The Location of *N*-Acetylgalactosamine in the Walls of *Bacillus subtilis* 168

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The *N*-acetylgalactosamine in the walls of *Bacillus subtilis* 168 occurs in two polymers. One of these contains *N*-acetylgalactosamine, glucose and phosphorus and is attached to the peptidoglycan through an alkali-labile bond; preliminary studies indicate that a repeating unit of this polymer is glucosyl-*N*-acetylgalactosamine 1-phosphate. *N*-Acetylgalactosamine is also associated with the peptidoglycan in a component that is not converted into the free sugar or other soluble compounds on treatment of the walls with alkali. The two polymers containing *N*-acetylgalactosamine are released on autolysis of the walls and can be separated by ion-exchange chromatography. As glucose 6-phosphate is produced by gentle hydrolysis of the wall with acid a third phosphate polymer, poly(glucose 1-phosphate), may occur in this wall. However, as no polymer with this structure could be separated from that containing galactosamine, its existence has not been established unequivocally. The methods described permit the study of variations in *N*-acetylgalactosamine content with respect to growth conditions.

The composition of the walls of *Bacillus subtilis* 168, and of morphological mutants derived from this organism, has been studied extensively (Young, 1966; Hughes & Tanner, 1968; Hughes, 1970*b*; Rogers *et al.*, 1971). The *rod* mutants described by Rogers and his collaborators (Rogers *et al.*, 1968, 1970; Rogers & McConnell, 1970) grow as chains or groups of round forms on minimal media, but on similar media supplemented with high concentrations of NaCl one class (A) grows as rods, whereas the second class (B) retains the round form. The composition of the walls of the *rod* mutants is closely similar to that of the walls of the parent strain grown under similar conditions, except that walls of the class (B) contain much less galactosamine (Rogers *et al.*, 1971). In both types of *rod* mutants and in the parent strain the amount of galactosamine in the walls of cells grown in minimal media is substantially increased by the presence of 0.8M-NaCl. Young (1965) has also shown that the galactosamine content of walls varies in cells grown under different conditions. The present study was undertaken to clarify the nature of the polymers containing galactosamine in the walls of *B. subtilis* strain 168.

Previous workers have reported that the galactosamine is associated with the glucosylated glycerol teichoic acid of the wall and that two fractions of teichoic acid, which differ in their galactosamine content (Young, 1966; Young *et al.*, 1968) can be isolated. It has also been reported that nearly all of

the galactosamine, glucose and P are extracted by treatment of walls with dilute NaOH solution, and that the galactosaminyl linkages are highly susceptible to acid hydrolysis (Hughes & Tanner, 1968). The acid lability of the galactosaminyl linkages is similar to that of the glycosyl 1-phosphate linkages in the teichoic acids and related phosphorylated polysaccharides that occur in several micrococci (Archibald *et al.*, 1968; Partridge *et al.*, 1971); we have studied the nature of the polymers in the wall of *B. subtilis* 168 to establish whether the galactosamine residues are of the sugar 1-phosphate type and whether they are components of the glycerol teichoic acid or of a separate polymer. It was also decided to determine whether the previously suggested heterogeneity of substitution of the teichoic acid by galactosamine could be explained structurally.

The present studies have shown that the wall of *B. subtilis* 168 contains the following components: peptidoglycan; teichoic acid, i.e. a glucosylated poly(glycerol phosphate); a component containing *N*-acetylgalactosamine, which is attached to that part of the peptidoglycan that carries the teichoic acid; a sugar 1-phosphate polymer, containing repeating units of glucosyl-*N*-acetylgalactosamine 1-phosphate, attached to a fraction of peptidoglycan which is not linked to teichoic acid; glucose 1-phosphate also occurs as a repeating unit of a polymer, but it is not known whether this is separate from that which contains the disaccharide 1-phosphate units.

Methods

Organism and culture

Materials. Spores of *Bacillus subtilis* 168 were given by Dr. H. J. Rogers, National Institute for Medical Research. Calf intestinal phosphomonoesterase (alkaline) and glucose 6-phosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Growth of bacteria and preparation of walls. *Bacillus subtilis* Marburg strain 168 *tryp* was grown as previously described (Hughes, 1970*b*). Walls containing autolytic enzyme were prepared by centrifugation after breakage of the bacteria by shaking with glass beads in a Braun homogenizer fitted with a cooling device. The crude walls were washed extensively with NaCl (0.9%) and water at 4°C before freeze-drying.

General procedures

Paper chromatography. Whatman no. 1 paper was used for descending chromatography. The following solvent systems were employed: *A*, propan-1-ol-aq. NH₃ soln. (sp.gr. 0.88)-water (6:3:1, by vol.); *B*, butan-1-ol-pyridine-water (6:4:3, by vol.); *C*, ethyl acetate-pyridine-acetic acid-water (5:5:1:3, by vol.).

Electrophoresis. Whatman 3MM chromatography paper was used with the buffer, water-acetic acid-pyridine (43:2:5, by vol., pH 5.3). The electrophoresis was carried out with a voltage gradient of 50 V/cm for 90 min. Compounds were detected and characterized on the paper after chromatography or electrophoresis with the following reagents: (a) periodate-Schiff for α -glycols (Baddiley *et al.*, 1956); (b) molybdate for phosphates (Hanes & Isherwood, 1949); (c) ninhydrin for amino compounds (Consden & Gordon, 1948); (d) alkaline AgNO₃ for reducing compounds (Trevelyan *et al.*, 1950).

Analytical methods. Phosphate was determined by the method of Chen *et al.* (1956). Glucose was determined by Glucostat reagent (Worthington Biochemical Corp., Freehold, N.J., U.S.A.). Glucose 6-phosphate was determined with glucose 6-phosphate dehydrogenase (Horecker & Wood, 1957). Amino sugars were determined by a modified Elson-Morgan method (Reissig *et al.*, 1955). Individual amino sugars were determined in a Jeol autoanalyser. Samples (3-6 mg) were hydrolysed with 4M-HCl for 16 h at 100°C in a sealed ampoule under vacuum. Acid was removed by vacuum distillation over KOH pellets before analysis. Amino acids were determined in a manner similar to that used for amino sugars but 6M-HCl was used in the hydrolysis. The values for amino sugars and amino acids quoted in the text have not been corrected for destruction during hydrolysis. Glycerol was determined by the method of Hanahan & Olley (1958).

Interaction of concanavalin A with the wall polymers

Fractions obtained by alkali extraction or by autolysis, and the various components obtained by further fractionation of these extracts, were examined by double diffusion in agar gel against concanavalin A. The conditions of these experiments were similar to those described previously (Archibald & Coopes, 1971). Glycogen was used throughout as a standard. The bands had formed before the plates were washed with water and no further bands were observed on washing. More sharply defined bands were observed by substituting agarose for agar and by substituting 0.05M-tris-HCl buffer, pH 7.3, for the buffer systems used previously.

Experimental and Results

Composition of the walls of Bacillus subtilis 168

Crude walls of *Bacillus subtilis* contained 3.2% of P, and were contaminated with protein and membrane fragments. Trypsin digestion and phenol extraction removed most of the protein, but little P and no galactosamine, showing that the galactosamine was not present as a glycoprotein but as a component(s) covalently attached to the wall. The molar ratio of galactosamine to P (1:7) in the purified walls was the same as that for the crude walls. As the unpurified walls contained active autolytic enzyme they were used for further study.

Autolysis of walls

Autolysis was effected by incubation of walls (500 mg) in 20mM-(NH₄)₂CO₃, pH 8.9 (100 ml), at 37°C for 16 h, when all of the galactosamine and at least 95% of P was converted into a soluble form. The insoluble residue was removed by centrifugation (9000g for 20 min) and the supernatant solution was evaporated to dryness in a rotary evaporator. The residue was dissolved in water, dialysed to remove salts and freeze-dried. This material (400 mg) was then dissolved in 0.01M-pyridine acetate buffer, pH 5.1, and applied in portions (135 mg, 2 ml) to columns (20 mm $\times$ 300 mm) of DEAE-cellulose, which had previously been equilibrated with the same buffer. Material was eluted from the columns with this buffer (100 ml) followed by a linear gradient (500 ml) of 0.01-5.0M-pyridine acetate buffer, pH 5.1 (molarities quoted are as pyridine concentrations). Fractions (10 ml) were collected and analysed for P. The P was located in a single broad peak (cf. Hughes, 1970*b*) and fractions corresponding to this peak were combined and then freeze-dried, after removal of the buffer salts on the rotary evaporator. This material contained all of the P that had been applied to the column and contained galactosamine and P in the same molar ratio (1:7) as found in the original wall and un-

Table 1. *Analysis of the wall autolysate and the material extracted from the walls by alkali*

The autolysate and alkali extract were each fractionated on columns of DEAE-cellulose, which were eluted with linear gradients of 0.01–5.0M-pyridine acetate buffer, pH 5.1, as described in the text. In each case a single broad peak of phosphate was obtained. Results are expressed relative to galactosamine.

	P	Glucosamine	Muramic acid	Galactosamine
Whole wall	7.0	1.4	1.2	1.00
Fractionated autolysate	7.0	0.8	0.63	1.00
Fractionated alkali extract	16.7	—	—	1.00

Fig. 1. *Separation of phosphorus-containing fractions from an autolysate of walls*

The fraction (220mg) obtained by DEAE-cellulose chromatography of the wall autolysate (400mg) as described in the text was run on a second column in pyridine acetate buffer, pH 5.1 (100ml), and material was then eluted with a linear gradient of 1.2–4.5M-pyridine acetate, pH 5.1 (600ml). Fractions (4ml) were collected at a flow rate of 15ml/h and analysed for P. Peak 1 was found by analysis to represent P_i .

fractionated autolysate, thus showing that all of the galactosamine was eluted in fractions that also contained P (Table 1). The fractionated material (220mg) was dissolved in 1.2M-pyridine acetate buffer, pH 5.1 (3ml), and applied to a column (25mm × 350mm) of DEAE-cellulose, which had previously been equilibrated against this buffer. The column was washed with the same buffer (100ml) and material was eluted with a linear gradient (600ml) of 1.2–4.5M-pyridine acetate buffer, pH 5.1, at a flow rate of 15ml/h; fractions (4ml) were collected and analysed for P. Three peaks corresponding to organic P were observed (Fig. 1). Material corresponding to peak 2 represented 11% of the total P and contained

galactosamine, glucose and P in the molar proportions 1:2:2; no glycerol or glycerol phosphates were present. Acid hydrolysis of this fraction gave glucosamine and muramic acid in equimolar quantities. As shown below, material corresponding to peak 2 includes a sugar 1-phosphate polymer that is distinct from the glycerol teichoic acid. The sugar 1-phosphate polymer contains approx. 50% of the galactosamine of the wall.

Material corresponding to peak 3 represented 89% of the total P and contained glucose, glycerol and P in the molar proportions 1:1:1. This fraction thus contained the glucosylated glycerol teichoic acid. The remainder of the galactosamine in the wall is associated with this material, and glucosamine and muramic acid were produced by hydrolysis of the fraction with acid. The analysis of this fraction after further purification on a DEAE-cellulose column is shown in Table 2. Analysis of early and late tubes corresponding to peak 3 differed in their P:galactosamine ratio (24:1 and 37:1 respectively) and it is concluded that this material is probably not homogeneous. Since it is eluted with the teichoic acid, the component containing galactosamine must either be highly charged or be attached to the teichoic acid-peptidoglycan complex.

Fractionation of autolysed walls thus gives two distinct phosphates: one of these is a glucosylglycerol teichoic acid that is combined with products derived from the peptidoglycan; the other is a sugar 1-phosphate polymer, also associated with peptidoglycan, but which does not contain glycerol. Galactosamine is present in both fractions.

Extraction of walls with alkali

A sample of walls (500mg) was suspended in 0.1M-NaOH (100ml) and stirred under N_2 at 37°C for 16h. The suspension was neutralized with 1M-HCl and insoluble material was removed by centrifugation (9000g for 20min); under these conditions more than 90% of the P was removed from the wall. The extract was desalted by dialysis against water, or in later experiments by passage through a column (20mm ×

Table 2. *Analysis of material obtained by autolysis of walls followed by fractionation on DEAE-cellulose*

The single peak of phosphate obtained on initial fractionation of the autolysate was further resolved by chromatography on a column of DEAE-cellulose, which was eluted with a linear gradient of 1.2–4.5M-pyridine acetate buffer, giving the three peaks of phosphate shown in Fig. 1. Results are expressed as molar proportions relative to muramic acid.

	Muramic acid	Glucosamine	Galactosamine	Glucose	P	Glycerol
Peak 2	1	1	5.6	11	11	—
Peak 3	1	1.8	1.1	24	25	24

750mm) of Sephadex G-100, and then freeze-dried. The extract contained 90% of the P but only 44% of the galactosamine originally present in the wall (galactosamine:P ratio in the extract, 1:16). The wall residue (275mg) contained the remainder of the galactosamine, which is thus present in a component that is not extracted by alkali. The extracted phosphate was separated from amino compounds by chromatography on DEAE-cellulose under the conditions used for the initial fractionation of the autolysate. Samples (50mg) of the phosphate peak were dissolved in 0.01M-pyridine acetate, pH 5.1 (2ml), and were fractionated on columns (15mm × 750mm) of Sephadex G-75 and G-100; in each case only partial separation of fractions was achieved. The ratio galactosamine:P increased with elution volume, indicating that the galactosamine was associated with the lower-molecular-weight components of the extract. Further samples (100mg) of the phosphate peak were fractionated on columns of DEAE-cellulose as described above for the fractionation of the autolysate. Elution with a linear gradient of 1.2–4.5M-pyridine acetate gave two fractions containing P corresponding in elution volume and content of P to peaks 2 and 3 obtained from the autolysate. Material corresponding to peak 2 from the autolysate contained galactosamine, glucose and P in the same molar proportions (1:2:2) and lacked glycerol. The alkali-extracted material did not contain glucosamine or muramic acid. The linkage of the sugar 1-phosphate polymer to peptidoglycan is therefore labile to dilute alkali.

Material corresponding to peak 3 contained the glucosylated glycerol teichoic acid, which was also free of peptidoglycan. Unlike that derived from the autolysate this fraction did not contain galactosamine. Since the amount of galactosamine in the sugar 1-phosphate polymer isolated by extraction with alkali is the same as that in the corresponding fraction of the autolysate, it follows that the galactosamine that is not extracted from the wall by alkali is the same as that in the glycerol teichoic acid fraction of the autolysate. It follows that this galactosamine is not held in the wall by linkage to the teichoic acid, but it may be a part of a component that

is attached by an alkali-stable bond to the part of the peptidoglycan that also bears the teichoic acid. The teichoic acid may be attached to this component or it may be directly attached to the peptidoglycan. The galactosamine in this component differs from that in the sugar 1-phosphate polymer in that it is not released by hydrolysis in 0.1M-HCl at 37°C during 16h or in 0.1M-HCl at 100°C during 10min, whereas under these conditions the sugar 1-phosphate polymer is completely degraded to products of low molecular weight.

Preliminary studies on the structure of the polymer containing galactosamine, glucose and phosphorus

Acid hydrolysis (2M-HCl for 3h at 100°C) of the polymer (extracted with alkali and purified on DEAE-cellulose), followed by neutralization, dephosphorylation and examination of the products by paper chromatography in solvent *B*, showed that glycerol was absent. After enzymic dephosphorylation paper chromatography in solvent *C* showed that the only reducing sugars were glucose and galactosamine. In the polymer the galactosamine is in fact *N*-acetylgalactosamine; this was shown by g.l.c. of the acetic acid produced by acid hydrolysis. The glucose, phosphate and *N*-acetylgalactosamine were present in the molecular proportions 2:2:1.

Gentle acid hydrolysis (0.1M-HCl for 10min at 100°C) of the polymer, followed by neutralization and examination of the hydrolysis products by paper chromatography, revealed only one component ($R_{\text{Glucosamine 6-phosphate}}$ 1.25 in solvent *C*). This component showed reducing properties and did not react with the ninhydrin reagent. High-voltage electrophoresis at pH 5.3 showed, however, that this fraction contained two components ($m_{\text{Glucose 6-phosphate}}$ 1.0 and 0.8), both of which were reducing phosphates.

Dephosphorylation of the fraction obtained by gentle acid hydrolysis also yielded two components having R_{Glucose} 1.0 and 0.8 (solvent *C*). Samples of these two compounds were purified further by preparative paper chromatography. The component with R_{Glucose} 1.0 was glucose, and the slower-moving component was a disaccharide of glucose and

N-acetylgalactosamine. Reduction with NaBH_4 showed that the amino sugar was at the reducing end of the disaccharide.

Samples of the two sugar phosphates were also purified by gel filtration on Sephadex G-10 followed by preparative high-voltage electrophoresis. The faster-moving component ($m_{\text{Glucose 6-phosphate}} 1.0$) contained glucose and P in equimolar amounts and was oxidized by glucose 6-phosphate dehydrogenase. The slower-moving component ($m_{\text{Glucose 6-phosphate}} 0.8$) contained equimolar proportions of glucose, *N*-acetylgalactosamine and phosphate. Glucose 6-phosphate and a glucosylgalactosamine phosphate are therefore repeating units either in separate polymers or in a mixed polymer containing both units. The ease of hydrolysis of the polymer or polymers in acid indicates that the repeating units are joined together by sugar 1-phosphate bonds.

Reaction of fractions with concanavalin A

The lectin concanavalin A reacts with polysaccharides and teichoic acids containing α -D-glucopyranosyl or α -D-mannopyranosyl substituents (or their 2-acetamido-2-deoxy derivatives) in which the hydroxyl groups at positions 3, 4 and 6 are unsubstituted (Goldstein *et al.*, 1965; Goldstein & Iyer, 1966; Goldstein *et al.*, 1967; Archibald & Coapes, 1971). The glycerol teichoic acid fraction isolated by alkali extraction or by autolysis of walls of *B. subtilis* 168 gave sharp precipitin bands on double diffusion against concanavalin A in agar and agarose gels. In each case only one band was observed, and this was sharply defined. Similar examination of the whole autolysate or of the unfractionated alkali extract also gave a single sharp band in each case. Neither preparation of the sugar 1-phosphate polymer interacted with concanavalin A. This difference in interaction with the lectin could form the basis of an alternative procedure for their separation, which might be more efficient than that described in the present study.

Discussion

Walls of *Bacillus subtilis* 168 grown in a casein medium contain *N*-acetylgalactosamine residues in two distinct fractions, one of which is a sugar 1-phosphate polymer containing *N*-acetylgalactosamine and glucose. The remainder of the *N*-acetylgalactosamine, about 50% of the total, is combined with a different part of the peptidoglycan. The nature of this latter *N*-acetylgalactosamine compound has not been established, but it differs from the sugar 1-phosphate polymer in that it is not readily hydrolysed by acid and is not extracted from the walls by treatment with dilute alkali.

Samples of the disaccharide 1-phosphate polymer have been isolated by ion-exchange chromatography of material extracted from the walls by treatment with dilute alkali (cf. Hughes *et al.*, 1968; Archibald *et al.*, 1969) or by autolysis (cf. Young, 1966). These samples are free from glycerol teichoic acid and differ from each other only in that the material obtained from autolysed walls is combined with components of the peptidoglycan. The polymer is thus not a part of the glycerol teichoic acid but is a separate polymer that is covalently attached to peptidoglycan by an alkali-labile linkage. It is completely hydrolysed by dilute acid under controlled conditions to a mixture of a glucose phosphate and a phosphate of glucosyl-*N*-acetylgalactosamine. The ease of this hydrolysis indicates that in the original polymer units are connected by sugar 1-phosphate linkages similar to those that occur in teichoic acids and sugar 1-phosphate polymers in certain micrococci (Partridge *et al.*, 1971). It is not yet known whether the glucose phosphate and glucosyl-*N*-acetylgalactosamine 1-phosphate units are present in the same polymer or in two separate polymers, but attempts to fractionate the material containing those units have not been successful.

Samples of the glycerol teichoic acid have been isolated after extraction of walls with alkali and by autolysis. The teichoic acid isolated from autolysed walls is combined not only with muramic acid and glucosamine components of the peptidoglycan but also with that *N*-acetylgalactosamine component of the wall that is not extractable in alkali. On the other hand teichoic acid obtained by alkali extraction does not contain amino sugars. In the wall therefore the teichoic acid is covalently attached to the peptidoglycan, either directly or through an intervening component containing galactosamine. This galactosamine component is resistant both to extraction by alkali and to hydrolysis under gentle acid conditions; it is therefore present in a component that is distinct from the sugar 1-phosphate polymer.

The teichoic acid and sugar 1-phosphate polymer are further distinguished by their interaction with concanavalin A. On double diffusion in agar or agarose gels the teichoic acid gives a single sharp precipitin band with concanavalin A, whereas the sugar 1-phosphate polymer does not give a precipitin reaction. These results are in contrast to those described by Doyle & Birdsell (1972), who report that autolysed walls of *Bacillus subtilis* 168 give two discrete precipitin bands with concanavalin A. These workers suggest that this is due to the presence of two major populations of glucosylglycerol teichoic acid, which might be the two sub-types previously described by Young (1966). It is noteworthy, however, that the two phosphate fractions described by Young (1966) have apparently identical composition and closely similar ion-exchange properties to the two

phosphate fractions that we have isolated from autolysed walls. As shown above, only one of these fractions is a glycerol teichoic acid; the other does not contain glycerol and is in fact a sugar 1-phosphate polymer. Our results therefore show that the wall contains at least two phosphate polymers that differ fundamentally from each other in composition and in structure. Only one of these polymers reacts with concanavalin A, and we do not observe two precipitin bands on interaction of concanavalin A with purified teichoic acid, with unfractionated alkali extract or with unfractionated autolysate.

It is noteworthy that, like previous workers (Hughes *et al.*, 1968), we have been unable to detect the presence of glucuronic acid residues in the walls. This suggests that the galactosamine associated with the teichoic acid-peptidoglycan complex is not present as teichuronic acid, although in view of the small amounts of material examined this conclusion should be regarded with some caution. It is known (Ellwood & Tempest, 1972) that, on growth under conditions of phosphate limitation, *B. subtilis* 168 synthesizes a teichuronic acid composed of *N*-acetylgalactosamine and glucuronic acid, and Hughes (1970a) has shown that teichuronic acid is not extracted by dilute alkali. This galactosamine compound is, however, clearly different from that present in the sugar 1-phosphate polymer, and the two forms are conveniently separated from each other by extraction of walls with alkali. These techniques should therefore be useful in examining more closely the variations in galactosamine content of walls of cells grown in different media, to establish whether these variations are due to differences in the amount of both or only one of the galactosamine-containing polymers.

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