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Control of Teichoic Acid and Teichuronic Acid Biosyntheses in Chemostat Cultures of *Bacillus subtilis* var. *niger*

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1. Quantitative determination of the anionic polymers present in the walls of *Bacillus subtilis* var. *niger* organisms undergoing transition, in a chemostat culture, from either Mg^{2+}-limitation to PO_4^{3-}-limitation or K^+-limitation to PO_4^{3-}-limitation showed that teichuronic acid synthesis started immediately the culture became PO_4^{3-}-limited and proceeded at a rate substantially faster than the rate of biomass synthesis. 2. Simultaneously, the cell-wall teichoic acid content diminished at a rate greater than that due to dilution by newly synthesized wall material, and fragments of teichoic acid and mucopeptide accumulated in the culture extracellular fluid. 3. Equally rapid reverse changes occurred when a PO_4^{3-}-limited *B. subtilis* var. *niger* culture was returned to being Mg^{2+}-limited. 4. It is concluded that in this organism both teichoic acid and teichuronic acid syntheses are expressions of a single genotype, and a mechanism for the control of synthesis of both polymers is suggested. 5. These results are discussed with reference to the constantly changing environmental conditions that obtain in a batch culture and the variation in bacterial cell-wall composition that is reported to occur throughout the growth cycle.

It was reported by Ellwood & Tempest (1967) that whereas the cell walls of Mg^{2+}-limited *Bacillus subtilis* var. *niger* contained a teichoic acid, this polymer was totally replaced by a teichuronic acid when the organisms were limited in their growth, in a chemostat culture, by the availability of PO_4^{3-}. Although it was reasonable to assume that the observed changes in cell-wall composition were entirely phenotypic, they could have been due to the selective growth of different genotypes, with the different limitations, and this is particularly likely since the chemostat is known to provide a strongly competitive selective environment (Powell, 1958; Meers & Tempest, 1968). The clearest way of distinguishing between these two possibilities was to study the kinetics of the transition process from fully Mg^{2+}-limited growth, for example, to fully PO_4^{3-}-limited growth, and vice versa. The results of this study, which showed not only that the changes in cell-wall composition were phenotypic but that they involved turnover of wall material, are given in this paper. A preliminary report has been published by Tempest & Ellwood (1968).

METHODS

Organism. B. subtilis var. *niger* (A.T.C.C. 9372) was maintained by monthly subculture on tryptic-meat-digest–agar slopes containing glucose (0·2%, w/v).

Growth conditions. Continuous cultures of *B. subtilis* were grown in 0·5l. chemostats (Herbert, Phipps & Tempest, 1965) with the temperature regulated at 35°, at pH 7·0. The Mg^{2+}-limited medium was that described by Tempest, Dicks & Meers (1967) but with the $NH_4H_2PO_4$ concentration lowered to 5·0mM; the K^+-limited medium was that described by Tempest, Dicks & Ellwood (1968) but with the $NH_4H_2PO_4$ concentration lowered to 5·0mM; the PO_4^{3-}-limited medium was also that described by Tempest *et al.* (1968) but with the $MgCl_2$ concentration lowered to 0·8mM.

Experimental. B. subtilis var. *niger* organisms were washed from the surface of a nutrient–agar slope into a Mg^{2+}-limited medium contained in a chemostat. The dilution rate was set to a value of about 0·2hr.$^{-1}$, and after a steady-state equilibrium had been attained (3–4 days) a large sample of culture (100–200ml.) was collected via the overflow tube; the medium in the reservoir bottle was then changed to one containing an excess of Mg^{2+} but a growth-limiting concentration of PO_4^{3-}. The dilution rate was maintained at about 0·2hr.$^{-1}$, and after the Mg^{2+}-limited medium in the feed line had been flushed into the growth chamber (about 30min.) collections from the effluent culture line were continued, the receiver bottles being changed hourly for the first 5–6hr. After the culture had fully equilibrated with the new PO_4^{3-}-limited environment a final sample was collected, the culture was returned to being Mg^{2+}-limited, and a corresponding series of samples was collected.

Bacilli were separated from each sample by centrifugation at 10000*g* for 15min., washed once with water and then disrupted in a Brown M.S.K. homogenizer with Ballotini grade 16 glass beads. Shaking for 10min. at the fastest

rate generally effected > 99% disruption, and the temperature could be kept at < 5° throughout. Isolation of the cell-wall fraction, purification and analysis were carried out as described by Tempest *et al.* (1968).

Analysis of culture extracellular fluids. Volumes (100ml.) of culture extracellular fluid, separated from the organisms by centrifugation at 10000*g* for 15min., and containing < 0·1 μg. dry wt. of organisms/ml., were freeze-dried and then dissolved in water (10ml.); 5ml. of this concentrated solution was placed carefully on top of a column (30cm. × 2cm.) of Sephadex G-25 and eluted with water. After passage of the void volume, successive volumes (4ml.) of eluate were collected until glucose, detected with a Clinistix reagent strip (Ames Co., Stoke Poges, Bucks.), emerged from the column. None of these samples contained material absorbing in the 260–280mμ range. They were combined, freeze-dried and weighed. They were then hydrolysed with either 2N-HCl or 1N-NaOH at 100° for 3hr., and analysed chromatographically for cell-wall degradation products (see Ellwood, Kelemen & Baddiley, 1963).

THEORY

If one considers the kinetics of bacterial growth in a chemostat (Herbert, Elsworth & Telling, 1956; Powell, 1965), organisms are contained in a culture of fixed volume (V) to which fresh medium is pumped at a constant rate (f), the dilution rate (D) being given by the ratio f/V. The net change in concentration of organisms (x) in the culture vessel with time will thus depend on the relative rates of bacterial synthesis (μ) and 'washout' (D) of organisms from the culture vessel; that is,

$$\text{Change} = \text{Growth} - \text{Washout}$$
$$dx/dt = \mu x - Dx,$$

or

$$(1/x)(dx/dt) = \mu - D \qquad (1)$$

When the culture is in a steady-state equilibrium condition, the growth rate and dilution rate are equal so that the net change in the concentration of organisms with time is zero. Now, if cell synthesis was made to cease suddenly the concentration of organisms in the culture would diminish (through washout) at a rate proportional to the dilution rate; that is,

$$x_t/x_0 = e^{-Dt} \qquad (2)$$

where x_0 and x_t are the concentrations of organisms initially and after some time interval t respectively. Similar considerations would apply to any cellular component that ceased to be synthesized, even though bacterial synthesis as a whole continued. Thus, if at the moment of changeover from Mg^{2+}-limited growth to PO_4^{3-}-limited growth teichoic acid synthesis by *B. subtilis* organisms ceased totally, but the polymer contained in the wall was not degraded, the cell-wall teichoic acid content would diminish at the rate indicated by equation 2 (and as shown by the broken line in Fig. 1). Again, if at the moment of changeover teichuronic acid synthesis started, and continued at a constant rate equal to the overall rate of biomass synthesis, its concentration would increase so that at any particular moment,

$$z_t/z_s = 1 - e^{-Dt} \qquad (3)$$

where z_t is the concentration of teichuronic acid at time t, and z_s the final steady-state teichuronic acid concentration in the bacterial cell walls; this rate is indicated by the dotted line in Fig. 1. In contrast, if the particular limitation in the chemostat favoured the selection of variant organisms (teichuronic acid-containing in one case and teichoic acid-containing in the other), then the rate of change in cell-wall composition after a change in the nature of the growth limitation would depend on the initial concentration of variant organisms in the culture and the growth rate. Thus, if the initial concentration of variant organisms was < 1% of the total population it would require at least 7 doubling-times (> 24hr. at a dilution rate of 0·2hr.$^{-1}$) to effect a complete change in population, considerably more time than predicted for a phenotypic change.

RESULTS

The concentration of Mg^{2+} in the Mg^{2+}-limited medium, and of PO_4^{3-} in the PO_4^{3-}-limited medium, was adjusted to provide a steady-state bacterial

Table 1. *Changes in culture concentration of* B. subtilis *var.* niger *organisms and composition of extracellular products during transition from* Mg^{2+}*-limited growth conditions to* PO_4^{3-}*-limited conditions in a chemostat*

The hydrolysis products were obtained by treating material of mol. wt. > 200, which had been separated from other constituents present in the culture extracellular fluids on a Sephadex G-25 column, with 2N-HCl at 100° for 3hr. The products were analysed chromatographically by the methods of Ellwood, Kelemen & Baddiley (1963). n.d., Not determined.

Time after change (hr.)	0	1	2	3	4	5	24
Dry wt. of organisms (mg./ml.)	3·42	3·14	3·20	3·27	3·25	3·12	3·29
Extracellular organic P (μg./ml.)	10*	10*	33	31	31	35	4
Hydrolysis products of material of mol.wt. > 200							
Inorganic phosphate	+	n.d.	n.d.	+	n.d.	n.d.	±
Glycerol	−	n.d.	n.d.	±	n.d.	n.d.	−
Glycerol phosphate	−	n.d.	n.d.	+++	n.d.	n.d.	−
Glycerol diphosphate	−	n.d.	n.d.	++	n.d.	n.d.	−
Amino acids	±	n.d.	n.d.	+	n.d.	n.d.	+

* Small difference between two large values.

Table 2. *Changes in the phosphorus and uronic acid contents of the walls of* B. subtilis *var.* niger *organisms during transition from* Mg^{2+}*-limited growth to* PO_4^{3-}*-limited growth and vice versa and from* K^+*-limited growth to* PO_4^{3-}*-limited growth in a chemostat culture*

The samples were collected, fractionated and analysed as described in the Methods section. The initial sample (shown as time 0) was collected over a 2hr. period before the change of medium, and subsequent samples were collected over hourly periods up to the time indicated. The change from PO_4^{3-}-limitation to Mg^{2+}-limitation was initiated 46hr. after the completion of the previous changeover experiment.

Time after change (hr.)	Wall components (μmoles/mg. dry wt. of bacterial cell wall): Wall-bound phosphorus	Wall-bound uronic acid
	Mg^{2+}-limitation → PO_4^{3-}-limitation	
0	1·64	0·20
1	1·23	0·48
2	0·72	0·61
3	0·39	0·73
4	0·31	1·14
5	0·25	1·31
24	0·20	1·10
	PO_4^{3-}-limitation → Mg^{2+}-limitation	
0	0·15	0·92
1	0·18	0·78
2	0·47	0·65
3	0·82	0·33
4	1·12	0·26
5	1·31	0·20
24	1·63	0·20
	K^+-limitation → PO_4^{3-}-limitation	
0	1·40	0·12
1	1·38	0·27
2	1·03	0·55
3	0·55	0·77
4	0·29	0·90
5	0·16	0·89
24	0·20	0·70

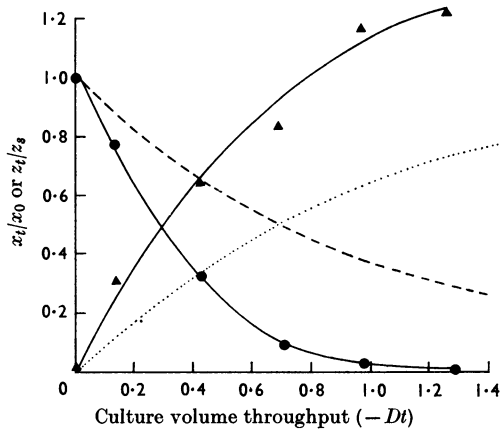


Fig. 1. Changes in the teichoic acid (●) and teichuronic acid (▲) contents of *B. subtilis* var. *niger* cell walls after changeover from conditions of Mg^{2+}-limitation to those of PO_4^{3-}-limitation, in a chemostat culture. The broken line represents the theoretical 'washout' rate, assuming that teichoic acid synthesis ceased immediately the environment became PO_4^{3-}-limited. The dotted line represents the theoretical rate of increase in teichuronic acid, assuming that its synthesis started immediately the environment became PO_4^{3-}-limited and continued at a constant rate.

concentration of about 3mg equiv. dry wt. of organisms/ml. when *B. subtilis* var. *niger* was grown at a dilution rate of about 0·2hr.$^{-1}$. Thus when the Mg^{2+}-limited feed medium was replaced by the PO_4^{3-}-limited medium the concentration of organisms in the chemostat culture changed only from 3·42 to 3·29mg./ml. (Table 1). Further, since the concentration of PO_4^{3-} in the Mg^{2+}-limited medium (and of Mg^{2+} in the PO_4^{3-}-limited medium) was decreased to a value that provided only a small excess, the transition period from one growth-limiting condition to the other after a change in the composition of the feed medium was minimal, and there was little fluctuation in the bacterial concentration in the culture throughout the whole changeover period (Table 1). The fact that the bacterial concentration in the culture remained approximately constant throughout the transition period also indicated that little or no lysis of the organisms in the culture had occurred, and this was further supported by the absence of significant amounts of lytic products of whole cells in the culture extracellular fluids (see Table 1).

When the cell walls of organisms undergoing transition from Mg^{2+}-limitation to PO_4^{3-}-limitation were analysed, their phosphorus content (indicative of teichoic acid) diminished, and their uronic acid content (indicative of teichuronic acid) increased, as shown in Table 2. The rates of change of cell-wall phosphorus and uronic acid are plotted in Fig. 1, along with the rates predicted from equations 2 and 3. The observed rates of change were substantially faster than the predicted rates, indicating (i) that they could not possibly be due to the upgrowth of variant organisms that were present initially in low concentration, (ii) that the changeover from teichoic acid synthesis to teichuronic acid synthesis was rapid and complete after PO_4^{3-} depletion, and (iii) that over and above a change in synthesis of polymer type, actual turnover of wall material must have occurred during the transition period; that is, some teichoic acid present in the walls of organisms at the moment of changeover from Mg^{2+}-limitation to PO_4^{3-}-limitation must have

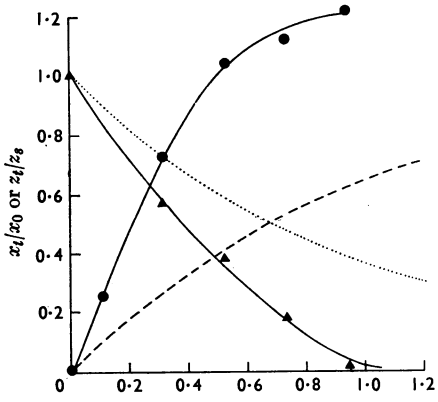
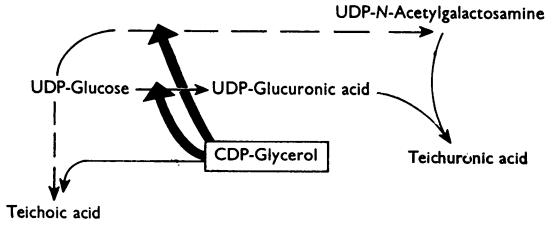


Fig. 2. Changes in the teichuronic acid (▲) and teichoic acid (●) contents of *Bacillus subtilis* var. *niger* cell walls after changeover from conditions of PO_4^{3-}-limitation to those of Mg^{2+}-limitation, in a chemostat culture. The dotted line represents the theoretical 'washout' rate, assuming that teichuronic acid synthesis ceased immediately PO_4^{3-} ceased to be growth-limiting. The broken line represents the theoretical rate of increase in teichoic acid, assuming that its synthesis started immediately PO_4^{3-} became non-limiting and continued at a constant rate.



Scheme 1. Possible system for the regulation of teichoic acid and teichuronic acid synthesis by the 'pool' CDP-glycerol content. The heavy arrows represent repressor activity.

been excised from the walls and replaced by teichuronic acid.

A study was made of the reverse change from PO_4^{3-}-limited growth to Mg^{2+}-limited growth of *B. subtilis* organisms in the chemostat (i.e., from a teichuronic acid- to a teichoic acid-containing wall). The rates of change of cell-wall teichuronic acid and teichoic acid contents were found to be just as rapid as those described above (see Fig. 2 and Table 2).

The cell walls of K^+-limited *B. subtilis* var. *niger* organisms were found to contain a teichoic acid that was similar to, but not identical with, that in the walls of Mg^{2+}-limited organisms. Again (Table 2) this teichoic acid was rapidly displaced by teichuronic acid when the culture was changed to being PO_4^{3-}-limited, but the rate of loss of teichoic acid from the walls of the organisms during the initial stages of changeover was less than that observed with Mg^{2+}-limited cultures.

Turnover of the cell walls would result in polymers being lost from the organisms to the extracellular fluid, where they might accumulate or be degraded, or even be assimilated by the growing organisms. Examination of the extracellular fluids from cultures of *B. subtilis* undergoing a transition from Mg^{2+}-limitation to PO_4^{3-}-limitation showed there to be an increase of organically-bound phosphorus (Table 1). On acid or alkaline hydrolysis, this material gave products that were characteristic of a glycerol teichoic acid (Table 1) showing unequivocally that this polymer, or a degradation product of it, had accumulated in the culture extracellular fluid during the changeover period. Further, fragments of the pentapeptide moiety of the cell-wall mucopeptide layer (containing diaminopimelic acid, alanine, glutamic acid and glycine) also were detected in acid hydrolysates of the 2–3 hr. sample but not in the initial and final steady-state samples (Table 1). However, no muramic acid was present in any of the samples.

DISCUSSION

If, in chemostat cultures of *B. subtilis* var. *niger*, the changeover from teichoic acid-containing cell walls to teichuronic acid-containing walls had involved the upgrowth of variant organisms that initially were present in a low concentration (1% of the total population or less) the time required for complete transition would necessarily have been greater than 24 hr.; in fact, it was virtually complete in 5 hr., showing that it must have involved purely phenotypic processes. But the nature of these control processes is not known; clearly, the fact that teichuronic acid was found only in the walls of PO_4^{3-}-limited organisms, whereas teichoic acid occurred in the walls of all *B. subtilis* var. *niger* organisms that had been grown in chemostat cultures in the presence of ample PO_4^{3-} (Ellwood & Tempest, 1967, 1968), suggests that PO_4^{3-} or some phosphorus-containing metabolite was a key regulating substance. Thus presumably teichuronic acid synthesis is repressed by some phosphorus-containing intermediate(s) involved in teichoic acid synthesis, and only under conditions of phosphate depletion is this intermediate(s) present intracellularly in a sufficiently low concentration to allow teichuronic acid synthesis to occur. And of course no teichoic acid synthesis would occur under these conditions. In this connexion, CDP-glycerol could

be the repressor and, say, UDP-glucose dehydrogenase the sensitive enzyme (Scheme 1); thus in the presence of ample phosphate, CDP-glycerol would be synthesized but not UDP-glucuronic acid and, therefore, not teichuronic acid.

The walls of exponential-phase, batch-grown, *Bacillus licheniformis* (N.C.T.C. 6346) organisms have been found to contain both teichoic acid and teichuronic acid (Hughes, 1966), and so too have the walls of similarly grown *B. subtilis* var. *niger* organisms, but not those of *B. subtilis* W23 (D. C. Ellwood & D. W. Tempest, unpublished work). These observations are compatible with those described above if one assumes that during exponential (unrestricted) growth, the 'pool' concentration of repressor substance became depleted, due to the rapid rate of synthesis of teichoic acid, thus permitting some synthesis of teichuronic acid to occur. Since the walls of *B. subtilis* W23 contain a ribitol teichoic acid, the precursor metabolite would be CDP-ribitol and, if this was a more potent inhibitor of teichuronic acid synthesis than CDP-glycerol, the absence of teichuronic acid from the walls of exponential-phase, batch-grown, *B. subtilis* W23 organisms would be explicable. But as expected, the walls of PO_4^{3-}-limited *B. subtilis* W23 organisms, like those of *B. subtilis* var. *niger* and *B. licheniformis*, contained teichuronic acid and no teichoic acid (Ellwood & Tempest, 1968).

In both the transition from teichoic acid synthesis to teichuronic acid synthesis (Fig. 1), and the transition vice versa (Fig. 2), there was an approximate equivalence between the amounts of old polymer lost from the walls and of new polymer synthesized; presumably at no time was there a naked mucopeptide cell wall. Indeed, turnover of wall seemed to involve the mucopeptide layer, or at least the pentapeptide part of the mucopeptide, since fragments of the pentapeptide also accumulated in the culture extracellular fluid during the transition period from Mg^{2+}-limitation to PO_4^{3-}-limitation (Table 1).

Teichoic acids may account for over 50% of the weight of the *B. subtilis* cell wall (Ellwood & Tempest, 1968), and yet they are rapidly and totally replaced by a teichuronic acid when the organisms are deprived of phosphate. Obviously the bacterial cell wall must be in a dynamic state during growth, but the large shifts in its chemical composition, in response to relatively small changes in the environment, suggest that the wall possibly is one of the most dynamic and phenotypically variable structures of the whole cell. The relevance of such variability to problems of vaccine production, as well as to problems of interpreting the structural complexity of cell walls of organisms grown in undefined and uncontrolled environments (that is, in batch cultures), is obvious.

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