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Cell Wall Metabolism in *Bacillus subtilis* Subsp. *Niger*: Effects of Changes in Phosphate Supply to the Culture

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Chemostat cultures of *Bacillus subtilis* subsp. *niger* WM were exposed to changes in the availability of phosphorus by means of a resuspension technique. Responses in wall metabolism were recorded by measuring the amounts of peptidoglycan and anionic polymers (teichoic or teichuronic acid) in the wall and extracellular fluid fractions. With respect to the wall composition, the effect of a change in orthophosphate supply was a complete shift in the nature of the anionic polymer fraction, the polymer originally present in the walls ("old" polymer) being replaced by the alternative ("new") anionic polymer. The peptidoglycan content of the walls remained constant. It was concluded that the incorporation of old polymer was completely blocked from the moment the orthophosphate supply was changed. However, from a measurement of the total amount of polymer in the whole culture during the course of the experiments, it was evident that synthesis of old polymer continued, but it was secreted. Synthesis of the new polymer started immediately, and it was incorporated exclusively into the wall. During adaption of the cells to the new environment, wall turnover continued in an identical fashion to that extant in steady-state cultures. It was concluded that the primary adaptive response to a change in orthophosphate supply occurred through a mechanism interacting with polymer incorporation and thus at the level of wall assembly at the membrane.

It has been reported previously (9, 19) that *Bacillus subtilis* varies the anionic polymer content of its wall, qualitatively and quantitatively, depending on the growth conditions. Furthermore, it has been found that the wall composition did not necessarily reflect the actual rate at which the organism synthesized anionic polymers (7); combined analyses of cell wall and extracellular fluid showed unequivocally that anionic polymers often were synthesized in larger quantities than could be deduced from their wall content. Under one set of growth conditions in the chemostat (phosphate limitation, high dilution rate) the organism synthesized an anionic polymer (i.e., teichuronic acid) without incorporating it into its wall. From these results, it was concluded that the cell possessed mechanisms to secrete anionic polymers which, although formed, did not serve as substrates for incorporation into the wall. Incorporation occurs through covalent linkage of the polymers to nascent peptidoglycan units on the membrane (12). A direct implication of the existence of secretion of anionic polymers is that, at least under certain growth conditions, a mechanism must be involved in the regulation of the wall composition, which acts at the level of wall assembly.

All experiments described in our previous papers were performed with steady-state chemostat cultures. Under these growth conditions, the net effect of a regulation mechanism was measured, whereas the underlying principles possibly may be manifest only when the balanced state of control is disturbed. Experiments designed to investigate such an effect are described here.

Cultures of *B. subtilis* subsp. *niger* WM were subjected to changes in the P_i supply, which was known to determine to a large extent the nature of the anionic polymer present in the cell wall (19). Experiments of this type, so-called limitation switch experiments, have been described in the literature (4, 8, 11), and indicated that the anionic polymer which initially was present (the old polymer) disappeared from the wall at a rate higher than was theoretically expected. This discrepancy was generally attributed to the occurrence of cell wall turnover, a process which is beginning to be understood (2, 6, 23), and which

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basically consists in the excision of peptidoglycan from the wall by the activity of hydrolytic enzymes. Parallel to the enhanced decline of the old polymer, in these turnover experiments there was an accumulation of the alternative anionic polymer (the new polymer) at a rate faster than expected.

When these experiments were initially carried out, however, the phenomenon of anionic polymer secretion as described above was not known so only the anionic polymer content of the wall was measured. The picture of anionic polymer metabolism which emerged from these experiments was seemingly too simple. Therefore, we decided to expand the field of polymer analysis to include the culture fluid. (F. Kruyssen, Ph.D. thesis, University of Amsterdam, Amsterdam, The Netherlands, 1979; W. de Boer, Ph.D. thesis, University of Amsterdam, 1979).

MATERIALS AND METHODS

Organism and growth conditions. *B. subtilis* subsp. *niger* WM was used throughout. For all details with regard to maintenance and cultivation of the organism see reference 19. All experiments were performed by using chemostat cultures which were grown with glucose as the carbon source, and at dilution rates (D) of between 0.2 and 0.3 h^{-1}, implying generation times (t_d) of between 2.5 and 3.5 h, according to this equation: $t_d = \ln 2/D$ (22).

Limitation switch experiments. To subject cultures to a sudden change in limitation, they were quickly transferred via a centrifugation step to a new environment which differed from the previous one only in the nature of the limiting nutrient (so-called limitation switch experiments). The procedure was as follows: 500 to 600 ml of a culture growing in a steady state under a certain limitation (2 to 3 g/liter [dry weight]) were harvested rapidly in centrifuge bottles (250 ml) and sedimented in a thermostated centrifuge (RC2-B; Sorvall Inc., Norwalk, Conn.), using a rotor that had been equilibrated at 37°C for 5 min at 5,000 × g at 37°C. The pellets were resuspended in about 200 ml of medium devoid of the required limiting nutrient (37°C; pH 7.0; polypropylene glycol added as an antifoam), and the suspension was transferred to a second chemostat. Stirring, air supply, temperature, and pH control were initiated, and the culture vessel was brought to its working volume by addition of the resuspension medium. Then, the growth medium (of the new limiting composition) was admitted at a controlled rate, identical to the one before the switch, and, consequently, overflow of culture occurred (t_o). Between harvesting and t_o about 20 min elapsed. Sterility was not maintained. The composition of the influent medium was based on the recipes described by Evans et al. (10). It is crucial to adjust the concentration of this medium with respect to the growth-limiting nutrient so that it supports a cell density closely similar to that in the culture at t_o. (This density can be estimated from the value in the original culture, the volume of the culture that had been centrifuged, and the working volume of the second chemostat.) Only then the culture may continue growth at a rate identical to the dilution rate. If, however, this is not the case (due to losses while transferring the culture, or to failure of the cells to continue growth immediately after the switch to the new limitation; see, e.g., Fig. 2c), the culture, since it is self-regulatory by nature (22), will adapt its density to the level supported in the new conditions. This adaptation takes place during an intermediate period of growth at a rate different from the dilution rate imposed.

Sampling. Two samples were taken circa every hour starting 30 min after t_o, each of which comprised an estimated maximal dry weight of 30 mg of cells. One (A) was collected on ice via the overflow port during a short period preceding the time that the direct sample (B) was taken from the culture. Both were centrifuged at 4°C (10 min at 12,000 × g). The supernatant of B was frozen, and used later on for analysis of extracellular compounds. The pellets of A and B were resuspended in ice-cold distilled water. The resuspended pellet of A was transferred quantitatively to a preweighed centrifuge tube, and both A and B were spun down once more. Pellet A was freeze-dried, weighed to obtain an accurate dry weight determination, and stored until required for the cell wall preparation. Pellet B was stored at −20°C and used as a source of enzymes.

Determination of wall polymers. Cell-bound wall polymers were determined as described previously (19). Wall polymers in the supernatant of the culture before and after the limitation switch were determined as described previously (7).

Preparation and assay of CDPglycerol pyrophosphorylase. To prepare CDPglycerol pyrophosphorylase (CTP:*sn*-glycerol-3-phosphate cytidylyltransferase, EC 2.7.7.39), cells (about 50 mg calculated dry weight) were suspended in 4 ml of TMD buffer (50 mM Tris-hydrochloride, 5 mM $MgCl_2$, 1.0 mM dithiothreitol, pH 8.0), and a crude extract was prepared by breaking the cells in a French pressure cell (Aminco, Silver Spring, Md.) set at high ratio and a pressure of 20,000 lb/in^2. The cell was rinsed once with TMD buffer, using an automatic refill device, and both fractions were pooled. Care was taken to keep the temperature as close as possible to 0°C. More than two-thirds of the cells were broken, as was apparent under a phase-contrast microscope. The enzyme suspension was centrifuged for 10 min at 8,000 × g to remove cell debris and for 60 min at 39,000 × g in a refrigerated centrifuge. The final supernatant was used as the enzyme preparation after overnight dialysis against TMD buffer. Protein content was determined according to the method of Lowry et al. (20). The enzyme was assayed according to the method of Anderson et al. (1), with some modifications. Incubation mixtures (50 μl each; in 2-ml tubes) were of the following composition: Tris-hydrochloride (20 mM, pH 8.0); magnesium acetate (2.5 mM); dithiothreitol (2.5 mM); CTP (4.0 mM); D,L-α-glycerol-3-P, disodium salt (50 mM); L-α-[U-^{14}C]glycerol-3-P, ammonium salt (RCA, Amersham, England, 21.5 mCi/mmol; 5.6×10^3 dpm/μl of incubation mixture), and enzyme (up to 50 μg of protein). They were kept at 37°C in a water bath for up to 30 min, and the reaction was stopped by immersing the tubes for 2 min in boiling water. The

samples were then quantitatively applied to Whatman no. 1 chromatography paper under a stream of cold air and eluted overnight in ethanol–1 M ammonium acetate, pH 3.8 (5:2; vol/vol). The paper was washed before use by eluting with 2 M acetic acid (analytical grade) and subsequent elution in double-distilled water. Phosphate esters (24) were made visible by successive spraying with $FeCl_3 \cdot 6H_2O$ (0.1% in 80% ethanol), drying at room temperature, and spraying with sulfosalicylic acid (1% in 80% ethanol). The esters appeared white on a lilac background. After intensive drying, radioactive spots on the chromatograms were traced by either visualization of included standards, by UV light (CDPGly) or by the specific color reaction (see above) of concomitantly run standards, or by overnight exposure of X-ray film (Eastman Kodak, Rochester, N. Y.) to the sheet, and subsequent development and fixation (DX 40 and FX 80, respectively, Eastman Kodak). The material that was to be counted (the whole chromatogram or just radioactive spots) was cut into pieces of about equal size, placed into scintillation vials with 10 ml of scintillation fluid (one package of Omnifluor [4 g; New England Nuclear Corp., Boston, Mass.] per liter of scintillation grade toluene), and counted in a Nuclear Chicago Isocap 300 liquid scintillator counter. Counting efficiency was 60%. Recovery of radioactivity from the chromatograms compared with the applied quantity amounted to at least 90%. The activity of the enzyme is given in nanomoles per minute per milligram of protein.

Theoretical considerations. In a chemostat culture, the decrease of a component which is no longer made from t = 0 h, will be described by $X_t = X_o \cdot e^{-Dt}$, where X_t and X_o are the concentrations of the component at times 0 and t h, respectively, and D is the dilution rate (h^{-1}). When a previously absent component is produced from t = 0 h at a rate finally giving a concentration Z_s, the increase of the concentration of this component in a chemostat culture will be described by $Z_t = Z_s \cdot (1 - e^{-Dt})$. More rapid decrease than predicted by the first equation indicates the conversion of the component to one no longer detected by the methods employed; a faster increase than predicted by the second equation indicates a higher rate of synthesis in the transient phase than is needed finally to maintain the concentration Z_s in the culture. For the derivation of the two equations see references 4 and 8.

Correction for carry-over. Centrifugation and resuspension of the cultures, as was done in these experiments, will result in a culture supernatant initially devoid of wall turnover and secretion products, but some carry-over of material originating from the extracellular fluid of the culture before the switch may occur. In our experiments, the first sample was drawn from the culture at 30 min after resuspension of the cells, so we did not have data on the extent of the carry-over of turnover products into the second culture. For this reason, we considered the amount of turnover products in the first sample as a background value, which was subsequently diluted in the chemostat after t = 0.5 h. Thus the background concentration present in the following samples (taken at time t after resuspension) was taken as being $C_t = C_{0.5} \cdot e^{-D(t-0.5)}$, where $C_{0.5}$ is the supernatant concentration in the first sample taken at 30 min after resuspension and D is the dilution rate in the chemostat (h^{-1}).

RESULTS

Evaluation of the procedures. The purpose of the experiments described here was to subject steady-state chemostat cultures to a sudden change in P_i supply. To allow accurate measurement to be made of the concentrations of relevant compounds in the extracellular fluid, it was necessary to resuspend the cultures. After preliminary experiments it was decided to insert a centrifugation step. However, it had first to be established that the organism was not hereby incapacitated. Figure 1 shows the control experiment with phosphate-limited cells, which, after centrifugation and resuspension in phosphate-free medium, were recultivated in a medium identical to the one used initially. From the dry weight determinations, it may be concluded that growth continued directly upon resuspension. It should be noted that the initial decrease in dry weight is not necessarily a consequence of washout. The dry weight figure indicated at zero time is calculated from the value existing in the original steady-state culture and the volume of cul-

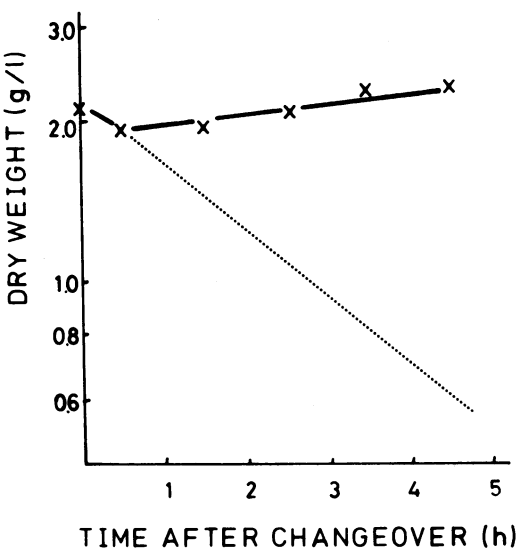

FIG. 1. *The effect of the limitation switch (changeover) procedure upon growth of B. subtilis var. niger WM: control experiment. Steady-state P_i-limited cells ($D = 0.23\ h^{-1}$) were subjected to the switch procedure as described in the text. They were then reinoculated into P_i-limiting medium of the same composition as had been used before. The dotted line indicates the decrease in cell density, if no growth at all would occur under the new growth conditions (so-called washout due to dilution of the culture with fresh medium). For derivation of the formula ($X_t = X_0 \cdot e^{-Dt}$) see the theoretical considerations in the text.*

ture which had been collected. Certainly some loss occurred during the procedure, and these losses were variable.

Figure 2 shows the results of comparable experiments which involved a change in the phosphate supply. Figure 2a shows an experiment in which the phosphate concentration of the medium was raised from 1.5 mM in phosphate-limiting medium to 7.5 mM in magnesium-limiting medium. The other two figures present experiments in which the concentration of this compound was lowered from circa 7.5 mM in both magnesium- and carbon-limiting media to 1.5 mM in the phosphate-limiting medium. The organism did not recover from the centrifugation step within several hours in the experiment of Fig. 2c, indicating that carbon-limited cells are more sensitive to damage during the procedure used than the phosphate- and magnesium-limited cells (Fig. 2a and b). We conclude, on the basis of these results, that both metal ion- and phosphate-limited cultures are able to respond quite adequately to the imposed changes in P_i supply, without an apparent intermediate period of nongrowth. Therefore, only carbon-excess cultures were used in the following experiments.

Effect of changes in P_i supply on the cell wall composition. Cell wall analyses were performed on samples harvested while the cultures were adapting to a new growth limitation. The kinetics of the change in wall composition can be visualized most directly by plotting the amounts of the anionic polymers (in percentages of the amount of cell wall) against time. This way, the decline of the old polymer can be compared with a theoretical line, based on the assumption that the polymer is no longer incorporated into the wall and that it (i.e., cells containing the old polymer) is washed out due to dilution of the culture with new medium (see above). Disappearance of teichuronic acid, in a switch from P_i-limited to P_i-excess conditions, clearly proceeded much faster than had been expected (Fig. 3). Moreover, it is evident that the increase in new anionic polymer proceeded at a higher rate than had been expected on the basis of theoretical considerations (see above; Fig. 3).

It must be realized that in this plot the variation in a specified polymer is brought into relation with the total amount of wall polymers (percentage). This implies that it must be established explicitly that this total is constant throughout the experiment. In the experiments reported in the literature, where the data were analyzed by using this plot (4, 8, 11), it was not shown conclusively that this condition (implying that both growth rate and wall content of the cells are constant) was satisfied. In our experiments these parameters were indeed found to be stable.

The quick replacement of the old anionic polymer which we found is thought to be due to

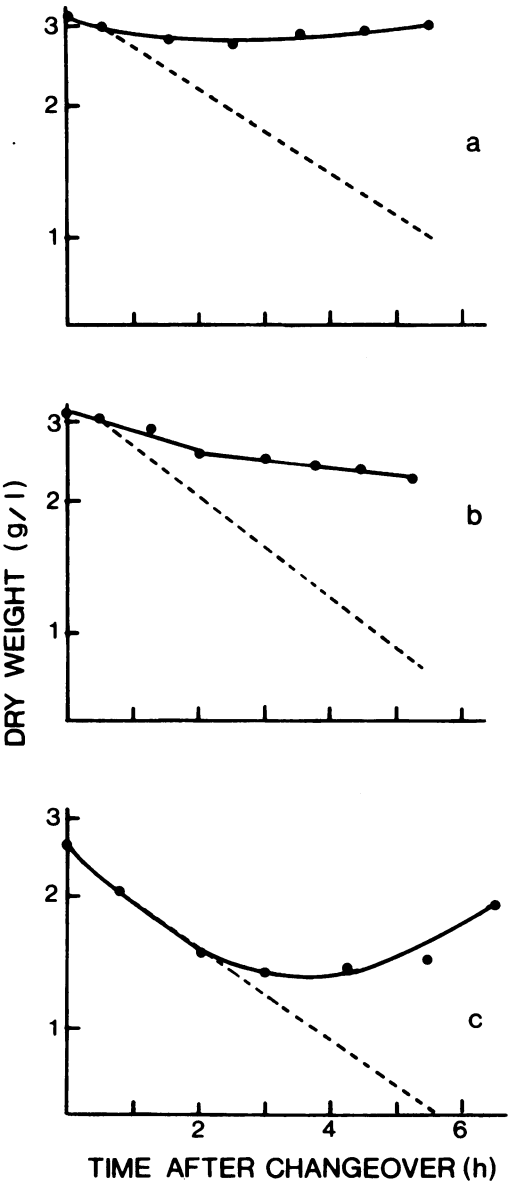

FIG. 2. *The effect of limitation switch on growth of B. subtilis var. niger WM. (a) P_i-limited culture (D = 0.23 h^{-1}), switched to magnesium limitation. (b) Potassium limitation (D = 0.26 h^{-1}), switched to P_i-limitation. (c) Carbon limitation (D = 0.23 h^{-1}), switched to P_i-limitation. The dotted lines indicate the theoretical washout (see legend to Fig. 1).*

cell wall turnover (see above). de Boer et al. (6) discussed thoroughly the kinetics of this latter process, based on experiments in which cells were labeled specifically in their peptidoglycan. Although the loss from the cells of the old anionic polymer followed kinetics strongly reminiscent of the model depicted in (6), rigorous treatment of these was not possible. Too few cell wall data were available to allow the turnover kinetics to be established with sufficient accuracy.

To obtain insight into the rates of the processes which are responsible for accumulation of wall polymers outside the cells (turnover and secretion), we analyzed also the extracellular fluids of cultures adapting to variations in the availability of phosphorus in the growth medium. To make possible a comparison between the amounts of polymer present in cell walls and in the culture fluid, all data below are given as concentrations (the amounts of polymers present in a culture volume containing 1 mg of cells).

Effect of changes in P_i supply on the metabolism of peptidoglycan. Figure 4 shows the peptidoglycan content of the wall and extracellular fluid fractions of cultures exposed to various changes in P_i supply. From the values obtained in the original steady-state cultures, one can estimate the rate of turnover under those culture conditions (7). Assuming that these rates of turnover were maintained in the course of the switch experiments, one can calculate the rate of accumulation of polymeric peptidoglycan in the supernatant (the dashed lines in Fig. 4). Obviously, there are no indications that essential changes occurred during the experiments in the process of turnover as it is encountered in steady state. (Possibly some suppression was observed in the experiment shown in Fig. 4b. Note, however, the high level of extracellular peptidoglycan in the starter culture, which may have been due not only to turnover, but also to secretion of the polymer [7].) This contrasts with the results reported by Ellwood and Tempest (8). Performing limitation switch experiments with *B. subtilis* var. *niger*, these authors could demonstrate turnover only in cultures in a transient state of growth (based on a qualitative analysis of the culture fluid), which suggests that these conditions induced turnover.

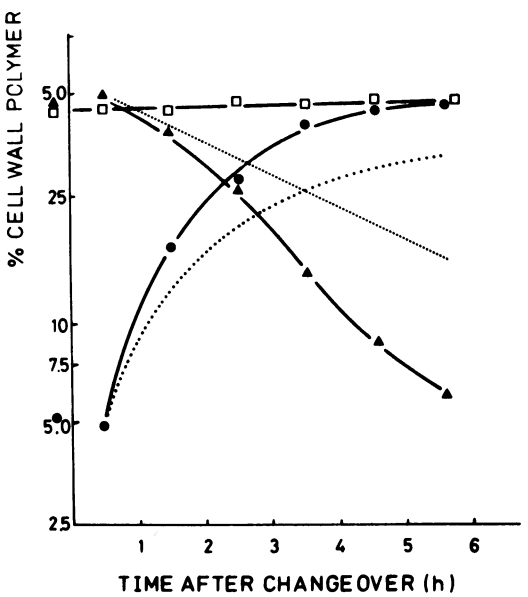

FIG. 3. *The effect of limitation switch on cell wall composition from P_i-limitation to magnesium limitation ($D = 0.23\ h^{-1}$). The dotted line indicates the calculated pattern of decrease of the old anionic polymer (teichuronic acid, ▲); the dotted curved line indicates the increase of the new anionic polymer (teichoic acid, ●). For derivations, consult the theoretical calculations in the text. The wall recoveries ranged between 20 and 24% of the cellular dry weight throughout the experiment. (□) Peptidoglycan. In this figure and the next figures, the data points indicated to the left of the ordinate refer to the steady state before the experiment.*

Effect of changes in P_i supply on the metabolism of the new anionic polymer. Figure 5 shows the increase of teichoic acid and teichuronic acid as the new anionic polymer in cultures adapting to switches in the P_i availability. By far the major part of the polymeric material found in the culture was recovered from the walls. The polymer in the supernatant fraction hardly accumulated during the first generation time after the start of the experiment, in spite of the rapidly increasing cellular content during that period.

Effect of changes in P_i supply on the metabolism of the old anionic polymer. Figure 6 (dotted line) indicates the decrease of old anionic polymer in the wall, calculated on the basis of the assumptions mentioned before. Since the concentration of the polymer in the extracellular fluid at time $t = 0.5$ h is taken to be zero (see above), this line also represents the expected decrease in the total concentration of the polymer in the culture, assuming that no synthesis takes place. Comparison of this theoretical line with a plot through the actual data points of total concentration leads to the conclusion that synthesis of the old anionic polymer continued after the change in P_i content of the growth medium. On the other hand, the decrease in wall-bound polymer suggests that no incorporation into the wall was possible.

Activity of CDPglycerol pyrophosphorylase in the course of switches in P_i supply.

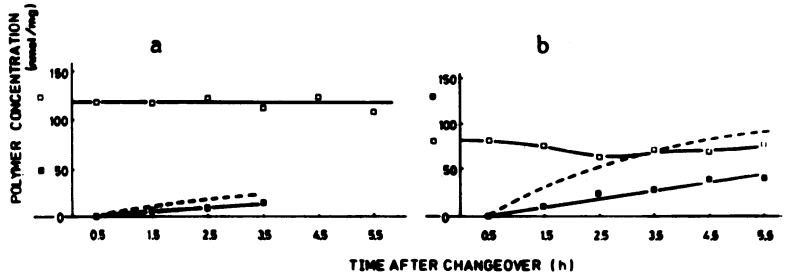

FIG. 4. *Concentration of peptidoglycan in the walls (□) and extracellular fluid (■) in the course of the limitation switch experiments. (a) Phosphate limitation to magnesium limitation ($D = 0.23\ h^{-1}$). (b) Magnesium limitation to phosphate limitation ($D = 0.30\ h^{-1}$). Dashed lines indicate the accumulation of peptidoglycan in the extracellular fluid, calculated from the value found in the starting culture (see the text).*

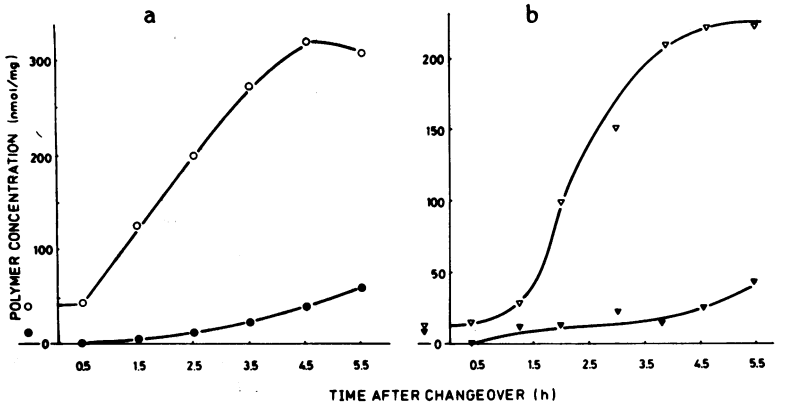

FIG. 5. *Concentration of new anionic polymer in the wall and extracellular fluid during limitation switch experiments. (a) Teichoic acid in a switch from phosphate limitation to magnesium limitation ($D = 0.23\ h^{-1}$). (b) Teichuronic acid in a switch from potassium to phosphate limitation ($D = 0.26\ h^{-1}$). (○, △) Concentrations in the wall fraction. (●, ▲) Concentrations in the extracellular fluid.*

CDPglycerol pyrophosphorylase, the only enzyme involved in the synthesis of precursors of anionic polymers which we found to be phenotypically variable in activity (Kruyssen, Ph.D. thesis) was measured in the experiments described in the previous paragraphs. Figure 7 shows the data of one experiment of each type of limitation switch.

When the culture was shifted from a high to a low P_i environment, a sharp decline in the activity of the enzyme was observed in the first 30 min (<0.16 generation time) to about 10% of the original activity. This residual activity was seemingly constant during the next part of the experiment.

When the P_i content of the medium was raised, an initial increase in enzyme activity was observed to a level similar to the residual activity specified above. During the period in which the process of wall change had been largely completed, no increase of the activity to the value usually found in cultures which grow in an excess of P_i (up to 100 nmoles/min per mg of protein) was found.

DISCUSSION

The cell wall serves various functions, many of which are associated specifically with growth (3, 26). The balance existing between the regulation of cell growth and the metabolism of the wall is clearly disturbed when growth is suppressed, as can be deduced from the occurrence, in many gram-positive organisms, of so-called wall thickening upon inhibition of, e.g., protein synthesis (16, 17). Many aspects of this wall thickening and of the interrelationships between protoplast growth and wall metabolism are only poorly understood at this time. Therefore, we used preferentially growing cultures in our experiments designed to elucidate the effects of changes in P_i supply on wall metabolism of *B. subtilis*. We wished to keep growth rate constant throughout the experiments, since this factor may affect the wall composition independent of the availability of P_i (19, 28). The dry weight curves of Fig. 1 and 2 demonstrate that the resuspension procedure we adopted met this criterion of constant growth rate.

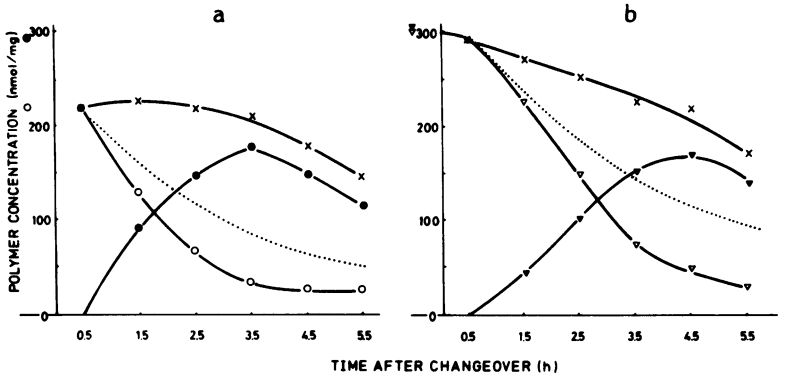

FIG. 6. *Concentration of old anionic polymer in the wall and extracellular fluid during limitation switch experiments. (a) Teichoic acid in a switch from magnesium to phosphate limitation ($D = 0.30\ h^{-1}$). (b) Teichuronic acid in a switch from phosphate to magnesium limitation ($D = 0.23\ h^{-1}$). (×) Total concentration of the old polymer in the culture (as the sum of the concentrations in wall (○, △) and extracellular fluid (●, ▲). For explanation of the dotted lines see the text.*

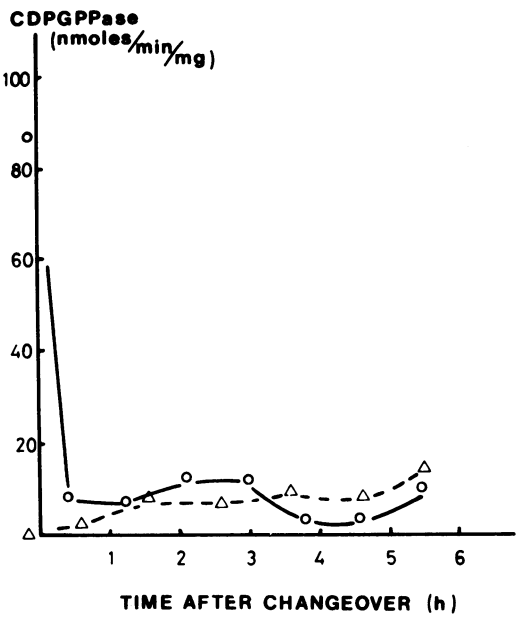

FIG. 7. *Activity of CDPglycerol pyrophosphorylase (CDPGPPase) during limitation switch experiments. The enzyme activity was tested in a P_i-limited culture which was subjected to a switch to magnesium limitation (△) and in a potassium-limited culture switched to P_i-limitation (○).*

The readiness with which the cultures responded to a change in P_i supply by incorporating a new anionic polymer into the wall may be compared to the data reported by Anderson et al. (2) who effected an environmental change without making use of a centrifugation step. These workers pulsed P_i into a P_i-limited chemostat culture and measured teichoic acid (with a more sensitive technique) in the walls of *B. subtilis* W23 over a period of 30 min. In several experiments with *B. subtilis* subsp. *niger* WM we detected significant amounts of new anionic polymer after 30 min (data not shown), which indicates that there was no question of a delayed response due to the resuspension manipulations. With respect to the response in the content of the old anionic polymer, no data are available in the literature that allow such a comparison. The limitation switch experiments reported (4, 8, 11, 18) were invariably carried out by connecting a chemostat culture in steady state to a vessel containing a growth medium of a different composition and therefore suffer the disadvantage that there is no clearly recognizable point in time when the new limitation becomes effective (simultaneously terminating an intermediate period of unrestricted growth). An extreme example is provided by Hussey et al. (18). They report that with cultures of *Bacillus licheniformis* the decrease in teichoic acid content of the wall (changing from a carbon to a phosphate limitation) did not occur within 5 h ($D = 0.2\ h^{-1}$) after the new medium vessel had been connected to the culture.

A clear result of the analyses carried out here is the observation that during the first couple of hours after a limitation switch the old anionic polymer was still being synthesized. From this observation a pertinent question arises as to whether incorporation of the old polymer ceases immediately after a change in P_i supply. Although this block in incorporation activity often is tacitly assumed by other workers, based upon the patterns of the decline of old polymer content in the wall, in fact, the techniques employed by other workers and ourselves do not allow this to be concluded unequivocally. The decline observed also may be the result of continuing in-

corporation, with a turnover that was altered qualitatively or quantitatively. The following considerations, however, refute the latter possibility.

One might suggest that after a limitation switch the covalent bonds between the old anionic polymer and peptidoglycan are subject to reactions, leading to the loss of the former only. (These reactions are essentially different from those causing turnover, since this process is characterized by hydrolytic reactions within the peptidoglycan molecule.) In this context, the teichoicase activities that have been reported by several authors (15, 27) might play a role. The detection of relatively large amounts of old anionic polymer in the extracellular fluid (see Fig. 6) seems to support this suggestion. Another observation, however, militates strongly against it. As can be derived from the data shown in Fig. 3, the ratio between the sum of the anionic polymers and peptidoglycan in the wall is constant. This was found in all experiments (data not shown). This constancy implies that the anionic polymers should be incorporated into the wall at an enhanced rate (relative to peptidoglycan) to compensate for the assumed specific excision of the old polymer. The incorporation process involves a reaction linking the anionic polymers to nascent peptidoglycan (12, 21), and, therefore, a mechanism which can account for the constant ratio that ultimately results between the anionic polymers and peptidoglycan is hard to envisage. The presumption that both wall assembly and turnover take place under these cultural conditions in a fashion identical to the processes encountered in cultures in steady state, on the contrary, yields directly an explanation of the observed constancy of this ratio, and therefore appears to be much more likely.

The considerations of the previous paragraph help one to understand better how the pattern of ingrowth of the new anionic polymer in the wall is effected. The discrepancy with the theoretically expected ingrowth, as observed in Fig. 3 and 5, should not be interpreted as showing that the rate of incorporation of the new anionic polymer into the walls during the course of the experiment is higher than in cells in the final steady-state culture. It is simply due to the specific properties of the turnover process, as it occurs in bacilli (see above); in this transient state the peptidoglycan, which is incorporated into the wall with the new anionic polymer bound to it, is not yet subject to turnover, and inevitably the rate of accumulation of this latter polymer in the wall is higher than in the final steady state.

The data we assembled on the accumulation of the "new" anionic polymer in the extracellular fluid fully support the assumption that turnover took place, as observed in bacilli growing in steady state: hardly any polymer was detected in the supernatant fraction until after a lag period, in which the new polymer undergrew and displaced the old polymer.

The conclusion regarding the absence of incorporation of the "old" anionic polymer, together with the observation that its synthesis continued in the course of the limitation switch experiments (Fig. 6) leads us to propose that direct export of the old anionic polymer from within the cell took place under these cultural conditions. As was stated above, the occurrence of such a process was concluded previously on the basis of results obtained with steady-state cultures, and was called secretion (7). For these latter cultures, it was suggested that direct secretion had been caused by a disparity between the rates of synthesis of the peptidoglycan and anionic polymer chains, and a loss to the medium of the surplus of the anionic polymer.

Secretion of the anionic polymer and incorporation of the other as found in the present experiments, seems to be a more complex phenomenon. In these transient-state cells, it appears that the wall assembly system is actively choosing only the new anionic polymer for attachment to the peptidoglycan, whereas the precursors of the old polymer are still made and polymerized. The occurrence of old polymer secretion, both during a switch from P_i-limited to P_i-excess growth conditions and vice versa, indicates clearly that secretion is not due to the intrinsic higher specificity of the wall assembly system for one of the polymers, but that incorporation of the one compound and not the other is actively regulated both ways.

It is not known whether anionic polymer secretion, as found above and in steady-state cultures of *B. subtilis* subsp. *niger* WM and *B. subtilis* W23 (7), is common among *Bacillus* species, since no reports are available on the quantitative analysis of culture supernatants for wall components. In this context it may be relevant that Hussey et al. (18) who performed a limitation switch experiment (from P_i excess to P_i limitation), using *B. licheniformis*, reported that the activity of CDPglycerol pyrophosphorylase dropped to zero, which precludes of course any synthesis of teichoic acid in the transient state of growth.

The mechanisms involved in the regulation of the activity of CDPglycerol pyrophosphorylase, as depicted in Fig. 7, are probably complex. However, since the change in the availability of

phosphate to the culture provokes a primary response at the level of the wall assembly system, as pointed out above, the activity of the enzymes catalyzing the synthesis of precursors does not seem to be crucial in the regulation of cell wall composition under these growth conditions, except from being a prerequisite for polymer synthesis. This conclusion was supported by the finding (Kruyssen, Ph.D. thesis) that another enzyme, UDPacetylglucosamine 4-epimerase (EC 5.1.3.7.), specific for the pathway of teichuronic acid biosynthesis, was present in the organism studied here, under both P_i-limited and P_i-excess growth conditions.

Since the early paper by Ellwood and Tempest (8) in which the problem of anionic polymer synthesis regulation in transient states was delineated, several reports were published focussing on the molecular aspects of the phenomenon (13, 18, 25). The firm proof presented here that, especially under conditions of environmental change, the actual wall composition does not necessarily reflect the activities of the machineries synthesizing the respective wall components, allows a different interpretation of the data reported in earlier papers (dealing with wall-bound polymers only) which were based explicitly on the assumption that the wall-bound polymer represented all anionic polymer synthesized during the experiment (25).

The picture of anionic polymer metabolism is still far from complete. In particular, the nature of the system regulating the wall composition (that is, the relative rates of incorporation of the respective anionic polymers, towards that of peptidoglycan) remains unknown, but the involvement of intermediates of phosphate metabolism and their direct interaction with the wall assembly machinery appears obvious. As a possibility it may be mentioned that the P_i supply might exert control on the synthesis of the linking unit (5) between peptidoglycan and the anionic polymers and thus determines which anionic polymer can be coupled to the nascent peptidoglycan. An indication in this direction was presented by Glaser and Loewy (14), who provided evidence that the activity of a membrane-bound enzyme in *B. subtilis* W23 involved in the biosynthesis of a lipid carrier, which is believed to be crucial in the coupling reaction between teichoic acid and peptidoglycan, is sensitive to the availability of phosphate in the growth environment. Further speculations regarding this matter must await more detailed knowledge about the reactions involved in the coupling of teichuronic acid to peptidoglycan in *B. subtilis*. Mechanisms regulating the synthesis of the precursors of anionic polymers by feedback coupling to the actual need apparently do not exist in the organism studied in this work. Under certain growth conditions, this absence may lead to overproduction of polymeric material which the cell disposes of by direct secretion.

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