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Germination and Peptidoglycan Solubilization in *Bacillus megaterium* Spores

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During initiation of *Bacillus megaterium* QM B1551 spore germination, trichloroacetic acid-soluble, nondialyzable peptidoglycan fragments with an average molecular weight of 20,000 were excreted. This solubilization of peptidoglycan was measured in vitro as the amount of trichloroacetic acid-soluble hexosamine released from a suspension of broken spores. $HgCl_2$, a potent inhibitor of initiation, had no effect on the in vitro solubilization of peptidoglycan. In vivo, $HgCl_2$ had no effect on peptidoglycan release from spores that had lost heat resistance, but $HgCl_2$ did block complete absorbance loss. These results suggest that mercury inhibits some reactions that normally occur before loss in heat resistance but not the subsequent peptidoglycan release, and mercury inhibits other reactions involved with complete absorbance loss.

Initiation of bacterial spore germination is generally assumed to consist of a sequence of reactions (8, 11) that are primarily degradative. One of these reactions is the excretion of nondialyzable hexosamine-containing peptidoglycan fragments (14) into the germination exudate. Strange and Dark (16, 18) described a spore lytic enzyme in spores of several *Bacillus* species, and in *B. cereus* the spore lytic enzyme caused initiation of urea-sensitized spores (7) or spores treated with reagents which rupture disulfide bonds (6, 7). It was suggested that the spore lytic enzyme was the germination enzyme (5-7) and that the hydrolysis of spore cortex was a primary event in initiation (5). The inhibitory effect of some heavy metal ions on *Bacillus* spore germination (11, 20) and the ability of thiolyglycolate or glutathione to reverse this effect (12) suggested that sulfhydryl groups are possibly involved in the initiation of spore germination. The *B. cereus* spore germination enzyme was inactivated by thiol-blocking agents or by oxidation, and this inactivation was reversed by reduction, which suggested that germination by the action of the lytic enzyme depended on thiol groups (6).

Because of our interest in the mechanism of initiation of germination in *Bacillus megaterium* QM B1551 spores, we investigated the role of peptidoglycan solubilization in this strain.

MATERIALS AND METHODS

Organism. *B. megaterium* QM B1551 was grown in supplemented nutrient broth as previously described (10). The spores were harvested after about 36 h, washed 10 times with distilled water, lyophilized, and stored at room temperature and ambient relative humidity.

Assays. Initiation of germination was measured spectrophotometrically at 30 C on heat-activated spores (10 min at 60 C) as previously described (10) and confirmed by observation under the phase-contrast microscope. In experiments where the concentration of spores was 10 mg/ml, samples of the spore suspension were diluted 50-fold at the indicated times into cold 4 mM $HgCl_2$, and the absorbances (660 nm) were determined within 10 s of the time of dilution. Loss of heat resistance during initiation was measured as described by Levinson and Hyatt (11) with minor modification. Samples (0.5 ml) of spore suspension were diluted into 9.5 ml of water at 70 C, incubated for 10 min, diluted in supplemented nutrient broth medium, and plated in triplicate on supplemented nutrient broth agar plates, and the plates were incubated for 24 h at 30 C.

Hexosamine was measured, after acid hydrolysis, in 3 N HCl for 4 h at 95 C, by the method of Ghuysen et al. (4) using glucosamine as a standard. Corrections for a difference in molar extinction coefficients between glucosamine and muramic acid were unnecessary by these methods (4). In addition, this acid hydrolysis procedure caused $<2\%$ destruction of glucosamine regardless of whether known amounts of glucosamine were hydrolyzed alone or in the presence of experimental samples. Free amino groups, reducing groups, N-terminal amino acids, and C-terminal amino acids were measured by the method of Ghuysen et al. (4). The reduced hexosamine was identified as previously described (4) with the following modifications. Samples were treated with 0.2 M KBH_4 for 16 h at room temperature, followed by dialysis in distilled water, lyophilization, and hydrolysis with 3 N HCl for 4 h at 95 C. The HCl was removed in vacuo

over KOH and the hydrolysates were analyzed by two-dimensional thin-layer chromatography on silica gel with ethanol-water (67:33 [vol/vol]) and ethanol-water-ammonia (67:23:10 [vol/vol/vol]). The R_f values for glucosamine, glucosaminitol, muramic acid and muramitol in ethanol-water were 0.08, 0.64, 0.44, 0.59, and in ethanol-water-ammonia were 0.3, 0.48, 0.16, and 0.17, respectively. Glucosaminitol and muramitol were prepared by reduction of glucosamine and muramic acid for 16 h in 0.2 M KBH_4 at room temperature followed by acidification to pH 1 with HCl and evaporation from methanol at least six times.

Samples for amino acid analysis were hydrolyzed in 6 N HCl for 18 h at 110 C under vacuum. HCl was evaporated under a stream of nitrogen and the amino acid composition was determined by using a Beckman model 120 C automatic amino acid analyzer at 54 C and a 59-cm column with Beckman AA15 resin. A 0.2 M citrate buffer (pH 3.25) was used for starting buffer which was then changed to 0.2 M citrate buffer-0.4 M NaCl (pH 4.12) before methionine was eluted and to 0.2 M citrate buffer-1.0 M NaCl (pH 6.4) before tyrosine was eluted. Under this elution program muramic acid was not separated from glutamic acid, and the diaminopimelic acid (Dpm) was eluted just before methionine. The glutamic acid values were corrected for the small amounts of muramic acid present by the method of Best and Mattingly (2). Because Dpm eluted at the time of the first change in buffer, the Dpm content of each sample was also determined in a separate run using 0.2 M citrate buffer (pH 3.49) and in each case the two determinations agreed to within 10%.

Release of hexosamine-containing material during initiation. Heat-activated spores (60 mg) were suspended in 6.0 ml of 0.1 M glucose-5 mM tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8.0) at 30 C. At intervals, 0.5-ml samples were removed and added to 25 μl of cold 100% (wt/vol) trichloroacetic acid. After incubation for 5 min on ice, the samples were centrifuged at 12,000 × *g* for 5 min and the amount of trichloroacetic acid-soluble hexosamine-containing material was determined. All values are expressed relative to the dry weight of spores at zero time.

In vitro hexosamine-releasing activity. The autolysis of spore peptidoglycan was measured in vitro as the amount of trichloroacetic acid-soluble hexosamine-containing material released from a suspension of broken spores. Lyophilized spores (100 mg) were broken with 300 mg of glass beads in a Wig-L-Bug (Crescent Dental Mfg. Co., Chicago, Ill.) 10 times for 30 s each with 1-min intermittent cooling periods on ice. The broken spores were rapidly suspended in 10 ml of 0.1 M Tris buffer (pH 8.0) and all assays were begun within 1 min from the time of adding buffer to the dry ruptured spores. At intervals, samples were precipitated with trichloroacetic acid and hexosamine in the trichloroacetic acid-soluble fractions was determined as above. The rate of peptidoglycan solubilization was constant with concentration of broken spores up to 14 mg/ml and was optimal at 37 C in 0.1 M Tris buffer (pH 8.0).

Extraction of spores. Spores were extracted with 0.1 N NaOH, 8 M urea-10% mercaptoethanol, or sodium dodecyl sulfate-dithiothreitol (SDS-DTT) as previously described (19).

Materials. Dipicolinic acid (DPA) and *p*-dimethylaminobenzaldehyde were purchased from Aldrich Chemical Co., *p*-hydroxymercuribenzoate and iodoacetic acid (titrated to pH 7.0 with 1 N NaOH) were purchased from Sigma Chemical Co., lysozyme was from Pentex and the glass beads were superbrite type 100-5005 (washed in 1 N NaOH followed by 1 N HCl and distilled water) from 3M Co. All other reagents were obtained from Calbiochem.

RESULTS

Total hexosamine content of spores. Lyophilized *B. megaterium* QM B1551 spores were broken in the Wig-L-Bug and suspended in 3 N HCl. After hydrolysis for 4 h at 95 C, the average amount of hexosamine (± standard deviation) was 148 ± 8 μg/mg of spore (dry weight). This value is almost 10 times higher than that estimated from Nelson and Kornberg's results (13) because their values were not corrected for the hexosamine destroyed by hydrolysis for 18 h in 6 N HCl. Using the same conditions as Nelson and Kornberg we found that the total hexosamine content of spores, measured by the Morgan-Elson reaction, was 19.2 μg of hexosamine per mg of spore, similar to the value of 17.5 to 20 μg of hexosamine per mg of spore that we calculated from their amino acid analysis results. Thus the apparent discrepancy was due to the acid lability of hexosamine, as also reported by others (21, 22).

Release of hexosamine-containing material during initiation. The amount of trichloroacetic acid-soluble hexosamine-containing material released during initiation of germination was measured (Fig. 1). The rate of release was slightly less than the rate of absorbance loss and, after 30 min, the amount released was 32 μg of hexosamine per mg of spore (dry weight) or about 22% of the total spore hexosamine. This trichloroacetic acid-soluble material obtained after 30 min of initiation was analyzed as shown in Table 1. Before HCl hydrolysis there was essentially no free hexosamine (<1 μg/mg of spore), but after HCl hydrolysis there was 32 μg/mg of spore. After dialysis of the trichloroacetic acid-soluble material, there was no detectable free hexosamine, but after acid hydrolysis there was 27 μg of hexosamine per mg of spore, indicating that about 85% of the hexosamine in the original trichloroacetic acid-soluble fraction was nondialyzable. The trichloroacetic acid-soluble material in the germination exudate after dialysis was also assayed for reducing groups (Table 1) and had 3.0 nmol

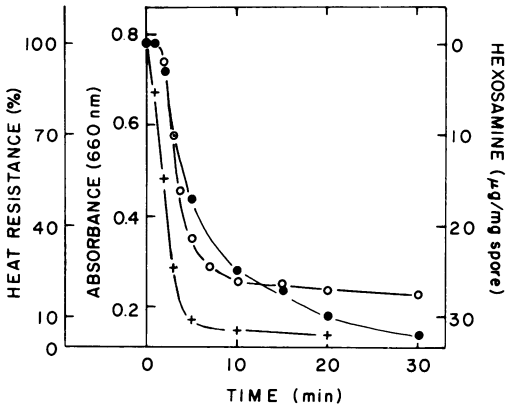

FIG. 1. *Release of hexosamine-containing material during initiation of B. megaterium spore germination. Heat-activated spores (60 mg) were suspended in 6 ml of 0.1 M glucose-5 mM Tris buffer (pH 8.0) at 30 C. At intervals, 0.5-ml samples were precipitated with 5% trichloroacetic acid and centrifuged, and the hexosamine content (●) in the trichloroacetic acid-soluble fractions was measured after acid hydrolysis as described in the Materials and Methods section. Also, at intervals, 0.1-ml samples of the spore suspension were diluted with 4.9 ml of cold 4 mM $HgCl_2$ and the absorbances (O) at 660 nm were measured within 10 s of the time of dilution. Heat resistance loss (+) was measured as described in Materials and Methods and is plotted as percent of the heat-resistant colony-forming units present at zero minutes of initiation.*

per mg of spore before acid hydrolysis and 192 nmol per mg of spore after hydrolysis, indicating an average polymer length of 64 hexosamine residues. These values have not been corrected for any differences in reducing power between free *N*-acetylglucosamine and *N*-acetylglucosamine residues that occur in terminal positions of large peptidoglycan polymers. Assuming a typical spore peptidoglycan structure (22) with one tetrapeptide per five hexosamine residues (see below), this corresponds to an average molecular weight of 20,000, which is higher than that of 15,300 in a previous report (15). To show that the trichloroacetic acid-soluble fraction contained most of the hexosamine material normally exuded during initiation, a germination exudate was prepared by centrifugation of the spores after 30 min of initiation but without the use of trichloroacetic acid. This hexosamine-containing material was 95% soluble in 5% trichloroacetic acid, <15% was lost by dialysis, and it contained 31.5 μg of hexosamine per mg of spore. After incubation with lysozyme (1 mg/ml) for 1 h at 37 C, >90% of the hexosamine was lost by dialysis (data not shown).

The amino acid composition of the germination exudate (Table 2) showed the presence of alanine, glutamate, and diaminopimelic acid in a ratio of 2.10:1.12:1.0 as expected for peptidoglycan. A sample of the dialyzed germination exudate was filtered through a Sephadex G-50 column. The void volume, containing 89% of the hexosamine, was dialyzed, lyophilized, and hydrolyzed in 6 N HCl for 18 h at 110 C and it contained only alanine, glutamate, glycine and Dpm in a ratio of 2.05:1.14:0.57:1.0 (Table 2). Except for glycine this would appear to be a typical peptidoglycan containing an Ala-Glu-Dpm-Ala tetrapeptide with an average of one tetrapeptide per five hexosamine residues, similar to that observed in *B. subtilis* (Table 7, reference 22). Although glycine has not been reported to be a component of spore peptidoglycan (17, 22), glycine was also found in the spore autolysates (see below) similar to that reported in *B. subtilis* spore autolysates (21). In these trichloroacetic acid-soluble fractions we found no evidence for the presence of a phosphomuramic acid polymer reported to be in the coats of these spores (9).

In vitro hexoamine-releasing activity. At-

TABLE 1. *Hexosamine and reducing groups in germination exudate*

Substances	Hydrolysis		Fraction[a]
	Before	After	
Hexosamine (μg/mg of spore)[b]	–	114	Trichloroacetic acid insoluble
	<1	32	Trichloroacetic acid soluble
	<1	27	Trichloroacetic acid soluble, dialyzed
Reducing group (nmol/mg of spore)[c]	3.0	192.0	Trichloroacetic acid soluble, dialyzed

[a] Heat-activated spores (20 mg) were incubated in 2.0 ml of 0.1 M glucose-5 mM Tris (pH 8.0) at 30 C for 30 min after which >90% of the spores were nonrefractile under the phase-contrast microscope. Trichloroacetic acid was added to a final concentration of 5% and after 5 min on ice, the suspension was centrifuged at 12,000 × *g* for 5 min and the supernatant fractions were removed. For the dialyzed sample, 0.5 ml of trichloroacetic acid-soluble fraction was first extracted three times with two volumes of ether to remove trichloroacetic acid and then dialyzed in 2.0 liters of distilled water at 4 C for 18 h.

[b] Hexosamine was determined on samples before and after acid hydrolysis as described in Materials and Methods.

[c] Reducing groups were determined on dialyzed samples before and after acid hydrolysis by the methods of Ghuysen et al. (4).

TABLE 2. *Amino acid analyses of B. megaterium germination exudate and spore peptidoglycan released in vitro*

Compound[a]	Germination exudate[b]		In vitro preparation[c]	
	Before G-50	After G-50	Before G-50	After G-50
Asp	0.50	0	0.50	0
Thr	0.20	0	0.25	0
Ser	0.19	0	0.22	0
Glu	1.12	1.14	1.18	1.20
Pro	0.21	0	0	0
Gly	0.52	0.57	0.37	0.63
Ala	2.10	2.05	2.25	2.18
Val	0.17	0	0.16	0
Dpm	1.00	1.00	1.00	1.00
Ile	0.10	0	0.13	0
Leu	0.09	0	0.16	0
Tyr	trace	0	0	0
Phe	trace	0	0.50	0
Lys	0.21	0	0.27	0
Hexosamine	5.35	4.95	5.80	5.20

[a] Amino acid composition was determined with a Beckman model 120C automatic amino acid analyzer as described in Materials and Methods. Hexosamine was determined by the methods of Ghuysen et al. (4). The values are presented as moles per mole of diaminopimelic acid as described by Warth (21).

[b] A trichloroacetic acid-soluble germination exudate (prepared as Table 1) was extracted three times with 2 volumes of ether and dialyzed in water. Samples were hydrolyzed for both amino acid and hexosamine analysis as described in Materials and Methods. Another sample was filtered through a Sephadex G-50 column in 0.05 M LiCl, the void volume, containing 89% of the hexosamine, was pooled, dialyzed in water, and lyophilized, and portions were hydrolyzed for amino acid or hexosamine analysis.

[c] The trichloroacetic acid-soluble material produced within 30 min in vitro (Fig. 2) was extracted three times with 2 volumes of ether, dialyzed in water, and analyzed as above.

tempts to measure a soluble *B. megaterium* lytic activity by conventional methods (7, 16) were unsuccessful. In addition, spore extracts, prepared by either sonic oscillation or the Wig-L-Bug, did not cause the release of trichloroacetic acid-soluble hexosamine-containing material from either *Micrococcus lysodeikticus* cell walls, prepared by the method of Fan and Beckman (3), or from a boiled suspension of spores broken in the Wig-L-Bug. The absence of a lytic activity in the soluble portion of mechanically disrupted spores was also reported for another strain of *B. megaterium* (16). Our attempts to solubilize a lytic activity from spores broken by either sonic oscillation or the Wig-L-Bug with 0.5% SDS, 0.1% Triton X-100, 3 M NaCl, or sodium deoxycholate were unsuccessful. However, the production of trichloroacetic acid-soluble hexosamine-containing material (peptidoglycan) was measured in vitro with a preparation of broken spores (Fig. 2). The rate of release of hexosamine was constant for 30 min at 37 C with the solubilization of 42 μg which was similar to the in vivo release. At zero time, the amount of trichloroacetic acid-soluble hexosamine-containing material was 11 μg of hexosamine per mg of spore and this value ranged from 9 to 12 μg in all of our experiments except for one in which it was 14 μg. The trichloroacetic acid-soluble material present at zero time was probably the result of mechanical shearing of the polymers during rupture in the Wig-L-Bug rather than enzymatic degradation since dry-ruptured spores suspended directly in 5% trichloroacetic acid had 9 μg of trichloroacetic acid-soluble hexosamine per mg of spore (data not shown). The possibility that this peptidoglycan solubilization represented the re-

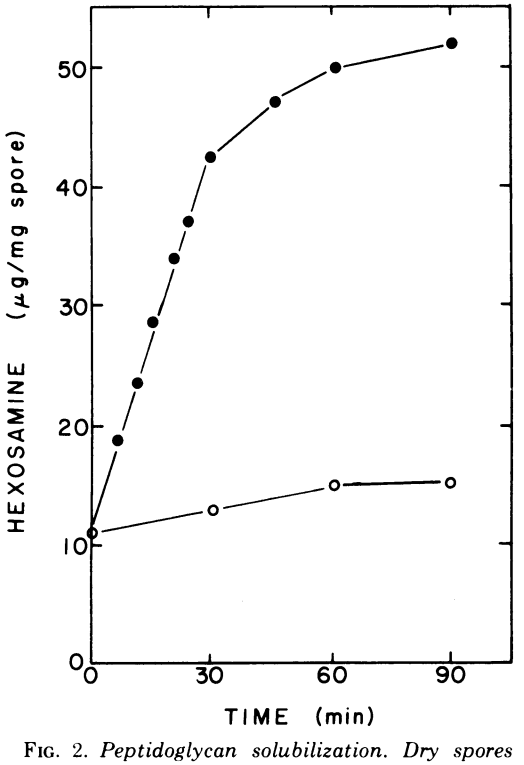

FIG. 2. *Peptidoglycan solubilization. Dry spores (100 mg) were broken in a Wig-L-Bug and suspended in 10 ml of 0.1 M Tris buffer (pH 8.0). The release of trichloroacetic acid-soluble hexosamine-containing material was measured at 37 C as described in Materials and Methods* (●) *and in a control that had been boiled for 20 min* (○).

lease of preformed spore peptidoglycan fragments seemed unlikely from the following observations. First, in a boiled control, only a net of 2 μg of hexosamine per mg of spore was solubilized within 30 min (Fig. 2). Second, if the assay was done in the presence of 5% trichloroacetic acid, only a net of 5 μg of hexosamine per mg of spore was solubilized within 30 min. Third, when the assay was tested on spores that had been either autoclaved in water or boiled for 30 min in propanol before disruption in the Wig-L-Bug, only a net of 1 and 3 μg of hexosamine per mg of spore, respectively, were solubilized within 30 min. These results are consistent with the idea that the peptidoglycan solubilization in the suspension of broken spores was the result of enzymatic action.

A portion of the trichloroacetic acid-soluble material produced by the in vitro assay after 30 min (see Fig. 2) was dialyzed and the amino acid composition was determined both before and after chromatography on Sephadex G-50 (Table 2). Like the germination exudate, this material contained amino acids other than those in typical peptidoglycan, and after gel filtration, only alanine, glutamate, glycine, and Dpm were present in ratios of 2.18:1.2:0.63:1.0. To estimate an average molecular weight, a sample of the same trichloroacetic acid-soluble material used above was dialyzed and the reducing groups before and after 3 N HCl hydrolysis (4 h at 95 C) were 5.4 and 286 nmol/mg of spore, respectively. Thus the average polymer length was 53 hexosamine residues per reducing group, which excluding glycine, corresponds to a molecular weight of about 16,600. These results show that the in vitro assay measured the release of peptidoglycan fragments of the same composition and slightly lower molecular weight than those produced in vivo.

In an in vitro assay for the peptidoglycan solubilization, there was an appearance of both reducing groups and free amino groups (Fig. 3). The trichloroacetic acid-soluble fraction obtained after 30 min of incubation in Fig. 3 was further analyzed by previously described methods (4). Derivatization with fluorodinitrobenzene followed by two-dimensional chromatography showed only alanine as the N-terminal amino acid in the peptidoglycan. After hydrazinolysis and chromatography (4), diaminopimelic acid or glutamic acid was undetectable, suggesting they were not C-terminal amino acids in the peptidoglycan. Thus the appearance of free amino groups probably resulted from an *N*-acetylmuramic acid-L-alanine amidase activity. The nature of the reducing groups was determined after reduction of spore autolytic digest with potassium borohydride. After two-dimensional chromatography, ninhydrin positive material corresponding in R_f to standard glucosamitol but not muramitol was observed, suggesting that the reducing groups formed during autolysis resulted from an *N*-acetylglucosamidase activity.

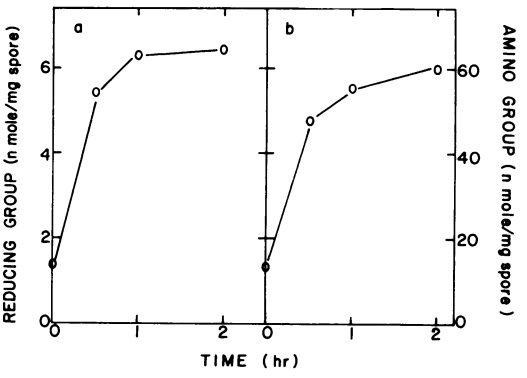

FIG. 3. *Appearance of reducing groups and free amino groups. A suspension of broken spores (10 mg/ml) in 0.1 M Tris buffer (pH 8.0) was incubated at 37 C and samples (0.5 ml) were precipitated with 5% trichloroacetic acid at indicated times. The trichloroacetic acid-soluble fractions were extracted three times with 2 volumes of ether, dialyzed in distilled water at 4 C for 18 h, and assayed for reducing groups (a) and free amino groups (b) as described in Materials and Methods.*

Effect of inhibitors. In the assay described in Fig. 2, the following compounds were tested for inhibition and found to cause <10% inhibition in each case: 10 mM iodoacetate or *N*-ethylmaleimide; 70 μM 2,4-dinitrophenol; 50 mM KCN, NaF, or Na_2HAsO_4; 5 mM phenylmethylsulfonyl fluoride; 10 mM ethylenediaminetetraacetic acid or DPA; 300 mM $CaCl_2$; 100 mM KBr; 10 mM $MgCl_2$ or $Co(NO_3)_2$. Although 0.25% SDS was reported to inhibit the *B. subtilis* lytic enzymes (21), we found no inhibition by SDS (up to 1%) on the *B. megaterium* spore peptidoglycan solubilization. An apparent inhibition by 4 mM $HgCl_2$ or 0.4 mM *p*-hydroxymercuribenzoate was the result of interference with the Morgan-Elson reaction by mercury. When samples were precipitated by 5% trichloroacetic acid containing excess DTT (16 mM) to complex the mercury, there was no inhibition. Alternatively, the $HgCl_2$ or *p*-hydroxymercuribenzoate could be removed by dialysis of the trichloroacetic acid-soluble fractions before acid hydrolysis and the solubilization of peptidoglycan was the same as in Fig.

2. These results showed that $HgCl_2$, an inhibitor of initiation of germination, did not inhibit the spore peptidoglycan solubilization nor did this solubilization require sulfhydryl groups.

Peptidoglycan release during initiation in the presence of $HgCl_2$. Because of the insensitivity of the peptidoglycan solubilization towards $HgCl_2$, the effect of $HgCl_2$ on in vivo release of peptidoglycan was studied. The addition of $HgCl_2$ at any time during initiation immediately stopped the decreases in absorbance (Fig. 4). Essentially no peptidoglycan had been released when $HgCl_2$ was added at 0, 1, or 2 min but peptidoglycan was released during subsequent incubation up to 30 min in $HgCl_2$. The amounts released represented, respectively, 6, 34, and 56% of the amount released in the control. If $HgCl_2$ was added at 3 min during initiation, the amount of peptidoglycan excreted was similar to the control. The amount of peptidoglycan eventually lost was roughly proportional to the heat resistance loss at the time of $HgCl_2$ addition as shown in Fig. 4. At 0, 1, 2, and 3 min the loss in heat resistance was 0, 16, 45, and 75%, respectively. It appears that spores that have lost heat resistance at the time of addition of $HgCl_2$ continued to degrade their peptidoglycan, but the completion of initiation (absorbance loss) was inhibited.

Peptidoglycan solubilization in extracted spores. It has been shown (19) that extraction of spores with NaOH, SDS-DTT or urea removes 4 to 46% of the total spore protein without causing loss in heat resistance. It was also shown that spores, treated with NaOH or SDS-DTT, initiated germination (as judged by absorbance decrease/30 min), but spores treated with urea initiated poorly in 0.1 M glucose-5 mM Tris buffer (pH 8.0). To test whether the initiation properties of these extracted spores might be related to peptidoglycan solubilization, an in vitro assay was done with extracted spores (Table 3). The results show that the initiation of germination was not related to only peptidoglycan solubilization. Since these extraction reagents are relatively strong protein denaturants that disrupt spore coats (1, 6, 19), the activity responsible for peptidoglycan solubilization must be inaccessible to these reagents and may be physically located in the spore cortex or core.

DISCUSSION

During initiation of *B. megaterium* QM B1551 spores, large-molecular-weight peptido-

TABLE 3. *Peptidoglycan solubilization in B. megaterium spores after various extraction procedures*

Extraction procedure[a]	μg of hexosamine/30 min per mg of spore[b]
None	32.0 ± 1.0
NaOH	27.0 ± 1.5
Urea	22.0 ± 1.5
SDS-DTT	21.0 ± 2.0

[a] Spores were extracted, washed, and lyophilized (19).

[b] Peptidoglycan solubilization was measured on suspensions of broken spores as described in Materials and Methods and Table 1. The values have been corrected for the amount of hexosamine at zero time which was 10 μg/mg of spore in each case and are the average (± standard deviation) of three determinations.

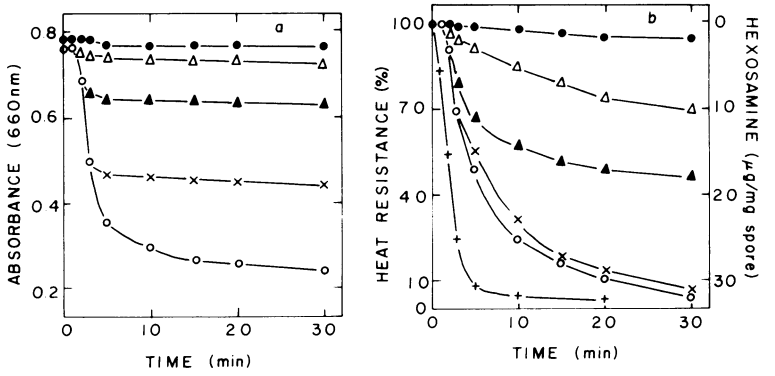

FIG. 4. *Effect of $HgCl_2$ on initiation and peptidoglycan release. Heat-activated spores (60 mg) were diluted into 6 ml of 0.1 M glucose-5mM Tris (pH 8.0) at 30 C and, in the control* (○), *samples were removed at the indicated times for measurement of (a) absorbance at 660 nm and (b) peptidoglycan release as described in the legend of Fig. 1. This same procedure was then repeated with samples to which $HgCl_2$ (4 mM final concentration) was added at 0 min* (●), *1 min* (△), *2 min* (▲) *or 3 min* (×). *Heat-resistance* (+) *was determined on the control sample as described in the legend of Fig. 1.*

glycan fragments were excreted into the germination exudate, as reported for other bacilli (17), at a rate slightly lower than absorbance loss. The composition of these fragments was similar to that of *B. subtilis* spore peptidoglycan (21, 22). The release of peptidoglycan fragments during initiation is presumably the result of lytic enzyme action (6, 17, 18), but our attempts to solubilize the lytic activity in *B. megaterium* spore extracts were unsuccessful as was reported for another strain of *B. megaterium* spores (16). Therefore, an assay based on the production of trichloroacetic acid-soluble peptidoglycan from suspensions of broken spores was designed. In this assay, about the same amount of peptidoglycan was solubilized as in vivo, and the polymers had the same composition as those produced in vivo with a slightly lower molecular weight of 16,600. The peptidoglycan solubilization did not require heavy metals, serine hydroxyl groups, or sulfhydryl groups and it was not inhibited by $HgCl_2$. We found both *N*-acetylglucosaminidase and *N*-acetylmuramic acid-L-alanine amidase activities in *B. megaterium* spores but we do not know if autolysis is the result of one or both or these activities. These same activities and the inability to determine if one or both of them cause autolysis has been reported for *B. subtilis* and *B. cereus* spores (21).

Of particular interest was the lack of inhibition by $HgCl_2$ which has been shown to inhibit initiation of germination (11). If $HgCl_2$ was added at any time during initiation, the decrease in absorbance stopped immediately. When $HgCl_2$ was added at 1 or 2 min there was continued release of peptidoglycan and if added at 3 min the peptidoglycan was released similar to the control. It appeared that the eventual loss of peptidoglycan in the presence of mercury was roughly proportional to the fraction of spores that had lost heat resistance at the time of addition of $HgCl_2$ and this peptidoglycan excretion occurred in the absence of continued absorbance loss. This is in contrast to an apparent sequence of initiation events of losses in heat resistance, absorbance, and then peptidoglycan shown in Fig. 1, but similar to results found for *B. cereus* (5, 8). These data suggest that mercury inhibits some reactions prior to loss in heat resistance but not the subsequent peptidoglycan release, and mercury blocks other reactions involved in the completion of initiation (absorbance loss). Also, complete peptidoglycan excretion did not insure complete initiation. Therefore, autolysis of the spore peptidoglycan did not appear to be the initial event in initiation of germination nor was complete autolysis sufficient to allow complete absorbance loss.

The activity responsible for peptidoglycan solubilization was present in spores extracted with SDS-DTT, 0.1 N NaOH or urea, suggesting that it may physically be located in the core or cortex. It was previously reported (19) that spores extracted with urea initiated germination very poorly compared to either untreated spores or spores extracted with 0.1 M NaOH or SDS-DTT. But autolysis in preparations from these extracted spores, i.e., the amount of peptidoglycan solubilized within 30 min, was not drastically different. Thus there was no correlation with the autolysis measured in extracted spores and the ability of these spores to initiate germination.

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