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CELL WALL–POLYPEPTIDE COMPLEXES IN *Bacillus subtilis**

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ABSTRACT

The cell surface of *Bacillus subtilis* contains several peptidoglycan-associated polypeptides. Cell walls were labeled with ^{125}I or ^{35}S, and the products were digested with lysozyme. When the digests were chromatographed on Sephacryl S-200, peaks of radioactivity corresponding to molecular weights of 240,000, 125,000, 20,000, 17,000, and 15,000 were observed. The walls solubilized by lysozyme were also subjected to sodium dodecyl sulfate–poly(acrylamide) gel electrophoresis, and radioactive bands corresponding to apparent molecular weights of 24,000, 22,000, and 19,000 were found. Isoelectric focusing of the digests revealed the presence of a component having an isoelectric point of 3.7, and, possibly, of minor components having isoelectric points of 4.7 and 6.1. Proteases, including trypsin, subtilisin, and pronase, removed some of the radioactivity from [^{35}S]-labeled walls. Significant proportions of label from [^{35}S]walls were solubilized by the peptide-bond-breaking agents cyanogen bromide and *N*-bromosuccinimide. Small proportions of radioactivity were released from labeled walls by hydroxylamine and trichloroacetic acid. Direct, amino acid analyses of the walls showed the presence of several amino acids not commonly regarded as constituents of peptidoglycan. Cell walls from a protease-deficient mutant, and from a wall preparation enriched in cell poles, contained similar proportions of amino acids. In addition, wall preparations from an autolysin-deficient mutant, and walls from protease hyper-producing strains, contained amino acids that could not be removed by rigorous extraction-procedures. The results suggest that the cell walls of *Bacillus subtilis* contain tightly, or covalently, bound protein molecules or polypeptides that are refractory to removal by denaturants.

*For a preliminary account of this work, see ref. 1.

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INTRODUCTION

Almost all Gram-positive bacteria possess certain common, structural features related to their cell surfaces. These include peptidoglycan and at least one kind of negatively charged heteropolymer, such as a teichoic acid. In contrast, the surfaces of Gram-negative bacteria are usually much more complex. Frequently, peptidoglycan components of Gram-negative bacteria are covalently complexed with protein or lipoprotein molecules (see a review by Braun[2]). In addition, some outer-membrane proteins of *Escherichia coli* bind strongly to peptidoglycan, even though covalent linkages are absent[3,4].

In efforts to explain why DNA–membrane complexes adhere to cell walls of *Bacillus subtilis*, we speculated that the walls contain protein molecules that could provide the specificity of interaction[5]. Previously, Brown *et al.*[6] demonstrated the difficulty in removing protein and nucleic acids from the walls of *B. subtilis*. Later, Doyle *et al.*[7] observed that denaturant-treated cell-walls were amenable to modification with agents that react with protein side-chains. The *B. subtilis* walls also incorporated sulfur, a non-peptidoglycan component[7]. The results suggested that the cell wall of *B. subtilis* contains either covalently, or tightly, bound protein molecules. We have now expanded the foregoing studies, and report on the partial characterization of the peptidoglycan-associated proteins[1].

EXPERIMENTAL

Bacterial growth. — *Bacillus subtilis* strain 168 (*trp*C2) and *B. subtilis* strain SR22 (*trpC2, spoA12,* Prt) were kindly provided by D. C. Birdsell, University of Washington, and have been used in this laboratory for several years[8,9]. *B. subtilis* strain W23 was provided by R. Yasbin. *B. subtilis* FJ6 (*metC3 thr-ts lyt-2*) was provided by J. Fein and H. Rogers[10]. The bacteria were maintained on AK sporulation agar (Baltimore Biological Laboratories). Batch cultures (1.400 L) of bacteria were cultivated with aeration in a gyratory shaker (200 rev/min) at 37° in the minimal salts medium of Spizizen[11], supplemented with L-tryptophan (50 μg/mL) and 0.08% (w/v) casein hydrolyzate. Prior to inoculation, D-glucose, which was autoclaved separately, was added to the medium to a final concentration of 0.5% (w/v). *B. subtilis* YN9 (*trpB3 purB6 metB5* Str^r Spo^+ Prt^h Lyt^-) and *B. subtilis* QB136 (*trpC2 leu8 sacU*^{h}*32* Spo^+ Prt^hLyt^-) were cultured as described previously[12] in minimal medium supplemented with the designated requirements. Growth was monitored in a Klett–Summerson colorimeter, using a number 55 filter.

Cell walls. — Washed cells from late-exponential-phase cultures were ruptured in a French pressure-cell (American Instruments Company) at[8,9] 20,000 lb.in.$^{-2}$. Cell walls were isolated by differential centrifugation (39,000*g* for 17 min), and washed six times in distilled, de-ionized water. The walls were subsequently subjected to rigorous extraction procedures that included successive washing with water, 5M lithium chloride, 3% (w/v) sodium dodecyl sulfate (SDS),

6M guanidine hydrochloride, and 8M urea[7]. The extracted cell-walls were stored in the freeze-dried form. Cell-wall samples from *B. licheniformis, B. stearothermophilus,* or *B. cereus* were obtained from J. B. Ward, N. Welker, and W. C. Brown, and were also subjected to the extraction procedures. Solubilization of the walls was performed by use of egg-white lysozyme (50 μg of enzyme per 5 mg of wall in 50mM phosphate, pH 6.5).

A wall preparation of *B. subtilis* SR22 was allowed to autolyze partially, prior to the extraction procedures, in order to enrich for cell poles. Fan *et al.*[13] observed that, when cell walls of *B. subtilis* were permitted to autolyze on microscope grids, the insoluble residues remaining were enriched in cell poles. We adapted the procedure of Fan *et al.*[13] to effect the production of large amounts of cell poles. *B. subtilis* SR22 cell walls (50 mg) were suspended in 20 mL of 50mM Tris–100mM potassium chloride buffer (pH 8.5), and incubated at room temperature with gentle stirring. When autolysis was 70% complete, as determined by loss of turbidity, the insoluble material was collected by centrifugation, and subjected to the regular extraction procedures. An electron-microscope examination of the preparation revealed that 60–70% of the fragments were identifiable as cell poles (R. J. Doyle and P. H. B. Carstens, unpublished observations).

A modification of the minimal medium of Spizizen[11] was prepared in which two of the salts, magnesium sulfate ($MgSO_4 \cdot 7\,H_2O$) and ammonium sulfate, were replaced by magnesium chloride and ammonium chloride[14]. In these experiments, carrier-free sodium [^{35}S]sulfate was added to the growth medium, and cell walls were obtained as already described. Samples from these batches were used in the electrophoresis and column chromatography experiments to be described.

Iodination of cell walls. — Extracted cell-walls were suspended to a density of 4 mg/mL in 5.0 mL of 0.5M sodium phosphate buffer (pH 7.5). Na[^{125}I] (5 μL, 100 μCi) was added to the suspension, with vigorous stirring. Iodination[15,16] was initiated by the addition of 1.0 mL of chloramine T (2 mg/mL in 0.5M sodium phosphate buffer, pH 7.5). After stirring for 3 min, the reaction was terminated by the addition of 1.0 mL of sodium hydrogensulfite (500 μg/mL). The iodinated walls were washed twice with distilled water, and then dialyzed against 0.1M sodium iodide in 100mM sodium phosphate (pH 8.6) until no radioactivity could be detected in the dialysis medium. Finally, the walls were dialyzed *versus* de-ionized water, and freeze-dried. Radioactivity was determined in a gamma spectrometer (Nuclear Chicago Corp.). When iodinated walls were solubilized with lysozyme, the digests were "desalted" in 4M sodium chloride in a column (1 × 25 cm) containing Bio-Gel P-2. The desalting procedure ensured the removal of all residual, iodide ions.

Poly(acrylamide) gel electrophoresis. — Lysozyme-digested cell-walls were dissolved in 1% sodium dodecyl sulfate–10mM sodium phosphate buffer (pH 7.0) to a concentration of 10 mg of cell wall per mL. The digests were then applied to 10% poly(acrylamide) disc gels (8 × 0.5 cm), and subjected to electrophoresis as described by Weber and Osborn[17]. The gels were frozen for 15 min at −20°, and

then sliced into ~1.1-mm slices by use of a horizontal gel-slicer (Hoefer Scientific Instruments). Each slice was incubated in a scintillation vial with 0.5 mL of Protosol (New England Nuclear) for 12 h at 22°. Following incubation, 10 mL of Aqueous Counting Scintillant ("ACS") Amersham, Arlington Heights, IL, was added to each vial, and the samples were assayed for radioactivity. Incubation was not necessary when [^{125}I]-labeled walls were used in the experiments. The positions of peaks of radioactivity were compared with the electrophoretic mobilities of protein standards, and the apparent molecular weights of the cell-wall proteins were calculated.

Sephacryl chromatography. — The labeled digests were subjected togel-exclusion chromatography on Sephacryl S-200 (Pharmacia, Piscataway, N.J.). The column (2.5 × 80 cm) was eluted with 0.05M Tris · HCl–1.0M sodium chloride–10mM sodium azide buffer (pH 7.5) at a flow rate of 20 mL/h. A calibration curve was obtained by use of ribonuclease, pepsin, aldolase, bacitracin, myoglobin, trypsin, ovalbumin, bovine serum albumin, thyroglobulin, and apoferritin. The void volume was determined by use of Blue Dextran (Pharmacia).

Isoelectric focusing. — Poly(acrylamide) gels were prepared for isoelectric focusing, by a modification of the procedure described by Drysdale *et al.*[18], in acid-cleaned tubes (10 × 0.5 cm i.d.). Lysozyme-digested cell-walls (1 mg) were dissolved in 100 μL of 20% (w/v) sucrose, and layered on top of the gel. Additional 100-μL layers of 15% and 10% sucrose were successively added to the gels. The gel experiments were conducted for 4 h at 1 mA/tube, with an electrophoresis constant-rate source (Canalco, Model 1400). The chambers were cooled to 4° with recirculating ice-water. The anolyte was a 10mM solution of H_3PO_4 (pH 2.2); the catholyte was a 20mM solution of NaOH (pH 12.0). The gels were removed from the tubes, frozen, and sliced into 5-mm portions. Each slice was added to a test tube with 0.8 mL of distilled, de-ionized water, and radioactivity was counted in a gamma spectrometer. After incubation for 2 h, the pH of each slice was determined.

Amino acid analyses. — Cell-wall samples (2.0 mg) were hydrolyzed[19] in 500 μL of 6M HCl in sealed, evacuated ampoules for 24 h at 110°. The samples were dried over KOH pellets in a vacuum desiccator, the dry hydrolyzate was taken up in 200 μL of loading buffer (0.2M sodium citrate, pH 2.2), and insoluble material was removed by centrifugation. Samples were analyzed with a Dionex D-300 amino acid analyzer equipped with a solvent–temperature-change programmer, utilizing single-column methodology with Na citrate eluants and a ninhydrin detection-system. The column (0.4 × 17 cm) was prepacked with Dionex DC-5A cation-exchange resin (6 μm diam.). In routine assays, duplicate runs agreed within ±2%. The limit of detection was ±0.2 nmol.

RESULTS

Amino acid composition of cell walls. — Walls from several strains of *B. sub-*

TABLE I

AMINO ACID COMPOSITION OF CELL WALLS OF *Bacillus strains*[a]

Amino acid	*168*	*168-trypsinized*	*168 subtilisin-treated*	*FJ6*	*QB136*	*SR22*	*SR22 Poles*	*YN9*
Asp	2.5	1.5	1.4	3.5	2.9	1.4	4.8	4.8
Thr	4.6	5.6	5.3	5.8	5.9	2.7	3.4	3.4
Ser	tr[b]	tr	tr	tr	tr	tr	tr	tr
Glu	600	410	520	nd[b]	590	440	290	nd
Gly	5.2	3.4	2.0	4.2	5.1	3 5	8.0	5.1
Ala	840	510	730	nd	780	600	420	nd
Cys	tr	tr	tr	tr	tr	tr	tr	tr
Val	5.1	2.3	4.5	8.0	4.3	4.3	7.1	7.1
Dap	570	390	480	nd	580	420	270	nd
Met	+[b]	+	+	+	+	+	+	+
Ile	0	0	0	0	0	0	0	0
Leu	3.1	1.6	2.2	2.2	3.3	1.8	1.9	1.8
Tyr	0.7	0	0	0.8	0.9	0.4	0.4	0.4
Phe	2 2	2.0	2.5	2.0	3.0	1.2	0.9	0.9
Lys	2 9	2.2	3.0	3.5	4.6	2.6	3.1	3.1
His	1.7	2.3	2.6	2.8	3.3	2 6	1.3	1.3
Arg	0.8	0.5	0	2.0	0.9	0.4	0.8	0.8

[a]Values shown are in nmol/mg. Extracted walls were assayed. Multiple runs were made on each sample, using a different amount of hydrolyzate for each run. [b]Trace that could not be quantitated, tr; nd, not determined; +, present, but could not be quantitated, because of the relatively high amount of Dap. The results are tabulated in the order of elution from the column.

tilis were prepared according to conventional techniques and extracted with a series of detergents, salts, and protein denaturants, as described earlier[7]. The walls, free from extractable protein, nucleic acid, or lipid, were hydrolyzed, and the hydrolyzates analyzed for amino acids. Wall samples were taken from the wild-type strain 168, from a protease-deficient strain (SR22), from a sample of SR22 walls highly enriched in poles, from an autolysin-deficient strain (FJ6), and from two protease hyper-producing strains (QB136 and YN9). The results are summarized in Table I. Several prominent features were revealed by the data. Present in all samples are amino acids that cannot be accounted for on the basis of peptidoglycan components. There is a paucity of serine and cystine and a lack of isoleucine. The molar ratios of Glu:Dap are almost constant, and close to unity for all samples. Similarly, the ratios of Glu:Dap are also reasonably close for all samples. The total amount of non-peptidoglycan amino acids[20] varies somewhat from wall to wall, ranging from 0.24% in strain SR22 to 0.39% in strains FJ6 and QB136. The wild-type strain 168 contained 0.33% of non-peptidoglycan amino acids, whereas treatment with trypsin and subtilisin lowered this proportion to 0.24 and 0.27%, respectively. There appears to be no correlation between the total proportion of wall-associated amino acids and the history of the strain. It is difficult to decide whether cell poles contained a unique profile of amino acids. In the poles, the proportions of Glu,

Ala, and Dap are much lower than those found in bulk walls of strain SR22. It is possible that the proportion of teichoic acid is much higher in the enriched poles. Further studies on more-highly purified poles will be necessary before conclusions can be drawn. In terms of non-peptidoglycan amino acids, the enriched-pole sample contained higher relative proportions of Asp, Thr, Gly, Val, Lys, and Arg. The composite view of the results of amino acid analyses is that the walls indeed contain small proportions of non-peptidoglycan amino acids, and that these constitute irreducible quantities under the conditions of extraction. In addition, it should be emphasized that the absolute proportions of amino acids present in the wall are small (see Table I). In order to obtain reproducible results, it was necessary to add much more wall hydrolyzate (>100 μg) to the column than if the samples were pure amino acid. For this reason, the presence of protein in cell walls may easily be overlooked when determining the composition of the wall.

Gel electrophoresis of labeled cell-wall digests. — Extracted cell walls, which had been obtained from *B. subtilis* 168 growing in the presence of ^{35}S, or that had been labeled with ^{125}I, were solubilized with lysozyme. The digests were subjected to SDS-gel electrophoresis in order to resolve any peaks of radioactivity. From protein standards, run in parallel, it was possible to obtain apparent molecular weights. The results for a ^{35}S-containing digest are shown in Fig. 1. Three peaks of radioactivity, respectively corresponding to molecular weights of 24,000, 22,000, and 19,000, were observed. Similarly, when the ^{125}I-labeled digest was electrophoresed, profiles of radioactivity were obtained that were identical to those from the ^{35}S-containing digest. The assumption is made that the peaks of radio-

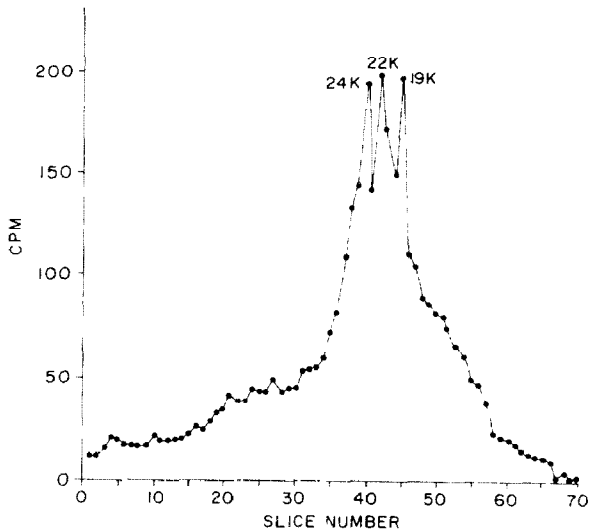

Fig. 1. Poly(acrylamide) gel electrophoresis of lysozyme-digested, ^{35}S-incorporated, cell walls [Lysozyme-digested, ^{35}S-incorporated cell walls were subjected to electrophoresis in 1% SDS–10% poly(acrylamide) gel. The gels were cut into 1.1-mm slices, and solubilized, and the radioactivity was determined by liquid scintillation counting.]

activity represent polypeptides or polypeptide–peptidoglycan-fragment complexes.

Gel-exclusion chromatography of labeled-wall digests. — A lysozyme digest of ^{125}I-labeled walls was passed through a calibrated column of Sephacryl S-200. The results, depicted in Fig. 2, showed that several peaks of radioactivity were present. Peaks corresponding to molecular weights of 240,000, 125,000, 20,000, 17,000, and 15,000 were observed. When a digest of ^{35}S-labeled wall was subjected to chromatography in the same column, similar results were obtained.

Fractions 31–40 (see Fig. 2) were pooled, dialyzed, freeze-dried, and rechromatographed. The component having a molecular weight of 240,000 appeared as only a trace, whereas the peak for mol. wt. 125,000 was dominant. These results suggest that the species of mol. wt. 240,000 may be a dimer of the component of mol. wt. 125,000. Rechromatography of the remaining fractions (see Fig. 2) did not result in any alteration of the elution characteristics.

A freeze-dried sample from peaks 31–40 was also subjected to SDS-gel electrophoresis. Peaks of mol. wt. 25,000, 19,000, and 18,000 were found, values similar to those determined by direct electrophoresis of whole digests. In addition, when the other major fractions from the Sephacryl column were electrophoresed, radioactivity–R_F profiles were always similar to those found for the unfractionated digest. Thus, it is possible that at least three polypeptides are associated with the cell wall. The polypeptides may associate to form complexes of high molecular weight (240,000 and 125,000). In the presence of SDS, the polypeptides are dissociated into individual components. The presence of 2-mercaptoethanol in the gel did not result in changes of positions of radioactivity. In both the gel electrophoresis and Sephacryl experiments, reproducible results were obtained only

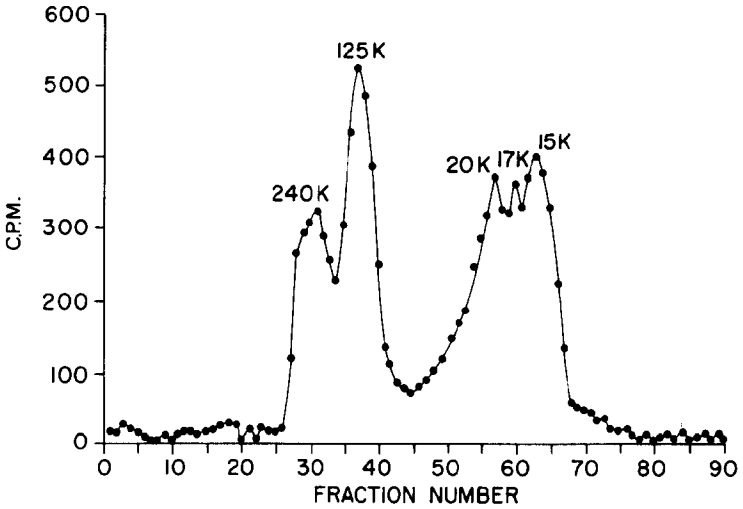

Fig. 2. Chromatography on Sephacryl S-200 of lysozyme-digested, ^{125}I-modified, cell walls. [Lysozyme-digested, ^{125}I-modified, cell walls (4 mg) were dissolved in 2 mL of 0.05M Tris–M NaCl, pH 7.5, and applied to a column of Sephacryl S-200. Fractions (5 mL) were collected, and 1-mL samples from each fraction were counted in a gamma spectrometer.]

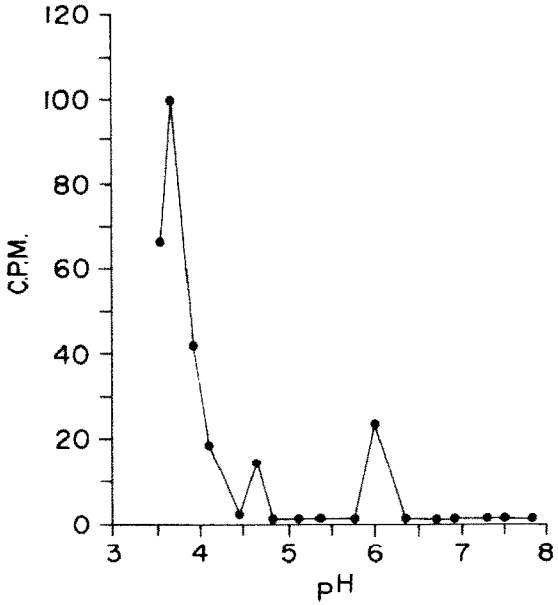

Fig. 3 Isoelectric focusing in poly(acrylamide) gels. [Lysozyme-digested, ^{125}I-labeled, cell walls (1 mg in 100 μL) were applied to gels, and run for 4 h, at 1 mA/tube, at 4°. The gels were frozen, and sliced into 5-mm slices, and the radioactivity was determined in a gamma spectrometer. The pH of each slice was subsequently determined.]

when standardized conditions of lysozyme digestion of the wall were used. The results reported herein reflect values obtained for "limit digests" of the walls.

Isoelectric focusing of cell-wall digests. — A convenient method for the separation of macromolecules having different charges is isoelectric focusing in gels. Accordingly, samples of ^{125}I-labeled, cell-wall digests were subjected to isoelectric focusing in poly(acrylamide) gels. Following resolution of a control, hemoglobin standard, the gels were sliced, and determinations of pH and radioactivity were made. A typical profile is shown in Fig. 3. Well resolved peaks of radioactivity, respectively appearing at pH values of 3.7, 4.7, and 6.1, were observed. Although constituting only a minor proportion of the total radioactivity, the last two peaks were consistently observed. The relationships between the fractions resolved by isoelectric focusing and by electrophoresis, or by gel chromatography, are unknown at present. The relatively low isoelectric point of one of the polypeptides (observed pH, 3.7; see Fig. 3) may reflect its association with the phosphorus-containing, teichoic acids of the cell wall. As a control, intact, but ^{125}I-labeled, walls were also subjected to the focusing procedures. No radioactivity entered the gels, suggesting that the wall must first be solubilized in order that the radiolabeled components may be resolved. Had the walls contained trapped and non-covalently bound iodide, it would have been expected that the radioactivity would have entered the gels.

Iodination of cell walls from various species of Bacillus. — Cell-wall samples

TABLE II

RADIO-IODINATION OF CELL WALLS FROM *Bacillus* SPP

Cell wall	*c.p.m./mg*
B. subtilis 168 (late exponential)	130,420
B. subtilis 168 (late exponential), control[a]	<200
B. subtilis SR22	268,290
B. subtilis W23	17,910
B. licheniformis	65,130
B. cereus	22,730
B. stearothermophilus	31,990

[a]Control walls represent the addition of sodium hydrogensulfite prior to the addition of chloramine T.

TABLE III

REMOVAL OF RADIOACTIVITY FROM ^{35}S-LABELED CELL-WALLS OF *Bacillus subtilis* 168[a]

Treatment[b]	*Soluble radioactivity (c.p.m.)*	
	4.5 h	*21 h*
Phosphate buffer (100mM, pH7.0)	64	70
Lithium chloride (7.0M in methanol)	57	51
(Ethylenedinitrilo)tetraacetic acid (50mM, pH 7.0)	85	86
Hydrochloric acid (300mM)	100	150
Cyanogen bromide (150mM in 300mM HCl)	3,275	5,150
N-Bromosuccinimide (100mM in 500mM acetate, pH 4.0)	8,126	14,200
Sodium acetate (500mM, pH 4.0)	21	48
Hydroxylamine (1.0M, pH 8.0)	525	700
Trichloroacetic acid (5%, w/v)	610	960
Lithium diiodosalicylate (200mM)	94	91
Sodium dodecyl sulfate (3%, w/v)	14	11
Lysozyme (50 μg/mL in 75mM phosphate, pH 6.5)	42,795	44,530

[a]Cell walls were extracted with protein solvents and detergents according to a previously described scheme[7]. [b]Cell walls (2.0 mg, 50,275 c.p.m.) were suspended in a total volume of 2.0 mL containing the extracting or cleaving agents shown. The walls were intermittently shaken, and incubated at 37°. When the walls were treated with hydrochloric acid, acetate buffer (pH 4.0), cyanogen bromide, or *N*-bromosuccinimide, the temperature of incubation was 22°. Determinations of radioactivity were made on the soluble portions of the mixtures.

from several species of *Bacillus* were subjected to the series of extractions and washes, as described earlier[7]. The walls were suspended in buffer, and mixed with sodium [^{125}I]iodide. Following the addition of chloramine T, the suspensions were stirred, sodium hydrogensulfite was added, and the walls were recovered by centrifugation. Attempts were made to maintain equal amounts of walls, and equal concentrations of labeling reagents. The walls were washed, dialyzed, and freeze-dried. Determinations of the radioactivity showed that labeling occurred on all of

the wall samples (see Table II). *B. subtilis* SR22, an extracellular, protease-deficient mutant[21], appeared to possess the greatest number of sites reactive with iodine.

Removal of radioactivity from cell walls labeled with ^{35}S. — A preparation of walls obtained from exponential-phase cells growing in the presence of [^{35}S]sulfate were subjected to additional extraction-procedures. Several extractants, including phosphate buffer, concentrated lithium chloride in methanol, (ethylenedinitrilo)tetraacetic acid, dilute hydrochloric acid, sodium acetate (pH 4), lithium diiodosalicylate, and SDS, did not remove significant amounts of radioactivity, even after 21-h incubations (see Table III). Trichloroacetic acid and hydroxylamine (a nucleophile) removed small proportions of the label. Reagents that break peptide bonds, such as cyanogen bromide[22] and N-bromosuccinimide[23], showed limited effectiveness in solubilizing the ^{35}S from the walls. In contrast, lysozyme, which solubilized the walls, also solubilized most of the material bearing the radioactivity. It appears that only those agents that are capable of breaking covalent bonds can be used to dissociate the label from the cell walls.

When the wall preparations were subjected to proteolytic enzymes, a rapid release of label occurred (see Fig. 4), suggesting that some peptide bonds were exposed, and were susceptible to the proteases. When trypsin was used ~19% of the radioactivity had been solubilized at the end of 21 h. Pronase and subtilisin respectively released ~37 and 51% during the same period of time.

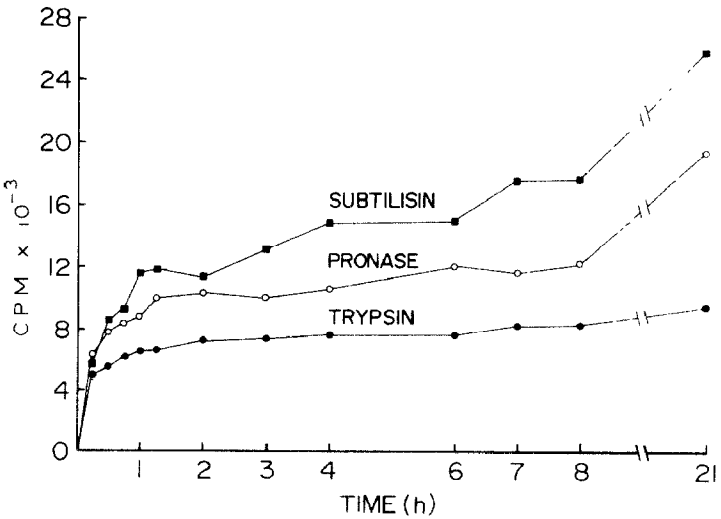

Fig. 4. Release by proteases of radioactivity from ^{35}S-labeled cell walls of *B. subtilis* 168. [Cell walls (2 mg/mL) in phosphate buffer (50mM sodium phosphate, pH 7.2) were incubated at room temperature in the presence of 50 μg of trypsin, pronase, or subtilisin/mL. Samples were removed at intervals, centrifuged, and the clear, supernatant liquors assayed for radioactivity. The radioactivity in the walls gave 25,137 c.p.m./mg.]

DISCUSSION

When cell walls of *B. subtilis* were subjected to a series of extractions with classical, protein solvents and denaturants, the walls remained amenable to radiolabeling with ^{125}I. On solubilization, the labeled walls yielded peaks of radioactivity that respectively corresponded to molecular weights of 240,000, 125,000, 20,000, 17,000, and 14,000, based on molecular sieving. Conversely, when cells were grown in the presence of ^{35}S, the walls contained label, and gave molecular-weight profiles similar to those found for extracted, iodinated walls. In addition, direct amino acid analyses of wall hydrolyzates showed that several non-peptidoglycan amino acids were non-extractable with the salts and denaturants (see Table I). Previously, we observed[7] that extracted cell-walls of *B. subtilis* were amenable to reagents modifying protein side-chains. On the basis of the results, we conclude that the cell walls of *B. subtilis* contain discrete, protein molecules. The origin of wall-associated polypeptide was not revealed in this study. Several possibilities exist that might explain the presence of polypeptide in the walls. (*i*) The protein may simply be insoluble in the denaturants or detergents, or the extraction procedures may have rendered the proteins insoluble by exposing hydrophobic side-chains. This possibility is viewed as unlikely, because lysozyme solubilized the proteins in a phosphate buffer. (*ii*) The wall proteins may be a result of interdigitation of membrane proteins in the peptidoglycan matrix. Other studies have suggested a strong, wall–membrane association[24,25]. This possibility is also considered to be remote, because *B. subtilis* secretes several proteins, such as the complex *N*-acetylmuramyl-L-alanine amidase[26], that presumably traverse the wall. Furthermore, the exclusion volume of cell walls of *B. subtilis* for proteins has been reported[27] to be of such a size as to accommodate a protein of molecular weight 70,000. (*iii*) The overall interpretation of the results presented herein suggests, but does not prove, that the walls contain covalently bound protein. Direct proof for a covalently linked, protein–peptidoglycan complex would require its isolation, and structural characterization of the linkage units.

Thus far, we have been unable to purify quantities of the polypeptides large enough to permit analysis of their linkages to peptidoglycan. In every case, following chromatography on Sephacryl S-200 (or on ion-exchange celluloses), it was observed that the peaks of radioactivity contained both phosphorus and amino sugar, indicating contamination with teichoic acid and glycan. The finding that a major peak of radioactivity from labeled walls possessed an isoelectric point of 3.7 (see Fig. 3) suggests an influence of a teichoic acid on the resolution of the band of radioactivity. The lack of significant quantities of protein which remain wall-associated further creates difficulties in attempts to purify the polypeptides. It was necessary to add considerable amounts of wall hydrolyzate to the analyzer in order to resolve the amino acids adequately (see Table I).

The presence of protein in the cell walls of Gram-positive bacteria may be much more widespread than has been generally recognized. It is presumed that

protein A is bound to an amino group on the cell wall of *Staphylococcus aureus*; however, a precise definition of the linkage has not yet been published[28,29]. Non-peptidoglycan amino acids have been found in the cell walls of *Streptococcus sanguis*[30,31], *S. salivarius* and *S. mutans*[32,33], *S. pneumoniae*[34], and *Mycobacterium tuberculosis*[35]. In addition, following lysozyme digestion, the cell walls of *B. licheniformis* were shown to possess an insoluble, polypeptide fraction that comprised almost 7% of the weight of the wall[36]. In Gram-negative bacteria, covalently bound, protein–peptidoglycan complexes are common[2]. Cell wall-associated, non-peptidoglycan amino acids have also been found in *Lactobacillus fermenti*[37]. When walls of *L. fermenti* are treated with lysozyme, a large fraction remains resistant to the enzyme. If the walls are first treated with trypsin, the walls become more susceptible to lysozyme[37]. Earlier, Braun[2] showed that lipoprotein imparted a partial resistance to the lysozyme in *E. coli* peptidoglycan.

Because cell poles of *B. subtilis* appear to resist turnover[38], we reasoned that proteins may be enriched in the polar areas. The results suggested (see Table I) that poles contain only a slightly higher proportion of non-peptidoglycan amino acids than bulk walls (see Table I). The amino acid composition of cell poles may be unrelated to their ability to resist autolysis.

The function(s) of the wall-bound proteins remains obscure. We presume that wall proteins can bind membrane, which, in turn, binds DNA. When cell wall extends, or grows out, from sites of septation, it offers a means for DNA–membrane complexes to accompany a rigid structure[5,24]. Thus, the wall may enhance the segregation of nascent chromosomes during cell division[5,24]. Cell-wall protein could also be important in the morphology of bacilli in the construction of bacteriophage binding-sites[39,40], and in the specificity of the binding of metals[41–43], or autolytic enzymes[26], or both. Furthermore, the presence of wall protein may modify cell-wall turnover in certain bacteria[12]. Our present studies are directed to the premise that cell-wall protein functions to interact with DNA–membrane complexes.

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