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OCCURRENCE OF *N*-GLYCOLYLMURAMIC ACID IN BACTERIAL CELL WALLS

A PRELIMINARY SURVEY*

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SUMMARY

The *N*-acyl substituent of muramic acid in the cell walls of some species of Actinomycetales and of the related family of Corynebacteriaceae has been studied. The *N*-glycolyl derivative, recently identified in *Mycobacterium smegmatis*, has been found in the three other species of Mycobacterium studied: *M. kansasii*, *M. tuberculosis* BCG, and *M. phlei*.

The classical *N*-acetyl derivative has been found in *Streptomyces albus* and *Corynebacterium fermentans*.

INTRODUCTION

We have shown recently[1,2] that the cell wall peptidoglycan of *Mycobacterium smegmatis* contains *N*-glycolyl muramic acid, instead of the usual *N*-acetyl derivative[3].

This finding prompted us to investigate if N-glycolylation was a peculiarity of this species or if it was encountered in other species of Mycobacterium and related genera. Among the Actinomycetales, we examined three others species of Mycobacterium: *M. kansasii*, *M. tuberculosis* BCG and *M. phlei*, and one species of Streptomyces: *S. albus*. We also studied one species of Corynebacterium: *C. fermentans*, as Corynebacteria are closely related to Mycobacteria in their antigenic properties (I. AZUMA, unpublished experiments), the general architecture of their cell wall and the analogies of the structure of their lipids and glycolipids (they are sometimes classified as Actinomycetales[4]).

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MATERIALS AND METHODS

(1) *Preparation of the bacterial cells*

M. kansasii Strain P_{21} was grown on Sauton's medium in Roux bottles for 30 days at 37°. *M. tuberculosis* BCG was grown on Sauton's medium in round flasks for 11 days at 37°. *M. phlei* Strain No. 356 A.T.C.C. was grown in BRODIE's medium in a gyratory shaker (New Brunswick Scientific Company, New Brunswick, N.J.) at 37° and harvested at the end of the exponential phase of growth. *C. fermentans* was grown in V.F. medium (meat and liver medium with 1 % glucose added) in a gyratory shaker at 37° and harvested at the end of the exponential phase. *S. albus* G was grown as described by LEYH-BOUILLE *et al.*[6].

(2) *Preparation of the cell walls*

Cell walls of *M. tuberculosis* BCG were prepared as described previously[1]. Cells of *M. phlei* and *C. fermentans* were disrupted using a Raytheon sonic oscillator of 10 kcycles; 5 g of cells (wet weight) were suspended in 25 ml of water and treated in the oscillator for 25 min. The cell walls were then obtained as described in ref. 1.

Cells of *M. kansasii* were first delipidated with acetone, alcohol–ether (1:1, v/v), chloroform and chloroform–methanol (2:1, v/v), extracted with water and dried with acetone. 1 g of delipidated cells was resuspended in 30 ml of water and sonicated for 25 min in the Raytheon 10-kcycles sonic oscillator. The unbroken cells were removed by centrifugation (15 min at 800 × *g*); the resulting supernatant was centrifuged at 27000 × *g* for 30 min. The pellet of cell walls thus obtained was then treated with trypsin and chymotrypsin and washed as described in ref. 1.

Cells of *S. albus* were disrupted with glass beads in a Bühler disintegrator (Tübingen, Germany). The walls were purified by differential centrifugation in water, heated for 7 min at 100°, treated at 37° for 2 h with trypsin (4 mg/ml) in 0.1 M phosphate buffer (pH 7.5) and finally washed several times with phosphate buffer and water.

(3) *Enzymes*

Chalaropsis endo-*N*-acetylmuramidase was a gift of Dr. N. A. HASH[7]. Streptomyces F_1 endo-*N*-acetylmuramidase and *N*-acetylmuramyl-L-alanine amidase were prepared as described by GHUYSEN *et al.*[8]. Streptomyces R_1 preparation, a mixture containing all the lytic enzymes secreted by *S. albus* G, was prepared as described by DIERICKX AND GHUYSEN[9].

Myxobacter AL_1 enzyme was prepared as described by ENSIGN AND WOLFE[10]; its activity was tested as described by ENSIGN AND WOLFE[11], but using a suspension of *Micrococcus lysodeikticus* cells instead of *Arthrobacter crystallopoietes*.

Helix pomatia gut juice, a commercial preparation, kindly given to us by the Industrie Biologique Française, Rue du Moulin de Gage, 92 Gennevilliers, France, was used as a source of *N*-acetylglucosaminidase.

Various commercial preparations of lysozyme, trypsin and chymotrypsin were used.

(4) *Mass spectrometry*

Mass spectrometry was performed with a AEI model MS9 mass spectrometer, after permethylation according to COGGINS AND BENOITON[12].

(5) *Analytical techniques*

The techniques used to study the chemistry of the cell walls and the chemical action of the enzymes are described by GHUYSEN *et al.*[13].

(6) *Chromatography*

Chromatograms were run on Whatman No. 1 paper in the following solvents: (1) butanol–acetic acid–water (5:1:2, by vol.), (2) isoamyl alcohol–pyridine–0.1 M HCl (2:2:1 ,by vol.).

(7) *Acylated amino sugars*

Acylated amino sugars and their oligomers were detected on paper chromatograms by the technique of SHARON[14].

RESULTS

(I) Enzymatic solubilization of the cell walls of M. kansasii, M. tuberculosis BCG, M. phlei and C. fermentans

The delipidated cell walls were first treated with 0.1 M HCl for 12 h at 60° to make them sensitive to lytic enzymes; following this treatment, most of the neutral sugars and mycolic acids could be removed by washing with water, acetone and ether.

The acid-treated walls were resuspended with a Potter grinder in 0.025 M final veronal buffer (pH 9.0) and Myxobacter AL_1 enzyme was added. On the average, 100 mg of walls were treated with 1400000 units of enzyme in a final volume of 30 ml.

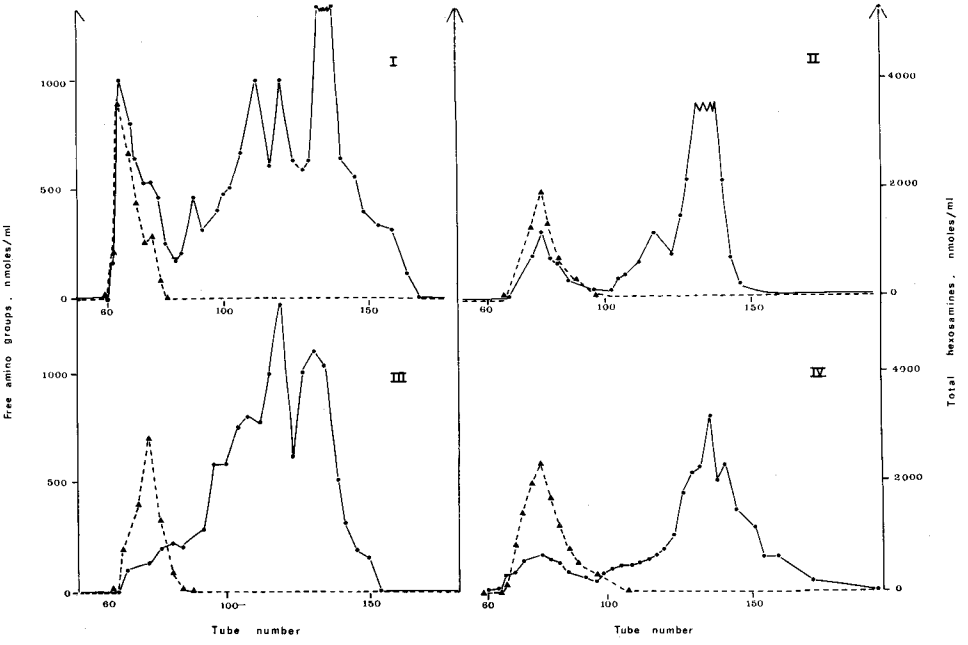

Fig. 1. Filtration of the lysate of 100 mg of cell walls by Myxobacter AL_1 enzyme on a Sephadex G-25 column (h = 75 cm, ϕ = 2.5 cm; V_o = 135 ml; $V_o + V_i$ = 350 ml) in 0.1 M acetic acid. Volume of the fractions, 2 ml. ●—●, free amino groups; ▲---▲, total hexosamines. (I) *M. kansasii*. (II) *M. tuberculosis* BCG. (III) *M.phlei*. (IV) *C. fermentans*.

After 24 h of incubation at 37°, the incubation mixture was centrifuged. The extent of solubilization of the cell wall peptidoglycan, estimated on the basis of the total hexosamines present in the pellet and in the supernatant (after acid hydrolysis by 4 M HCl for 6 h at 100°) was 85 % for *M. kansasii*, 65 % for *M. tuberculosis* BCG, 80 % for *M. phlei* and 93 % for *C. fermentans*.

(*II*) *Fractionation of the lysates*

The supernatants were concentrated to 3 ml in a rotating evaporator and filtered through a column of Sephadex G-25. The elution patterns are given in Fig. 1. For each column, the last peak corresponds to amidated tetrapeptides Ala–Glu–Dpm*–Ala and tripeptides Ala–Glu–Dpm** and the intermediary peaks to more complex peptides which have not yet been studied. The first peak contains the whole of the hexosamines. It is essentially composed of the glycan moiety of the peptidoglycan, to which are still attached some peptides, phosphorus and neutral sugars[1].

(*III*) *Isolation of the disaccharides from the glycans of the three Mycobacteria*

An aliquot (40 μmoles of total hexosamines) of the glycan-containing fractions of Columns I, II and III of Fig. 1 (first peak) was lyophilized and redissolved in 40 ml of 0.03 M ammonium acetate (pH 6.3); lysozyme (1 mg) was added and incubation took place at 37° for 20 h. Under these conditions, enzyme action was maximal as judged from the increase in reducing groups and the appearance of material giving the Morgan–Elson reaction after 30 min of heating in alkaline buffer[13].

The lysozyme-treated glycans were then filtered on a Sephadex G-15 column (Fig. 2).

The material contained in the last peak of each column was chromatographed in Solvent 1, and revelation with Sharon's reagent showed a single component, whose R_{GlcNAc} of 0.70 is that of the disaccharide *N*-acetyl glucosaminyl-1 → 4-*N*-glycolylmuramic acid[2]. Considering the sensitivity of the revelation procedure, it can be stated that if the classical disaccharide *N*-acetylglucosaminyl-1 → 4-*N*-acetylmuramic acid is present, its concentration is less than 10 % of the preceding disaccharide. Moreover, treatment of the disaccharides with *Helix pomatia* gut juice (0.1 μmole of disaccharide and 2 μl of *Helix pomatia* gut juice in 0.2 ml of 0.1 M ammonium acetate of pH 5.0 for 20 h at 37°) gave rise to two compounds revealed by Sharon's reagent after chromatography in Solvent 1: the R_{GlcNAc} of these compounds, 1 and 1.36, respectively, is identical with that of *N*-acetylglucosamine and *N*-glycolylmuramic acid[2].

Paper chromatography of the material contained in the preceding peak of each column showed, after revelation with Sharon's reagent, a main compound tentatively identified as a dimer of the disaccharide described above[1,2].

(*IV*) *Preparation of the disaccharide of S. albus G*

Cell walls were solubilized with Chalaropsis enzyme (in 0.01 M acetate buffer of pH 4.5; ratio enzyme to substrate = 1:100; 24 h at 37°) which degrades the glycan into disaccharide peptide units. As judged by the Morgan–Elson reaction after 30 min of heating in borate buffer, 85 % of the hexosamines were transformed into disaccha-

* Dpm, diaminopimelic acid.

** J. WIETZERBIN-FALSZPAN AND I. AZUMA, unpublished experiments.

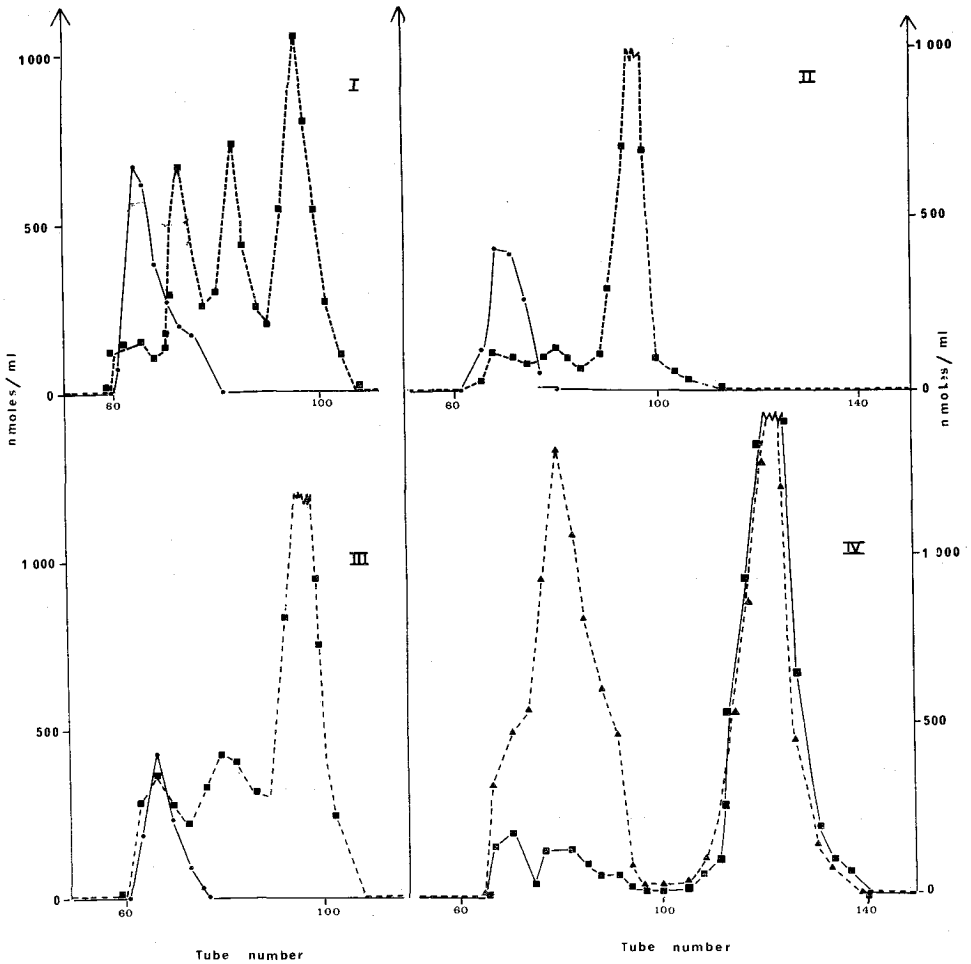

Fig. 2. I–III. Filtration of the lysozyme hydrolysate of glycan moieties (40 μmoles of total hexosamines) from *M. kansasii* (I), *M. tuberculosis* BCG (II) and *M. phlei* (III) on a Sephadex G-15 column (h = 75 cm, ϕ = 2.5 cm, V_o = 130 ml, $V_o + V_i$ = 320 ml) in 0.1 M acetic acid. Volume of the fractions, 2 ml. ●—●, free amino groups; ■—■, Morgan–Elson-reactive products after 30 min of heating at 100° in alkaline buffer, expressed as *N*-acetylglucosamine[13]. IV. Filtration on the same Sephadex G-15 column of the hydrolysate by R_1 enzymes of the glycan moiety (40 μmoles of total hexosamines) from *C. fermentans*. ■—■, Morgan–Elson-reactive products after 7 min of heating at 100° in alkaline buffer, expressed as *N*-acetylglucosamine[13]; ▲---▲, total hexosamines.

ride-peptides. After further treatment with *S. albus* G *N*-acetylmuramyl-L-alanine amidase (in 0.03 M acetate buffer of pH 5.5), about 300 nmoles of N-terminal L-alanine were present per mg of cell walls (*S. albus* G cell walls contain 550 nmoles of glutamic acid per mg). By gel filtration of the degradation products thus obtained on two Sephadex G-50 and G-25 columns connected in series[6], in 0.1 M LiCl, a fraction of K_D = 0.75, composed of disaccharide units, was separated; 270 nmoles of disaccharides were obtained per mg of cell walls. Comparing these data with the glutamic acid content of the cell wall and assuming that there is a disaccharide unit per glutamic acid residue, the yield of disaccharide is 50 %.

The disaccharide-containing fraction gave a single spot of $R_{GlcNAc} = 1$ by paper chromatography in Solvent 1 and revelation by Sharon's reagent; it thus seems to be the classical disaccharide *N*-acetylglucosaminyl-1 → 4-*N*-acetylmuramic acid. (For the reasons stated above, if the disaccharide containing *N*-glycolylmuramic acid was present, its concentration would be less than 10%.) *Helix pomatia* gut juice hydrolyzed it into two components of $R_{GlcNAc} = 1.92$ and 1 which are the R_{GlcNAc} values of *N*-acetylmuramic acid and *N*-acetylglucosamine, respectively.

The disaccharide of *S. albus* G thus seems to be the classical disaccharide *N*-acetylglucosaminyl-1 → 4-*N*-acetylmuramic acid.

(V) Mass spectrometry of the dissaccharides

The various disaccharides were analyzed by mass spectrometry after permethylation.

The permethylated disaccharides of *M. kansasii*, *M. tuberculosis* and *M. phlei* have the same mass spectrum as the disaccharide *N*-acetylglucosaminyl-1 → 4-*N*-glycolylmuramic acid obtained from *M. smegmatis*[2].

The mass spectrum of the permethylated disaccharide of *S. albus* G is identical with that of the disaccharide *N*-acetylglucosaminyl-1 →4-*N*-acetylmuramic acid obtained from *Micrococcus lysodeikticus*[2].

As both spectra have already been published[2], they will not be reproduced here.

(VI) Isolation of the monosaccharide residues of the glycan of C. fermentans

The glycan of *C. fermentans*, isolated after Myxobacter degradation of the walls (first peak of Column IV in Fig. 1, see paragraph II), is not sensitive to any of the following muramidases: lysozyme, Chalaropsis enzyme and *S. albus* G F_1 enzyme; the reason for this resistance to muramidases is not yet understood. It must be noted, however, that, perhaps due to an insufficient treatment of the cell walls with diluted HCl, the glycan-containing peak of the effluent of the column of Fig. 1 shows a ratio of neutral sugars to amino sugars of four: this might be a reason for the resistance of the glycan to muramidases (in the corresponding peak in Columns I, II and III this ratio is smaller than one).

The hexosamine constituents of the glycan were identified as glucosamine and muramic acid after total acid hydrolysis (4 M HCl, 4 h, 100°) by chromatography in Solvent 2, electrophoresis in borate buffer using the technique described by MALEY AND MALEY[15] and chromatography in a Technicon amino acid analyzer.

Treatment of an aliquot of this fraction with the *S. albus* G R_1 preparation (40 μmoles of total hexosamines were incubated with 45 mg of lyophilized R_1 in 60 ml of 0.066 M phosphate buffer (pH 6.3) for 24 h at 37°) led to the appearance of 60% of the hexosamines as free *N*-acylhexosamines (as determined by the Morgan–Elson reaction after 7 min of heating in alkaline buffer).

The incubation mixture was concentrated to 3 ml in a rotating evaporator and filtered through the column of Sephadex G-15 described in paragraph III (Fig. 2). The last peak contains all the material giving the Morgan–Elson reaction after 7 min of heating in alkaline buffer. Chromatography of the material of this peak in Solvent 1 and revelation by the technique of Sharon show that it contains two *N*-acylhexosamine constituents whose R_{GlcNAc} of 1 and 1.92 are identical with that of *N*-acetylglucosamine and *N*-acetylmuramic acid, respectively.

Thus, at least 60 % of the glycan of *C. fermentans* is made up of *N*-acetylglucosamine and *N*-acetylmuramic acid.

DISCUSSION

Although our study includes only a few species of Actinomycetales and a single species of Corynebacteriaceae, some conclusions can be drawn.

(1) *N*-Glycolylmuramic acid has been found in the four species of Mycobacterium so far studied: thus, it might be a new criterion of taxonomic importance for this genus. GUINAND AND MICHEL[16] have also found *N*-glycolylmuramic acid in one species of Nocardia: this stresses again the similarity between the genera Mycobacterium and Nocardia.

(2) *N*-Glycolylmuramic acid was not found in the cell walls of *S. albus* G nor *C. fermentans*. The presence of *N*-glycolylmuramic acid is thus, for the moment, restricted to the genera Mycobacterium and Nocardia of the Actinomycetales.

(3) It was first thought that the glycan moiety of the peptidoglycan was identical in all bacteria, the only known variation being the presence of an *O*-acetyl substituent on C-6 of *N*-acetylmuramic acid[3]. The present study, as well as the recent demonstration of the presence of muramic acid lactam in spores of *Bacillus subtilis*[18,19] and *Bacillus sphaericus*[20] shows that, in fact, variation can also occur in the glycan part of the peptidoglycan.

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