Deficiency of Autolytic Activity in *Bacillus subtilis* and *Streptococcus pneumoniae* is Associated with a Decreased Permeability of the Wall

By RUSSELL WILLIAMSON† AND J. BARRIE WARD*

Division of Microbiology, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

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Autolytic-deficient mutants of *Bacillus subtilis* which grow as chains of non-separated bacilli have been isolated by a procedure involving filtration of mutagenized cultures through glass-sinter filters. The mutants obtained were some 80–90% deficient in both autolysins, *N*-acetylmuramoyl-**L**-alanine amidase and endo-**β**-*N*-acetylglucosaminidase. Treatment of one of these mutants with 5 M-LiCl extracted only about 20% of the protein obtained from an equivalent amount of the autolysin-containing bacilli. Similarly, a reduction of 40–60% in LiCl-extractable protein was obtained with autolytic-deficient *Streptococcus pneumoniae* whether these organisms lacked the autolytic enzyme or were phenotypically deficient by growth in ethanolamine-containing medium. Chromatography on Sephadex G-100 of the protein extracted from *B. subtilis* and *S. pneumoniae* revealed that the major difference between autolytic-deficient and parent organisms was a decrease in proteins of high molecular weight. Smaller differences were observed in a second fraction which contained low molecular weight material and proteins such as the autolysins, whose elution from the column was retarded by interaction with the Sephadex. Further examination of the fractionated protein from *B. subtilis* by sodium dodecyl sulphate-polyacrylamide gel electrophoresis confirmed that the major difference between the extracts was in the amount of protein present and did not result from marked changes in the size of the extracted proteins. These observations suggest that autolysin deficiency in *B. subtilis* and *S. pneumoniae* results in a change in the porosity (permeability) of the bacterial wall.

INTRODUCTION

Autolytic enzymes (autolysins) are now known to occur widely in bacteria where they are responsible for the hydrolysis of peptidoglycan. As a group of enzymes they have a range of specificities which result in the cleavage of bonds in both the glycan and peptide side-chains of the polymer (for review, see Rogers, 1979). Two autolysins have been isolated and purified from *Bacillus subtilis* – an endo-**β**-*N*-acetylglucosaminidase and an *N*-acetylmuramoyl-**L**-alanine amidase (Herbold & Glaser, 1975; Taylor et al., 1980) – whereas in *Streptococcus pneumoniae* only an amidase has been described (Höltje & Tomasz, 1976).

Much of the earlier work on autolysins was concentrated on the degradative aspects of their action under conditions of unbalanced growth, when the uncontrolled action of these enzymes leads to degradation of peptidoglycan with concomitant lysis of the organism. Thus, under balanced growth the potential lethal effects of autolysins must be closely regulated. This aspect of their activity has received considerable attention in recent years. In addition, biochemical and genetic studies have provided evidence of a role for autolysins in the bactericidal effects of antibiotics known to inhibit peptidoglycan synthesis, in the turnover of

† Present address: The Rockefeller University, York Avenue, New York, N.Y., U.S.A.
peptidoglycan during growth and in the separation of daughter cells after cell division has been completed. Other studies have suggested that autolytic activity is required for both sporulation and transformation. These aspects of the function of autolysins have been reviewed by Rogers (1979). More recently, Fein (1979) has provided evidence for the involvement of autolytic enzymes in flagellar morphogenesis. Autolysin-deficient mutants of *B. licheniformis* and *B. subtilis* were non-flagellate and non-motile. If, however, the mutants were grown under conditions where autolytic activity was regained, then the organisms also became flagellate and motile. These observations led Fein to postulate that localized hydrolysis of peptidoglycan is required for the assembly and insertion of the basal structures of the flagella into the cell wall.

The action of autolysin necessary to bring about these various effects may be localized as described above for flagellar morphogenesis or may occur predominantly at the wall surface as in the case of turnover. This paper reports an additional consequence of autolysin deficiency in *B. subtilis* and *S. pneumoniae* which suggests that a deficiency of these enzymes in some way affects the porosity of the wall. Such a change would be of importance in defining the molecular sieving properties of the wall (Gerhardt & Judge, 1964; Scherrer & Gerhardt, 1971) and would presumably also influence the release of extracellular proteins. Moreover, in contrast to the aspects of autolysin deficiency described above, these changes in porosity would probably affect the bulk of the peptidoglycan rather than specific or localized areas.

**METHODS**

**Organisms.** *Bacillus subtilis* 168 trpC2 (Spizizen, 1958) and MB21 metC3 leu-8 (Hranueli et al., 1974) were obtained from Dr P. J. Piggot of this department. *Bacillus subtilis* strain FJ3 metC3 Lyt1 (Fein & Rogers, 1976), an autolysin-deficient mutant derived by transformation of strain MB21, was obtained from Dr J. E. Fein. *Streptococcus pneumoniae* R6, a derivative of the Rockefeller University strain R36A, and strain CW1, an autolysin-deficient transformant of the wild-type (Waks & Tomasz, 1978), were also used.

**Media.** Cultures of *B. subtilis* were grown in medium (TYE) containing Difco Tryptone (1%, w/v), Difco yeast extract (1%, w/v), K2HPO4 (0-5%, w/v) and glucose (0-4%, w/v). The cultures, shaken for aeration, were grown in Erlenmeyer flasks containing up to one-fifth of their volume of medium and incubated at 35 °C, unless stated otherwise. The pneumococci were grown without aeration at 37 °C in a chemically defined medium (Höltje & Tomasz, 1976) at an initial pH of 8-0, with either choline or ethanolamine. Difco yeast extract (0-1%, w/v) was added as required to enable growth of the mutant.

**Mutagenesis.** A 1 ml portion of *B. subtilis* 168 culture grown to exponential phase was incubated with ethyl methanesulphonate (EMS; 1-5%, w/v) for 2 h with gentle shaking and then the bacteria were recovered on a membrane filter (0-45 μm porosity, 2-5 cm diam.). The organisms were washed twice with 60 mM-sodium phosphate buffer, pH 7-0 and inoculated into fresh medium.

**Transformation procedure.** The recipient strain, *B. subtilis* MB21, was grown to competence (Bott & Wilson, 1968) and transformed by saturating concentrations of DNA (about 5 μg ml-1) isolated by the method of Marmur (1961).

**Selection procedure for the isolation of autolytic-deficient mutants of *B. subtilis.*** Cultures (5 x 5 ml) of *B. subtilis* 168, previously treated with EMS and grown at 45 °C to mid-exponential phase (approximately 5 x 107 colony-forming units ml-1) in TYE medium containing heat-killed *B. subtilis* (1 mg ml-1), were filtered under vacuum through glass sinters (porosity 3, 20–30 μm; 1 cm diam.). Bacteria remaining on the sinter were washed twice with prewarmed medium (10 ml), resuspended in fresh medium (5 ml), and incubated at 45 °C.

**Preparation of labelled walls and amidase indicator plates.** Sodium dodecyl sulphate (SDS)-inactivated walls of *B. subtilis* and *Micrococcus luteus* were prepared by the procedure of Fein & Rogers (1976). [3H]Choline-labelled walls of *S. pneumoniae* were prepared as described by Hakenbeck et al. (1978), and these were also inactivated with SDS. Procion-conjugated walls of *B. subtilis* were prepared and incorporated at 1 mg ml-1 into double-layer agar plates (Forsberg & Rogers, 1971). The medium (TYE) used for these plates was buffered at pH 9-5 with 25 mM-diethanolamine/HCl. The amidase indicator plates were incubated aerobically at 45 °C for 24 h, then anaerobically at the same temperature.

**Preparation of samples for assay of autolytic activity.** Cultures grown to late-exponential phase (*A*46b 1.6–1.8) were harvested, after rapid cooling with ice to minimize autolytic activity, by centrifugation (12 000 g for 5 min at 4 °C). All subsequent procedures were carried out in the cold, unless stated otherwise. Autolysin and protein
extracts were obtained by the method of Brown (1973). Freeze-dried bacteria (100 mg) were resuspended in 50 mM-Tris/HCl, pH 7.0 (2 ml) containing 0·2 mM-phenylmethylsulphonyl fluoride (PMSF) and 5 M LiCl for 1 h on ice.

Extracts or fractions were assayed for autolytic activity against SDS-inactivated walls by the binding or dilution techniques of Fan & Beckman (1972). The *B. subtilis* extracts (25–500 μl) were diluted 20-fold with 50 mM-succinate/NaOH, pH 5.5 containing 0·2 mM-PMSF, and incubated with *M. luteus* or *B. subtilis* walls (1 mg) on ice for 15 min to allow binding of the autolytic enzymes. The walls were recovered by centrifugation (39 000 g for 2 min at 4 °C), resuspended in the appropriate prewarmed buffer (4 ml succinate/PMSF buffer, pH 5.5 for *M. luteus* and 2 ml ammonium carbonate/PMSF buffer, pH 9.5 for *B. subtilis*) and the turbidity decrease was measured at 380 nm. One unit of activity was defined as the loss of 0·1% of the initial turbidity min⁻¹ during the period of maximum decrease. The activity against *M. luteus* walls was specifically due to the endo-β-N-acetylgalactosaminidase (Fan & Beckman, 1973b) and the lysis of *B. subtilis* walls at pH 9.5 was primarily due to the N-acetylmuramoyl-L-alanine amidase (Fan & Beckman, 1972). The dilution technique was only used when assaying fractions obtained from the Sephadex G-100 separations of LiCl extracts. Samples (100 μl) of the *B. subtilis* fractions were added to *M. luteus* or *B. subtilis* walls (1 mg) in succinate/PMSF (4 ml) or ammonium carbonate/PMSF (2 ml) buffers, respectively, and incubated at 35 °C for 20 h. Samples (100 μl) of the *S. pneumoniae* fractions were assayed using ³H-labelled walls as described by Høltje & Tomasz (1976). The fractions from the wild-type strain and ethanolamine-grown R6 were incubated for 30 min, whereas those from the autolytic-deficient mutant CW1 were incubated for 16 h.

Fractionation of LiCl extracts. The 5 M LiCl extracts (2 ml) were fractionated on a column (1.55 × 65 cm) of Sephadex G-100, eluted with 5 mM-succinate/NaOH, pH 5.5 containing 0·02 mM-PMSF. Fractions of approximately 1·2 ml were collected and the elution volumes were expressed according to the formula: \( V_p = (V_e - V_o)/(V_t - V_o) \), the values of \( V_e \) (void volume) and \( V_t \) (total volume available to small molecules) having been determined by the elution of Blue Dextran 2000 and [¹⁴C]-glycine respectively. The proteins in pooled fractions from the Sephadex G-100 columns were separated by SDS-polyacrylamide gel electrophoresis using concentrations of acrylamide and N,N′-methylenebisacrylamide of 5·4 % and 0·22 % and 13 % and 0·9 % in the stacking and separating gels, respectively. Electrophoresis was at a constant current of 8 mA for 16 h. The gels were stained with Coomassie brilliant blue (Fairbanks et al., 1971), and destained in methanol/acetic acid/water (3:1:6, by vol.).

Assays of other enzymes and estimation of DNA and protein concentrations. α-D-Glucosidase (EC 3.2.1.20) was measured as described by Pollock (1961), except that the release of p-nitrophenol was determined at 400 nm (Halvorson & Ellias, 1958). Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was assayed as described by Frasal & Freese (1974). The maximum amounts of these enzymes were determined using lysozyme lysates obtained from lyophilized exponential-phase organisms. The bacteria (100 mg) were suspended in 50 mM-sodium phosphate buffer, pH 7·0 (2 ml), and incubated at 35 °C for 30 min with lysozyme (10 μg) and deoxyribonuclease (2 μg). Insoluble material was removed by centrifugation (39 000 g for 10 min at 4 °C) and enzyme activity present in the supernatant was assayed. One unit of enzyme activity was defined as the amount of enzyme catalysing the transformation of 1 μmol substrate min⁻¹.

The amounts of DNA were measured by the method of Giles & Myers (1965), using calf thymus DNA as the standard. Protein was assayed by the Lowry method using bovine serum albumin as the standard.

**Materials**. Bovine serum albumin, bovine cytochrome c, deoxyribonuclease (EC 3.1.21.1), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), ethyl methanesulphonate, and phenylmethylsulphonyl fluoride were purchased from Sigma. Lysozyme (EC 3.2.1.17) was obtained from Armour Pharmaceutical Co., Eastbourne, U.K. p-Nitrophenyl-α-D-glucoside was supplied by Koch-Light. [methyl-³H]Choline chloride (82 Ci mmol⁻¹; 3·03 TBq mmol⁻¹) was purchased from New England Nuclear.

### Results

**Isolation of autolytic-deficient mutants**

Cultures of *B. subtilis* 168 that had been treated with EMS (about 9 % survival) were filtered through glass sinters to enrich for chain-forming organisms, as described in Methods. Under the conditions used, about 5 % of the autolytic-deficient mutant FJ3 was retained on the sinter, whereas all of the wild-type strain passed through. The enrichment technique was repeated six times, and the proportion of chain-forming organisms increased throughout the procedure, as judged by phase-contrast microscopy of exponential-phase bacteria. Possible autolytic-deficient mutants were obtained by screening the bacteria from the last filtration step on amidase indicator plates at 45 °C. Under these conditions wild-type control colonies produced large, clear zones around them, whereas autolytic-deficient mutants produced faint
Table 1. Autolytic activities in LiCl extracts obtained from B. subtilis Lyt+ strains and some autolytic-deficient mutants

<table>
<thead>
<tr>
<th>Strain</th>
<th>Phenotype</th>
<th>Protein concn (mg ml⁻¹)</th>
<th>Amidase activity* (units ml⁻¹)</th>
<th>Glucosaminidase activity† (units ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>168</td>
<td>Wild-type, Lyt+</td>
<td>7.71</td>
<td>102.1</td>
<td>80.7</td>
</tr>
<tr>
<td>RW11</td>
<td>Lyt+</td>
<td>7.25</td>
<td>97.3</td>
<td>77.3</td>
</tr>
<tr>
<td>RW2</td>
<td>Lyt+</td>
<td>1.94</td>
<td>9.3</td>
<td>8.7</td>
</tr>
<tr>
<td>RW3</td>
<td>Lyt-</td>
<td>2.24</td>
<td>23.3</td>
<td>14.0</td>
</tr>
<tr>
<td>RW10</td>
<td>Lyt-</td>
<td>1.79</td>
<td>19.3</td>
<td>14.0</td>
</tr>
</tbody>
</table>

* Assayed against B. subtilis walls at pH 9.5. † Assayed against M. luteus walls at pH 5.5.

or no zones. About 11% of the colonies obtained from organisms after six cycles of enrichment appeared to be deficient in lytic activity and grew in chains. Twenty of the 147 isolates which produced the smallest zones of lysis were grown to late-exponential phase at 45 °C, and then allowed to autolyse in ammonium carbonate buffer, pH 9.5. Two of these mutants (designated RW2 and RW3) showing the slowest rates of lysis were chosen for further study.

DNA from strain RW3 (Lyt- Trp-) was used at a saturating concentration to transform B. subtilis MB21 (Lyt+, Leu-, Met-). The proportion of leucine-independent organisms which were also found to be autolytic-deficient was 5.3%. A randomly selected colony from these transformants was designated RW10. An isogenic Lyt+ strain (designated RW11) was obtained by transformation using a non-saturating concentration of DNA from RW3, again selecting initially for leucine-independent bacteria.

Autolytic activity of the wild-type and autolytic-deficient mutants

Amidase and glucosaminidase activities were measured in 5 M-LiCl extracts obtained from the various B. subtilis strains (Table 1). The mutants were some 80–90% deficient in both autolytic enzyme activities. Approximately fourfold less protein was extracted from each of the Lyt- mutants than from the Lyt+ strains. Thus, there appeared to be a correlation between a lack of autolytic activity and a low release of protein by 5 M-LiCl.

To test if the above observation was also applicable to other bacteria, samples of S. pneumoniae wild-type (R6) and autolytic-deficient organisms (CW1, a Lyt- mutant, and the wild-type grown in ethanolamine, EA-R6, which is non-permissive for lysis) were treated with 5 M-LiCl. The amounts of protein extracted, in mg ml⁻¹, and the percentage extracted relative to the wild-type were: R6, 3.26 (100%); CW1, 2.10 (64%); EA-R6, 1.43 (44%). Thus, again, significantly less protein was extracted from the Lyt- organisms.

Fractionation of proteins extracted with 5 M-LiCl

B. subtilis extracts. A sample of the protein extracted from the Lyt+ strain RW11 was applied directly to a column of Sephadex G-100, eluted with 5 mM-succinate/PMSF, and fractions were assayed for protein, amidase and glucosaminidase activities (Fig. 1a). Two major peaks of protein were detected. The second of these, which contained about 55% of the total protein applied to the column and both autolytic enzymes, appeared to be retarded on the column. When a sample from an equivalent amount of the Lyt- strain RW10 was chromatographed in the same manner, only the second peak was detected (Fig. 1b), and as expected this contained much less autolytic enzyme activity. Although this peak contained about 80% of the protein eluted from the column, the amount present was reproducibly only about 40% of that found in the second peak of the Lyt+ strain RW11 extract. The absence of the first peak from the proteins extracted from the autolytic-deficient mutant suggested that its wall might be less permeable than that of the isogenic wild-type. Alternatively, it seemed possible that the observed differences were a consequence of changes in the structure and accessibility of the cytoplasmic membrane in the autolytic-deficient organisms. However,
Fig. 1. Fractionation on Sephadex G-100 of 5 M-LiCl extracts from *B. subtilis* Lyt+ strain RW11 (a) and the autolytic-deficient mutant RW10 (b). Samples were assayed for protein (▲) and autolytic activity against *M. luteus* (○) and *B. subtilis* (○) walls. Fractions were pooled as shown. The *K*_D values of bovine serum albumin (BSA) and cytochrome c (Cyt c) are indicated.

Fig. 2. SDS–polyacrylamide gel electrophoresis of proteins extracted by 5 M-LiCl from *B. subtilis* Lyt+ strain RW11 and Lyt− strain RW10. Tracks 1–4 contained the pooled fractions indicated in Fig. 1. Bovine serum albumin (mol. wt 67 000), ovalbumin (45 000) and trypsin inhibitor (21 500) were used as standards.
Fig. 3. Fractionation on Sephadex G-100 of 5 m-LiCl extracts from S. pneumoniae strain R6 (a), ethanolamine-grown organisms EA-R6 (b) and the autolytic-deficient mutant CW 1 (c). Samples were assayed for protein (▲) and for N-acetylmuramoyl-L-alanine amidase activity against S. pneumoniae walls (●). The $K_D$ values of bovine serum albumin (BSA) and cytochrome c (Cyt c) are indicated.

the addition of 1-butanol (1% v/v) to the extraction buffer, conditions previously shown to perturb the cytoplasmic membrane of protoplasts and mycoplasma (Razin & Argaman, 1963), produced no significant change in the amount or distribution of proteins extracted from either strain RW 11 (Lyt+) or the autolytic-deficient mutant RW 10.

Fractions were pooled as indicated (Fig. 1), dialysed against distilled water, concentrated and centrifuged (20000 g for 5 min) to remove insoluble material, most of which was found in the pooled fractions containing the autolytic enzymes. Samples of the concentrated material were subsequently examined by SDS–polyacrylamide gel electrophoresis (Fig. 2). Although fraction 1 from the Lyt− strain RW 10 extract contained little protein when compared with the first peak of the Lyt+ strain RW 11 extract, electrophoresis of the fractions from both strains revealed proteins of a similar molecular weight range. Thus, the major difference observed was in the amount of protein extracted although changes were apparent in the distribution of individual polypeptides.

S. pneumoniae extracts. Samples of extracts from equivalent numbers of the wild-type strain R6 and the autolytic-deficient S. pneumoniae were chromatographed on Sephadex G-100, and fractions were assayed for protein and amidase activity (Fig. 3). A similar column profile to that for the B. subtilis Lyt+ strain was observed with strain R6 and, again, the autolytic enzyme was associated with the second peak. The distribution of proteins in extracts from both strain CW 1 and EA-R6 showed a loss of the first peak. Moreover, the extract from strain EA-R6 had a reduced amount of protein in the peak associated with the autolytic enzyme.

Proteins solubilized from disrupted B. subtilis. The distributions of proteins extracted from B. subtilis strains Lyt+ RW 11 and Lyt− RW 10 were clearly different when fractionated on Sephadex G-100. Therefore, the amount and distribution of the total soluble proteins of each strain were examined to determine whether these were similar.

The soluble fraction from Lyt+ strain RW 11 (100 mg) was recovered by centrifuging after disruption of the organisms in a French press, and chromatographed on Sephadex G-100
Autolysin deficiency and wall permeability

Fig. 4. Fractionation on Sephadex G-100 of proteins obtained from B. subtilis strain RW 11 by disruption in the presence (△) and absence (Δ) of 5 M-LiCl.

(Fig. 4). The bulk of the protein had an apparent molecular weight greater than 70000 although some was eluted just ahead of the salt volume. This latter peak was small in comparison with that obtained by fractionation of the LiCl extract from an equivalent amount of Lyt+ strain RW11 (Fig. 1a), and it seemed possible that some protein had remained bound to the wall fragments from the disrupted organisms. When the bacteria were disrupted in buffer containing 5 M-LiCl and the soluble material was chromatographed in the same manner, only the last peak increased in size (Fig. 4). This suggested that the LiCl had released proteins which were normally bound to the wall, or had become so during breakage of the bacteria, and that these proteins were retarded on Sephadex G-100 under the conditions used.

The distribution of proteins obtained from Lyt− strain RW10, disrupted in the absence of LiCl, appeared almost identical to that observed with those from Lyt+ strain RW11. However, the addition of LiCl to the autolytic-deficient mutant during breakage resulted in only a 20% increase in the size of the retarded peak, whereas the increase was some threefold with Lyt+ strain RW11. It appears, therefore, that the mutant walls contained much less extractable protein.

Measurement of α-D-glucosidase and glucose-6-phosphate dehydrogenase activities in LiCl extracts of B. subtilis

Since the decrease in extractable protein from autolytic-deficient organisms in comparison with Lyt+ strains seemed to be due partly to a decrease in wall permeability, the extracts obtained from B. subtilis were tested for the presence of intracellular enzymes. Glucose-6-phosphate dehydrogenase and α-D-glucosidase were chosen for this purpose. The molecular weight of glucose-6-phosphate dehydrogenase has been estimated as 350000 (Ujita & Kimura, 1975), and that of the glucosidase as about 43500 by fractionation on Sephadex G-100 in the present studies.

In initial experiments the glucosidase was found to be inhibited by Tris (1 mM giving 50% inhibition) (see Larner & Gillespie, 1956) and so 3-(N-morpholino)propanesulphonic acid (MOPS)/PMSF buffers were used for these experiments. In addition, both enzymes were inhibited by high concentrations of LiCl (>0.1 M) and so 0.5 M-LiCl was used for these extractions to allow a suitable dilution during the assays. (The lower concentration of LiCl had little apparent effect on the total yield of extracted protein from either strain, although 5 M-LiCl was required to extract the maximum amounts of amidase.) Lysozyme lysates of both strains were indistinguishable in terms of protein concentration and the activity of the two enzymes (Table 2), whereas the LiCl extract from autolytic-deficient mutant RW10
Table 2. Total and LiCl-extracted α-D-glucosidase and glucose-6-phosphate dehydrogenase activities of B. subtilis strains RW11 and RW10

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Strain</th>
<th>Protein concn (mg ml⁻¹)</th>
<th>Glucosidase activity (10⁶ x units ml⁻¹)</th>
<th>Glucose-6-phosphate dehydrogenase activity (10³ x units ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme lysate</td>
<td>RW11 (Lyt⁺)</td>
<td>17.35</td>
<td>27920</td>
<td>1840</td>
</tr>
<tr>
<td></td>
<td>RW10 (Lyt⁻)</td>
<td>17.45</td>
<td>26520</td>
<td>1970</td>
</tr>
<tr>
<td>0.5 M-LiCl extract</td>
<td>RW11 (Lyt⁺)</td>
<td>7.37</td>
<td>2440</td>
<td>54.0</td>
</tr>
<tr>
<td></td>
<td>RW10 (Lyt⁻)</td>
<td>1.83</td>
<td>660</td>
<td>14.3</td>
</tr>
</tbody>
</table>

contained about fourfold less protein and enzyme activities compared with the amounts extracted from Lyt⁺ strain RW11. Moreover, in both strains the proportion of the total glucose-6-phosphate dehydrogenase extracted was approximately threefold lower than the proportion of glucosidase released. Therefore, the walls of the mutant were less permeable to the cytoplasmic enzymes than the Lyt⁺ walls, and in both cases less of the higher molecular weight enzyme was extracted, confirming that the permeability of the walls was decreased to proteins of increasing molecular size.

If the walls of Lyt⁺ strain RW11 had been degraded to some extent by limited autolysis prior to extraction, the walls would presumably have become more permeable and thus caused more proteins to be released when compared with the Lyt⁻ strain RW10. However, this was unlikely, since treatment of organisms by the standard extraction technique but without LiCl released only small amounts of protein and glucosidase from both organisms.

DISCUSSION

Lithium chloride has been used to extract autolytic enzymes from a variety of Gram-positive organisms, including B. licheniformis (Robson & Baddiley, 1977) and B. subtilis (Fan & Beckman, 1971, 1972, 1973a, b; Brown, 1973; Fein & Rogers, 1976). None of these authors reported the amounts of protein released by the extraction method, even when wild-type and autolytic-deficient mutants were compared (Fan & Beckman, 1971, 1973a; Fein & Rogers, 1976; Robson & Baddiley, 1977). However, Brown et al. (1976) reported the specific activities of the amidase from B. subtilis strain BR151 and one of its thermosensitive morphological mutants. Extraction of this mutant after growth under conditions where it was approximately 80% deficient in amidase activity released only 37% of the amount of protein extracted from the organism when it had full autolytic activity. The amounts of protein extracted from the autolytic-deficient mutants in the present studies were always some four- to fivefold lower than that from the wild-type for the bacilli, and about twofold lower for the pneumococci. It seems likely that the lower amount of protein extracted from the Lyt⁻ organisms was due to a change in wall permeability. This conclusion is supported by the differences observed in the distribution of the extracted proteins after chromatography on Sephadex G-100, the absence of marked changes when the extraction was carried out in the presence of butanol, and the small amounts of intracellular enzymes extracted with LiCl.

The proteins extracted from the Lyt⁺ strain RW11 were fractionated into two major peaks. However, there was no equivalent first peak in the profile of proteins extracted from the autolytic-deficient mutant RW10, although the bulk of the proteins obtained from disrupted organisms of both types had apparent molecular weights greater than 100000. This suggests that significant amounts of proteins with molecular weights greater than about 70000 could not be extracted through the wild-type wall, and that only smaller amounts of these proteins could be extracted through the wall of the Lyt⁻ bacilli. Essentially similar results were obtained with the pneumococci except that the apparent exclusion limit was significantly
lower. The greater decrease in permeability of the autolytic-deficient bacilli compared with that of the wild-type in comparison with the decrease in permeability of the Lyt- pneumococci may indicate that the longitudinal wall of the bacilli was more affected than the new or old septal regions.

In contrast to the results reported here for the Lyt- B. subtilis strain, Hughes et al. (1975) reported that intracellular, soluble proteins of molecular weights less than 70000 could pass into the external medium through walls of an autolysin-deficient strain of B. licheniformis grown to stationary phase. However, no comparison was made with a Lyt+ strain or with exponential-phase organisms. Proteins of molecular weight less than 70000 have been extracted with 2 M-LiCl from toluene-treated, exponential-phase B. megaterium 899 (Taku et al., 1975). Similarly, the membrane-associated alkaline phosphatase of B. subtilis (Ghosh et al., 1971), which has a molecular weight of 69000 (Glenn & Mandelstam, 1971), was extracted from intact organisms with 5 M-NaCl (Wood & Tristram, 1970). Experiments with dextrans and polyglycols have indicated that the number average molecular weight (\(M_n\)) exclusion limit of B. subtilis peptidoglycan was about 60000 (Hughes et al., 1975), and that of walls obtained from exponential-phase B. megaterium was 57000 (Gerhardt & Judge, 1964), although this value was later revised to a range between 70000 and 120000 (Scherrer & Gerhardt, 1971). Thus, although the apparent exclusion limit of walls from various bacilli appears to be similar, the observations made in the present study suggest that a reduction in those enzymes capable of hydrolysing specific linkages in peptidoglycan results in a marked reduction in the porosity of the wall. Whether this arises as a consequence of changes in the packing of the glycan and peptide chains of peptidoglycan must remain open to speculation.

It also appears from the experiments described that a range of proteins is capable of binding to the walls of B. subtilis or S. pneumoniae, and that these are generally retarded on Sephadex G-100 when applied in a high salt concentration. Included among these in B. subtilis are the amidase and glucosaminidase, which have molecular weights of 50000 (Herbold & Glaser, 1975) and 188000 (Taylor et al., 1980), respectively. The retention observed is probably due to interactions with the dextran, which may resemble the interaction responsible for the association of these proteins with the walls. Lysozyme is also retained on columns of Sephadex (Whitaker, 1963, and references listed therein) and agarose (Fernandez-Sousa et al., 1978), and it has been suggested that the enzyme forms a weak complex with the polysaccharide (Whitaker, 1963). There are also examples of other enzymes, generally those which catalyse reactions involving carbohydrates, being similarly retarded on Sephadex (Andrews, 1970; Yamane et al., 1973). Thus, this retardation on the dextran under the conditions used may be a useful step in the purification of autolytic enzymes.

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REFERENCES


