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Absence of Correlation Between Rates of Cell Wall Turnover and Autolysis Shown by *Bacillus subtilis* Mutants

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Bacillus subtilis mutants with reduced rates of cell wall autolysis reached a constant rate of wall turnover after a longer lag than the standard strain but eventually showed the same turnover rate. In reverse, a turnover-deficient mutant autolysed at a slightly higher rate than the standard strain. Consequently, there is no correlation between the rates of cell wall turnover and autolysis.

The cell walls of gram-positive bacteria turn over during cell growth as peptidoglycans are continually synthesized and degraded. In *Bacillus subtilis*, in which this process has been particularly well studied, wall turnover is a pseudo-first-order rate process which depends on the enzymatic activities of cell wall hydrolases (2, 8) and extracellular proteases (5), and on the growth rate, i.e., rate of cell expansion (4; H.-Y. Cheung, L. Vitković, and E. Freese, manuscript in preparation). Cells of the *Bacillus* species also autolyse if their wall synthesis is inhibited (8) after growth has reached the stationary phase or under conditions dissipating the proton-motive force (6). Mutants which exhibit greatly reduced rates of autolysis for both whole cells and isolated cell walls (1, 3) and those with reduced rates of wall turnover (7, 10), have been given the genotype *lyt*. Because autolysis and turnover both involve hydrolysis of peptidoglycan bonds, it was believed that their rates were controlled by the same enzymes. However, we have not found published data on the turnover of *lyt* mutants deficient in autolysis nor on autolysis for the turnover-deficient mutants. Using both of these mutant types, we show here that the rates of turnover and autolysis are not correlated. Therefore, it is clear that different *lyt* mutations represent alterations in different genes, as has been already demonstrated for *lyt-1* and *lyt-2* mutations (3).

The *B. subtilis* strains used here were selected for having widely varying rates of autolysis or turnover so that a correlation or lack of it would be immediately apparent. The following is additional genetic background on these mutants. Strain 60015 (*metC7 trpC2*) is our standard laboratory strain. Strain 62253 [*metB5 purB6 str trpB3 amyE(M) amyR1 pap-9 pro(L)* = strain YN9 of Y. Yoneda, Tokyo University] has a reported Lyt$^-$ phenotype (measured with isolated cell walls) due to a pleiotropic mutation called *pap*, which also causes hyperproduction of extracellular protease and α-amylase (1, 11). Jolliffe et al. did not find a reduced autolytic activity in this strain (5), whereas we observed a threefold lower cell autolysis rate than in our standard strain (60015). Strain 62255 [*metB5 purB6 str amyE(M) amyR2 pap-9 pro(L)* = strain YY88 of Y. Yoneda] was derived by transformation of strain NA64 [*metB5 purB6 amyE(M) amyR2 pro(L)*] of K. Yamaguchi, Kanazawa University, with DNA of YN9; this strain was reported to have a Lyt$^-$ phenotype (1, 11) because wall preparations autolysed slower than those of the parent strain. However, we found that the cells of this strain autolysed 1.6 times faster than the cells of our standard strain. Strains 62271 (*metC3 lyt-1* = FJ3) and 62272 (*metC3 lyt-2* = FJ6) are independent isolates made by J. Fein, McGill University, Montreal, which are 90 to 95% deficient in two main cell wall hydrolases, *N*-acetylmuramyl-L-alanine amidase, and endo-β-*N*-acetyl-glucosaminidase (3). Strain 62355 (*thyA thyB trpC2 xin-15 lyt-15* = Nil5 of D. Karamata, Institut de Génétic et de Biologié Microbiennes, Lausanne) is deficient in cell wall turnover (7). The strains 62253, 62255, and 62355 were obtained from the *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio (as 1A182, 1A311, and 1A504), and the strains 62271 and 62272 were gifts from H. J. Rogers, National Institute for Medical Research, London.

Synthetic medium contained 10 mM ammonium sulfate, 5 mM potassium phosphate, 100 mM morpholinopropanesulfonate (pH adjusted to 7.0 by KOH), 2 mM $MgCl_2$, 0.7 mM $CaCl_2$, 50 μM $MgCl_2$, 50 μM $FeCl_3$, 1 μM $ZnCl_2$, 2 μM thiamine, 85 mM D-glucose, and the following amino acids (final concentration in micrograms per milliliter): L-Ala (112), L-Arg (100), L-Asp (166), L-Cys (10), L-Gly (94), L-His (10), L-homoserine (25), L-Ile (50), L-Leu (50), L-Met (10), L-Phe (10), L-Pro (10), L-Ser (132), L-Thr (10), L-Trp (25), L-Tyr (10), L-Val (50), and L-Glu (20 mM). The medium was supplemented with 2 mM thymine for strain 62355 or 2 mM hypoxanthine for strains 62253 and 62255.

Unless stated otherwise, cells were precultured at 37°C in synthetic medium to an optical density at 600 nm (OD_{600}) of about 0.5 and inoculated into the experimental culture at an OD_{600} of 0.03.

Turnover of cell wall was measured by the method of Pooley (7) with some modifications. *N*-acetyl-D-[^{14}C]glucosamine ([^{14}C]GlcNAc) (final concentration, 0.1 μM; 56 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) was added to an exponentially growing culture in synthetic medium, and the cells were shaken for 0.5 to 0.7 doubling time. The cells of a 10-ml culture were then collected on a moist (washed with 37°C water) membrane filter (pore size, 0.45 μm and 2.5-cm diameter; type HA; Millipore Corp., Bedford, Mass.), rapidly washed three times with 20 ml of warm (37°C) synthetic medium supplemented with 10 mM nonradioactive GlcNAc and immediately suspended in 10 ml of warm synthetic medium containing 2 mM GlcNAc. During continuing growth, 0.25-ml samples (in duplicate) were withdrawn at 15-min intervals and added to an equal volume of ice-cold 10% trichloroacetic acid (TCA) containing 100 mM GlcNAc. After more than 30 min at 0°C, the TCA-precipitat-

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TABLE 1. Cell wall turnover rates, autolysis rates, and extracellular protease activities of different *B. subtilis* strains[a]

Strain	Relevant marker	Doubling time (h)	Protease activity[b]	Rates (h^{-1})	
				Turnover	Autolysis
60015	Standard	0.39	53[c]	0.91[d]	0.19[e]
62253	*pap-9*	0.47	69	0.92	0.059
62255	*pap-9*	0.48	162	0.53	0.30
62271	*lyt-1*	0.50	59	0.64	0.061
62272	*lyt-2*	0.50	52	0.87	0.072
62355	*lyt-15*	0.47	104	0.22	0.26

[a] The strains were grown in synthetic medium at 37°C to an OD_{600} of 0.5, and the turnover rates of pulse-labeled cells or the autolysis rates as well as the protease activities were determined as described in the text. Each value is an average of two to six independent experiments.

[b] For protease units see text.

[c] The standard deviation was ±7.0%.

[d] The standard deviation was ±2.0%.

[e] The standard deviation was ±29%.

ed material was collected on a membrane filter and washed three times with 2 ml of ice-cold 5% TCA containing 10 mM GlcNAc. The membrane filters were dried under a heat lamp and placed into scintillation vials containing 15 ml of Aquasol (New England Nuclear Corp., Boston, Mass.), and their radioactivity was measured in a liquid scintillation counter (model 1216 Rackbeta II; LKB Instruments, Inc., Wallac OY, Finland). The logarithm of the counts per minute or of the percent of the initial counts per minute was plotted against the time, and the points lying in the region of a linear decline were used to calculate the best straight line of natural logarithm (radioactivity remaining in cell wall) versus time. The resulting first order rate constants (in $hours^{-1}$) are given in Table 1.

Cell autolysis was measured similarly to the method of Sayare et al. (9). The cells of the 20-ml culture, grown in the synthetic medium to an OD_{600} of 0.5, were rapidly collected on a membrane filter, washed three times with ice-cold distilled water, and suspended, by using a Vortex mixer, in 20 ml of 0.3 M sodium phosphate buffer (pH 7.0; 37°C) in a 125-ml Erlenmeyer flask. This suspension was shaken at 120 strokes per min at 37°C, and the decrease in OD_{600} was monitored with a Gilford spectrophotometer every 30 min for up to 4 h. The rate of autolysis (in $hours^{-1}$) was calculated from the slope of the natural logarithm (OD_{600}) decrease versus time.

Extracellular protease activity was measured in cell-free supernatants of cells growing exponentially in synthetic medium. When the OD_{600} was 1.0, cells were removed by centrifugation at 13,000 × *g* for 10 min at 4°C. A 3-ml amount of this supernatant was immediately mixed with 1 ml of synthetic medium containing hide powder azure (3 mg/ml) (Sigma Chemical Co., St. Louis, Mo.), and the mixture was shaken (to keep the insoluble substrate in suspension) for 3 h at 37°C. The undegraded substrate was removed by centrifugation at 37,000 × *g* for 10 min, and the absorbance of the supernatant was measured at 595 nm. One unit of protease activity was defined as the amount causing the release of 0.001 absorbance units of dye per h per OD_{600} of the original culture.

First, we compared the wall turnover of our standard strain (60015) with that of the *lyt-2* mutant (62272) which had been reported to have the lowest rate of autolysis of all known *lyt* mutants (3). The strains were grown at 37°C in synthetic medium where they multiplied with doubling times of 0.39 and 0.50 h, respectively. When the OD_{600} was about 0.05, [^{14}C]GlcNAc was added to label the cultures for 60 min. After that time, the cells were collected on a membrane filter, extensively washed, and then resuspended in warm (37°C) synthetic medium supplemented with 2 mM nonradioactive GlcNAc. The subsequent turnover measurement showed to our surprise that the *lyt-2* mutant turned over its cell wall at the same rate as the standard strain (Fig. 1). The only difference between the two strains was that the *lyt-2* mutant showed a 50-min longer lag than the standard strain.

Pooley had measured turnover with uniformly labeled cells at 45°C (7). To ascertain that our results did not depend on our particular experimental conditions, we measured turnover also at 45°C. At that temperature, the culture of the standard strain reached, after about 25 min, a constant turnover rate of 1.71 h^{-1} (curve not shown), which agreed with the data of Pooley (7). The *lyt-2* mutant (62272) showed the same turnover rate as the standard strain but after a longer lag period (Fig. 2). This result did not depend on the method of measuring turnover because the decrease of cell-bound radioactivity was mirrored by the increase of the radioactivity in the culture medium. The two measurements agreed well with each other, as the constancy of the sum of the two values throughout the experiment shows. Moreover, the rate of turnover, measured by the radioactivity of the cells retained on the filter (Fig. 2), had the same value as that measured by precipitation of cell-bound radioactivity with 5% TCA (data not shown). When the turnover was measured

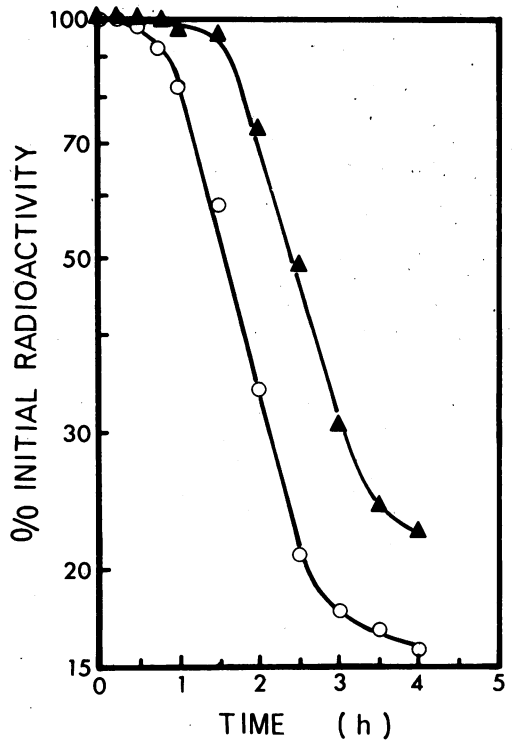

FIG. 1. Growth and cell wall turnover of the standard strain (60015) and the *lyt-2* mutant (62272) of *B. subtilis*. Cells were grown at 37°C for 60 min (about two doublings) in synthetic medium containing 1.8 μM [^{14}C]GlcNAc. When the OD_{600} was about 0.15, the cells were collected, washed, and suspended in the synthetic medium containing 2 mM nonradioactive GlcNAc. Cell wall turnover was determined as described in the text. The percentage of initial radioactivity remaining in TCA-precipitated samples of 60015 (○) and 62272 (▲) was plotted against the time. The initial values were 34,400 cpm per ml for 60015 and 37,100 cpm per ml for 62272.

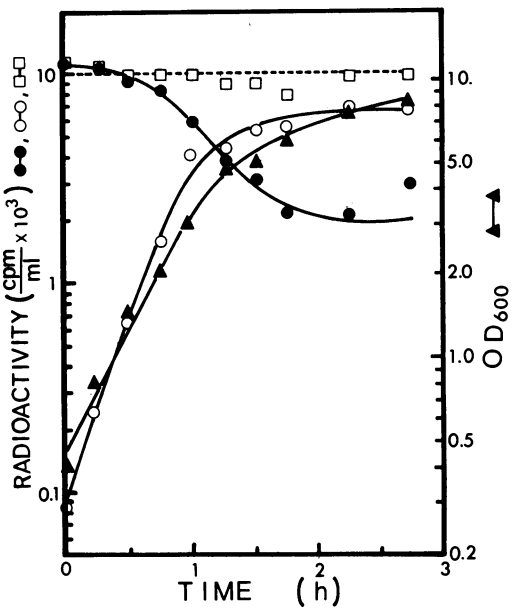

FIG. 2. Growth and cell wall turnover of the *lyt-2* mutant at 45°C. Cells were grown at 45°C for five doublings in synthetic medium containing 1.8 μM [^{14}C]GlcNAc. When the OD_{600} was 0.44, the cells were collected on a membrane filter and washed with and resuspended in the same volume of warm (45°C) synthetic medium containing 5 mM nonradioactive GlcNAc. Growth was followed by measuring the OD_{600} (▲). At the indicated times, the cells in 0.25-ml samples (in duplicate) were collected on a membrane filter, and the radioactivity in the filtrate was measured (○); the cell-bound radioactivity was also measured after washing the filters three times with 2 ml of ice-cold synthetic medium containing 10 mM GlcNAc (●). The constancy of the total radioactivity, i.e., the sum of the radioactivity in the filtrate and in the washed cells (□), shows that losses due to the washing procedure were negligible.

at different temperatures (between 33 and 45°C), the turnover rates observed for the standard strain (60015), the *lyt-1* (62271) mutant, and the *lyt-2* (62272) mutant (data shown for 37°C in Table 1) were similar, and the rates increased roughly proportionally to the growth rates (data not shown).

When wall turnover was measured for all strains (at 37°C), rates between 24 and 101% of the rate of the standard strain were observed (Table 1); the doubling times varied only little (from 0.47 to 0.61 h). The lowest turnover rate was observed for the *lyt-15* (62355) mutant for which Pooley had also reported the lowest turnover (7).

Jolliffe et al. have reported an inverse correlation between the concentration of extracellular proteases and the rate of cell wall turnover (5). To determine whether such a correlation was responsible for the different turnover rates observed here, the extracellular protease activities were also measured. Although strains with high extracellular protease activities had somewhat reduced turnover rates, the results (Table 1) show clearly that there was no quantitative relationship between protease activity and turnover rate. For example, the strain (62252) with the highest protease activity (three times that of the standard strain) had only a 42% lower turnover rate than the standard strain, whereas the strain (62355) with the lowest turnover rate had only a two times elevated protease activity. Thus, the protease activity is not the only factor that determines the turnover rate.

To measure autolysis, cells were grown to an OD_{600} of 0.5 and washed, and the rate of the natural logarithm (OD_{600}) decrease was calculated. The rates varied from 31 to 158% of the rate of the standard strain (Table 1). It is apparent that the rates of wall turnover were not at all correlated with the rates of cell autolysis. In particular, the turnover-deficient strain (62355 = *lyt-15*) autolysed 37% faster than the standard strain, and the autolysis-deficient strain (62253 = *pap-9*) had a normal turnover.

The results show that cell autolysis and wall turnover rates depend to different degrees on the activities of wall hydrolases. Wall turnover is controlled by certain hydrolases (2, 8), by proteases (5) which may degrade some of the hydrolases more than others, and by the rate of cell expansion, i.e., cell growth (Cheung, Vitković, and Freese, in preparation). Until mutations altered in the structural genes coding for wall hydrolases have been isolated, it will not be possible to establish a more precise biochemical basis for the control of turnover and autolysis by the different enzymes.

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