

## Proton Motive Force May Regulate Cell Wall-Associated Enzymes of *Bacillus subtilis*

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**Bacterial metabolism excretes protons during normal metabolic processes. The protons may be recycled by chemiosmosis, diffuse through the wall into the medium, or bind to cell surface constituents. Calculations by Koch (J. Theor. Biol. 120:73-84, 1986) have suggested that the cell wall of gram-positive bacteria may serve as a reservoir of protons during growth and metabolism, causing the wall to have a relatively low pH. That the cell wall may possess a pH lower than the surrounding medium has now been tested in *Bacillus subtilis* by several independent experiments. When cultures of *B. subtilis* were treated with the proton conductors azide and carbonylcyanide *m*-chlorophenylhydrazone, the cells bound larger amounts of positively charged probes, including the chromium ( $\text{Cr}^{3+}$ ) and uranyl ( $\text{UO}_2^{2+}$ ) ions and were readily agglutinated by cationized ferritin. In contrast, the same proton conductors caused a decrease in the binding of the negatively charged probe chromate ( $\text{CrO}_4^{2-}$ ). Finally, when levansucrase was induced in cultures by the addition of sucrose, the enzyme was inactive as it traversed the wall during the first 0.7 to 1.0 generation of growth. The composite interpretation of the foregoing observations suggests that the wall is positively charged during metabolism, thereby decreasing its ability to complex with cations while increasing its ability to bind with anions. This may be one reason why some enzymes, such as autolysins, are unable to hydrolyze their substrata until they reach the wall periphery or are in the medium.**

During chemiosmosis, protons are extruded through the cytoplasmic membrane and acidify a narrow region near the membrane. The protons subsequently return to the cytoplasm by mechanisms that extract work in the form of ATP creation or powering transport events. While outside the cell membrane, the local pH may be reduced by 3 to 4 U (17). It can be shown by physicochemical calculations based on the Debye-Hückel and Gouy-Chapman theories that the pH lowering is only a distance of a few nanometers if no cations are being pumped into the cell, but because potassium is needed in quantity by the growing cell the pH may be lowered throughout the 25-nm thickness of the gram-positive wall. The first evidence that this had important consequences for the cell was the study of Jolliffe et al. (12) which showed the activities of wall autolysins of *Bacillus subtilis* were increased (i.e., no longer inhibited) when chemiosmosis was blocked by adding proton conductors. The present paper brings two additional kinds of evidence on the influence of proton extrusion on wall physiology. The first comes from experiments showing that when the cell is metabolizing and extruding protons, positively charged probes ( $\text{Cr}^{3+}$ ,  $\text{UO}_2^{2+}$ , cationized ferritin) display a weak affinity for the wall, whereas a negatively charged probe ( $\text{CrO}_4^{2-}$ ) binds the bacteria with a relatively high affinity. In contrast, when the proton motive force is dissipated, the positively charged probes readily complex with the cell surface, whereas the negatively charged probe binds with a decreased affinity. The second series of experiments describe the kinetics of release of an exoenzyme. Levansucrase (LVS), a protein with a molecular weight of 50,000 induced by sucrose, is secreted by the cytoplasmic membrane and takes nearly a generation to become liberated from the cell. This is ex-

pected for the passage of a large protein through the thick wall by the inside-to-outside wall growth process (18). What is of issue and what is shown here is that the enzyme does not function while in the wall but becomes active once it has been secreted into the growth medium. This lack of activity is consistent with its known pH optimum (13). Moreover, it and other exoenzymes, including autolysins are shown to be relatively stable under acidic conditions.

(Parts of this research were presented at the 1991 American Society for Microbiology Annual Meeting [14a].)

### MATERIALS AND METHODS

**Bacterial strains.** *B. subtilis* 168 (*trpC*) and *B. subtilis* QB13 [*sacU(H)32 trpC2*] were maintained on AK (Difco Laboratories, Detroit, Mich.) sporulation plates. *B. subtilis* QB13 was provided by the Bacillus Stock Center, Ohio State University.

**Growth conditions.** Cells were grown in minimal salts of Spizizen (33) without citrate, supplemented with tryptophan (50  $\mu\text{g}/\text{ml}$ ), glycerol (5  $\text{mg}/\text{ml}$ ), and acid-hydrolyzed casein (0.02  $\text{mg}/\text{ml}$ ). Cultures were incubated with aeration at 37°C in a gyratory shaker at 200 rpm.

**Binding of chromate ( $\text{CrO}_4^{2-}$ ) and chromium ( $\text{Cr}^{3+}$ ) to *B. subtilis*.** Overnight cultures of *B. subtilis* 168 (*trpC2*) were washed twice in 50 mM HEPES [*N*-(hydroxyethyl)piperazine-*N'*-(ethanesulfonic acid)] (Sigma Co., St. Louis, Mo.), pH 6.0, containing 1 mg of glucose per ml and adjusted to an optical density of 0.80 at 540 nm. Cells (1.0 ml), labelled at time zero with sodium chromate ( $^{52}\text{Cr}$  [50  $\mu\text{Ci}/\text{ml}$ , specific activity, 2.63 Ci/mmol; Amersham, Arlington Heights, Ill.]) were mixed constantly at either room temperature or 4°C. The final concentration of the chromate in the reaction mixture was 10  $\mu\text{M}$ . The suspension was divided and carbonylcyanide *m*-chlorophenylhydrazone (CCCP) (40  $\mu\text{M}$ ;

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Sigma) was added into one portion 10 min after the initial labelling. Buffer was added to the other portion. All additions of CCCP or sodium chromate were less than 5% of the total volume. Samples (50  $\mu$ l) were taken at various time intervals after labelling, deposited onto 0.45  $\mu$ M filters, type HA (Millipore Co., Bedford, Mass.), and washed twice with 5.0 ml of HEPES, pH 6.0, containing 1 mg of glucose per ml. The filters were placed into plastic vials and counted in a Beckman 5500 gamma counter. The binding of chromium ( $^{52}\text{Cr}^{3+}$  [9.0  $\mu$ Ci/ml, specific activity, 500  $\mu$ Ci/mmol; NEN Research Products, Boston, Mass.]) to *B. subtilis* 168 was determined in the same manner as chromate except that 0.1 M KCl containing 1.0 mM glucose (pH 5.6) was used as a solvent. The use of buffers near neutrality for  $\text{Cr}^{3+}$  binding was impossible because the cation tended to precipitate.

**Electron microscopy.** For labelling of cells with uranyl ion, the bacteria were centrifuged, washed twice in distilled deionized water, and divided into two portions. The control cell portion was suspended in distilled water, whereas the second portion was suspended in 1 mM sodium azide. Both the control and the sodium azide-treated cells were then suspended in aqueous 1.0 mM uranyl acetate for 10 min at room temperature. After incubation, both samples were washed five times in distilled water. Washed cells were fixed for 60 min at 22°C in 5% glutaraldehyde in 50 mM HEPES buffer (pH 7.0), washed in buffer, dehydrated through an ethanol-propylene oxide series, and embedded in Epon 812 (32, 34). Microscopy was performed with either a Philips EM300 or EM400T electron microscope operating under standard conditions at 60 kV with the cold trap in place. Energy-dispersive X-ray spectroscopy verified that uranyl acetate (U signal) was the only contrasting agent in the wall preparations.

**LVS induction.** An overnight culture (3 ml) of *B. subtilis* QB13 was used to inoculate 200 ml of prewarmed medium. LVS was induced by the addition of sucrose to give a final concentration of 100 mM in exponentially growing cells (approximately 100 Klett units). Additions of sucrose were less than 5.0% of the total volume. Growth was monitored by use of a Klett-Summerson colorimeter (540 filter). Chloramphenicol (50  $\mu$ g/ml) was added to culture samples (30 ml) at various times after induction to inhibit protein synthesis. Each sample was centrifuged at 6,000  $\times g$  for 10 min, and the supernatants were placed into dialysis bags. The samples were dialyzed overnight at 4°C against 5 liters of 50 mM phosphate buffer, pH 6.0. Dialyzed samples were concentrated to approximately 1.0-ml volumes with polyethylene glycol (Calbiochem Corp., La Jolla, Calif.) prior to enzyme assays. In some experiments, phenylmethylsulfonyl fluoride, at a 0.5 mM final concentration, was used in the dialysis media to prevent proteolysis of the LVS. Controls established that the phenylmethylsulfonyl fluoride had no effect on the enzyme.

**Induction of LVS by protoplasts.** Protoplasts of strain QB13 were generated by a modification of the method of Chang and Cohen (5). Samples (30 ml) were collected, centrifuged, and washed twice with 2 $\times$  SMMP buffer (SMMP is 20 mM maleate, 20 mM  $\text{MgCl}_2$ , 0.8 M mannitol) and diluted with an equal volume of 4 $\times$  Penassay broth (BBL, Cockeysville, Md.). Cells were incubated for 30 min at 37°C in SMMP (3 ml) containing lysozyme (50  $\mu$ g/ml). The protoplasts were centrifuged at 4,000  $\times g$  and incubated at different time intervals in SMMP (3 ml) containing 0.8 M sucrose instead of mannitol. After incubation in sucrose, chloramphenicol (50  $\mu$ g/ml) was added, the samples were centrifuged, and the supernatants were retained. The super-

natant samples were dialyzed against 50 mM phosphate buffer (pH 6.0) and concentrated with polyethylene glycol. To monitor protoplast lysis, strain QB13 cultures had been initially labeled with thymidine ([methyl- $^3\text{H}$ ]thymidine [1  $\mu$ Ci/ml]; ICN, Irvine, Calif.). Samples (0.1 ml) from the protoplast supernatants were placed in vials and counted in a liquid scintillation counter. All supernatant samples contained less than 1.0% of the radioactive count of the initial culture. Therefore, the protoplasts retained their integrity during handling procedures.

**LVS assay.** Enzyme activity was assayed by a modification of the method of Germaine et al. (10) in which the formation of alcohol-insoluble levan was determined. In a final volume of 450  $\mu$ l, the reaction mixture contained 1 mM sucrose (2.5  $\mu$ Ci of [ $^{14}\text{C}$ ]sucrose and unlabelled sucrose as needed), 50 mM phosphate buffer (pH 6.0), and the enzyme mixture. Samples (50  $\mu$ l) were spotted onto Whatman 3MM filter paper disks (2.4 cm in diameter) after a 15-min incubation at room temperature. The enzymatic reaction was terminated by placing the filters in absolute methanol (20 ml per disk) for 15 min. The disks were washed twice more for 15 min each with methanol, placed in scintillation vials, and counted in a liquid scintillation counter.

**Detection of exocellular levan.** An overnight culture of *B. subtilis* QB13 was used to inoculate 300 ml of prewarmed minimal salts solution. LVS was induced by the addition of sucrose to exponentially growing cells. Samples (5.0 ml) were taken at various times before and after induction. The samples were immediately incubated for 5 min at 80°C to prevent further levan production by the enzyme. Ethanol (2.0 ml) and water (1.0 ml) were added to the samples (1.0 ml each) to precipitate the polysaccharide. The samples were centrifuged, and the procedure was repeated. The residual material was dissolved in 3.0 ml of distilled water. Levan production was determined by the anthrone reaction (26) in which fructose served as a standard.

**SDS-PAGE.** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) employing a discontinuous buffer system (Tris-HCl glycine, pH 8.3) was performed by the method of Laemmli (21). Samples containing equal quantities of protein were loaded onto 6% gels. Gels were visualized with Coomassie brilliant blue 250 or were silver stained by the method of Merrill et al. (24). After electrophoresis, unfixed gels were incubated overnight at 37°C in 50 mM sodium acetate buffer (pH 5.5) containing 1% (vol/vol) Triton X-100 and 10 mg of sucrose per ml to allow renaturation of the LVS and the synthesis of levan (30). The gel was then fixed for 1 h in 12.5% (wt/vol) trichloroacetic acid, washed with distilled water, and oxidized for 2 h in 1% (wt/vol) periodic acid (Sigma). After several changes in 15% acetic acid for 2 h, the gel was placed in Schiff's reagent (Sigma) and incubated in the cold and dark for 1 to 2 h (14). Frequent changes of 15% acetic acid were used to destain the gel.

**Assay for wall-bound and extracellular enzymes.** (i) **Autolysins.** Samples of freeze-dried *B. subtilis* 168 cell wall (10 mg/ml) were suspended in either 5, 1.0, or 0.2 mM cold acetic acid. As a control, one sample was suspended in cold distilled water. All samples were stirred for 30 min, freeze-dried, and ultimately suspended in solutions of 20 mM ammonium carbonate (pH 7.7) or 0.1 M sodium acetate (pH 5.5). Autolysin activity was monitored spectrophotometrically at 540 nm by the loss of turbidity of the wall fractions.

(ii) **Amylase.** Samples (1 mg/ml) of *B. subtilis* amyase (Boehringer-Mannheim, Indianapolis, Ind.) were dissolved in 50 mM KCl and incubated at 4°C for 30 min under different

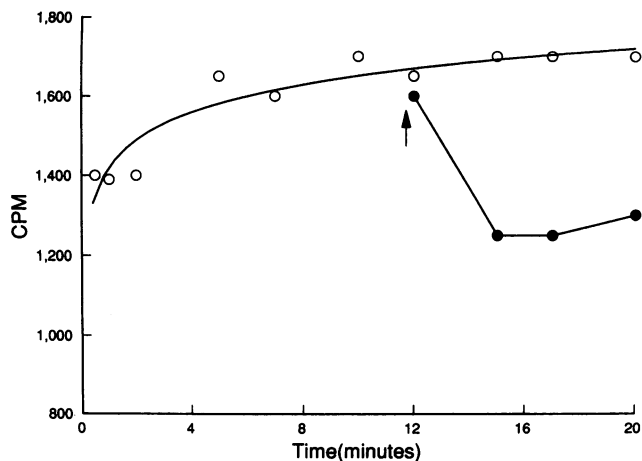


FIG. 1. Binding of chromate ( $\text{CrO}_4^{2-}$ ) by *B. subtilis* in the presence and absence of an uncoupling agent. Open circles, binding of  $^{52}\text{CrO}_4^{2-}$ ; solid circles, binding of chromate upon addition of CCCP at the arrow.

pH conditions. After incubation, the hydrogen ion concentration of each sample was readjusted to a pH of 6.0. Amylase activity was then monitored at 405 nm with *p*-nitrophenyl- $\alpha$ -D-maltoheptaoside as a substrate (Sigma).

(iii) **Protease.** Subtilisin (Sigma) samples ( $10^{-4}$  mg/ml) were dissolved in 50 mM KCl and adjusted to various pH conditions with glacial acetic acid. After incubation for 30 min at 4°C, the pH of each sample was readjusted to 7.2. Portions (3 ml) were placed into tubes containing hide powder azure (12 mg) and 1.0 ml of 50 mM KCl. The tubes were incubated at 37°C for 3 h with occasional blending in a Vortex mixer. After incubation, the turbidity of each tube was measured spectrophotometrically at 595 nm. All assays for proteins were performed according to the method of Bradford (4).

## RESULTS

**Binding of chromate and chromium to *B. subtilis*.** Chromate is a bivalent anion which should bind well to positively charged cell walls. Suspensions of *B. subtilis* 168 were mixed with sodium chromate ( $^{52}\text{Cr}$ ), and samples were removed, washed, and assayed for radioactivity. The results (Fig. 1) show that the binding of chromate to the cells was rapid. Control experiments showed that equimolar concentrations of sulfate and phosphate did not interfere with the binding. Other control experiments showed that the binding was as rapid at 1 to 3°C as at 22°C. The results are taken to support the view that chromate is not transported into *B. subtilis* but binds to the cell surface. When CCCP was added to the cells, there was a loss of chromate binding (Fig. 1). The loss would be expected if the cell walls had become more negatively charged upon dissipation of proton motive force. Similarly,  $\text{Cr}^{3+}$  would be expected to bind better to cells which have had their membranes deenergized. The basic assumption is that  $^{52}\text{Cr}^{3+}$  does not enter the cell via a stereospecific uptake system. Experiments confirmed that the acquisition of  $\text{Cr}^{3+}$  was independent of temperature, suggesting that the cation was binding to the cell surface. When CCCP was added to a  $\text{Cr}^{3+}$ -labeled *B. subtilis* suspension, there was enhanced binding of the cation (Fig. 2). The enhanced binding would

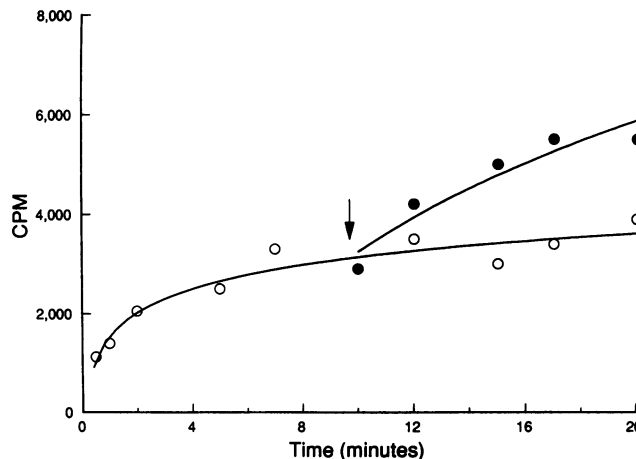


FIG. 2. Binding of chromium by *B. subtilis*. Open circles, binding of  $^{52}\text{Cr}^{3+}$ ; solid circles, binding of  $\text{Cr}^{3+}$  after addition of CCCP at the arrow.

be predicted if CCCP caused the wall to become less protonated and therefore more electronegative.

**Binding of cationized ferritin.** A suspension of *B. subtilis* was mixed with cationized ferritin for several minutes. The suspension was divided, and sodium azide was added to one portion, whereas buffer was added to the other portion. Within a few minutes after the addition of azide, the cells began to aggregate, whereas the control cells remained suspended. The results are presented as first-order rate plots describing the change in turbidity (absorbance) of the suspensions plotted on a logarithmic scale versus time (Fig. 3). It is clear that when sodium azide was added to the suspension, a high rate of aggregation occurred. The loss in opacity was not due to cellular lysis, because lysis of cell suspensions in the absence of the ferritin probe was less than 10% when azide was added. At higher temperatures, lysis is more rapid.

**Binding of uranyl ion ( $\text{UO}_2^{2+}$ ) by *B. subtilis*.** The positively charged uranyl ion would be expected to complex more

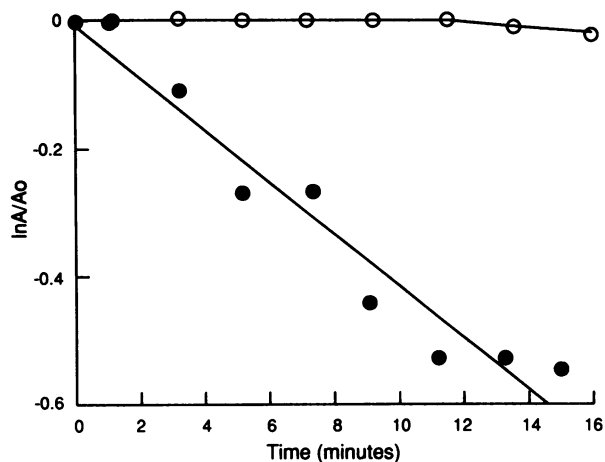


FIG. 3. First-order rate plot of the aggregation of *B. subtilis* induced by cationized ferritin in the presence (solid circles) and absence (open circles) of sodium azide.  $A_{540}$  readings were made in 1-cm cuvettes.  $A$ , absorbance;  $A_0$ , absorbance at zero time.

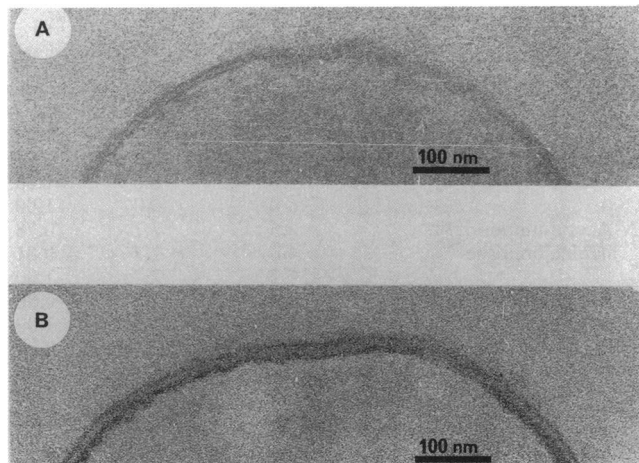


FIG. 4. Thin section showing the binding of uranyl ion ( $\text{UO}_2^{2+}$ ) by *B. subtilis* cells in the absence (top) and presence (bottom) of sodium azide.

strongly with highly negatively charged complementary sites. A thesis of this paper is that the walls of vegetative cells are positively charged because of the binding of protons as the protons are extruded from the membrane. Azide and CCCP, which prevent the production of protons, should give rise to a cell surface capable of enhanced binding of the positively charged uranyl ion. Thin sections of cells mixed with uranyl acetate in the presence or absence of sodium azide show that the azide-treated cells stain more densely than the control cells (Fig. 4). The results are consistent with the view that agents which dissipate the proton motive force increase the binding of positively charged molecules to the cell surface.

#### Time course for appearance of LVS in the growth medium.

When an exponential culture of *B. subtilis* QB13 was mixed with 100 mM sucrose, LVS activity was detectable in the supernatant only after about 0.75 generations (Fig. 5). In some experiments, cell walls were isolated from cultures

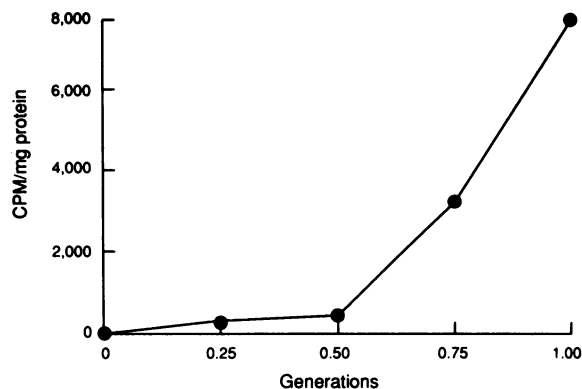


FIG. 5. Extracellular LVS activity in *B. subtilis* QB13 after the addition of sucrose. Sucrose (100 mM) was added to an exponential culture, and supernatants were obtained during growth. At the indicated times, chloramphenicol (50  $\mu\text{g}/\text{ml}$ ) was added to the cultures. Samples (30 ml) were then centrifuged, and the supernatants were dialyzed against 50 mM phosphate buffer (pH 6.8). All soluble fractions were concentrated with polyethylene glycol and assayed for LVS activity with radioactive sucrose.

after addition of sucrose and the walls were then washed four to five times with diluted phosphate buffer. When analyzed for LVS activity, the walls were invariably free of the enzyme. Presumably, the washing procedures caused the solubilization of any LVS trapped within the wall matrix. In addition, when soluble LVS preparations were added to wall suspensions, there was no tendency for the walls to bind or complex the enzymes. Finally, wall preparations had no effect on LVS activity when the enzyme was added to wall suspensions.

The extracellular form of the LVS of *B. subtilis* has a molecular weight of approximately 50,000 (3, 27). If there is a lag for the enzyme to be exteriorized, then electrophoresis of the culture supernatants should reveal the protein only after >0.75 of a generation. An exponential culture was mixed with sucrose (100 mM final concentration), and samples were removed at intervals and centrifuged. The samples were dialyzed and concentrated by use of polyethylene glycol. Electrophoresis revealed that a protein with a molecular weight of about 50,000 appeared in the supernatants only after a generation in the presence of sucrose (results not shown). These results are consistent with the data in Fig. 5 which showed that LVS enzymatic activity could be detected only after a delay of approximately 0.75 of a generation postsucrose treatment. To confirm that the band at 50,000 was indeed LVS, an SDS-PAGE gel was washed extensively with 1% Triton X-100 (14) to remove the SDS. Enzyme activity returned when the detergent was removed (30). When the gel was soaked in sucrose, washed, and developed with periodic acid-Schiff's reagent, the band comigrating with the protein at a molecular weight of 50,000 yielded a positive stain (14). Schiff's-positive material would be expected if the LVS converted the sucrose into levan. Finally, under the conditions employed in this study, LVS activity could not be detected at a pH of <4.5. Dedonder (7), using a more highly purified enzyme, was able to detect activity at a pH of >4.4.

#### Detection of levan in sucrose-induced cultures of *B. subtilis*.

Levan, a polyfructose product of LVS, should be present in cultures very shortly after induction of LVS by sucrose, provided the cell wall does not prevent enzymatic activity. It is assumed that LVS is induced and secreted through the cytoplasmic membrane and cell wall. Sucrose should be able to penetrate the wall and serve as a substrate for LVS trapped within the peptidoglycan matrix. If the wall matrix has a low pH during metabolism and secretion of LVS, then it is unlikely that levan would be found in the culture medium until LVS had been exteriorized and exposed to the higher pH of the medium. Figure 6 shows that levan in supernatants could be detected by chemical methods only as the cell population approached 1 generation after the addition of sucrose. In other experiments, when radioactive sucrose was added to the cells, extracellular radioactive polysaccharide could not be detected in culture supernatants until the bacteria had been in the presence of the sucrose for at least 0.7 of a generation. These results support the view that as LVS penetrates the wall matrix it is inactive. Only upon the appearance of the enzyme on the cellular exterior or in the growth medium does it assume an active conformation.

**LVS production in protoplasts.** When sucrose is added to suspensions of *B. subtilis* protoplasts, no significant lag in detection of LVS in the medium would be expected. This is because the cell wall is absent, and any secreted LVS should be directly diluted into the suspending medium. Figure 7 shows that LVS can be detected 5 min after addition of the sucrose. Protoplasts incubated with sucrose plus 20  $\mu\text{g}$  of

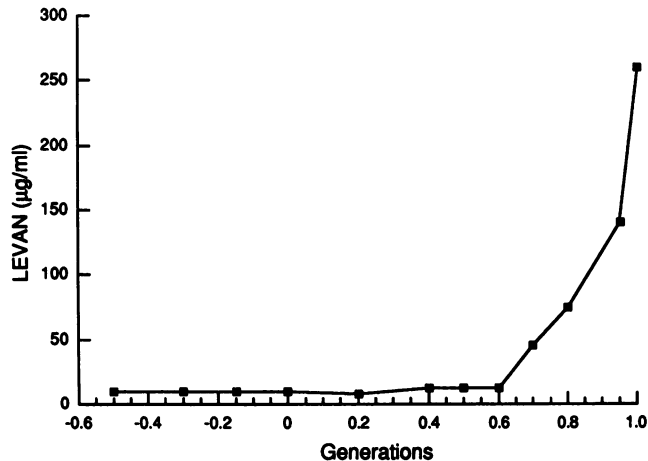


FIG. 6. Detection of exocellular levan. Exponential cultures of *B. subtilis* QB13 were induced with 100 mM sucrose. Samples (5 ml) were taken at intervals before and after induction, heated to 80°C for 5 min, and tested for levan production by the anthrone reaction.

chloramphenicol per ml did not secrete detectable LVS. The minimum time required for appearance of LVS in the suspending medium (5 min) represents approximately 0.15 of a generation for whole cells in the medium.

**Extracellular enzyme activities after exposure to a low pH.** During metabolism, the cell wall of *B. subtilis* should have a pH primarily dictated by the pKs of carboxylate (from peptidoglycan) and phosphate (from teichoic acid) as the result of proton pumping. At present, it is difficult to define the wall pH of metabolizing cells but it can be estimated to be between 3 and 5 from acid-base titration curves (reference 20 and unpublished observations). A prediction is that low

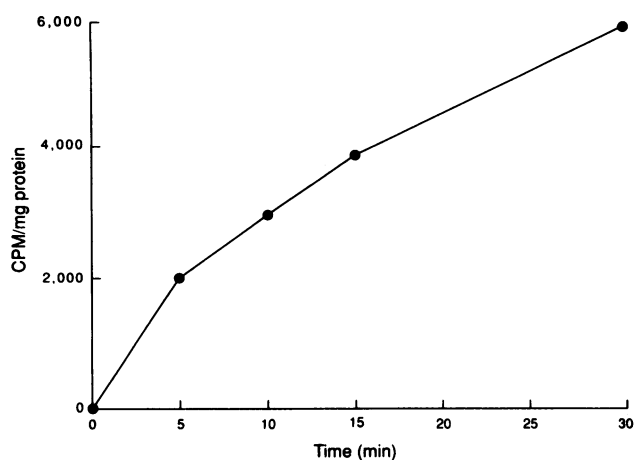


FIG. 7. Production of LVS by *B. subtilis* protoplasts. *B. subtilis* QB13 was grown to a Klett reading of 150 (late-exponential cells) in minimal medium. The cells were harvested by centrifugation, washed twice in SMMP containing 0.8 M mannitol, and finally suspended in the same SMMP solution. Lysozyme (50 µg/ml) was added, and the suspension was incubated at 37°C for 30 min. Protoplasts were collected in SMMP containing 0.8 M sucrose. Samples were withdrawn at intervals and mixed with chloramphenicol (50 µg/ml). Protoplasts were sedimented, and the supernatants were subjected to dialysis in 50 mM phosphate buffer (pH 6.0). The retentate materials were assayed for LVS activities.

TABLE 1. Effects of low-pH treatment on extracellular enzymes of *B. subtilis*<sup>a</sup>

Enzyme	pH:		Relative activity	
	Preincubation	Assay		
α-Amylase	3.8	6.0	0.73	
	4.0	6.0	1.00	
	6.0	6.0	1.00	
<i>N</i> -Acetyl-muramoyl-L-alanine amidase	3.6	7.7	0.78	
	4.0	7.7	0.81	
	7.7	7.7	1.00	
<i>N</i> -Acetylglucosaminidase	4.0	5.5	0.86	
	5.5	5.5	1.00	
	LVS	3.6	6.8	0.94
LVS	3.8	6.8	1.00	
	4.0	6.8	1.00	
	6.8	6.8	1.00	
	Subtilisin	3.8	7.2	0.65
	4.0	7.2	0.75	
	7.2	7.2	1.00	

<sup>a</sup> Enzyme solutions or cell walls were incubated for 30 min in acetic acid-KCl solution at the indicated hydrogen ion concentrations. Cell walls were freeze-dried and then assayed for amidase or glucosaminidase. Enzymes were adjusted to the assay pH values shown in the table, and then substrata were added. Enzyme activities were measured at room temperature.

pH values would not irreversibly inactivate the wall-bound enzymes or exo-enzymes. This is in contrast to many cellular enzymes which are denatured at low pH values. Suspensions of *B. subtilis* cell walls were prepared in diluted acetic acid solutions (low pH) and freeze-dried to remove the volatile acetic acid. The walls were then suspended in buffers and permitted to autolyze. At pH 7.7, a hydronium ion concentration which permits amidase activity only (13), there was only a slight decrease after suspension of the walls in acetic acid solutions and lyophilization (Table 1). Autolysis at pH 5.5, which permits *N*-acetylglucosaminidase activity (13), similarly showed that the enzymes were not denatured. Finally, solutions of subtilisin and α-amylase from *B. subtilis* were acidified for 30 min at 4°C. The enzyme solutions were then brought to pH 7.2 and 6.0, respectively, and assayed for their enzymatic activities. Supernatants containing LVS from sucrose-induced cultures were concentrated, dialyzed, and acidified by acetic acid for 30 min at 4°C. The enzyme solution was then brought to pH 6.0 and assayed for LVS activity. The results (Table 1) show that acid treatments do not irreversibly denature autolysins, LVS, subtilisin, or α-amylase.

## DISCUSSION

Several independent experiments showed that surface characteristics of *B. subtilis* depend on the energized state of the membrane. The results suggest that the cell wall, made up of peptidoglycan and teichoic acid, is less negatively charged when the bacteria are metabolizing a carbon source and creating a proton motive force. When the cells are poisoned with uncoupling agents, the cell wall then becomes more negatively charged and the wall pH approaches that of the surrounding medium. Metabolizing cells bind more anionic chromate than cells possessing deenergized membranes. Moreover, the addition of azide or CCCP to *B. subtilis* results in the enhanced binding of cationized ferritin, uranyl ion, and the Cr<sup>3+</sup> cation. All of these results are predictable if the wall is a reservoir for protons during growth.

In a quite different approach, it was shown that LVS, an inducible enzyme, is inactive until it becomes exteriorized or is secreted into the growth medium. The enzyme is large enough that it cannot rapidly diffuse through the cell wall. Small enzymes, such as staphylococcal nuclease (molecular weight, 16,800) are not impeded by the wall (25). Presumably, the LVS is passively carried through the wall to the cell surface as peptidoglycan-teichoic acid is laid down on the inner face of the wall (2, 8, 19, 21, 26, 28, 29). In this regard, Coxon et al. (6) showed that *B. subtilis*, when pulsed with a radioactive amino acid, did not secrete labelled protein in the medium until nearly a generation had lapsed. Furthermore, there is a lag in the release of soluble protein antigens of *Mycobacterium tuberculosis* as the cells leave the lag phase and enter the exponential growth phase (1).

At any one time during exponential growth of a culture of *B. subtilis*, amidase is probably found randomly distributed in the wall matrix (11). During its lifetime in the wall, the enzyme is inactive but activity can be rapidly achieved by dissipation of the proton motive force (9, 12, 15). If the cell wall were able to bind secreted protons, then the wall would possess a low pH, thereby preventing the enzymatic activity of LVS, amidase, and other enzymes. Loss of proton motive force would quickly result in loss of bound protons in the wall, and autolysis would ensue. Thus, the energized membrane may regulate the activities of some extracellular enzymes in *B. subtilis* by providing the wall with protons. Walls exhibit a greater tendency to bind to protons than cations (22). An interpretation of the results is shown in Fig. 8. In this model, LVS and autolysin (or other enzymes) in the wall matrix would be inactive because of the surrounding low pH. Only when the enzymes had become surface exposed would they become active. Protons on the cell surface would be displaced by  $K^+$  or  $Na^+$  from the medium. As long as the cell is metabolizing, the wall matrix would have a source of hydrogen ion to effectively compete with cations in the medium.

A prediction of the foregoing model is that most secreted *B. subtilis* enzymes would be generally resistant to low pH. The results showed that pH values of  $<4$  do not readily inactivate LVS, amylase, subtilisin, amidase, or glucosaminidase. Another prediction is that the catalytic product of LVS, levan, would be found in the growth medium only when the enzyme had adequate time to reach the cell exterior. Experiments showing the kinetics of levan production confirmed that levan is not produced during the time LVS is in the wall matrix. Finally, a further prediction is that cell wall at a near neutral pH would have no effect on LVS activity. This was borne out by experimentation.

There are other possibilities for enzyme inactivity before the enzyme is exteriorized or surface exposed. The enzyme may not be able to fold properly while in the cell wall. This seems unlikely because washed cell walls are hydrolyzed at hydrogen ion concentrations permitting enzyme activity. There is no evidence that walls, once isolated from intact bacteria, are degraded by endogenous autolysins only on the outer surface where an active conformation would be expected if complete exteriorization is essential for activity. In fact, when walls of exponential cells are radiolabelled and the cells are treated with azide to induce autolysis, there is clear evidence that the newly labelled wall inner face and the old wall outer face are hydrolyzed at the same rates (16).

It seems possible that the protoplast of a gram-positive intact cell is surrounded by a sea of wall-bound protons. The resulting pH dictated by wall-proton complexes would be low enough to regulate enzymatic activities. This may be a

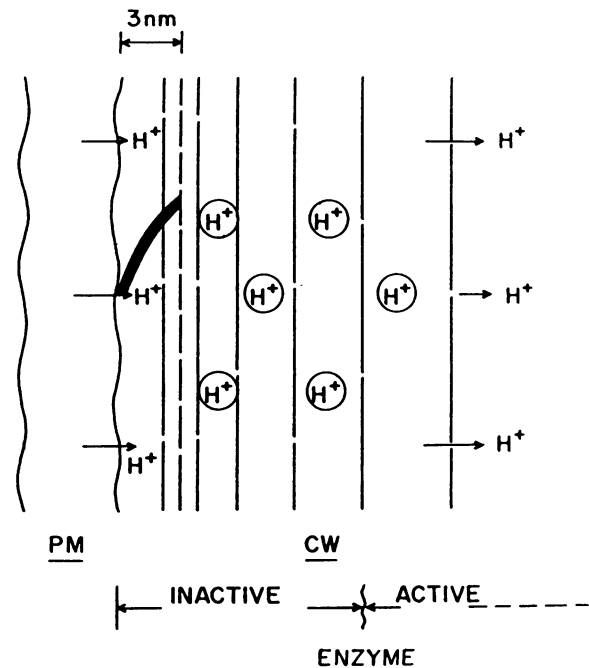


FIG. 8. Regulation of enzymes and autolysin during growth. Protons extruded by energized membrane are bound by carboxylate and phosphate groups of the wall matrix. The wall will assume a pH which is dictated by the  $pK_a$ s of the carboxylate and phosphate groups. This low pH will prevent autolysin from degrading cell wall (CW) as the cell wall is pushed toward the outside by newly added wall (26, 28). Therefore, the wall acts as a reservoir for protons. As the wall is pushed to the cell exterior, protons are titrated away. The increase in pH and the surface stress assumed by the wall permit activity of autolysin, resulting in turnover of wall material. Hence, other secreted proteins, such as LVS, may remain inactive close to the cytoplasmic membrane. As wall material is pushed outward, the enzyme is unable to pass through the wall matrix because of its size. When the proton gradient within the wall is dissipated, activated autolysin degrades wall material. The thick curved line reflects the distance from the energized membrane that protons would be expected to diffuse before combining with wall (for a theoretical discussion of diffusion of protons from membrane, see reference 17). The figure also suggests that wall at the very periphery is more stressed, and therefore more susceptible to enzymes, such as autolysins. The more highly stressed wall is depicted by the long, relatively uninterrupted, vertical lines. PM, plasma membrane.

heretofore unrecognized function of the cell wall of *B. subtilis*.

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