teichuronic acid in walls of *B. licheniformis* grown under a variety of conditions, it is pertinent to raise the question of whether 2 forms of teichuronic acid synthetase exist, each of whose activity is regulated independently of the other.

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