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Cell Wall Metabolism in *Bacillus subtilis* subsp. *niger*: Accumulation of Wall Polymers in the Supernatant of Chemostat Cultures

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Cell wall polymers were measured both in the cells and in the cell-free medium of samples from steady-state chemostat cultures of *Bacillus subtilis*, growing at various rates under magnesium or phosphate limitation. The presence of both peptidoglycan and anionic wall polymers in the culture supernatant showed the occurrence of wall turnover in these cultures. Variable proportions of the total peptidoglycan present in the culture samples were found outside the cells in duplicate cultures, indicating that the rate of peptidoglycan turnover is variable in *B. subtilis*. Besides peptidoglycan, anionic wall polymers were detected in the culture supernatant: teichoic acid in magnesium-limited cultures and teichuronic acid in phosphate-limited cultures. In several samples, the ratio between the peptidoglycan and the anionic polymer concentrations was significantly lower in the extracellular fluid than in the walls. This divergency was attributed to the occurrence of direct secretion of anionic polymers after their synthesis.

Fragments of cell wall peptidoglycan have been demonstrated in the culture supernatant of exponentially growing *Bacillus subtilis* (21, 23) and a number of other bacterial species (2, 4, 15, 17). Chemical analysis showed that these extracellular products had arisen from the hydrolytic action of wall-bound autolytic enzymes on peptidoglycan in vivo (21). Thus, in *B. subtilis* cultures mainly free glycan chains and cross-linking peptides were found, apparently released by the action of the main autolytic enzyme *N*-acetylmuramyl-L-alanine-amidase (EC 3.5.1.28) (3) on wall peptidoglycan. Accessory wall polymers, such as the anionic polymers teichoic acid (TA) and teichuronic acid (TUA) (19), appeared in the supernatant by virtue of their covalent linkage to peptidoglycan.

In a previous report (9), we showed that analysis of the way by which a specific radioactive peptidoglycan label was lost from *B. subtilis* by turnover provided some insight into the mechanisms of wall growth in this organism. Based on kinetic data, the conclusion (1, 23) was reinforced that wall turnover in bacilli occurs from the outer wall layer and that wall surface enlargement takes place by spreading of peptidoglycan (9). The rate of wall turnover calculated from those previous experiments, however, referred merely to the peptidoglycan amino sugar backbones, since in *B. subtilis* specific wall labeling is feasible only by using the glycan precursor *N*-acetylglucosamine (25).

We now have measured the rates of turnover of both peptidoglycan fragments (glycan strands and cross-linking peptides) and of the anionic polymers by determining their concentrations in both the wall and the cell-free medium of chemostat cultures of *B. subtilis*. The use of chemostat cultures offered several advantages in these studies. The existence of a stabilized situation in a steady-state chemostat culture growing at a specified rate allows one to obtain the rate of loss of wall components simply from the measured concentrations. It has been suggested that the nature of the anionic wall polymer would exert a large influence on wall turnover (16); experiments with radioactive labeling as described above did not support such a hypothesis (9). In chemostat cultures, turnover can now be measured in exponentially growing cultures containing either TA or TUA as the anionic wall polymers by growing the cells under magnesium or phosphate limitation, respectively (19). The results indicated that peptidoglycan and anionic polymers were lost at dissimilar rates from the cells. This phenomenon was detected under several growth conditions. It is argued that a part of the polymerized anionic polymers is not incorporated into the wall structure under these circumstances but secreted directly to the extracellular fluid. A rationale for this phenomenon

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is proposed along with some remarks on the role of autolytic enzymes in the regulation of the rate of wall polymer synthesis in vivo (W. R. de Boer, Ph.D. thesis, University of Amsterdam, Amsterdam, The Netherlands, 1979; F. J. Kruyssen, Ph.D. thesis, University of Amsterdam, 1979).

MATERIALS AND METHODS

Strains. *B. subtilis* subsp. *niger* WM as described previously (9) and *B. subtilis* W23 were used.

Media and growth conditions. Cells were grown under phosphate or magnesium limitation in 0.5- or 1-liter chemostats in the media described by Evans et al. (10), using 75% of the concentrations specified. For details on cultivation of the cells and the control of the culture, see Kruyssen et al. (19). The maximal specific growth rate in each medium was $\mu = 0.75$ to 0.80 h^{-1} (generation time ± 50 min).

Isolation and analysis of cell walls. The methods used during the isolation and purification of cell walls, and the determination of the contents of peptidoglycan, TA, and TUA were described by Kruyssen et al. (19). The amounts of the different wall polymers have been expressed as nanomoles per milligram of cellular dry weight and were derived from measurement of the component monomers (i.e., peptidoglycan stands for diaminopimelic acid [A_2pm], TA for phosphorus, and TUA for uronic acid; for details see below).

Wall polymers in supernatants of chemostat cultures. Samples of 20 ml (equivalent to 10 to 75 mg of cellular dry weight) were taken from steady-state chemostat cultures. The samples were cooled rapidly on ice and centrifuged at 4°C for 10 min at 12,000 × *g*. The supernatant was decanted and stored at −20°C. Supernatant samples taken from steady states at a series of increasing growth rates were thawed and extracted twice with petroleum ether (20 ml; boiling range, 60 to 80°C) to remove the polypropylene glycol used as an antifoaming agent during the cultivation of the cells. Material which formed a layer at the interface between the two liquids was included in the water phase. The extracted sample was cooled at 0°C on ice, and 2 ml of 100% trichloroacetic acid was added. After incubation at 0°C for 20 min, the precipitate was removed by centrifugation at 4°C (20 min, 48,000 × *g*), leaving a clear supernatant. The sticky precipitate was dissolved in 2 ml of 0.1 N NaOH. The supernatant was divided into two portions; 12 ml was extracted three times with ether (25 ml) to remove the trichloroacetic acid (S-samples). The remaining 10 ml was dialyzed against running tap water (18 h) and a large volume of double-distilled water (5 h) (SD-samples). Both S- and SD-samples were lyophilized and then dissolved in 5.0 ml of water. Supernatant concentrations of wall polymers were expressed as nanomoles per milligram, i.e., the amount of monomeric units (A_2pm, phosphate, uronic acid) present in a culture volume containing 1 mg of cells.

Analytical methods. Total phosphorus was determined as described by Chen et al. (6); the oxidation procedure was adapted to process small amounts of material as described previously (19). Uronic acid in SD-samples was measured with the harmine reagent (Koch-Light, Colnbrook, England), after hydrolysis in concentrated sulfuric acid essentially according to Wardi et al. (27). SD-samples containing teichoic acid as well as teichuronic acid showed an absorption at the wavelength used before the addition of the reagent, probably due to the action of the acid on glucose residues present in the teichoic acid. Final absorption was corrected for such disturbances. Uronic acid could not be determined in S-samples since the residual glucose, used as the carbon source, caused too high blanks. For analysis of peptidoglycan components, S- or SD-samples were hydrolyzed in 6 N HCl for 6 h at 100°C in air-tight stoppered tubes; the hydrolyzed samples were frozen in liquid nitrogen, and the HCl was removed by lyophilization over NaOH pellets (two times). Muramic acid ($MurNH_2$), glucosamine ($GlcNH_2$), alanine (Ala), glutamate (Glu), and A_2pm present in the samples were determined by using an amino acid analyzer (Locarte, London, England), and an elution system based on citrate buffers. Galactosamine ($GalNH_2$) was determined in samples hydrolyzed as described above either from the amino acid spectra or with a galactose oxidase test system (Galax, Kabi A. G., Stockholm, Sweden). Glycerol was measured after hydrolysis in HCl (see above) and dephosphorylation with alkaline phosphatase (*Escherichia coli* APase, pH 9.0), by the method of Wieland (28). Hexose was determined by use of the anthrone reagent as described by Jermyn (18).

Theoretical considerations. The amounts of the different wall components in the supernatant can be used to calculate the percentage of the cell-bound wall lost per generation time. Suppose that a nanomoles of a wall polymer per milligram is found in the supernatant and that b nanomoles of a wall polymer per milligram is found in the cells. Assuming that all the polymers present in the medium are the result of the enzymatic action (by autolytic enzymes) on the cell-bound wall, it can be stated that $\kappa \cdot b$ nmol mg^{-1} h^{-1} is lost from the cell wall (κ is a rate constant; dimension, h^{-1}). Loss of supernatant-located polymers from the culture by washout will be $D \cdot a$ nmol mg^{-1} h^{-1} ([26]; D is the dilution rate in the chemostat [h^{-1}]), so the rate equation will be:

$$\frac{da}{dt} = -D \cdot a + \kappa \cdot b$$

In a steady-state chemostat culture, $D = \mu = (\ln 2/\tau)$ (μ = specific growth rate, h^{-1}; τ = doubling time, h) and the concentration of wall polymers is constant (that is, $[da/dt] = 0$; see also reference 26), so the fraction of cellular wall lost per hour (κ) will be:

$$\kappa = \mu \cdot \frac{a}{b} \, h^{-1}$$

and per generation time the percentage of the cell-bound wall polymers lost to the medium (k) will be:

$$k = \kappa \cdot \tau \cdot 100\% = \frac{a}{b} \cdot \ln 2 \cdot 100\%.$$

RESULTS

Determination of wall polymers in supernatants of chemostat cultures. (i) Peptido-

glycan. In *B. subtilis*, A_2pm and $MurNH_2$ are compounds occurring only in the peptidoglycan. We have used A_2pm values in the comparison between the amounts of cell-bound and supernatant peptidoglycan, since $MurNH_2$ is subject to considerable (and rather variable) losses during hydrolysis in HCl (19). No A_2pm was found in supernatants before acid hydrolysis, indicating its bound nature. Generally, a molar proportionality of A_2pm with the amino sugars $MurNH_2$ and $GlcNH_2$ was similar to that in the cell wall (for exceptions see particular experiments). The organisms used excreted Ala and Glu as free amino acids, making the molar ratio of the amino acids within the peptide cross-links in the supernatants difficult to assess. Differences between Ala and Glu values before and after hydrolysis pointed to a A_2pm/Glu/Ala ratio of approximately 1:1:1.5, as found in the walls.

When culture supernatants were dialyzed, it appeared that over 90% of the A_2pm, Ala, and Glu present in the samples was lost (Fig. 1). For $MurNH_2$ and $GlcNH_2$, the percentage that was lost by dialysis from supernatant samples showed a relation to growth rate. In samples taken from cultures grown at low growth rates, about 60% of the two amino sugars was lost, whereas even lower percentages were lost from samples from faster growing cultures (Fig. 1).

(ii) TA. TA in the supernatant was measured by determinations of total phosphate in dialyzed culture supernatants (SD-samples). In magnesium-limited cultures, this phosphate accounted for 80 to 90% of the organically bound phosphate found in nondialyzed supernatants (data not shown). The supernatants showed a very low absorption at 260 nm after dialysis, and it was concluded that nucleic acids did not contribute significantly to the amount of phosphorus found. In several samples a close molar proportionality was found between phosphorus and glycerol, as predicted by the structure of TA (8). The presence of bound hexose in the preparations was demonstrated quantitatively by the formation of color in the anthrone reaction.

(iii) TUA. TUA was also determined in the culture supernatants after dialysis. Identification of the polymer as TUA was threefold. It was identified by the specific color reaction of uronic acids, by the determination of $GalNH_2$ in the amino acid analyzer, and by the specific enzymatic assay for $GalNH_2$. The ratio between uronic acid and $GalNH_2$ was 1:0.6 to 1.0, which was also found in determinations on whole walls (19). No significant amounts of A_2pm or amino sugars were detected in the material precipitated after addition of trichloroacetic acid.

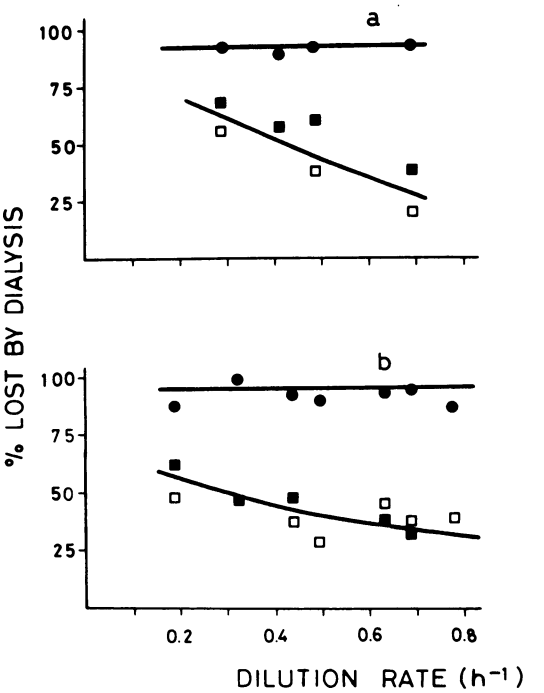

FIG. 1. *Loss of turnover products from supernatant samples by dialysis. Peptidoglycan components present in supernatant samples from phosphate-limited (a) and magnesium-limited (b) cultures were measured as described in the text before and after dialysis of the samples.* ●, *A_2pm;* ■, *$MurNH_2$;* □, *$GlcNH_2$.*

Wall polymers in supernatants of phosphate-limited cultures. The amounts of wall polymers present in the cell wall and supernatant fractions were measured in samples from phosphate-limited chemostat cultures of *B. subtilis* subsp. *niger* WM. Samples were taken from steady-state cultures at several dilution rates. The results from one of these cultures are shown in Fig. 2. In all samples, approximately equal amounts of A_2pm were found in the supernatant (A_2pm_x) and the wall fraction (A_2pm_i) of the cells (i.e., $A_2pm_x/A_2pm_i = 1$), as shown in the ratio between the solid and hatched parts of the left bars in the histograms of Fig. 2. In a number of duplicate cultures (samples taken at $D = 0.2$ to 0.3 h^{-1}), variable amounts of extracellular A_2pm were found, whereas the cellular content was relatively constant (see reference 19). As a consequence, the A_2pm_x/A_2pm_i ratio ranged from 0.5 to 1.0 in the various cultures. When the dilution rate was below 0.5 h^{-1}, TUA was present in the cells and in the cell-free supernatant in equal concentrations (the middle bar in the histograms of Fig. 2). At higher dilution rates, TUA was replaced by TA in the walls, notwithstand-

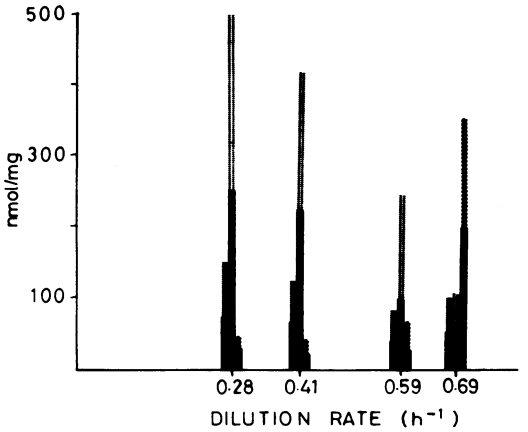

FIG. 2. *Wall polymers in the cells (black part of the bars) and the supernatant (hatched part of the bars) in a phosphate-limited chemostat culture of B. subtilis subsp. niger WM at several dilution rates. The amounts of peptidoglycan (left bar), TUA (middle bar), and TA (right bar) in the different samples are shown.*

ing the maintenance of the phosphate-limiting conditions (19). Analysis of the supernatant fluids of such cultures revealed, however, that the external TUA concentration (TUA_x) decreased to a much lesser degree than did the cellular teichuronic acid content (TUA_i). This is reflected in the increasing TUA_x/TUA_i ratio in samples taken at higher dilution rates: ±1.0 at $D < 0.5\ h^{-1}$; 1.6 at $D = 0.59\ h^{-1}$ and 3.8 at $D = 0.69\ h^{-1}$. As stated above, no alteration occurred in the fraction of the peptidoglycan (A_2pm) found outside the cells. As a consequence, the extracellular TUA concentration was much higher than would be expected from the extracellular A_2pm concentration and the ratio between these two components as found in the walls. Thus, the ratio between A_2pm and uronic acid in the samples taken at high dilution rates was 0.55 in the supernatant (A_2pm_x/TUA_x) and 2.4 in the wall fraction (A_2pm_i/TUA_i). Under these growth conditions, less than 10% TUA was present in the walls, and mainly TA was found as the anionic polymer; TA was also detected in the supernatant ($TA_x/TA_i = 0.7$, right bars in Fig. 2). It should be stressed here that all samples were taken from steady-state chemostat cultures, implying that these data refer to stabilized metabolic states within the cultures.

A single phosphate-limited culture of *B. subtilis* W23 was analyzed (Fig. 3). Wall content and composition did not differ significantly from those in *B. subtilis* subsp. *niger* WM at the lower dilution rates. The switch from TUA to TA in the walls was at a somewhat higher dilution rate in the *B. subtilis* W23 culture. Very little A_2pm was found in any of the supernatant samples ($A_2pm_x/A_2pm_i < 0.1$). $MurNH_2$ was found in equal proportions to A_2pm in the supernatants. Extracellular $GlcNH_2$ was found in approximately eight times higher concentration than was A_2pm_x. Forty percent of this material was lost by dialysis (A_2pm_x over 90%); the origin of this material was not investigated further. In contrast with the low extracellular A_2pm concentrations, very high amounts of teichuronic acid were demonstrated in the supernatants, giving a ratio of $TUA_x/TUA_i = 4.9$ in the sample taken at $D = 0.12\ h^{-1}$. This ratio dropped to 1.3 at intermediate dilution rates and to 1.9 in a sample at high growth rate ($D = 0.72\ h^{-1}$) which contained a substantial amount of TA in the walls.

Wall polymers in supernatants from magnesium-limited cultures. In a magnesium-limited culture of *B. subtilis* subsp. *niger* WM, it was found that the amount of A_2pm in the culture supernatant (A_2pm_x) increased with increasing growth rate (Fig. 4). Thus, the A_2pm_x/A_2pm_i ratio was 0.1 at a dilution rate of $0.18\ h^{-1}$ and rose to 0.7 at $D = 0.77\ h^{-1}$. The extracellular TA concentration (TA_x) was relatively constant at 150 to 200 nmol mg^{-1} throughout the range of growth rates tested. This amount actually corresponded to an increasing fraction of the total TA in the culture because the wall content de-

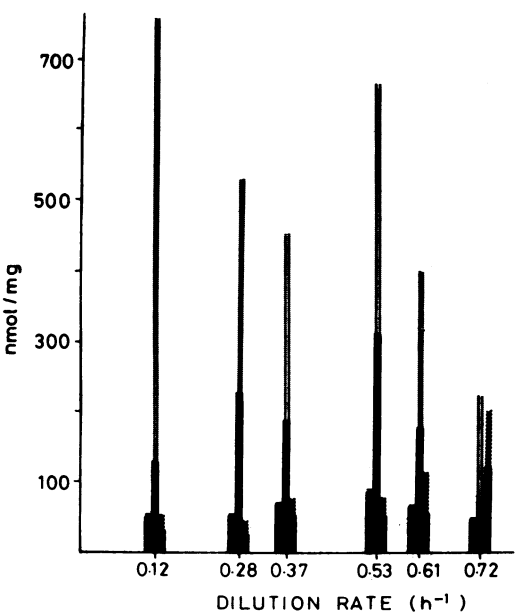

FIG. 3. *Wall polymers in the cells (black part of the bars) and supernatant (hatched part of the bars) in a phosphate-limited chemostat culture of B. subtilis W23 at several dilution rates. The amounts of peptidoglycan (left bar), TUA (middle bar), and TA (right bar) in the different samples are shown.*

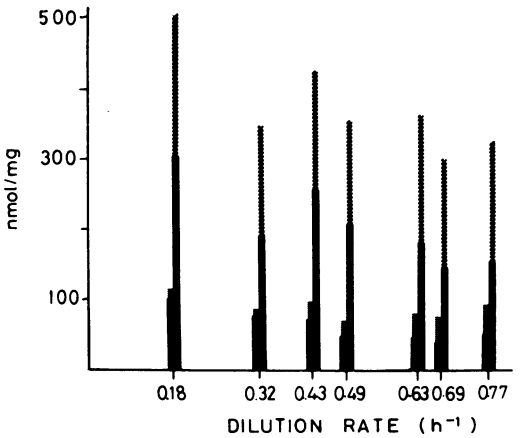

FIG. 4. *Wall polymers in the cells (black part of the bars) and supernatant (hatched part of the bars) in a magnesium-limited chemostat culture of B. subtilis subsp. niger WM at several dilution rates. The amounts of peptidoglycan (left bar) and TA (right bar) in the different samples are shown.*

creased at higher growth rates (19). Therefore, the ratio between cellular and extracellular TA increased from 0.7 at $D = 0.18\ h^{-1}$ to 1.1 at near-maximal growth rate ($D = 0.77\ h^{-1}$).

From the data shown above, it follows directly that in the samples taken at a low dilution rate a totally different ratio was found between peptidoglycan (A_2pm) and the anionic polymer in the culture supernatant and in the cellular walls (although less extreme than between A_2pm and TUA in the phosphate-limited culture of *B. subtilis* W23). For instance, in the sample taken at $D = 0.18\ h^{-1}$, a molar ratio of 3.0 was found in the walls between phosphate (TA) and A_2pm; in the supernatant TA_x/A_2pm_x was 16.5. So, as described above, the supernatant anionic polymer concentration was much higher than would be expected from the supernatant peptidoglycan concentration.

In some duplicate cultures ($D = 0.2$ to $0.3\ h^{-1}$), higher rates of peptidoglycan turnover (i.e., a higher A_2pm_x/A_2pm_i ratio) were found. As was found in the phosphate-limited cultures (see above), this variation was caused mainly by the different amounts of A_2pm_x, the cellular A_2pm content being rather constant at a given dilution rate in duplicate cultures (19). The TA_x/TA_i ratio was fairly constant in duplicate cultures. As a consequence, the above-mentioned differences between the ratios of cell-bound and external wall components were smaller; e.g., in a sample from a magnesium-limited culture taken at $D = 0.29\ h^{-1}$, the ratio between TA and peptidoglycan in the cells was 3.3 (almost as in Fig. 4), whereas in the supernatant this ratio was 6.9 (in Fig. 4 at $D = 0.32\ h^{-1}$, 15.9). These data clearly indicated that peptidoglycan and TA (or TUA in the phosphate-limited cultures) were lost from the cells at dissimilar rates.

Rate of turnover. As shown below the rate of loss from the cells of the different wall components in steady-state chemostat cultures (expressed as the percentage of the wall polymers lost per generation time) can be obtained from the ratio between the supernatant and the cellular concentrations, multiplied by ln2. From Table 1, it is clear that peptidoglycan and the anionic polymers were lost at very different rates in the cultures described in detail above.

DISCUSSION

Data reported in this paper show that peptidoglycan and anionic polymers were present in different ratios in the cell walls and in the culture supernatant of *B. subtilis* grown under several conditions in chemostat culture. Such dissimilarity was not to be expected a priori. Cell wall turnover in *B. subtilis* is caused by autolytic enzymes (mainly *N*-acetylmuramyl-L-alanine amidase [3]) which act randomly on bonds within the peptidoglycan in the outer layer of the wall (9). Anionic polymers will appear in the supernatant because of their covalent coupling to the muramic acid moiety in peptidoglycan. Several possible reasons for the observed disparity between wall and supernatant contents of these two components may be considered.

Firstly, peptidoglycan and anionic polymers might be lost at dissimilar rates by preferential excision of those peptidoglycan fragments containing an anionic polymer. This would imply that, at the membrane, newly made peptidoglycan chains are heavily loaded with anionic polymers and that specific excision of many of the anionic polymers (linked to small peptidoglycan fragments) would occur in the turnover-sensitive layer of the wall (see reference 9). As was shown clearly by pulse-label experiments no peptidoglycan (and, most likely, no anionic polymers) is lost from the turnover-resistant inner wall layers (9). So all peptidoglycan-anionic polymer complexes released must originate from excision in the outer layers, at the rates indicated by k in Table 1 for peptidoglycan and the anionic polymer. It was discussed previously (9), however, that a significantly different rate of excision of anionic polymers and peptidoglycan from the turnover-sensitive layers of the wall is improbable. So, we regard as most likely that turnover proceeded at approximately identical rates for peptidoglycan and the anionic polymers as has been found previously in *B. subtilis* W23 (21) and *Staphylococcus aureus* (29).

TABLE 1. *Rate of loss of wall polymers in chemostat cultures of B. subtilis at several dilution rates*

Organism	Limitation	Dilution rate (h^{-1})	Rate of loss of wall polymers (k)[a]		
			Peptidoglycan	TA	TUA
B. subtilis subsp. *niger* WM	Magnesium	0.18	8.3	46	
	Magnesium	0.32	9.1	58	
	Magnesium	0.43	22	43	
	Magnesium	0.49	29	50	
	Magnesium	0.63	46	71	
	Magnesium	0.67	57	73	
	Magnesium	0.77	46	73	
	Phosphate	0.28	68		68
	Phosphate	0.41	59		69
	Phosphate	0.59	94	50	109
	Phosphate	0.69	69		260
B. subtilis W23	Phosphate	0.12	6.1		336
	Phosphate	0.28	3.0		91
	Phosphate	0.37	1.0		99
	Phosphate	0.53	1.4		81
	Phosphate	0.61	1.5	60	82
	Phosphate	0.72	2.0	46	128

[a] k is defined as the percentage of the wall-bound polymers lost from the cells per generation time; see text for details.

Another explanation for the different ratios of wall polymers in cells and supernatant might be the masking of peptidoglycan turnover by reutilization of a part of the turnover products by the cells. Release, and, possibly, reutilization of free A_2pm occur during wall turnover in *Bacillus megaterium* growing in rich medium but not in mineral medium (21). Endopeptidases, needed for the degradation of wall peptides, have only been demonstrated in sporulating *Bacillus sphaericus* (14) and *B. subtilis* (13), and not in vegetative cells, making the presence of such enzymes under our growth conditions unlikely. Reutilization of amino sugars from the turnover products also must be excluded, since almost complete loss from the cells of (tritiated) *N*-acetylglucosamine-derived wall label was observed in turnover experiments (9).

Therefore, we suggest that the aberrant ratios between peptidoglycan and the anionic polymers are not caused by anomalies in the mechanisms of wall turnover, but by the direct secretion of a fraction of the anionic polymers after their polymerization.

The occurrence of secretion of anionic wall polymers indicates that these wall components are synthesized in the cytoplasmic membrane at a higher rate than is needed for filling all the attachment sites on the emerging peptidoglycan chains. Detachment of the anionic polymer chain from the polymerization locus is apparently not dependent on the concomitant transfer to peptidoglycan. It might be suggested that the anionic polymers are detached after a "critical length" has been reached; the contemporaneous availability of a peptidoglycan attachment site determines whether either linkage or secretion will follow. The secretion of TUA in phosphate-limited cells, under the conditions in which TA is incorporated into the walls (Fig. 2), may indicate, in addition, a different affinity of the enzyme systems linking the anionic polymers to the peptidoglycan attachment site for both compounds. The secretion of anionic polymers under conditions in which both TA and TUA are synthesized will be discussed more extensively elsewhere (20).

Throughout our experiments on wall turnover in *B. subtilis*, we were hampered by the irreproducibility of the rate of peptidoglycan turnover between duplicate cultures (9), a phenomenon also noted by other authors (21). In the experiments shown here, it was found that in samples from different cultures grown under the same limitation and at an identical rate, the supernatant concentrations of peptidoglycan were not the same. A contribution of some directly secreted peptidoglycan cannot be excluded here. But results from labeling experiments, in which turnover is measured exclusively, made it clear that the rate of turnover of wall-bound peptidoglycan was variable between duplicate cultures (9). The cellular content of peptidoglycan was rather constant at a specified dilution rate (19); therefore, the presence of variable amounts in the supernatant implies that the total amount of peptidoglycan synthesized per milligram of cells per hour is higher in cells

showing a high rate of turnover and lower when turnover is less rapid. Variable losses by turnover apparently can be compensated for by an adaption of the rate of peptidoglycan synthesis and wall assembly to the incidental turnover rate. Such a flexible response suggests that polymerization activity is regulated directly by the actual need for new peptidoglycan polymers, as determined by the rates of wall growth and turnover.

Several reviewers (11, 12) have proposed that newly polymerized peptidoglycan chains are already attached to the existing walls by transpeptidation reactions before polymerization is completed. When such intimate coupling does exist, it is probable that the rate at which peptidoglycan chains can be made is not only dependent on the rate of supply of precursors, but also on the rate at which chain growth is permitted within the existing peptidoglycan structure; this latter factor could be governed by the radial and lateral movements during wall surface expansion. Speculatively, it is proposed that the autolytic amidase acts not only in the outer layers of the wall as a hydrolytic enzyme (9), but also as a transamidase in the layers of the wall nearer to the cytoplasmic membrane (as already proposed by Chatterjee et al. [5]), functioning in the "spreading" mechanisms which lead to surface enlargement of the wall (1, 9, 23, 24). In this system, a higher autolysin content or activity in the walls would result in a faster movement of new peptidoglycan chains (or conglomerates of chains) away from the site of synthesis by a higher transamidase activity, permitting a higher rate of polymerization and attachment of new chains. So, variable losses from the wall by an inconstant autolysin content or activity are compensated for by the direct adjustment of the rate of attachment of new peptidoglycan chains, made possible by the variation of the autolysin itself. The intimate relation between the rate of peptidoglycan synthesis, the rate of wall surface expansion, and, finally, the rate of turnover by the involvement of the autolysins in all three processes could be one of the methods by which the cell can regulate its equilibrium amount of cellular wall.

No published data are available to test this hypothesis; it has been found by Pooley (cited in reference 7) that in *B. subtilis* Ni15 (showing a low rate of turnover [23]) overall peptidoglycan synthesis was stimulated by the external addition of a crude autolysin preparation. It was shown (23) that wall turnover was greatly accelerated under these conditions. These data indicate that peptidoglycan synthesis may be regulated, at least partially, by the rate of wall turnover; the translation system, however, remains unknown.

Returning to the secretion of anionic polymers, it can now be stated that polymerization of these wall components is apparently not regulated by the actual need for anionic polymers by the growing wall, as suggested above for peptidoglycan. TA is transferred as completed polymers to the attachment sites on peptidoglycan (30), which in itself makes a feedback control of the polymerization by the rate of wall growth less likely. It is plausible that wall assembly is directed to the delivery of a wall with a constant ratio between peptidoglycan and the anionic polymer. Since anionic polymers probably can be attached only to peptidoglycan chains synthesized concomitantly, and not to pre-existing walls (22, 30), a slightly excessive anionic polymer synthesis might be advantageous to the cells under growth conditions showing variable turnover activities. This would permit the cells to respond rapidly, at the wall assembly level, to variations in the rate of peptidoglycan synthesis as a result of a varying autolysin activity and ensure the complete filling of peptidoglycan with anionic polymers under all circumstances.

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LITERATURE CITED

1. **Anderson, A. J., R. S. Green, A. J. Sturman, and A. R. Archibald.** 1978. Cell wall assembly in *Bacillus subtilis*: location of wall material incorporated during pulsed release of phosphate limitation, its accessibility to bacteriophages and concanavalin A, and its susceptibility to turnover. J. Bacteriol. **136**:886–899.
2. **Blümel, P., W. Uecker, and P. Giesbrecht.** 1979. Zero order kinetics of cell wall turnover in *Staphylococcus aureus*. Arch. Microbiol. **12**:103–110.
3. **Brown, W. C.** 1977. Autolysins in *Bacillus subtilis*, p. 75–84. *In* D. Schlessinger (ed.), Microbiology—1977. American Society for Microbiology, Washington, D.C.
4. **Chaloupka, J., and P. Křečková.** 1974. Characterization of degradation products of the cell wall released during growth and sporulation of *Bacillus megaterium*. Folia Microbiol. (Prague) **19**:292–300.
5. **Chatterjee, A. N., R. J. Doyle, and U. N. Streips.** 1977. A proposed functional role for bacterial N-acetylmuramyl-L-alanine amidases. J. Theor. Biol. **68**:385–390.
6. **Chen, P. S., T. Y. Toribara, and H. Warner.** 1956. Microdetermination of phosphorus. Anal. Chem. **28**: 1756–1758.
7. **Daneo-Moore, L., and G. D. Shockman.** 1977. The bacterial cell surface in growth and division, p. 597–715. *In* G. Poste and G. Nicholson (ed.), Cell surface reviews, vol. 4. North-Holland Publishing Co., Amsterdam.
8. **de Boer, W. R., F. J. Kruyssen, J. T. M. Wouters, and C. Kruk.** 1976. The structure of teichoic acid from *Bacillus subtilis* var. *niger* WM as determined by ^{13}C

nuclear-magnetic-resonance spectroscopy. Eur. J. Biochem. **62**:1–6.
9. **de Boer, W. R., F. J. Kruyssen, and J. T. M. Wouters.** 1981. Cell wall turnover in batch and chemostat cultures of *Bacillus subtilis*. J. Bacteriol. **145**:50–60.
10. **Evans, C. G. T., D. Herbert, and D. W. Tempest.** 1970. The continuous cultivation of micro-organisms. 2. Construction of a chemostat, p. 277–327. *In* J. Norris and D. Ribbons (ed.), Methods in microbiology, vol. 2. Academic Press, Inc., New York.
11. **Fiedler, F., and L. Glaser.** 1973. Assembly of bacterial walls. Biochim. Biophys. Acta **300**:467–485.
12. **Ghuysen, J. M.** 1977. Biosynthesis and assembly of bacterial cell walls, p. 463–596. *In* G. Poste and G. Nicholson (ed.), Cell surface reviews, vol. 4. North-Holland Publishing Co., Amsterdam.
13. **Guinand, M., G. Michel, and G. Balassa.** 1976. Lytic enzymes in sporulating *Bacillus subtilis*. Biochem. Biophys. Res. Commun. **68**:1287–1293.
14. **Guinand, M., M. J. Vacheron, G. Michel, and D. J. Tipper.** 1979. Location of peptidoglycan lytic enzymes in *Bacillus sphaericus*. J. Bacteriol. **138**:126–132.
15. **Hebeler, H. B., and F. E. Young.** 1976. Mechanism of autolysis of *Neisseria gonorrhoeae*. J. Bacteriol. **126**: 1186–1193.
16. **Herbold, D. R., and L. Glaser.** 1975. Interaction of N-acetylmuramic acid L-alanine amidase with cell wall polymers. J. Biol. Chem. **250**:7231–7238.
17. **Hisatsune, K., S. J. de Courcy, Jr., and S. Mudd.** 1967. Studies on the carbohydrate-peptide fraction of the centrifugal supernatants of *Staphylococcus aureus* cultures. Biochemistry **6**:586–594.
18. **Jermyn, M. A.** 1975. Increasing the sensitivity of the anthrone method for carbohydrate. Anal. Biochem. **68**: 332–335.
19. **Kruyssen, F. J., W. R. de Boer, and J. T. M. Wouters.** 1980. Effects of carbon source and growth rate on cell wall composition of *Bacillus subtilis* subsp. *niger*. J. Bacteriol. **144**:238–246.
20. **Kruyssen, F. J., W. R. de Boer, and J. T. M. Wouters.** 1981. Cell wall metabolism in *Bacillus subtilis* subsp. *niger*: effects of changes in phosphate supply to the culture. J. Bacteriol. **146**:867–876.
21. **Mauck, J., L. Chan, and L. Glaser.** 1971. Turnover of the cell wall of gram-positive bacteria. J. Biol. Chem. **246**:1820–1827.
22. **Mauck, J., and L. Glaser.** 1972. On the mode of in vivo assembly of the cell wall of *Bacillus subtilis*. J. Biol. Chem. **247**:1180–1187.
23. **Pooley, H. M.** 1976. Turnover and spreading of old wall during surface growth of *Bacillus subtilis*. J. Bacteriol. **125**:1127–1138.
24. **Pooley, H. M.** 1976. Layered distribution, according to age, within the cell wall of *Bacillus subtilis*. J. Bacteriol. **125**:1139–1147.
25. **Rogers, H. J., P. F. Thurman, C. Taylor, and J. N. Reeve.** 1974. Mucopeptide synthesis by rod mutants of *Bacillus subtilis*. J. Gen. Microbiol. **85**:335–350.
26. **Tempest, D. W.** 1970. The continuous cultivation of micro-organisms. I. Theory of the chemostat, p. 259–276. *In* J. Norris and D. Ribbons (ed.), Methods in microbiology, vol. 2. Academic Press, Inc., New York.
27. **Wardi, A. H., W. S. Allen, and R. Varma.** 1974. A simple method for the detection of hexuronic acids and pentoses. Anal. Biochem. **57**:268–273.
28. **Wieland, O.** 1974. Glycerin. UV-Methode, p. 1448–1453. *In* H. Bergmeyer (ed.), Methoden der enzymatischen Analyse, 3rd ed. Verlag Chemie, Weinheim, Germany.
29. **Wong, W., F. E. Young, and A. N. Chatterjee.** 1974. Regulation of bacterial cell walls: turnover of cell wall in *Staphylococcus aureus*. J. Bacteriol. **120**:837–843.
30. **Wyke, A. W., and J. B. Ward.** 1977. Biosynthesis of wall polymers in *Bacillus subtilis*. J. Bacteriol. **130**:1055–1063.