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Relation Between Cell Wall Turnover and Cell Growth in *Bacillus subtilis*

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The kinetics of cell wall turnover in *Bacillus subtilis* have been examined in detail. After pulse labeling of the peptidoglycan with *N*-acetylglucosamine, the newly formed peptidoglycan is stable for approximately three-quarters of a generation and is then degraded by a process that follows first-order kinetics. Deprivation of an auxotroph of amino acids required for protein synthesis results in a cessation of turnover. If a period of amino acid starvation occurs during the lag phase of turnover, then the initiation of turnover is delayed for a period of time equivalent to the starvation period. During amino acid starvation, new cell wall peptidoglycan is synthesized and added to preexisting cell wall. This peptidoglycan after resumption of growth is also subject to degradation (turnover). It is suggested that cell wall turnover is dependent on cell growth and elongation. Several possible control mechanisms for cell wall autolytic enzymes are discussed in light of these observations.

The cell wall peptidoglycan of many gram-positive microorganisms turns over during logarithmic growth (3-6, 11, 12, 21, 22, 26, 27, 31). A number of observations suggest that peptidoglycan turnover is not required for normal cell growth (13); however, the phenomenon is of interest because it may provide clues to the mode of cell wall growth and assembly and some insight into the regulation of cell wall autolytic enzymes.

Previous work with *Bacillus subtilis* has shown that after pulse labeling of the peptidoglycan, there is a lag period, equivalent in time to 0.5 to 1.0 generations, during which the newly synthesized peptidoglycan is immune from turnover. After this lag period, the peptidoglycan is degraded by a process that follows first-order kinetics, and the products of peptidoglycan turnover (glycan strands and peptide bridges) appear in the growth medium (21). If any fraction of the peptidoglycan in this organism is resistant to turnover, it must represent less than 5% of the total cell wall (12, 21). Although the loss of radioactivity from the cell wall is about 50% per generation, it has been pointed out by Pooley (26, 27) that since there is a lag between the time at which peptidoglycan is synthesized and the time at which it is degraded, the loss of peptidoglycan from the cell is only 10 to 20% of the mass of the cell wall per generation.

Cell wall turnover has also been demonstrated in a variety of other gram-positive organisms including *Streptococcus aureus* (31), *Lactobacillus acidophilus* (3, 11), and *B. megaterium* (4-6, 21), each of which shows somewhat different kinetic characteristics than the turnover observed with *B. subtilis*.

In this communication we have examined some of the kinetics of cell wall turnover in *B. subtilis*, and its relation to cell growth. The results appear to place important restrictions on any model of cell wall expansion in this organism.

MATERIALS AND METHODS

The bacterial strains used were either the Marburg strain of *B. subtilis* (ATCC 6051) or a derivative of this strain, *B. subtilis* B42, kindly made available by L. Mindich, which requires tryptophan, histidine, and glycerol for growth (24). The cells were grown either in antibiotic medium 3 (Difco) or in minimal medium (1), supplemented for *B. subtilis* B42 with 20 μg of histidine, tryptophan, and glycerol per ml and with 0.2% glucose as a carbon source. All cultures were grown at 37°C in a rotary water bath shaker.

For turnover experiments, cells were labeled with either *N*-acetyl-D-[^{14}C]glucosamine (GlcNAc) (50 mCi/mmol), obtained from Amersham-Searle, or with *N*-acetyl-D-[^{3}H]glucosamine (4 Ci/mmol), obtained from New England Nuclear Corp., as described in the individual experiments. After the indicated labeling periods, the cells were collected by filtration on a 0.45-μm membrane filter (Millipore Corp.), washed with medium at 37°C, and suspended in prewarmed medium at 37°C. Aliquots of the culture were taken at intervals to monitor cell growth by measurement of the optical density at 600 nm (OD_{600}) and for radioactive peptidoglycan content.

The latter was measured by first adding aliquots of the culture to an equal volume of 10% trichloroacetic acid at 0°C. The cells were then collected by filtration on 0.45-μm membrane filters and washed with 5% trichloroacetic acid. The radioactivity on the filter was determined in a liquid scintillation counter using 3a70 (Research Products International) as a counting fluid. As also reported by others (26), better than 90% of the radioactivity incorporated into trichloroacetic acid-insoluble material by *B. subtilis* from radioactive GlcNAc can be converted into acid-soluble form with lysozyme or with the *B. subtilis* *N*-acetylmuramic acid L-alanine amidase.

B. subtilis B42 shows no lag in growth when filtered and transferred to fresh minimal medium. *B. subtilis* ATCC 6051 shows considerable lag under these conditions. Therefore, experiments with this organism were carried out with preconditioned medium, where no lag is observed (21).

RESULTS

In previous work from this laboratory, in which we studied peptidoglycan turnover, we used an extremely cumbersome method of analysis, in which cell walls were isolated from suitably labeled cells, to assess the degree of peptidoglycan turnover. Recent work has shown that GlcNAc can be used as a suitable label that labels only the cell wall peptidoglycan and that trichloroacetic acid-insoluble radioactivity in GlcNAc-labeled cells represents almost exclusively peptidoglycan (26). These observations have allowed us to carry out a detailed examination of the kinetics of peptidoglycan turnover in relation to cell growth.

After labeling of the cells with GlcNAc, peptidoglycan turnover started with a lag, and this turnover could be interrupted by the addition of chloramphenicol, although a small fraction of the peptidoglycan was still degraded after addition of chloramphenicol (Fig. 1). A possible interpretation of these observations would be that at any given time a small fraction of the cell wall is "committed" to be degraded and that addition of chloramphenicol prevents this commitment but does not prevent the hydrolysis of that portion of the peptidoglycan which is already committed to be degraded.

One of the disadvantages of using chloramphenicol in this system is that it is not readily reversible. The data in Fig. 2 were obtained by using a mutant of *B. subtilis*, designated *B. subtilis* B42, which is an auxotroph for both histidine and tryptophan. Removal of histidine and tryptophan from a culture of *B. subtilis* B42 prevented turnover of the cell wall peptidoglycan, and this turnover started again when histidine and tryptophan were added to the culture. Thus the effect of amino acid deprivation on cell wall turnover was reversible. The

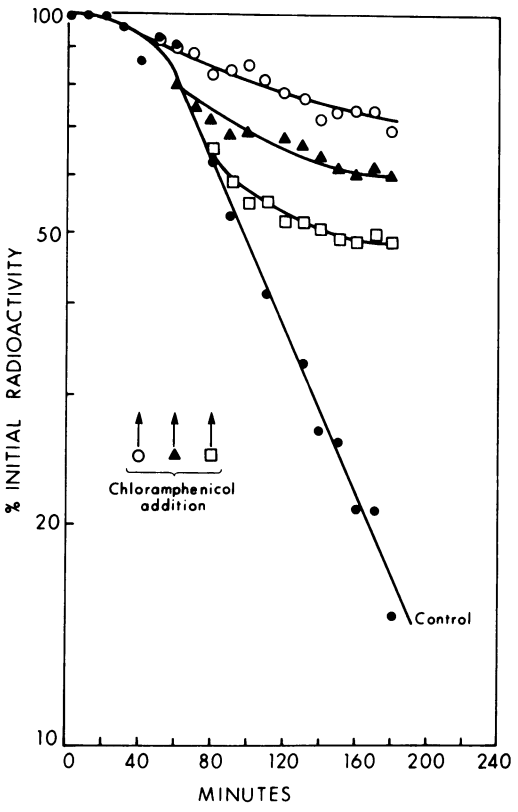

FIG. 1. *Effect of chloramphenicol on turnover of cell wall peptidoglycan. A 50-ml culture of B. subtilis ATCC 6051 was incubated at OD_{600} = 0.3 with 50 μCi of [³H]GlcNAc for 5 min. The cells were filtered and suspended in fresh medium, either in the absence of chloramphenicol or with the addition of chloramphenicol (50 μg/ml) at the times indicated. The generation time of the control culture was 60 min. Zero time on the graph is immediately after the culture is transferred to nonradioactive medium.*

cessation of turnover due to amino acid deprivation was not specific, since a similar but slower effect could be observed if *B. subtilis* B42, a glycerol auxotroph, was placed in glycerol-free medium (Fig. 3).

If the peptidoglycan of *B. subtilis* B42 was pulse labeled with [³H]GlcNAc and then at a later time with [¹⁴C]GlcNAc, the turnover of the ³H-labeled peptidoglycan started before the turnover of the [¹⁴C]GlcNAc (Fig. 4A). When the turnover was delayed by a period of amino acid starvation during the lag phase and then after growth was reinitiated, turnover would start but the ³H-labeled peptidoglycan would still initiate turnover before the ¹⁴C-labeled peptidoglycan (Fig. 4B). Thus the order in which the peptidoglycan was degraded was not affected by the amino acid starvation. This was

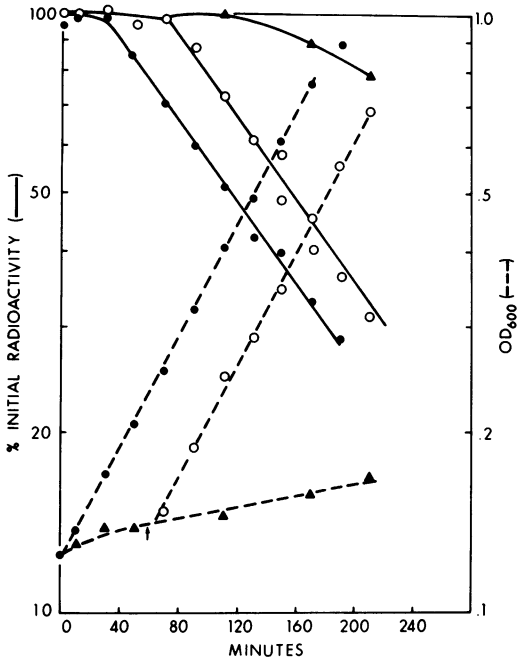

FIG. 2. *Effect of amino acid deprivation on cell wall turnover. A 22-ml culture of B. subtilis B42 in minimal medium was grown to* OD_{600} = 0.23 *and labeled for 10 min with 10 μCi of [³H]GlcNAc (4 Ci/mmol) and then transferred to unlabeled medium for 25 min. The cells were isolated by filtration and suspended in 70 ml of prewarmed medium lacking histidine and tryptophan. This was divided into three samples; one received histidine and tryptophan at zero time (●), the second received these amino acids at 60 min (○), and the third did not receive amino acids (▲). Radioactivity in peptidoglycan was determined in 0.5 ml of these cultures. The initial radioactivity was 18,500 dpm/0.5 ml. Solid lines indicate radioactivity; broken lines indicate* OD_{600}.

particularly remarkable because during amino acid starvation peptidoglycan synthesis continues at a reduced rate and results in cell wall thickening (13, 14, 23). In the case of *B. subtilis* B42, the apparent rate of peptidoglycan synthesis after 80 min of tryptophan and histidine starvation was approximately 50% of the rate of peptidoglycan synthesis observed before removal of histidine and tryptophan (Fig. 5).

Amino acid starvation during the lag period resulted in a delay in the initiation of start of turnover that was identical to the time of amino acid starvation for periods up to 120 min (Fig. 6). This implies that the deposition of peptidoglycan during the starvation period does not influence the time at which turnover will start for preexisting labeled peptidoglycan and strongly suggests that initiation of turnover depends on the rate of cell growth and not simply on the rate of peptidoglycan deposition. The observation made previously that the rate of turnover per generation is independent of the generation time is also consistent with this assumption (21, 22).

Since new peptidoglycan is added to the cell wall during amino acid starvation, it becomes important to determine whether this peptidoglycan is subject to normal turnover once the cell growth is resumed. Peptidoglycan synthesized during amino acid starvation was subject to normal turnover, and peptidoglycan synthesized at different times during amino acid starvation was degraded sequentially in the order in which it was synthesized (Fig. 7). These data therefore suggest that peptidoglycan synthesized during amino acid starvation is normally linked to the cell wall and, when cell growth resumes, is processed normally during lateral expansion so that it ultimately becomes available for degradation. There was no increase in the level of *N*-acetylmuramic acid L-alanine

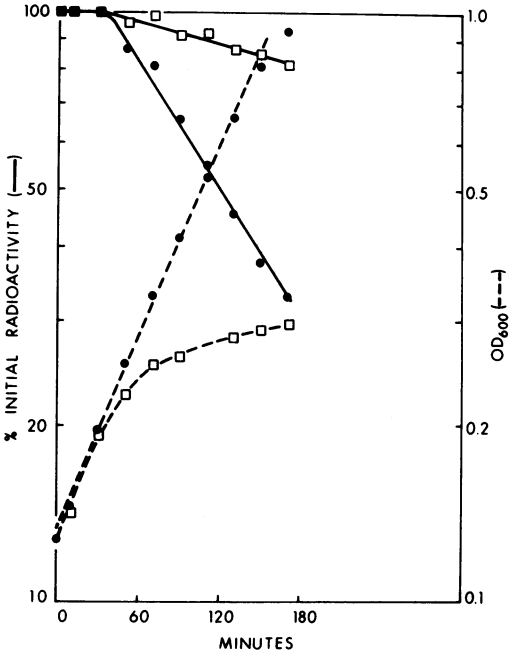

FIG. 3. *Effect of glycerol limitation on peptidoglycan turnover. A 18-ml culture of B. subtilis B42 was labeled for 10 min with 10 μCi of [³H]GlcNAc, transferred to 18 ml of complete minimal medium, and allowed to grow for 25 min. The cells were collected by filtration and suspended in 50 ml of minimal medium with no glycerol. One half of the culture received glycerol at zero time (●), and the other half remained without glycerol (□). Solid lines represent radioactivity; broken lines represent* OD_{600}.

amidase during amino acid starvation (Table 1). Thus, the cells appear not to have a large pool of latent amidase, and the peptidoglycan synthesized during the amino acid starvation period cannot carry with it any *N*-acetylmuramic acid L-alanine amidase synthesized at the same time (25). Therefore, the degradation of the peptidoglycan synthesized during amino acid starvation is catalyzed by enzymes synthesized either before or after the peptidoglycan is made.

Pooley has proposed a specific mechanism for cell wall turnover whereby only the outermost layers of the peptidoglycan are available to the autolytic enzymes (26, 27). This model is based on the observation that in a strain of *B. subtilis* that is deficient in autolytic activity, peptidoglycan only becomes available to a crude preparation of autolytic enzyme added to the medium

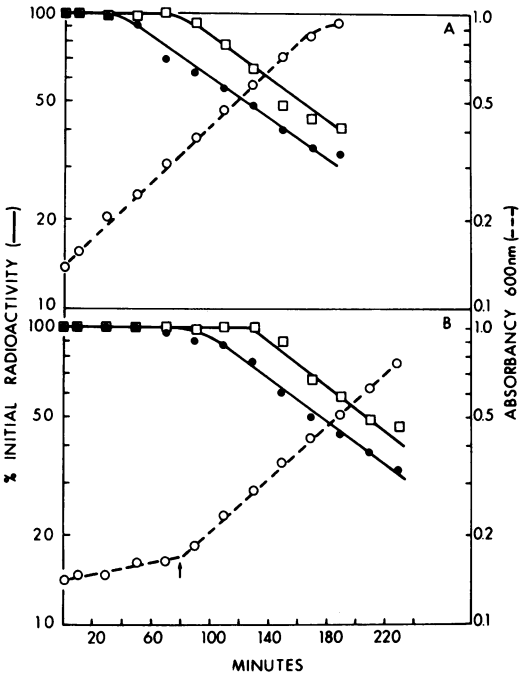

FIG. 4. *Effect of amino acid starvation on the sequence of peptidoglycan turnover. A culture of B. subtilis B-42 (25 ml) was labeled at OD_{600} = 0.23 for 10 min with 15 μCi of [^{3}H]GlcNAc. The cells were collected by filtration, suspended in 25 ml of medium, and, after 30 min of incubation, labeled for 5 min with 4 μCi of [^{14}C]GlcNAc (50 mCi/mmol). The cells, after filtration and washing, were suspended in 70 ml of minimal medium without histidine and tryptophan. The culture in (A) received amino acids immediately; the culture in (B) received them after 80 min. Symbols: ●, ^{3}H; □, ^{14}C; ○, OD_{600}. 100% initial radioactivity was 25,900 dpm of ^{3}H and 6,600 dpm per ml of ^{14}C.*

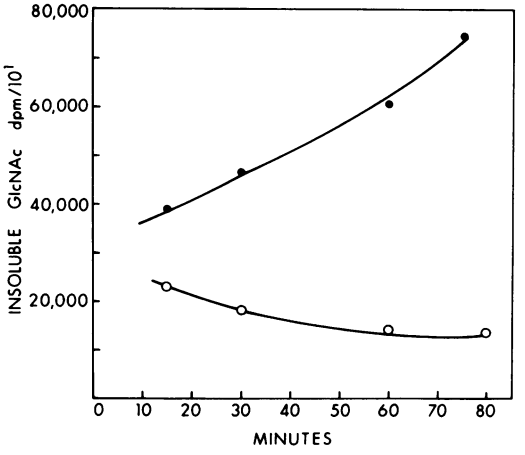

FIG. 5. *Incorporation of amino sugar into peptidoglycan during amino acid starvation. A 25-ml culture of B. subtilis B42 at OD_{600} = 0.2 was filtered and suspended in 25 ml of minimal medium minus histidine and tryptophan. To one half of the culture, histidine and tryptophan were added at zero time. At the times indicated, 1-ml aliquots were incubated with shaking with 0.2 μCi of [^{3}H]GlcNAc for 10 min, and the radioactivity insoluble in 5% trichloroacetic acid was determined. The doubling time of the control culture was 80 min. Symbols: ●, Control culture; ○, amino acid-starved culture.*

after a lag period, similar to that observed for turnover in normal strains.

The data in Fig. 8 are complementary to those obtained by Pooley. They show that *B. subtilis* was resistant to the addition of pure autolytic enzyme (*N*-acetylmuramic and L-alanine amidase) (Fig. 8A), but that the bacteria became sensitive to this enzyme if they were placed in 25% sucrose. This was true both with normal cells (Fig. 8B) and with cells to which chloramphenicol had been added and in which no peptidoglycan turnover was taking place (data not shown). In the experiment in Fig. 8A, the cells were exposed to about 100 times more amidase added externally than the amidase present in the cells, yet this large excess of enzyme had no effect on the cells' peptidoglycan. Taken together, observations suggest that the peptidoglycan in logarithmically grown *B. subtilis* is not intrinsically resistant to the enzyme, but that some other cellular component or structure prevents cell lysis. Data leading to the same conclusion in *Pneumococci* have been presented by Tomasz and Waks (29).

When cell walls prepared from *B. subtilis* by mechanical rupture are incubated with the homologous *N*-acetylmuramic acid L-alanine amidase, the enzyme fails to distinguish between recently synthesized and old cell wall (17). It is

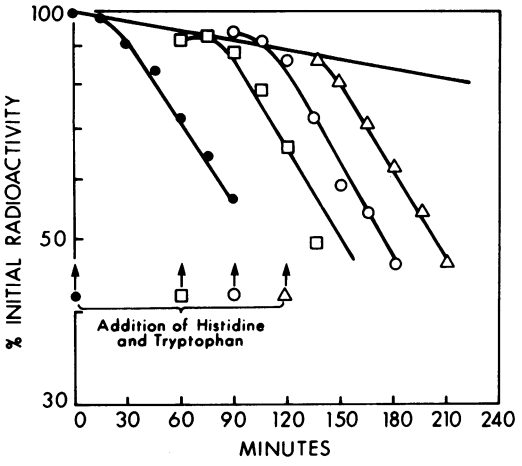

FIG. 6. *Effect of different times of amino acid starvation on peptidoglycan turnover. A 60-ml culture of B. subtilis B42 at OD_{600} = 0.19 was labeled with [^{3}H]GlcNAc (15 μCi) for 10 min. The cells were transferred to complete minimal medium and allowed to grow for 60 min. The culture was then transferred to amino acid-free medium. At the times indicated, histidine and tryptophan were added to aliquots of the culture, and radioactivity remaining in peptidoglycan was determined. The generation time of cells after addition of amino acids was 80 min. The line at the top shows the radioactivity in peptidoglycan in a culture that did not receive histidine or tryptophan. (Symbols have been omitted for clarity). 100% initial radioactivity = 13,700 dpm.*

possible that the mechanical disruption has distorted the cell wall structure so that the normal arrangement of the peptidoglycan is no longer present. In an attempt to circumvent this difficulty, we examined the ability of *N*-acetylmuramic acid L-alanine amidase to degrade peptidoglycan in cells that had been boiled in sodium dodecyl sulfate, and in some cases also extracted with chloroform-methanol (2:1). These cells appeared to retain an intact appearance when examined by phase microscopy and were readily lysed by the enzyme (Fig. 9). The ^{14}C label was in peptidoglycan that was already available for turnover, whereas the ^{3}H label was in newly synthesized peptidoglycan. There was some preference for the lysis of "old" cell wall, which was already in the process of turnover, as compared with new cell wall, which was synthesized immediately before harvesting of the cells, but the twofold difference in rate does not account for the total or nondifference in turnover observed for the same portion of the cell walls in the whole cell. Thus, at the time at which the cells used in the experiment in Fig. 9 were harvested, the ^{14}C-labeled peptidoglycan had already lost 50% of the initial radioactivity, whereas the ^{3}H-labeled peptidoglycan was still 20 min away from the start of turnover.

Work in several laboratories has suggested that either lipoteichoic acids or cardiolipin may be inhibitors of the cell wall autolytic enzymes (8–10, 18). Whether these molecules are relevant to the present observations is not known.

Penicillin, as well as other antibiotics that inhibit peptidoglycan synthesis, leads to cell lysis in growing cultures, which is due to the cells autolytic enzymes (25, 28, 30). We exam-

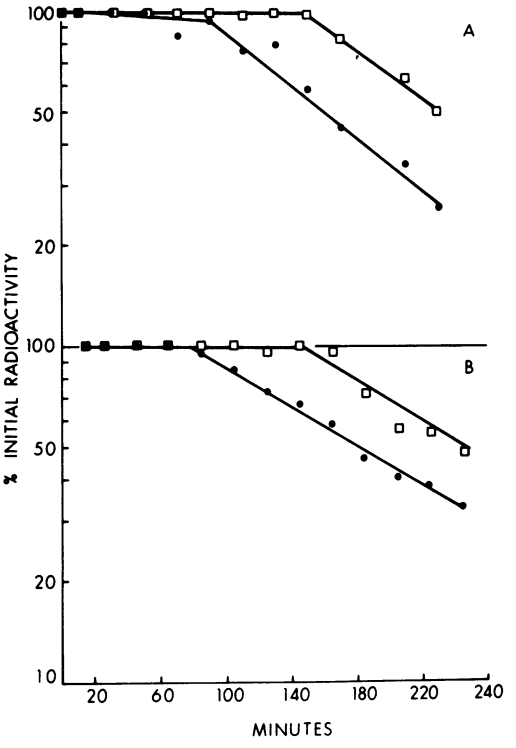

FIG. 7. *Turnover of peptidoglycan synthesized during amino acid starvation. A 12-ml culture of B. subtilis B42 at OD_{600} = 0.25 was filtered, and the cells were suspended in 25 ml of amino acid-free medium. After 10 min the cells were labeled for 10 min with 10 μCi of [^{3}H]GlcNAc; then they were incubated for 50 min in amino acid-free medium, labeled for 10 min with 2 μCi of [^{14}C]GlcNAc, and transferred to complete medium. After transfer to complete medium, samples were taken to determine radioactivity in peptidoglycan. In (B), the labeling with [^{3}H]GlcNAc was identical to that in (A). The culture was then allowed to remain in amino acid-free medium for 60 min. Amino acids were then added, and after 5 min the culture was labeled with 2 μCi of [^{14}C]GlcNAc for 10 min, filtered, and suspended in complete medium, and aliquots were taken to determine the radioactivity in peptidoglycan. The generation time of the culture was 70 min. Symbols: ●, ^{3}H; □, ^{14}C.*

TABLE 1. *Activity of N-acetylmuramic acid L-alanine amidase during amino acid starvation*[a]

Time (min)	Control culture		Amino acid-starved culture	
	OD_{600}	Amidase U/ml of culture	OD_{600}	Amidase U/ml of culture
0	0.42	1.06	0.42	1.1
45	0.61	1.5	0.48	1.06
75	0.83	2.4	0.52	0.99
110	1.0	2.8	0.55	1.06

[a] A 75-ml culture of *B. subtilis* B42 at OD_{600} = 0.2 was labeled with 10 μCi of [^{3}H]GlcNAc for 70 min. The cells were collected by filtration and placed in medium without histidine or tryptophan. One half of the culture was kept in this medium, and to the other half was added histidine and tryptophan. At the times indicated, the cells from 6 ml of each culture were collected by centrifugation, washed with 6 ml of 0.05 M hydroxyethyl piperazine-N'-2-ethanesulfonic acid (pH 8.0), 0.02 M $MgCl_2$, and 0.001 M 2-mercaptoethanol, suspended in 0.3 ml of the same buffer containing 0.1 % deoxycholate, and incubated at 37°C for 90 min. This incubation solubilized at least 90% of the peptidoglycan in the control culture. A 70-μl amount of the solution was then assayed for *N*-acetylmuramic acid L-alanine amidase by the fluorescamine method in the presence and absence of antibody against the enzyme (20). One unit of enzyme solubilizes 5 μg of cell wall/h.

ined the rate of cell wall lysis after penicillin addition by measuring the decrease in the radioactive peptidoglycan retained on a membrane filter in the presence of 10% trichloroacetic acid. The data in Fig. 10 show that penicillin only caused a minor increase in the rate of peptidoglycan degradation, and that cell lysis, as judged by the rapid decrease in the optical density of the culture, must reflect a very localized rapid peptidoglycan degradation, at one or more areas of the cell surface, rather than a rapid activation of essentially inactive cell wall lytic enzymes that would lyse all the available peptidoglycan.

The *N*-acetylmuramic acid L-alanine amidase from *B. subtilis* uses the teichoic acid in the cell wall as an allosteric ligand, and tight binding to the cell wall is dependent on the presence of teichoic acid (16, 17). If cell wall lysis occurs primarily on the cell surface, concanavalin A, which also binds to the same teichoic acid (18), should compete with the amidase and interfere with cell wall turnover. In fact, cell wall turnover occurred at the same rate in the presence and absence of concanavalin A (Fig. 11). Control experiments using [^{3}H]acetyl-concanavalin A (15) show that under these culture conditions, 1 ml of *B. subtilis* ATCC 6501, at OD_{600} = 0.3, cells will bind 1 μg of concanavalin A. This experiment suggests that the *N*-acetylmuramic acid L-alanine amidase molecules responsible for cell wall turnover are not anchored to the most exposed teichoic acid molecules on the cell surface.

DISCUSSION

The data presented in this paper lead us to the following general conclusions:

(i) Turnover of peptidoglycan is directly related to cell growth and therefore to the rate of lateral expansion of the peptidoglycan. We clearly do not know the location on the cell surface where turnover is taking place. The attractive notion presented by Pooley (26) that the exterior surface of the cell wall is the site of turnover is difficult but not impossible to reconcile with two of our observations, namely that no turnover takes place during a period of cell wall thickening and that concanavalin A does not interfere with cell wall turnover. It is possible that concanavalin A and the amidase can bind simultaneously to the cell surface teichoic acid and that the latter result does not conflict with the model presented by Pooley.

It should be pointed out that independent observations by Archibald and Coapes (2) suggest that the cell wall polymers are initially linked to the preexisting cell wall at the site closest to the plasma membrane and are then translocated to the exterior surface of the cell during further growth.

If cell wall polymers are all attached to the preexisting cell wall at a site near the membrane, then cell expansion will require translocation of cell wall material to the more exterior layers of the cell wall. A hypothetical transpeptidation mechanism that would account for such a translocation has been proposed (13).

(ii) The control of cell wall autolysins appears to depend on the structural and spatial arrangements of the cell surface and does not depend on the simultaneous synthesis of peptidoglycan and its autolytic enzyme. This conclusion is mainly derived from the data in Fig. 7 and Table 1, which show that peptidoglycan synthesized in the absence of autolytic enzyme synthesis is subject to normal turnover. Therefore, the autolytic enzymes must be able to move from one peptidoglycan area to another, and the simultaneous synthesis of peptidoglycan and autolytic enzyme is not required.

(iii) There are no structural features of the peptidoglycan per se that influence turnover in the sense that those parts of the peptidoglycan that are in vivo not subject to turnover are readily available to the autolytic enzymes in

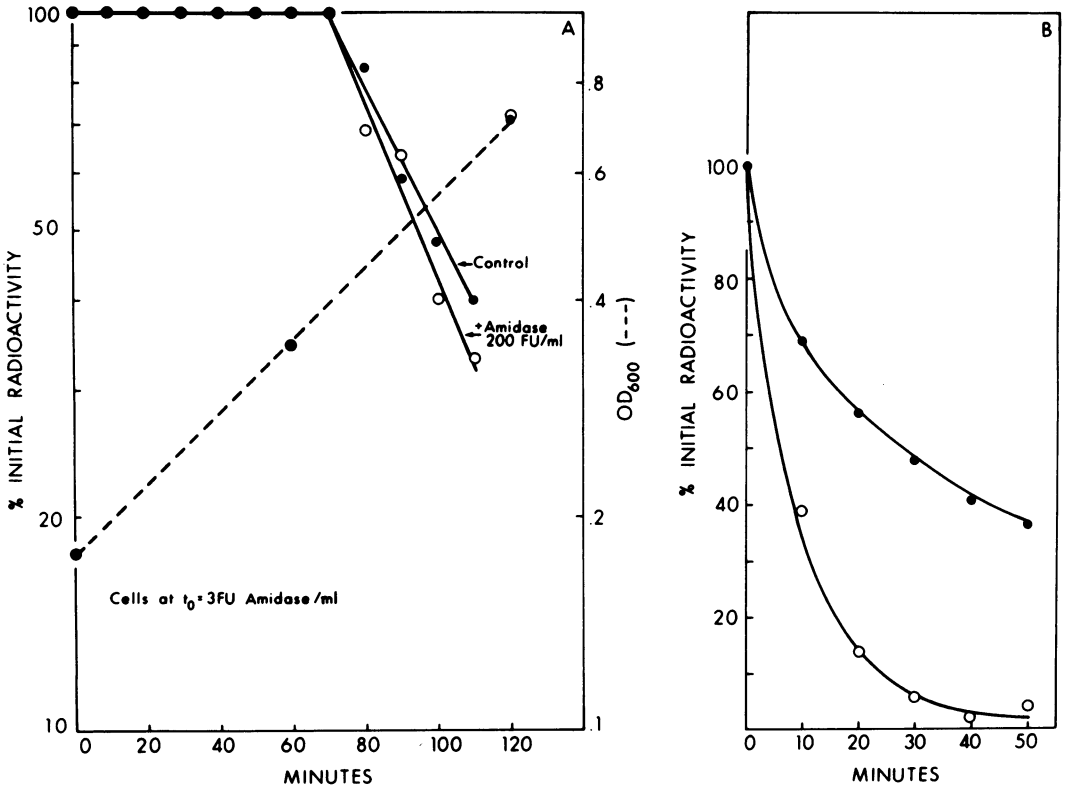

FIG. 8. *Effect of N-acetylmuramic acid L-alanine amidase on peptidoglycan turnover. (A) A 10-ml culture of B. subtilis ATCC 6501 at OD_{600} = 0.18 was labeled for 10 min with 10 μCi of [³H]GlcNAc. The cells were filtered and suspended in 10 ml of preconditioned medium. After 5 min, one half of the culture received N-acetylmuramic acid L-alanine amidase obtained from B. subtilis ATCC 6051 (17) to give a final concentration of 200 U/ml of amidase (20). One unit of amidase hydrolyzes 5 μg of B. subtilis cell wall/h under optimum conditions. In the presence of culture medium this quantity of enzyme could hydrolyze 6.5 mg of cell wall/h per ml, which is approximately 20 times the amount of cell wall contained in 1 ml of culture at OD_{600} = 0.20. At the end of the experiment, the cells were removed by centrifugation and the medium was assayed for enzyme activity. Essentially all of the enzymes added to the culture could be recovered in the supernatant fluid. Symbols: ●, Control culture; ○, culture with amidase; ——, radioactivity; - - -, OD_{600}. Initial radioactivity = 1.3 × 10^5 dpm/ml. (B) A 25-ml culture of B. subtilis ATCC 6051 in antibiotic medium 3 was labeled at OD_{600} = 0.22 with 10 μCi of [³H]GlcNAc for 30 min. The cells were collected by filtration and allowed to grow in unlabeled medium for 15 min (OD_{600} = 0.65). The cells were then collected by centrifugation at 40,000 × g for 10 min at 4°C, washed with 0.05 M hydroxyethyl piperazine-N'-2-ethanesulfonic acid (pH 8.0), 0.02 M $MgCl_2$, and 25% (wt/vol) sucrose, and finally suspended in 0.75 ml of the same buffer containing 10^{-4} M phenylmethylsulfonyl fluoride and 1 μg of deoxyribonuclease type 1 (Sigma). The cells were incubated in this buffer either alone (●) or with 400 U of N-acetylmuramic acid L-alanine amidase per ml (○). The initial radioactivity was 7.5 × 10^5 dpm/ml. Note that the ratio of amidase to cells is 55 times higher in the experiment in (A) than in (B).*

vitro. We can consider several possible alternatives to explain this paradox; the most likely would be that during cell wall purification or during any of the other procedures that allow cell wall degradation, a component is removed from the cell wall that in certain localized areas prevents peptidoglycan degradation. It would appear that the best candidate for such a regulatory component would be a constituent of the cell membrane, and its function would be dependent on the three-dimensional arrangement of the cell wall and the cell membrane. In other systems, the cell wall autolytic enzymes have been shown to be inhibited by lipoteichoic acids and by cardiolipin (8–10, 18). We have had no success in demonstrating such a inhibition with the enzymes obtained from *B. subtilis* (16, 20), but these difficulties may represent differences in the handling of these complex amphipatic molecules. The location of these molecules

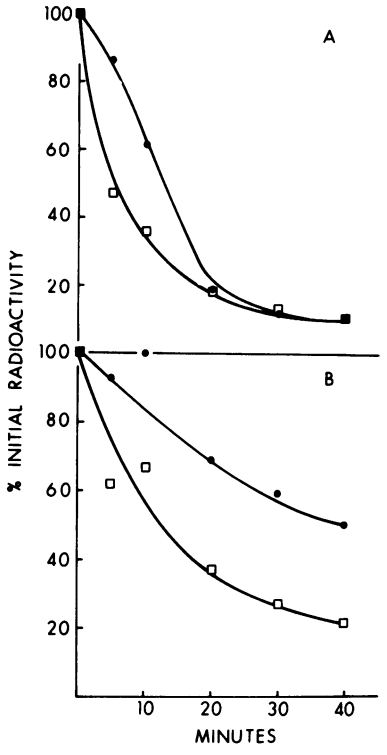

FIG. 9. *Digestion by N-acetylmuramic acid L-alanine amidase of new and old peptidoglycan. A 70-ml culture of B. subtilis ATCC 6051 in antibiotic medium 3 was labeled at OD_{600} = 0.2 for 10 min with 4 μCi of [^{14}C]GlcNAc. After 30 min, the culture was labeled for 10 min with 20 μCi of [^{3}H]GlcNAc, and the cells were collected by filtration, suspended in 50 ml of 1% sodium dodecyl sulfate, and heated at 100°C for 5 min. The cells were washed by centrifugation with sodium dodecyl sulfate and again heated at 100°C for 5 min. The cells were finally collected by filtration, washed with a large excess of distilled water, and suspended in 2.5 ml of 0.05 M hydroxyethyl piperazine-N'-2-ethanesulfonic acid (pH 8.0), 0.02 M $MgCl_2$, and 0.001 M 2-mercaptoethanol. (A) 0.15 ml of these cells was lysed with 28 U of amidase at 37°C, in a final volume of 0.18 ml of the same buffer. (B) 1 ml of the cells was first dried under vacuum and then extracted two times with 3 ml of chloroform methanol (2:1), dried under vacuum again, and then lysed as in (A). Symbols: □, ^{14}C; ●, ^{3}H.*

make them ideal candidates as regulators of cell wall lysis, and some evidence is available to suggest that they extend outward from the membrane into the cell wall (19).

As a highly speculative model, we would suggest that the membrane is in fact interdigitated into the peptidoglycan layer and thus prevents peptidoglycan degradation. This interdigitation may be the result of the fact that nascent peptidoglycan chains may be attached to both the membrane and the peptidoglycan. Cell wall thickening as during amino acid starvation results in maximum interdigitation and no turnover. Lateral expansion results in the random formation of peptidoglycan areas that do not contain any membrane and become available to the autolytic enzymes.

Cell wall thickening in the absence of lateral expansion can be considered to represent either a normal initial step in cell wall growth or a totally abnormal cell wall synthesis that the cell utilizes under nongrowing conditions, perhaps to prevent the accumulation of nucleotide precursors. The data presented in this paper support the first possibility, since cell wall material deposited during amino acid starvation undergoes normal turnover when growth resumes and therefore must be able to spatially fit into the normal pattern of cell wall expan-

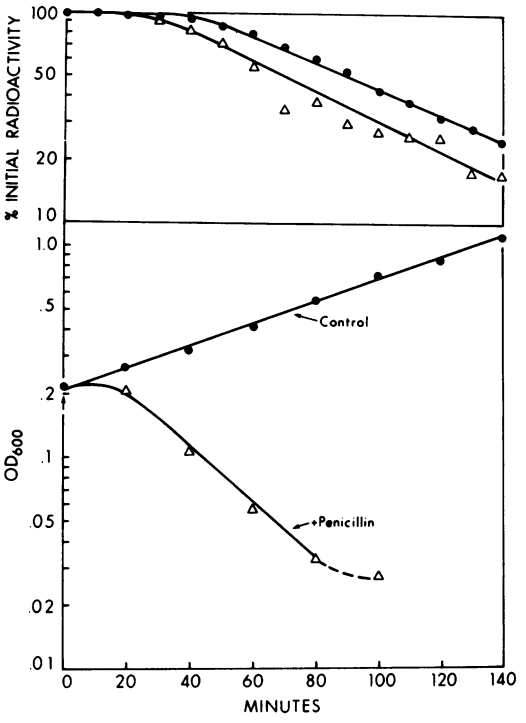

FIG. 10. *Effect of penicillin on the rate of peptidoglycan solubilization. B. subtilis ATCC 6051 was grown in minimal medium to OD_{600} = 0.4, and 70 ml of this culture was labeled with 60 μCi of [^{3}H]GlcNAc for 5 min. The cells were collected by filtration and suspended in twice the original volume of preconditioned medium. One half of the culture was kept as a control, and to the other half was added 100 μg of penicillin per ml. The upper lines show the radioactivity in peptidoglycan; the lower curves show the OD_{600} of the culture.*

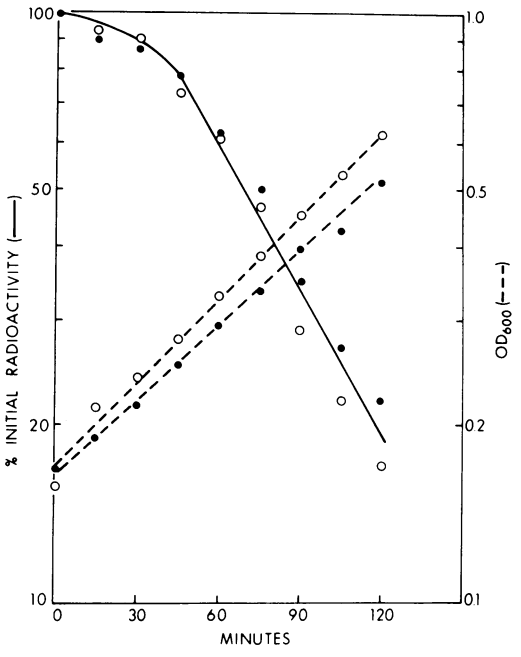

FIG. 11. *Effect of concavalin A on peptidoglycan turnover. B. subtilis ATCC 6051 was grown in minimal medium with glycerol as a carbon source to* OD_{600} *= 0.13. A 25-ml amount of this culture was labeled with 20 μCi of [³H]GlcNAc for 10 min and filtered, and the cells suspended in fresh medium and allowed to grow for 30 min. After 30 min, one half of the culture served as a control and the other half received concavalin A (Miles) to a final concentration of 0.1 mg/ml. Symbols: ●, Control cells; ○, cells with concanavalin A; ——, radioactivity; - - -,* OD_{600}.

sion. This interpretation clearly is only valid for cell wall synthesized during short periods of amino acid deprivation and may not apply to cell wall synthesized during prolonged starvation periods.

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