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Cell Wall Turnover in Batch and Chemostat Cultures of *Bacillus subtilis*

WIM R. DE BOER,† FRED J. KRUYSSEN,‡ AND JAN T. M. WOUTERS*

Laboratorium voor Microbiologie, Universiteit van Amsterdam, 1018 WS Amsterdam, The Netherlands

Wall turnover was studied in *Bacillus subtilis*. The loss of radioactively labeled wall polymers was followed during exponential growth in batch and chemostat cultures. Turnover kinetics were identical under all growth conditions; pulse-labeled wall material was lost with first-order kinetics, but only after exponential growth for 1 generation time after its incorporation. Similarly, continuously labeled cells showed an accelerating decrease in wall-bound radioactivity starting immediately after removal of the labeled precursor and also reached first-order kinetics after 1 generation time. A mathematical description was derived for these turnover kinetics, which embraced the concept of "spreading" of old wall chains (H. M. Pooley, J. Bacteriol. **125**:1127-1138, 1976). Using this description, we were able to calculate from our experimental data the rate of loss of wall polymers from cells and the fraction of the wall which was sensitive to turnover. We found that about 20% of the wall was lost per generation time and that this loss was affected by turnover activity located in the outer 20 to 45% of the wall; rather large variations were found with both quantities and also between duplicate cultures. These parameters were quite independent of the growth rate (the specific growth rate varied from 1.3 h^{-1} in broth cultures to 0.2 to 0.3 h^{-1} in chemostat cultures) and of the nature of the anionic polymer in the wall (which was teichoic acid in cultures with an excess of phosphate and teichuronic acid in phosphate-limited chemostat cultures). Some implications of the observed wall turnover kinetics for models of wall growth in *B. subtilis* are discussed.

In many bacterial species wall polymers are lost from cells during exponential growth (4, 9, 15, 20-22, 25, 26, 30). Wall turnover is caused by wall-bound enzymes (autolysins), which act hydrolytically on one of the bonds within the peptidoglycan (13, 23, 25). The solubilized peptidoglycan and (in gram-positive bacteria) the covalently attached anionic polymers (teichoic acid, teichuronic acid) can be recovered from the supernatant fluid (8, 25).

In this paper, we describe a quantitative assessment of wall turnover in *Bacillus subtilis*. We measured the rate of loss of radioactively labeled (pulse-labeled or continuously labeled) wall material from cells during growth under various conditions in batch and chemostat cultures.

In previous investigations with other *Bacillus* strains, it was shown that a specific pulse-label introduced into the wall is available for turnover only after a lag of between 0.5 and 2 generation times (1, 20, 21, 25, 26). This indicates a partial susceptibility of the wall to turnover; Pooley hypothesized that, as new wall material is deposited on the inner surface and then migrates outward radially in bacilli (1), the hydrolytic activity of the autolysins is confined to the outer layers of the wall (26, 27). Once they are susceptible to turnover, pulse-labels are lost with apparent first-order kinetics (1, 20, 25, 26), suggesting a random excision of older and newer chains from the turnover-sensitive fraction of the wall.

During the lag period, mingling of older and newer wall chains apparently does not occur, since two sequential pulse-labels (^3H and ^{14}C, respectively) incorporated into the wall were lost after identical lag periods and in the same order in which they had been incorporated (21). Thereafter, both labels were lost with identical half-lives, again showing the equality of wall chains of different ages toward turnover in the sensitive layers.

The rate of turnover (i.e., the rate at which wall chains are set free by turnover) can be calculated from the first-order rate constant by which the pulse-label is lost only when the proportion of the turnover-sensitive layer in the wall is known. To be able to calculate this pro-

† Present address: Gist-Brocades N.V., Microbiology Research Laboratory, 2600 MA Delft, The Netherlands.

‡ Present address: Department of Biochemistry and Microbiology, University of Victoria, Victoria, British Columbia, V8W 2Y2, Canada.

portion, another aspect of wall turnover must be considered as well.

Pooley (26, 27) showed that with cells whose walls had been continuously labeled, the rate of decrease in wall labeling accelerated after transfer of the cells to the chase medium, before apparent first-order kinetics were established. In this initial period, no decrease in the production rate of radioactive turnover products was observed in the chased culture compared with a culture in which the labeling was continued, which implies that the specific radioactivity of the wall material in the turnover-sensitive layers remained constant in both the original cells and their daughters. These data showed that newly made wall is spread in an orderly fashion over the cells, so that the radioactive wall originally present covers an increasing surface over old and new cells. This "spreading" concept was confirmed recently by data from Anderson et al. (1), who showed that a phage receptor (teichoic acid) which was incorporated pulse-wise into phage-resistant (teichoic acid-free) *B. subtilis* cells occurred at the outer surfaces of all of the cells after a period of growth of approximately 1 generation time. Thus, the teichoic acid-containing wall chains apparently spread out over the original cells and the newborn cells.

From this model of wall growth, a quantitative description of the kinetics of wall turnover in *B. subtilis* could be derived, which is described below. With this model, it is possible to calculate the proportion of the turnover-sensitive layers in the wall and the rate of production of wall polymers as a consequence of turnover directly from the data obtained with the label-chase experiments described above. A preliminary account of this work has been published (14).

Using this method, we could compare the rate of wall turnover in cultures grown under a variety of cultural conditions; the use of a chemostat permitted the investigation of wall turnover in cultures of cells containing either teichoic acid (phosphate-excess conditions) or teichuronic acid (phosphate limitation) in their walls (24a).

(These results were taken from a thesis submitted by W. R. de Boer to the University of Amsterdam.)

MATERIALS AND METHODS

Strain. We used *B. subtilis* subsp. *niger* WM, a spontaneously occurring mutant from *B. subtilis* subsp. *niger* ATCC 9372, which forms white instead of reddish colonies on peptone agar containing 1% glucose.

Media. For a complex growth medium, we used nutrient broth (Difco Laboratories, Detroit, Mich.), occasionally supplemented with 1% glucose. Chemostat cultures were grown in the mineral medium of Evans et al. (18), using glucose as the carbon source under glucose limitation, potassium limitation, or phosphate limitation. To maintain a culture at an optical density at 540 nm (E_{540}) of 3 (approximately 1 mg of cellular dry weight per ml), the overall medium concentration of each nutrient was one-quarter that specified by Evans et al. (18). When a culture E_{540} of 0.3 was used, only the concentration of the limiting nutrient in this medium was decreased tenfold.

Growth conditions. Batch cultures were grown in 100-ml conical flasks which contained 15 ml of culture and were placed in a 37°C shaking water bath. Growth was monitored by measuring the E_{540} with a Vitatron DCP photometer; therefore, the flasks were provided with side arms which fit into the cuvette holder of the photometer. Continuous cultures were grown in Bioflo C 30 fermentors (New Brunswick Scientific Co., New Brunswick, N.J.) equipped with pH regulating devices (2 M NaOH was used as the titrating fluid) and antifoam pumps for the continuous addition at a low rate of a sterile Silicone AF (Dow Chemical) emulsion in water (20%, wt/vol), when necessary. The temperature used was 37°C, the pH used was 7.0, and aeration was obtained by stirring vigorously while air was pumped through the cultures. The culture volume was 200 to 300 ml, depending on the fermentor used, and the medium pump was set to obtain dilution rates between 0.2 and 0.3 h^{-1}. Bacterial densities in the chemostats were measured by reading the E_{540} of properly diluted culture samples. Cultures were used when all parameters had been stable for at least 2 days. In a steady-state system, the specific growth rate (μ) of the cells in a chemostat is equal to the dilution rate (D) (29): $\mu = D = \ln 2/\tau$, where τ is the doubling time (in hours). Growth rates of chemostat and batch cultures are expressed as specific growth rates (per hour).

Cell wall labeling. Cells were labeled with either *N*-acetyl-D-[^{3}H]glucosamine ([^{3}H]GlcNAc) (3 Ci/mmol) or *N*-acetyl-D-[^{14}C]glucosamine ([^{14}C]GlcNAc) (60 mCi/mmol) (3). Radioactive compounds were added to the cultures without carrier GlcNAc. Pulse-labels were applied for a period of 0.1 generation time to batch cultures (10 μCi in 20 ml) and chemostat cultures (50 to 100 μCi in 200 to 300 ml). Continuously labeled cells were obtained in a chemostat culture by connecting to the medium pump a flask containing 1.3 liters of the medium used plus 250 μCi of [^{3}H]GlcNAc. This medium was pumped into the culture for 15 to 18 h (approximately 5 generation times). In several experiments, a pulse-label (10 μCi of [^{14}C]GlcNAc) was added at 0.1 generation time before the chase.

Chasing procedures. Radioactivity was chased in either of two ways. The first method involved the addition of a large excess of unlabeled GlcNAc (for concentrations, see below). In the second method, cells were removed from the labeled medium by centifugation. Therefore, labeling was carried out in a high-cell-density chemostat culture (E_{540}, ca. 3). A sample (20 ml) was centrifuged rapidly (1 min, 39,000 × *g*) at 35°C in a Sorvall RC2-B centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). The cells were resuspended in 20 ml of prewarmed medium (37°C, brought to pH 7.0 with NaOH) lacking the growth-limiting nutrient, and the suspension was transferred to a second chemostat already containing 180 ml of the resuspension medium.

To this culture, which now had a low optical density (E_{540}, ca. 0.3), medium containing the limiting nutrient at a 10-fold-lower concentration was added via the medium pump. The volume was maintained at 200 ml, and the pump was set to obtain a dilution rate identical to that in the original culture. Optical density remained constant after such medium changes, indicating the immediate initiation of growth of the cells in the second chemostat. Cultures labeled in nutrient broth at an E_{540} of ca. 1 were resuspended similarly after centrifugation in nutrient broth-containing flasks at an E_{540} of ca. 0.2.

Approximately 10 min was needed for this chase procedure, which was 0.04 to 0.07 generation time in the chemostat cultures used and 0.25 generation time for the nutrient broth cultures. More than 95% of the radioactivity initially present in the chased cultures was bound to the cells. More than 85% of the label was rendered soluble and could no longer be precipitated by 10% trichloroacetic acid after digestion of the cells with lysozyme, indicating specific labeling of the walls, as described previously by others (21, 26).

Measurement of wall turnover. From batch cultures, 200- to 500-μl samples were taken and filtered with membrane filters (diameter, 2.5 cm; pore size, 0.2 μm; Millipore Corp., Bedford, Mass.). Each filter was washed three times with 1.0 ml of 0.9% NaCl before it was prepared for counting. From chemostat cultures 1.5-ml samples were taken. Cell-bound radioactivity was measured after filtration of 1.0-ml samples, as described above. Trichloroacetic acid treatment of the samples (final concentration, 5%; 20 min; 0°C) before filtering did not lower the amount of cell-bound radioactivity significantly and therefore was omitted. To obtain comparable figures for both types of cultures, the cell-bound radioactivity of chemostat culture samples was corrected for washout by multiplication of the counts with e^{Dt} (29). The washout rates (D) of total radioactivity in the chemostat cultures were determined by counting duplicate 0.1-ml culture samples directly. When C_t and C_{t_0} are total radioactivity per fixed culture volume at times t and t_0 (the start of the chase period), respectively, D is obtained from $C_t = C_{t_0} \cdot e^{-D(t-t_0)}$.

Counting of radioactivity. Culture samples (0.1 ml) were mixed with 8 ml of a counting fluid containing toluene and Triton X-100 (1:1, vol/vol). The toluene contained one package (4 g) of Omnifluor (New England Nuclear Corp., Boston, Mass.) per liter. Membrane filters were dried at 50°C and counted in 5 ml of the toluene-Omnifluor counting fluid. Counting was performed with a Nuclear-Chicago Isocap 300 liquid scintillation counter.

Materials used. The chemicals used for the preparation of media and buffers were of analytical grade (E. Merck AG, Darmstadt, Germany). Glucose (Denkro-m) was from Corn Products Co., Utrecht, The Netherlands. Radioactive GlcNAc was obtained from Amersham Searle, Amersham, England. Toluene was from the Packard Instrument Co., Inc., Downers Grove, Ill., and Triton X-100 was from Koch-Light, Colnbrook, England. Lysozyme was from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Wall turnover in batch cultures in complex media. After transfer to unlabeled medium, pulse-labeled *B. subtilis* subsp. *niger* WM retained wall-bound radioactivity for approximately 1.5 generation times when it was grown in a complex medium at a specific growth rate of 0.9 h^{-1} (nutrient broth) to 1.3 h^{-1} (nutrient broth containing 1% glucose) (Fig. 1). The loss of radioactive wall polymers, as measured by the number of cell-bound counts per milliliter of culture, followed apparent first-order kinetics thereafter.

Wall turnover in potassium-limited chemostat cultures. Growth in potassium-limited chemostat cultures pulse-labeled with [^{3}H]GlcNAc (specific growth rate, 0.3 h^{-1}) resulted in patterns of wall turnover qualitatively identical to those exhibited in the rapidly growing batch cultures described above (Fig. 2a). Thus, the pulse-label was lost with apparent first-order kinetics only after a lag of about 1 generation time. In identically grown continuously labeled cultures (specific growth rate, 0.24 h^{-1}), the loss of radioactivity from the cells started immediately after transfer to fresh unlabeled medium (Fig. 2b). After an initial phase of accelerating loss of radioactive label, apparent first-order kinetics were attained in these cultures as well.

Wall turnover in glucose-limited chemostat cultures. Wall turnover results in the loss from cells of polymers with high contents of carbon and energy. We investigated whether growth under glucose limitation in chemostat cultures imposed some restraints upon this proc-

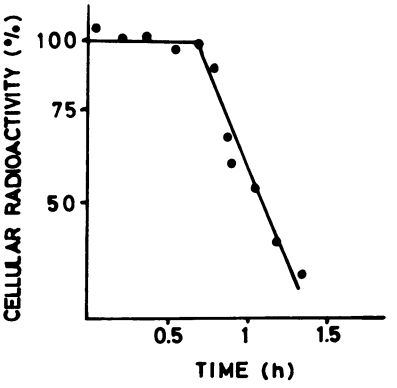

FIG. 1. *Wall turnover in complex medium. Bacteria were grown to an E_{540} of 0.4 in nutrient broth containing 1% glucose (specific growth rate, 1.3 h^{-1}) and pulse-labeled with [^{3}H]GlcNAc. The cells were isolated by centrifugation and reincubated at an E_{540} of 0.2 in nutrient broth, and wall turnover was followed.*

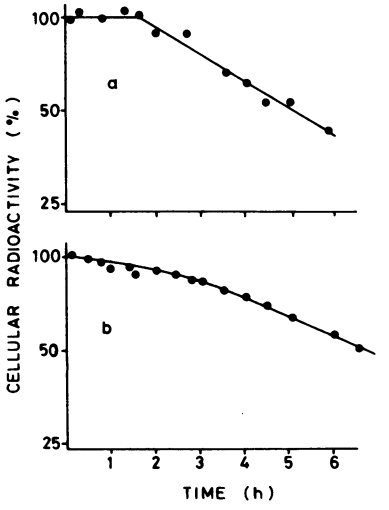

FIG. 2. *Wall turnover in potassium-limited chemostat cultures. (a) Bacteria growing in a potassium-limited chemostat (E_{540}, 3.1; specific growth rate, 0.3 h^{-1}) were pulse-labeled by adding [^{3}H]GlcNAc and chased by centrifugation, as described in the text. Further growth was under potassium limitation at an E_{540} of 0.31 and a specific growth rate of 0.30 h^{-1} in a second chemostat. The initial cellular radioactivity (100%) was 1.3 × 10^5 dpm/ml. (b) Bacteria growing in a potassium-limited chemostat (E_{540}, 3.2; specific growth rate, 0.22 h^{-1}) were labeled continuously for 15 h with [^{3}H]GlcNAc and chased by centrifugation, as described in the text. Further growth was under potassium limitation at an E_{540} of 0.24 and a specific growth rate of 0.24 h^{-1}. The initial cellular radioactivity (100%) was 1.6 × 10^4 dpm/ml.*

ess. As Fig. 3 shows, wall turnover occurred in cells in glucose-limited cultures; the extent to which wall polymers were lost from these cells did not differ from the extent of loss under other growth conditions (see below).

The glucose-limited cultures were chased by adding nonradioactive GlcNAc (final concentration, 0.2 mM). This molar excess of at least 1,500-fold still permitted the continuing incorporation of radioactivity during 0.4 generation time in both cultures shown in Fig. 3. During this period, 30% (Fig. 3, line a) and 15% (Fig. 3, line b) of the plateau value of radioactivity finally established were incorporated into the cells. Higher GlcNAc concentrations could not be used, since it was necessary to prevent the risk of a specific incorporation in these media with a very low glucose concentration (11). However, more than 70% of the label was incorporated into wall material made within 0.1 generation time, whereas the residual 30% was distributed in wall made in 0.4 generation time, when the specific radioactivity of the incorporated wall chains obviously declined sharply. Therefore, we did not expect that the label incorporated after the chase would interfere with the turnover kinetics.

Wall turnover in phosphate-limited chemostat cultures. When grown in the media described above, *B. subtilis* subsp *niger* WM was shown to possess walls which had more or less identical compositions (24a); on a dry weight basis, 40 to 60% of the wall was peptidoglycan, and the remainder was covalently attached teichoic acid. In phosphate-limited chemostat cultures, almost no teichoic acid was found (<5% of the walls), and the walls contained a phosphorus-free anionic polymer, teichuronic acid. The role of anionic polymers in the action of autolytic enzymes on walls has been emphasized by several authors (24), but the in vivo effect on turnover of the presence in the wall of teichuronic acid instead of teichoic acid has never been investigated.

We measured the turnover of teichuronic acid-containing walls by growing and labeling the bacteria in phosphate-limited chemostat cultures. It should be noted that in the walls of phosphate-limited cells, amino sugars appear in peptidoglycan (*N*-acetylmuramic acid and GlcNAc) and in teichuronic acid (*N*-acetylgalactosamine [GalNAc]); at low growth rates, approximately 50% of the wall-bound amino sugars are present in teichuronic acid. External GlcNAc

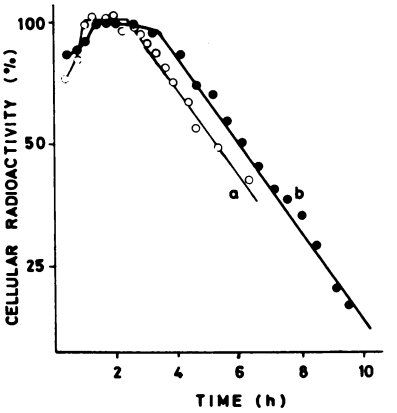

FIG. 3. *Wall turnover in glucose-limited chemostat cultures. Glucose-limited chemostat cultures were pulse-labeled with [^{3}H]GlcNAc and chased by adding unlabeled GlcNAc to a final concentration of 0.18 mM, and wall turnover was measured as described in the text. Line a: E_{540}, 0.29; specific growth rate, 0.19 h^{-1}; pulse, 100 μCi; initial cellular radioactivity (at the start of the chase), 4.0 × 10^5 dpm/ml. Line b: E_{540}, 0.32; specific growth rate, 0.31 h^{-1}; pulse, 50 μCi; initial cellular radioactivity (at the start of the chase), 1.1 × 10^5 dpm/ml.*

is metabolized via the joint precursor glucosamine 1-phosphate (2), so all three amino sugars were labeled in these experiments.

A pulse-labeled phosphate-limited culture lost its radioactivity with apparent first-order kinetics only after a lag (Fig. 4a), as in the cultures described above. Wall turnover in a double-labeled phosphate-limited culture clearly showed the different ways by which the pulse-label and the continuous label were lost from the cells (Fig. 4b). The rate constants of the first-order decrease that was reached finally were identical for the two types of labels.

In the experiment shown in Fig. 4a, the chase was performed by adding nonradioactive GlcNAc. In this culture a much higher concentration could be used than in the glucose-limited cultures described above (Fig. 3), because of the high residual glucose concentrations in the phosphate-limited chemostat, which prevented the breakdown of the GlcNAc via glycolysis (11) and its subsequent aspecific incorporation into cell constituents. Only a small increase in the radioactivity of the cells was observed during the chase (<10%).

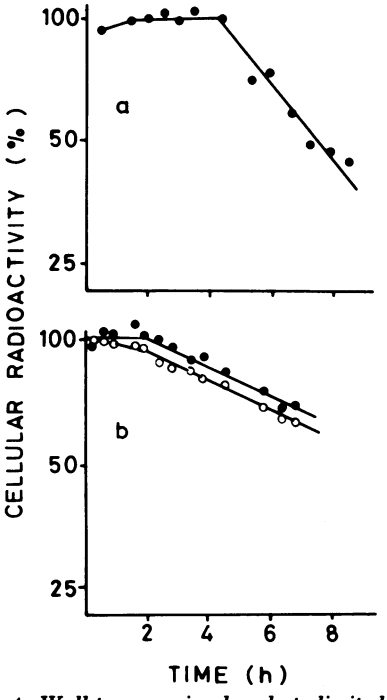

FIG. 4. *Wall turnover in phosphate-limited chemostat cultures. (a) Bacteria in a phosphate-limited chemostat (E_{540}, 0.33; specific growth rate, 0.19 h^{-1}) were pulse-labeled with 100 μCi of [^{3}H]GlcNAc and chased with nonradioactive GlcNAc (2.0 mM). At the start of the chase, the cells contained 8.6 × 10^4 dpm/ml. (b) Bacteria growing in a phosphate-limited chemostat (E_{540}, 2.8; specific growth rate, 0.23 h^{-1}) were labeled continuously for 17 h with [^{3}H]GlcNAc; 10 μCi of [^{14}C]GlcNAc was added 20 min before harvesting as a pulse-label. The cells were chased by centrifugation as described in the text. Further growth (in the absence of GlcNAc) was under phosphate limitation at an E_{540} of 0.23 and a specific growth rate of 0.22 h^{-1}; 100% radioactivity was 1.2 × 10^4 dpm/ml for ^{3}H (○) and 1.7 × 10^3 dpm/ml for ^{14}C (●).*

There was considerable variation between the durations of the lags exhibited by the pulse-labels, as well as between the first-order rate constants in duplicate cultures (Fig. 4a and 4b). Such variations were not specific for phosphate-limited cultures, but were observed also under the other growth conditions employed (data not shown).

Calculation of parameters involved in turnover. We show the derivation of several parameters involved in wall turnover below. We applied these equations to the experiments shown here to obtain the fraction of the total cellular wall lost per hour (κ) or per generation time (k) and the fraction of the wall sensitive to turnover (X_B).

Table 1 summarizes the results of these calculations, for which the values of μ, λ, Y_0, X_0, and X_f' (for definitions, see Appendix) were obtained from the experimental data. As Fig. 5a shows, considerable variation was observed for values of κ obtained at specific growth rates between 0.19 and 0.31 h^{-1} for chemostat-grown cultures. Such a result was not unexpected in view of the previously noted differences in lag times (θ; see Appendix) and λ values in cultures having similar growth rates (Table 1). We could make no distinction between the differently grown organisms, and all κ values were between 0.03 and 0.08 h^{-1}. Cultures grown at high growth rates in broth media showed much higher κ values.

However, multiplication of these data with the respective generation times yielded equal k values for slow- and fast-growing cells (Fig. 5b). Within rather wide limits, k values of all cultures clustered around 20%.

Our calculations yielded X_B values of 25 to 44% in the slow-growing cultures; again, no distinction could be made between cells grown with different limitations in the chemostats (Fig. 5c). In cultures growing rapidly in complex media, somewhat lower X_B values were found (16 to 22%).

DISCUSSION

Our results show that in *B. subtilis* subsp. *niger* WM wall turnover follows identical patterns in cells grown under widely different conditions in batch and chemostat cultures; in addition, patterns in cells with teichoic acid as

TABLE 1. *Kinetic parameters of wall turnover in B. subtilis subsp. niger WM under several growth conditions*

Growth conditions[a]	Label[b]	μ (h^{-1})	Parameters calculated[c]				
			λ (h^{-1})	θ (h)	κ (h^{-1})	k (%)	X_B (%)
Nutrient broth	Pulse	0.94	1.01	1.08	0.22	16	22
Nutrient broth + 1% glucose	Pulse	1.29	1.46	0.81	0.27	14	18
Glucose limitation	Pulse	0.19	0.28	3.33	0.087	32	32
Glucose limitation	Pulse	0.31	0.24	2.40	0.080	18	34
Potassium limitation	Pulse	0.30	0.19	1.96	0.084	20	43
Potassium limitation	Continuous	0.24	0.16	4.00	0.044	13	27
Phosphate limitation	Pulse	0.19	0.20	3.87	0.062	23	31
Phosphate limitation	Pulse	0.22	0.09	3.03	0.041	13	43
Phosphate limitation	Continuous	0.22	0.08	4.28	0.027	9	32

[a] Labeling and chase were as described in the text.
[b] Pulse, Pulse label for 0.1 generation time; continuous, continuous label for ca. 5 generation times.
[c] For definitions and derivations of the parameters, see text.

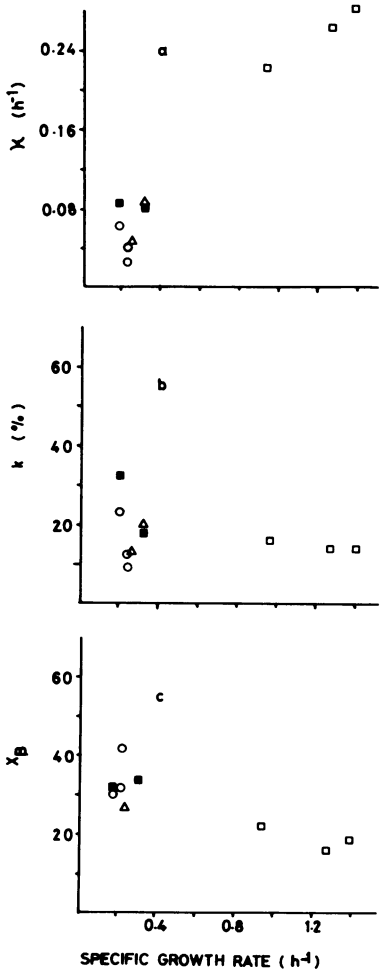

FIG. 5. *Relationship between the specific growth rate and the parameters of wall turnover* κ, k, *and* X_B *(for definitions, see text). The data shown were obtained from cultures grown under different conditions. Symbols:* □, *complex medium;* ■, *glucose limitation;* △, *potassium limitation;* ○, *phosphate limitation.*

their anionic wall polymer (phosphate-excess cultures) were identical to patterns in cells with teichuronic acid (phosphate-limited cultures).

In all cultures, pulse-labeled wall material was lost only after a lag of about 1 generation time. Thus, the duration of this lag was related directly to the growth rate. The strict preservation of the labeled wall material in this period clearly indicates that there is a general underlying growth-related principle.

Although direct evidence is still lacking, it is assumed that there is close contact between the inner surface of the wall, to which new wall chains are linked (1), and the cytoplasmic membrane (21). In this region of the wall, the hydrolytic action of autolysins, which are the agents of wall turnover, may be inhibited by membrane components, such as lipoteichoic acid and phospholipids, which have been shown to be potent inhibitors of *N*-acetylmuramyl-L-alanine amidase (EC 3.5.1.28; the main *B. subtilis* autolysin) in vitro (12). During cell growth, newly made wall chains (in our experiment represented by pulse-labeled wall) migrate outward (1) and finally reach the surface layers, where, possibly due to the absence of membrane autolysin inhibitors, hydrolysis of the susceptible bonds within the peptidoglycan may take place.

The lag before turnover of pulse-labeled wall is determined by the rate of migration of the label through the wall and by the proportion of the turnover-resistant layer within the wall. The sharp subsequent changeover to the first-order rate of decrease of label shows that there is only a small transitional region between the turnover-resistant and the turnover-sensitive layers within the wall. The first-order kinetics argue against models describing turnover as the sequential and integral degradation of very thin layers at the outside of the wall, but rather indicate the presence of a sizable outer layer from which random excision of all chains takes place, irrespective of their age.

Spreading of wall material, as defined originally by Pooley (26, 27), was taken into account in the quantitative description of wall turnover (see Appendix). The resulting kinetic equations describe very closely the experimental data obtained with continuously labeled cells. Indeed, the loss of radioactivity from the cells accelerated right after the start of the chase, as was predicted from the occurrence of wall spreading (26) (see equations 2 and 4 in the Appendix). An equivalent set of kinetic equations, in which the concept of wall spreading was not incorporated, was obtained by substitution of equation 2 by $v_t = \kappa \cdot X_0$ and proceeding further (see Appendix). We found that the results obtained from this procedure yielded a much less adequate description of the experimental results than the one outlined in the Appendix (Fig. 6b). Thus, our results support the hypothesis that wall material is spread over old and new cells during surface expansion (26) of *B. subtilis* under all growth conditions.

In phosphate-limited cells approximately 50% of the labeled amino sugars are present in peptidoglycan, and the remainder are located in teichuronic acid. Turnover kinetics in these cultures were identical to those in phosphate-excess cultures, in which all of the label was present in peptidoglycan. This is strong evidence that peptidoglycan and teichuronic acid are lost from the cells at identical rates, since overall first-order kinetics, as we observed, were not met when the turnover-sensitive fraction of the wall contained two or more populations with significantly different half-lives. Furthermore, the equally sharp transition after an initial lag observed in both pulse-labeled phosphate-limited cells and cells grown otherwise showed that labels from peptidoglycan and teichuronic acid arrived in the turnover-sensitive layer at the same moment and that no specific excision of anionic polymer linked peptidoglycan fragments occurred. These conclusions agree with the data of others, who showed that peptidoglycan and teichoic acid were lost from cells at identical rates (25, 30).

The k values (Fig. 5b) were not the same in different cultures grown at the same rate, and the reproducibility of k between duplicate cultures was low. One possible explanation is that the amount of autolysin activity per cell is constant, but that this activity is exerted on a variable amount of wall material in cells. However, the cellular wall content in the type of cultures used here varied much less than the values found for k (24a). Therefore, it is more likely that the inconstancy of k was primarily due to a variable activity of the autolysins in the walls. Related data have been reported by Brown (5), who noticed marked differences between the amounts of extractable autolytic enzymes in duplicate (batch) cultures of *B. subtilis*; Brown did not mention whether these differences also resulted in variations in the action of the enzymes in vivo. Measurements of the wall turnover products (peptidoglycan, anionic polymers) in the supernatants of chemostat cultures also revealed the occurrence of variable rates of turnover between duplicate cultures; this matter is discussed elsewhere (de Boer et al., J. Bacteriol., submitted for publication).

The mechanisms by which surface enlargement of bacilli is accomplished are largely unknown. Spreading of wall material after its initial linkage involves the breakage or transposition of covalent bonds within the peptidoglycan; such processes can be understood more readily by accepting the presence of larger subunits within the walls (sheets, bundles) which are mutually connected by a limited number of peptide bridges (26) and are spatially stabilized by interjacent anionic polymers, as proposed by Burge et al. (6, 7). Surface extension could then be achieved by movement of the subunits relative to each other, made possible by transamidation of L-alanine moieties to adjacent *N*-acetylmuramic acid residues. This transamidation activity could be carried out by the autolytic *N*-acetylmuramyl-L-alanine amidase in the hydrophobic regions of the walls (10). In this way a relatively low transamidase activity may result in a large surface enlargement, leaving the majority of the *N*-acetylmuramyl-L-alanine bonds intact, which would benefit greatly the smooth and orderly progress of wall growth.

Meanwhile, during radial migration, long peptidoglycan complexes synthesized at the membrane (17) could be broken into pieces by a second *B. subtilis* autolysin, an endo-β-*N*-acetylglucosaminidase (5, 19; C. Taylor, H. J. Rogers, and J. B. Ward, Soc. Gen. Microbiol. Q. 7:73–74, 1980). The shorter overall chain length would promote the lateral mobility of the wall material. In this respect, it is interesting that a wide range of chain lengths have been found in the peptidoglycan of *B. subtilis* (S. M. Fox, J. B. Ward, and M. G. Sargent, Proc. Soc. Gen. Microbiol. 4:90, 1977).

In the outer layers of the wall, autolysins are active as hydrolytic enzymes. A decrease in the concentration of the hydrophobic membrane components, such as lipoteichoic acid, might enable complexing of the *N*-acetylmuramyl-L-alanine amidase with wall teichoic (or teichuronic) acid, which is important for its hydrolytic activity on peptidoglycan (24). Since free glycan chains and peptides are the main degradation products of peptidoglycan found in the extracellular fluids (25; de Boer et al., submitted), it is

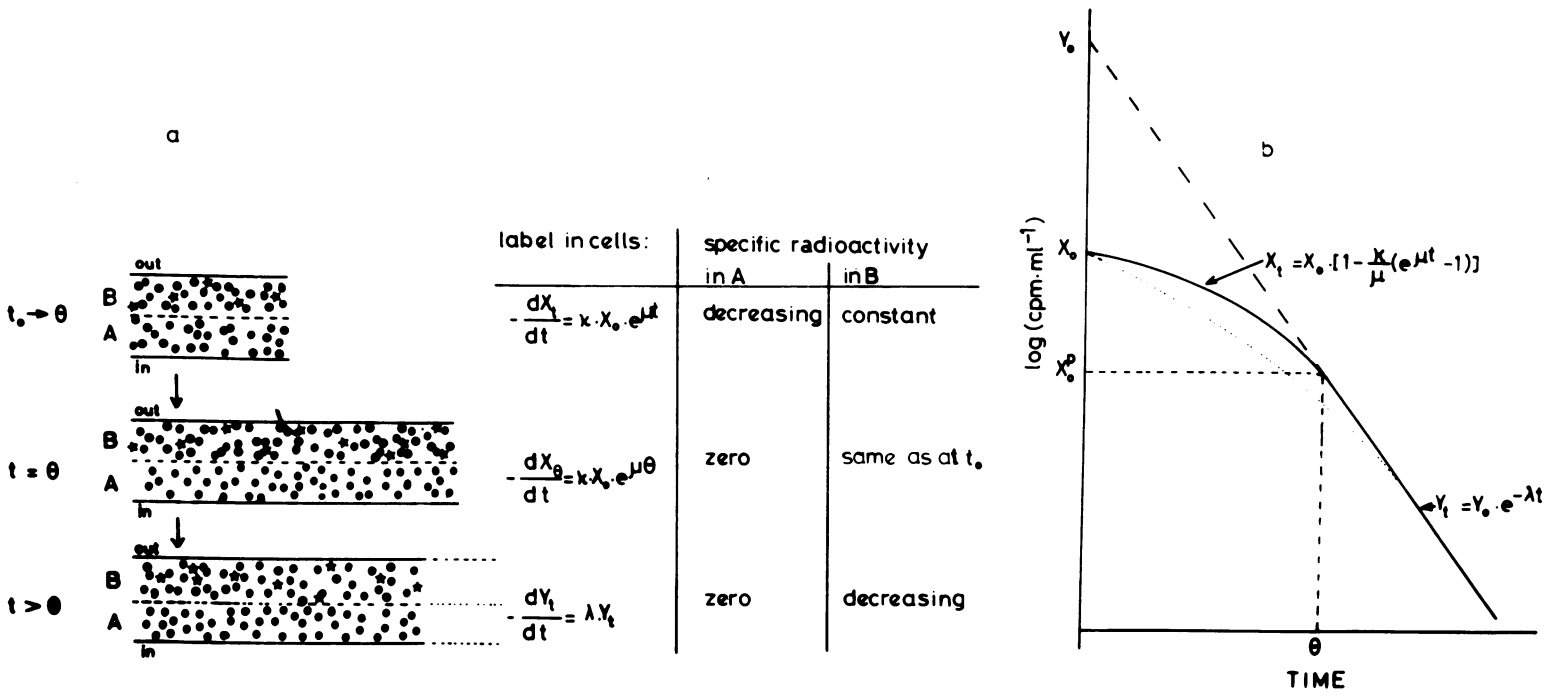

FIG. 6. *Wall turnover kinetics in B. subtilis. (a) Representation of the outward migration and concomitant spreading of old (radioactive) wall material, as described in the text. Black dots represent labeled wall chains, open dots represent nonlabeled wall chains, and stars show the localization of hydrolytically active autolysins in the turnover-active (B) wall layer. The upper drawing shows a part of a wall that has been labeled continuously; the middle drawing shows this wall fragment after growth for a period of θ hours (when all radioactivity has migrated to the B-layer); and the lower drawing shows the situation thereafter. (b) Time course of the decrease in cellular radioactivity (counts per minute per milliliter of culture) after a chase in continuously labeled cells (solid line), as given by equations 4 and 6. The dotted line shows the decrease in the initial phase (as in the solid line, calculated from X_o, Y_o, μ, and λ [see Appendix]), when no wall spreading was taken into account (i.e., substitution of equation 3 by $v_t = \kappa \cdot X_o$). This procedure resulted in values of θ that were much higher than those derived from the kinetics of pulse-labels in double-labeled cells. For further details and definitions of the parameters, see text.*

likely that all *N*-acetylmuramyl-L-alanine bonds are susceptible to (hydrolytic) attack by amidase.

The question may arise as to whether wall material still proceeds with spreading and surface enlargement (by transamidation activity) after arrival in the turnover-active layer B (see Fig. 6a), or that only hydrolysis of the *N*-acetylmuramyl-L-alanine bonds occurs. When transamidation and hydrolysis of *N*-acetylmuramyl-L-alanine bonds are controlled by two non-coexistent enzyme activities, as described above, it could be expected that only hydrolysis would occur in layer B, which implies that there is some kind of fixation of wall chains after arrival in layer B, since no lateral spreading (as in layer A) would be possible. It might be suggested that surface enlargement of the outer wall layers occurs by side-long attachment of new chains. In this way, one could explain the fact that segregation of wall label occurs some time after the start of the chase, despite initial spreading of the labeled wall, as has been demonstrated by Pooley et al. (28). These authors suggested that localized insertion of new chains caused label segregation in a strain (*B. subtilis* Ni15) with low turnover activity. In a previous publication, however, it had been demonstrated (26, 27) that wall spreading did occur in this strain, which could easily randomize a localized insertion pattern. The conflicting data on localized versus diffuse incorporation of new chains in the literature (16) might be reconciled by realizing that the results depend on the relationship between the rate at which the process can be measured and the rate of the spatial randomization of the labeled wall polymers.

APPENDIX

Derivation of the kinetic equations describing wall turnover in *B. subtilis*. (i) Definition of the parameters. As shown above, a specific pulse-label introduced into the wall is not subject to turnover for approximately 1 generation time (θ) (dimension, hours) after its incorporation. Thus, newly made wall chains are placed in a fraction of the wall immune to turnover (fraction A). After this lag, the pulse-labeled wall material is lost from the cells; the decrease in cellular label per milliliter of culture shows first-order kinetics (rate constant, λ [dimension, hour^{-1}]) until very low levels of radioactivity are left in the cells. This indicates that the pulse-label has now reached a second fraction in the wall (fraction B), from which older and newer chains are excised randomly by autolysins, irrespective of their age. From its incorporation until time θ, the labeled wall material is spread over old and newborn cells, as described above.

(ii) Decrease in wall-bound label during a chase of continuously labeled cells. In exponentially growing cells (specific growth rate [μ]) the rate of release of wall material from the cells by turnover per milliliter of culture at time t (v_t) (in nanomoles per milliliter per hour) is given by

$$v_t = \kappa \cdot X_t \tag{1}$$

where κ is a constant (dimension, hour^{-1}) and X_t is a measure of the amount of cell-bound wall material per milliliter of culture at time t. In a growing culture (specific growth rate μ), the amount of cell-bound wall material per milliliter of culture increases exponentially, and the rate of release of wall chains increases in the same manner:

$$v_t = v_0 \cdot e^{\mu t} = \kappa \cdot X_0 \cdot e^{\mu t} \tag{2}$$

We now turn to the situation where cells with continuously labeled walls are transferred to nonradioactive medium. At the start of the chase period (t_0), X_0 (the amount of cell-bound label per milliliter at t_0) is a direct measure of the amount of wall material bound to the cells per milliliter, provided the specific radioactivity within the wall fraction and the bacterial dry weight are known.

From the wall turnover kinetics and the wall growth model described above, we propose that the radioactive wall material that is present in the turnover-resistant (A) layers of the continuously labeled cells at t_0 migrates to the turnover-sensitive (B) layers along the following two routes: (i) by spreading mechanisms, the B layers of cells formed after t_0 are formed from wall material already present in the A layers of the cells extant at t_0; and (ii) material lost by turnover from the B layers is replenished, as shown schematically in Fig. 6a. Thus, until time θ the B layers of all cells (old and new) preserve a constant specific radioactivity of wall chains, and, consequently, the increase in the number of cells per milliliter is coupled to a similar increase in the rate of release of radioactive wall chains per milliliter of culture.

We can now derive the time course of the cellular radioactivity per milliliter of culture from t_0 to θ (X_t) (in counts per minute per milliliter) as expected from the above considerations, since

$$v_t = -\frac{dX_t}{dt} = \kappa \cdot X_0 \cdot e^{\mu t} \tag{3}$$

Integration yields an expression for X_t:

$$X_t = X_0 \cdot \left[1 - \frac{\kappa}{\mu} \cdot (e^{\mu t} - 1)\right] \tag{4}$$

At time θ, all radioactively labeled wall material has migrated to the turnover-sensitive B layers (Fig. 6a), and nonradioactive chains synthesized after t_0 enter the B layers, causing a lowering of the specific radioactivity. Wall material is removed from the B layers at random; i.e., no preference is exhibited for older (labeled) or younger (nonlabeled) chains, as derived from the first-order decrease in the cellular radioactivity per milliliter after θ (now called Y_t, to discriminate from X_t):

$$v_t = \lambda \cdot Y_t = -\frac{dY_t}{dt} \tag{5}$$

or, integrated:

$$Y_t = Y_0 \cdot e^{-\lambda t} \tag{6}$$

where λ is the first-order rate constant (dimension, hour^{-1}) and Y_t is the amount of cellular label per milliliter after time θ (in counts per minute per milliliter); Y_0 is the extrapolated value of Y_t at t_0 (Fig. 6b).

(iii) Calculation of θ and κ. From the experiments with continuously labeled cells, the parameters X_0, λ, μ, and Y_0 can be obtained. Because of the smooth transition at time θ from the curve described by equation 4 to the exponential decrease given by equation 6, the direct determination of θ is not possible simply from the experimental data. At time θ, however, there is continuity in the cellular labeling, so

$$X_\theta = Y_\theta \tag{7}$$

Also, the rates of release of label are the same in equations 3 and 5:

$$v_\theta = -\frac{dX_\theta}{dt} = -\frac{dY_\theta}{dt} \tag{8}$$

When equations 3 and 5 are inserted into equations 7 and 8, two equations with κ and θ as unknowns are obtained. From equation 7:

$$\kappa = \frac{\mu}{e^{\mu\theta} - 1} \cdot \left(1 - \frac{Y_0}{X_0} \cdot e^{-\lambda\theta}\right) \tag{9}$$

and from equation 8:

$$\kappa = \lambda \cdot \frac{Y_0}{X_0} \cdot e^{-(\lambda+\mu)\cdot\theta} \tag{10}$$

Eliminating κ from equations 9 and 10 yields

$$Y_0 \cdot \left[\frac{\lambda}{\mu} \cdot (1 - e^{-\mu\theta}) + 1\right] - X_0 \cdot e^{\lambda\theta} = 0 \tag{11}$$

from which θ can be calculated by using numerical methods [e.g., the Newton method to find a solution for $f(x) = 0$]. Once θ is known, the rate constant of the turnover process (κ) can be obtained by using equation 9 or 10.

(iv) Calculation of the turnover-sensitive fraction of the wall. The fraction of X_0 belonging to the turnover-sensitive B layers (X_0^B) can be calculated by invoking its condition at time θ, when all of the label is contained within the B layers (Fig. 6a); thus, from equation 6,

$$X_\theta^B = Y_0 \cdot e^{-\lambda\theta} \tag{12}$$

Between t_0 and time θ, the total amount of wall material in the B layers has increased by

$$X_\theta^B = X_0^B \cdot e^{\mu\theta} \tag{13}$$

Elimination of X_θ^B yields an expression for X_0^B:

$$X_0^B = Y_0 \cdot e^{-(\lambda+\mu)\cdot\theta} \tag{14}$$

or with equation 10,

$$X_0^B = \frac{\kappa}{\lambda} \cdot X_0 \tag{15}$$

The percentage of the wall sensitive for turnover (X_B) is now

$$X_B = \frac{\kappa}{\lambda} \cdot 100\% \tag{16}$$

(v) Percentage of the cellular wall polymers lost per generation time. The product $\kappa \cdot X_0$ (in counts per minute per milliliter per hour) is directly proportional to the rate of wall production caused by turnover activity. Provided that X_0 has been converted to the correct dimensions in all cases (e.g., nanomoles of wall chains per milligram of cells), the turnover rates of different cultures or the rates under different cultural conditions may be compared. When it is necessary to compare different growth rates, a parameter which gives the wall turnover per generation time is preferable.

At t_0, the cellular radioactivity (in counts per minute) per milliliter of culture (X_0) is related to the amount of cell-bound wall polymers per milligram of cells. Thus, the total amount of wall polymers lost in 1 generation time per milligram of cells (x_τ) can be obtained by integration of the rate of loss at t_0 (v_0) between times t and $t + \tau$ (τ is the generation time in hours), or from equation 3 at $t = 0$:

$$x_\tau = \int_t^{t+\tau} \kappa \cdot \alpha \cdot X_0 \cdot dt = \kappa \cdot \tau \cdot \alpha \cdot X_0 \tag{17}$$

where α is the conversion factor of X_0 to dimensions of nanomoles of wall per milligram. The percentage of X_0 lost per generation time (k) is now obtained from

$$k = \kappa \cdot \tau \cdot 100\% \tag{18}$$

(vi) Pulse-label experiments. From Fig. 6a, it can be concluded that a pulse-label applied at t_0 is available for turnover (or reaches the B layers) at time θ. Thereafter, the amount of cellular label per milliliter decreases, following the first-order kinetics described by equation 6. At time θ the loss of label has not yet occurred, and the radioactivity per milliliter is still X_0^P, the amount introduced originally by the pulse-labeling procedure. Thus, equation 6 can be rewritten,

$$X_0^P = Y_0 \cdot e^{-\lambda\theta} \tag{19}$$

from which θ can be calculated directly:

$$\theta = \frac{1}{\lambda} \cdot \ln\left(\frac{Y_0}{X_0^P}\right) \tag{20}$$

Once θ is known, the equations for the calculation of κ, k, and X_B are identical to those used for continuously labeled cultures.

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