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Presence of fucosamine in teichuronic acid of the alkalophilic *Bacillus* strain C-125

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Cell walls of the alkalophilic *Bacillus* strain C-125 are composed of γ-peptidoglycan, teichuronic acid and a polymer of glucuronate and glutamate. An amino sugar that was a main component of the teichuronic acid did not correspond to any of the commercially available hexosamines. The amino sugar was purified into crystalline form from the hydrolysate of the teichuronic acid by ion-exchange chromatography and then partition chromatography on a cellulose column. The amino sugar was identified as D-fucosamine (2-amino-2,6-dideoxy-D-galactose) by 400 MHz n.m.r. spectrometric analysis, measurement of optical rotation and elemental analysis.

INTRODUCTION

Previously we have analysed the cell walls of alkalophilic strains of *Bacillus* (Aono & Horikoshi, 1983). The amounts of acidic compounds (e.g. uronic acids and acidic amino acids) found in the cell walls of group 2 strains were enhanced when the strains were cultured at an alkaline pH. This result suggested that the acidic polymers in the outermost layers in the group 2 bacteria should have a function in supporting growth at an alkaline pH. The acidic polymers were extracted from *Bacillus* sp. strain C-125 (classified in group 2) and separated chromatographically into two fractions (Aono, 1985). One is an unknown polymer composed of mainly glucuronic acid and glutamic acid. The other is composed of glucuronic acid, galacturonic acid and an unidentified compound. This compound was ninhydrin-positive, reducing-ability-positive and Elson–Morgan-reaction-positive after separation by cellulose t.l.c. Therefore the compound was thought to be some kind of amino sugar, and the polymer containing this amino sugar was assumed to be a teichuronic acid. This amino sugar could not be identified by cellulose t.l.c. as one of the usually available amino sugars from commercial sources.

The hydrochloride salt of the amino sugar was purified into crystalline form from a hydrolysate of the teichuronic acid. A preliminary analysis of the amino sugar suggested that it was a hexosamine. Analysis of the 400 MHz n.m.r. spectrum and measurement of the optical rotation revealed that the amino sugar was D-fucosamine. This paper presents data on purification and characterization of the amino sugar.

MATERIALS AND METHODS

Purification of the amino sugar

The teichuronic acid was prepared from cell walls of the alkalophilic *Bacillus* strain C-125 by extraction with 5% (w/v) trichloroacetic acid as described previously (Aono, 1985). This organism, which is a producer of extracellular xylanase (Honda *et al.*, 1985), has been deposited as FERM 7344 at Fermentation Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry, Tsukuba, Japan. A 60 mg portion of the teichuronic acid was hydrolysed in 5 ml of 4 M-HCl at 100 °C for 5 h. This hydrolysate was concentrated to dryness in a rotary evaporator at 43 °C. The residue was dissolved in 5 ml of deionized water and filtered. The filtrate was passed through a column (0.5 cm × 6 cm) of Dowex 1-X4 (200–400 mesh; Cl^- form). The column was washed with 50 ml of deionized water. The effluent and wash were combined and applied to a column (1 cm × 11 cm) of Dowex 50W-X4 (200–400 mesh; H^+ form). The column was washed with 50 ml of deionized water and then 100 ml of 0.05 M-HCl at a flow rate of 45 ml/h. A linear gradient of 0.05–0.8 M-HCl (200 ml) was applied to the column at a flow rate of 14 ml/h. Fractions (2 ml) containing amino sugar were pooled, concentrated to dryness in a rotary evaporator and dried *in vacuo* over NaOH. The residue was dissolved in 0.2 ml of water and loaded on to a column (2.5 cm × 55 cm) of Whatman CC-31 cellulose that had been equilibrated with ethyl acetate/pyridine/water/acetic acid (5:5:3.1, by vol.). The column was eluted with 600 ml of the same solvent. Fractions (5 ml) containing the amino sugar were pooled, washed with diethyl ether and concentrated to dryness. The residue was redissolved in a small amount of water. The amino sugar was obtained as crystals on the addition of acetone. Crystallization was repeated once more. The crystal was washed with acetone and then diethyl ether, and dried.

Structural analysis of the amino sugar

A 7 mg portion of the amino sugar purified as described above was dissolved in 0.7 ml of ^2H_2O. P.m.r. spectra were measured in a JEOL JHN-FX 400 FT n.m.r. spectrometer (400 MHz). Tetramethylsilane was used as an external standard.

The sample (6.3 mg) was dissolved in 1 ml of distilled water. The optical rotation of the solution was measured at 25 °C in a 10 cm-pathlength quartz cell in a Perkin–Elmer model 241 MC polarimeter.

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Chemical analysis

Amino sugar was determined by the Elson–Morgan reaction as described previously (Aono, 1985).

Avicel cellulose t.l.c. of the sample was carried out as described previously (Aono & Horikoshi, 1983), with the solvents ethyl acetate/pyridine/water/acetic acid (5:5:3:1, by vol.) and phenol/aq. 1% (v/v) NH_3 (2:1, v/v).

RESULTS AND DISCUSSION

Purification of the amino sugar

The teichuronic acid dealt with in this paper is composed of glucuronic acid, galacturonic acid and *N*-acetylfucosamine (Aono, 1985). Recovery of fucosamine was about 90% under the conditions of hydrolysis, whereas both the uronic acids were destroyed to form a dark-brown insoluble material and were removed by filtration. Fucosamine hydrochloride was eluted from the column of Dowex 50 at 0.18–0.23 M-HCl (Fig. 1). Cellulose t.l.c. revealed that this fraction contained a small amount of other ninhydrin-positive compounds but no uronic acids. Fucosamine (60 μmol) was purified by cellulose column chromatography and eluted at 340–390 ml of the solvent.

The eluent of the amino sugar was concentrated and gave a small volume of pale-yellow solution. The yellowish contaminant was precipitated by the addition of acetone to a final concentration of 80% (v/v) and removed by centrifugation. Acetone was added to the colourless supernatant to a final concentration of 98% (v/v). Fucosamine hydrochloride was precipitated as white needle-like crystals.

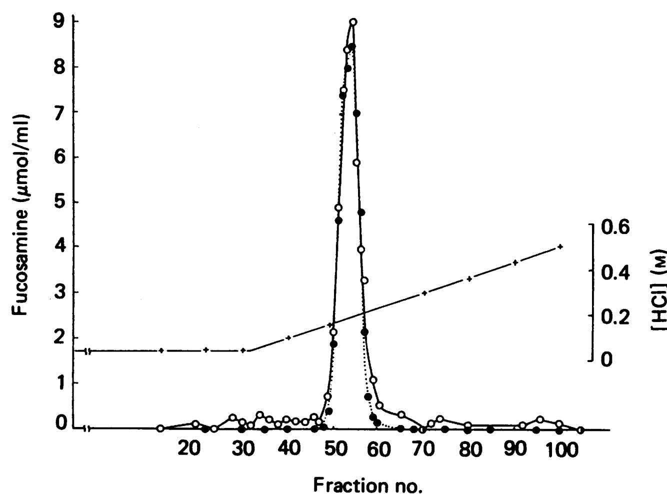

Fig. 1. Cation-exchange chromatography of the hydrolysate of the teichuronic acid

Acidic hydrolysate of the teichuronic acid after removal of HCl was passed through a small column of Dowex 1 (Cl^- form) and loaded on a column (1 cm × 11 cm) of Dowex 50W-X4 (200–400 mesh; H^+ form). The column was eluted with deionized water, 0.05 M-HCl and then a linear gradient of HCl as described in the Materials and methods section. Fractions (2 ml) were collected and assayed for amino sugars (●) by the Elson–Morgan reaction and amino group (○) by the ninhydrin method with glucosamine as a reference standard. Concentration of HCl (+) was determined by titration.

Identification of the amino sugar as D-fucosamine

Fig. 2 shows the p.m.r. spectrum of the amino sugar measured at 400 MHz. Doublet signals for methyl groups were found at 1.16 and 1.21 p.p.m. relative to tetramethylsilane. Three doublet signals and six quartet signals for methyne groups were found between 3.0 and 5.4 p.p.m. These signals were assigned, as shown in Table 1, with their multiplicities and spin-coupling constants as shown in Table 2. This assignment was confirmed by decoupling experiments (results not shown). Moreover, the decoupling experiment revealed that a signal for H-1 of the β-anomer had been hidden by a large signal for $^1H^2HO$ and was present at the region 4.7–4.8 p.p.m. The doublet signals for H-4 and the quartet signal for H-5 of the α-anomer were broader than the other signals. These were caused by the small spin-coupling between H-4 and H-5. The value of the coupling constant $J_{4,5}$ was not determined accurately and was estimated to be below 0.5 Hz.

These data suggested, on the basis of the rule of Karplus (1959), that the basal conformation of protons and carbons of the sample should be as shown in Fig. 3. This structure is identical with that of fucose. The p.m.r. spectrum of authentic D-fucose was recorded under the same conditions to compare it with that of the sample. The values for chemical shift and spin-coupling constant obtained by measuring the n.m.r. spectrum of fucose were identical with those previously reported for fucose measured at 300 MHz in ^2H_2O (De Bruyn *et al.*, 1976). Fucose and the sample had similar values for chemical shift and spin-coupling constant except for the chemical-shift values of H-2, as shown in Table 2. These chemical-shift values of H-2 of the sample were 0.4 p.p.m. lower than those of fucose. This difference in the chemical-shift values of H-2 suggested that the C-2 position of the sample should be amidated. Therefore the sample could be identified as 2-amino-2,6-dideoxy-galactose, namely fucosamine.

This suggestion was supported by the fact that the amino sugar was Elson–Morgan-reaction-positive. The following elemental analysis was shown: C, 35.13; H, 6.77; N, 6.82% (calc. for fucosamine hydrochloride, $C_6H_{14}ClNO_4$: C, 36.10; H, 7.07; N, 7.02%). The isomerism of fucosamine purified from the teichuronic acid was determined by measurement of the optical rotation of the sample: $[\alpha]_D^{25}$ +89.0° (*c* 0.62% in water) was found. This value was in good agreement with the value +91.0° previously reported for synthetic D-fucosamine hydrochloride (Zehavi & Sharon, 1964). These results confirmed that the amino sugar discussed in this paper was D-fucosamine hydrochloride.

As far as we know, fucosamine is not available commercially and its n.m.r. spectrum has not been measured. We had to purify large amounts of the amino sugar in order to analyse it structurally. Although fucosamine appears rarely as a structural component of cell walls, D- and L-forms of fucosamine are known to occur in antigenic capsular polysaccharides or lipopolysaccharides of a number of bacteria. The D-form of fucosamine is known to be present in the polysaccharides of *Bacillus licheniformis*, *Bacillus subtilis* (Sharon *et al.*, 1964) and *Bacillus cereus* (Wheat *et al.*, 1964), the lipopolysaccharides of *Chromobacterium violaceum*

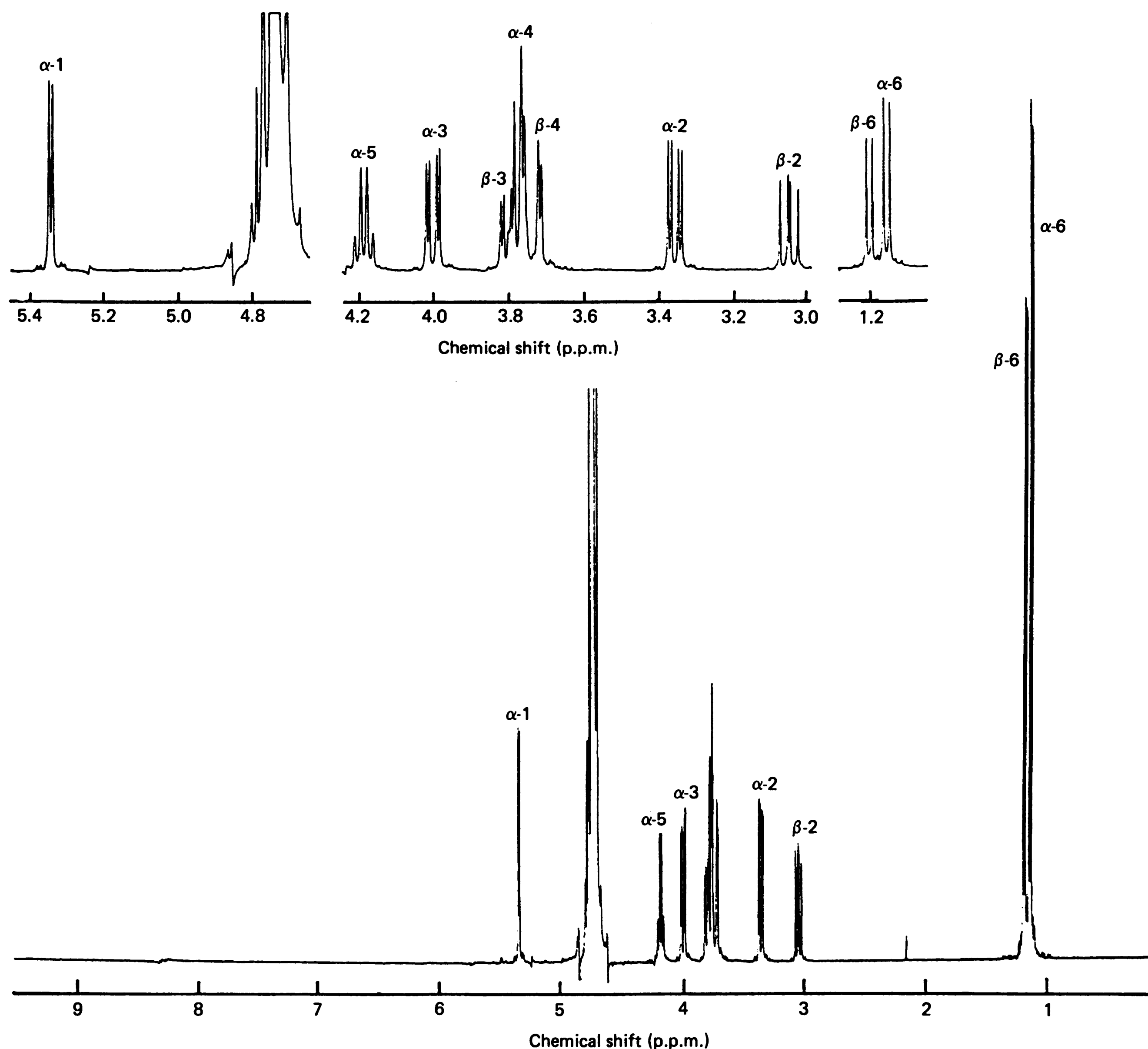

Fig. 2. 400 MHz p.m.r. spectrum of the amino sugar hydrochloride purified from the teichuronic acid

Table 1. Assignment of chemical-shift values of the amino sugar purified from the teichuronic acid

Each signal found in Fig. 2 was assigned with its multiplicities and spin-coupling constant. The assignment was certified by the double-resonance method. Chemical-shift values of authentic D-fucose were obtained from the n.m.r. spectrum measured under the same conditions as were those of the sample.

Sugar	Chemical shift (p.p.m.)					
	H-1	H-2	H-3	H-4	H-5	H-6
α-Anomer of the sample	5.34	3.36	4.00	3.76	4.19	1.16
β-Anomer of the sample	4.7–4.8	3.05	3.80	3.72	3.78	1.21
α-D-Fucose	5.21	3.77	3.86	3.81	4.21	1.21
β-D-Fucose	4.56	3.45	3.64	3.75	3.82	1.25

Table 2. Spin-coupling constants of fucosamine purified from the teichuronic acid and of authentic D-fucose

Sugar	Spin-coupling constant (Hz)				
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6}$
α-Anomer of the sample	3.79	10.9	3.45	< 0.5	6.72
β-Anomer of the sample	8.62	10.9	3.45	< 0.5	6.72
α-D-Fucose	3.79	10.3	3.45	0.69	6.55
β-D-Fucose	7.93	10.2	3.45	0.69	6.55

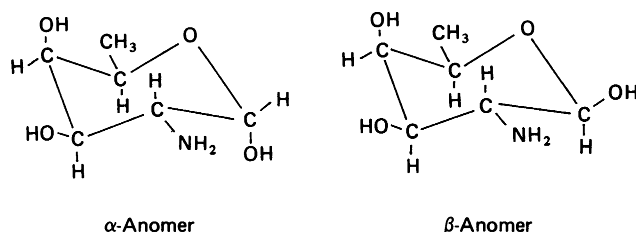

Fig. 3. Proposed structure for the amino sugar purified from the teichuronic acid

(Crumpton & Davies, 1958) and *Pseudomonas aeruginosa* (Knirel *et al.*, 1982) and the capsular polysaccharides of *Staphylococcus aureus* (Liau *et al.*, 1974). The L-form of the sugar is present in the antigenic capsular polysaccharides of *Bacteroides fragilis* (Kasper *et al.*, 1983), *Streptococcus pneumoniae* (Daoust *et al.*, 1981) and *Pneumococcus* type V (Barker *et al.*, 1961). These antigenic capsular polysaccharides are well known to be strain-specific structures and to differ in different strains belonging to even the same species. Fucosamine is not always present in any of the strains of the bacteria mentioned above. For example, D-fucosamine has been found in *Staphylococcus aureus* M strain and a mutant T derived from its strain H, but has not been found in Smith diffuse strain (Haskell & Hanessian, 1964; Wu & Park, 1971; Liau *et al.*, 1974; Fournier *et al.*, 1984). Therefore it should be not very significant taxonomically that D-fucosamine exists in the cell walls of the alkalophilic *Bacillus* strain C-125. D-Fucosamine, however, was extracted from the polysaccharides of *Bacillus cereus*, *Bacillus subtilis* and *Bacillus licheniformis* (Sharon *et al.*, 1964; Wheat *et al.*, 1964). These facts suggest that *Bacillus* strain C-125 should belong to one of these or related species.

Type 8 capsular polysaccharide of *Staphylococcus aureus* was reported to contain unidentified isomers of *N*-acetylfucosamine and *N*-acetylgalactosaminouronic acid, and was suggested to be similar to teichuronic acid in composition (Fournier *et al.*, 1984). The structures of the polysaccharides extracted from the species of *Bacillus* mentioned above are not known, though they might be teichuronic acids of some kind. However, the presence of D-fucosamine in teichuronic acids has not been clearly indicated. D-Fucosamine was subsequently recovered from the hydrolysate of a kind of teichuronic acid and proved to be a main component thereof (Aono, 1985). It may be noted that the amount of the teichuronic acid containing D-fucosamine as a main constituent is enhanced in the cell walls of the organism grown at an alkaline pH.

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