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IDENTIFICATION OF 6-DEOXY-D-TALOSE IN AN EXTRACELLULAR POLYSACCHARIDE PRODUCED BY *Butyrivibrio fibrisolvens* STRAIN X6C61

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ABSTRACT

An extracellular polysaccharide made by the obligately anaerobic ruminal bacterium *Butyrivibrio fibrisolvens* was purified by anion-exchange chromatography and found to contain an unknown carbohydrate component. This unknown component was purified to homogeneity by preparative paper chromatography and column chromatography on Dowex 50W-X4-immobilized calcium ions. Reaction in the cysteine–sulfuric acid test suggested that the unknown component was a 6-deoxyhexose, a supposition that was confirmed by electron impact and chemical ionization mass spectrometric studies of alditol acetate derivatives prepared from the unknown sugar. On the basis of results obtained by analytical paper chromatography with three different solvent systems, the unknown was tentatively identified as 6-deoxytalose. The unknown was confirmed as being 6-deoxy-D-talose by g.l.c. analysis of the acetylated glycosides prepared from it and chiral (−)-2-octanol. These were compared with the corresponding sets of diastereometric glycosides of 6-deoxy-D- and -L-talose.

INTRODUCTION

The rumen, or forestomach, of ruminant animals contains a large and metabolically diverse assortment of anaerobic microbial species. Collectively, these species hydrolyze the wide variety of plant polysaccharides ingested by these animals, and ferment their constituent sugars[1].

Butyrivibrio fibrisolvens is one of the ruminal bacterial species most commonly isolated, with various strains displaying considerable versatility in their ability to hydrolyze and ferment plant polysaccharides[2]. Interestingly, it has been reported that 33 of 37 strains of *B. fibrisolvens* actually produce significant amounts of extracellular polysaccharides (EPS) when grown in pure culture[3], although the function and amount of these EPS made by this organism *in rumino* are still

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unknown. An intriguing question is: how do these EPS survive in an environment that contains[1] a heterogenous assortment of polysaccharide-hydrolyzing enzymes? Assuming that these EPS do indeed serve some function(s) for *B. fibrisolvens,* it would be highly advantageous to prevent somehow, or minimize, their degradation by other ruminal micro-organisms. One possible means of accomplishing this might be to incorporate rare or unusual monosaccharides into their structures such that recognition and hydrolysis, by other enzymes is prevented. Indeed, the EPS from eighteen strains of *B. fibrisolvens* contain substantial amounts of 4-*O*-[1-carboxyethyl]-D-galactose, an unusual acidic sugar not yet found elsewhere in Nature[3,4]. Similarly, 12 strains of *B. fibrisolvens* produce EPS that contain L-altrose[3,5], another sugar thus far uniquely found in the EPS made by isolates classified in the genus *Butyrivibrio*.

Stack *et al.*[6] reported that the EPS made by *B. fibrisolvens* strain X6C61 can be separated into two distinct polysaccharides by anion-exchange chromatography. The major polysaccharide, EPS-II, was found to contain L-iduronic acid. Although commonly found in certain mammalian connective-tissue polysaccharides[7], L-iduronic acid has only rarely been found in microbial polysaccharides[8,9], and never in plants. The other EPS, designated EPS-I, did not contain L-iduronic acid, but did contain an additional unidentified carbohydrate component[6]. The present study describes the isolation and characterization, and identification as 6-deoxy-D-talose of this component.

EXPERIMENTAL

Organism, and conditions of growth. — *Butyrivibrio fibrisolvens* strain X6C61, used for all studies, was originally isolated from the rumen of a sheep by N. O. van Gylswyk, National Chemical Research Laboratory, Pretoria, Republic of South Africa, using a xylan-containing medium. Cultures were routinely grown to stationary phase at 37° on 1% D-glucose in the chemically defined medium of Cotta and Hespell[10].

Purification and fractionation of EPS. — Crude EPS was obtained from concentrated, cell-free culture supernatant liquors following extraction with phenol as previously described[5]. Crude EPS was separated by anion-exchange chromatography on DEAE-Sephadex A-25 (Pharmacia, Piscataway, NJ) into two components, previously designated EPS-I and EPS-II, with a linear gradient of buffered NaCl (0–2.0M)[6]. Purified EPS-I was dialyzed against water at 4°, lyophilized, and used for all subsequent studies.

Purification of the unknown component from EPS-I. — EPS-I was hydrolyzed under N_2 in 2M trifluoroacetic acid (TFA) at 20 mg/mL for 1 h at 110°, and spotted directly onto sheets (23 × 57 cm) of Whatman No. 1 filter paper for preparative paper chromatography (p.c.). Sheets were developed in the descending mode for 17–20 h with 10:3:3 (v/v) 1-butanol–pyridine–water as the solvent. After thorough drying, a thin strip was cut from the center of the sheet and stained for carbohydrate

by means of the alkaline silver nitrate dip method[11]. The corresponding sections of the sheet containing the unknown were cut out, eluted with water, and the eluate was lyophilized. The yellow, syrupy material was redissolved in the minimal volume of water, and 0.2 mL was applied to the top of a column (1 × 28 cm) of Dowex AG 50W-X4 resin in the calcium form, prepared as described by Angyal *et al.*[12]. The column was eluted with water at 0.3 mL/min, the eluate being monitored with a refractive index detector. Fractions (2.0 mL) were collected, and analyzed for carbohydrate by g.l.c., after reduction and acetylation of small volumes of every third fraction. Fractions containing only the unknown compound were identified, pooled, and lyophilized.

Analytical paper chromatography. — A preliminary identification of the unknown sugar was made on the basis of its chromatographic behavior following analytical p.c. on sheets of Whatman No. 1 paper with solvents IV (1-butanol saturated with water), V (2-butanone saturated with water), and VI [4:1 (v/v) 1-butanol–ethanol, saturated with borate buffer at pH 8.9] of Krauss *et al.*[13]. In all cases, chromatography was performed in the descending mode, with standards corresponding to L-rhamnose, L-fucose, and 6-deoxy-D-glucose (Sigma Chemical Co., St. Louis, MO) also spotted as references.

G.l.c.–m.s. studies. — Small aliquots (0.2 mg) of the purified unknown were converted into alditol acetates by using both $NaBH_4$ and $NaBD_4$ to reduce the aldehydic group. Both electron impact (e.i.) and chemical ionization (c.i.) mass spectra were obtained from each preparation with a Finnegan 4535/TSQ g.l.c.–m.s. equipped with a DB-225 fused-silica capillary column (0.25 μm × 30 m; J & W Scientific, Rancho Corodova, CA). Injections were split 15 to 1, and runs were isothermal at 210°. Eluting compounds were ionized at 70 eV for e.i. spectra. Spectra were compared to those obtained from alditol acetates prepared from L-rhamnose and D-glucose.

Determination of the absolute configuration of the unknown. — The absolute configuration of the unknown was deduced by analyzing the pattern of peaks shown by the mixture of acetylated glycosides prepared from the unknown and chiral (−)-2-octanol, using the procedure of Leontein *et al.*[14] as modified by Stack and Ericsson[15]. The glycosides were analyzed by g.l.c. isothermally at 210°, using a Hewlett–Packard 5890A gas–liquid chromatograph equipped with the same DB-225 capillary column already mentioned. Results were compared with those obtained from similarly derivatized L-rhamnose, L-fucose, 6-deoxy-D-glucose, 6-deoxy-D-allose, 6-deoxy-D-talose, and 6-deoxy-L-talose. Authentic samples of the last three compounds were kindly provided by Dr. T. Reichstein, University of Basel, Basel, Switzerland.

Miscellaneous methods. — Alditol acetates of various EPS samples or column fractions were prepared essentially as described by Albersheim *et al.*[16]. The color reaction of the unknown was also investigated, using the cysteine–sulfuric acid reaction described by Dische[17], and compared to that obtained from other selected sugars.

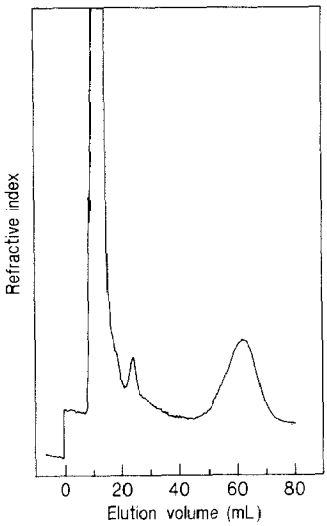

Fig. 1. Elution profile of the p.c.-eluted unknown eluted with water from a column (1 × 28 cm) of Dowex 50W-X4 in the calcium form. Fractions 25–35 (corresponding to 50–70 mL elution volume) were pooled, lyophilized, and designated as the purified unknown component.

RESULTS AND DISCUSSION

Stack *et al.*[6] reported that the crude EPS fraction obtained from *B. fibrisolvens* strain X6C61 could be separated by anion-exchange chromatography into two distinct polysaccharide fractions. The first of these, designated EPS-I, did not contain any uronic acids (or other acidic sugars) and did not bind to DEAE-Sephadex at pH 7. The relative neutral sugar composition of EPS-I deduced from alditol acetate data was reported as mannose (0.12), glucose (0.67), galactose (1.00), and rhamnose plus the unknown (1.71). The alditol acetates of rhamnose and the unknown were only partially resolvable by capillary g.l.c., such that even a preliminary characterization of the unknown (by g.l.c.–m.s.) was not attemped in this earlier study[6].

The unknown could, however, be readily separated from rhamnose and the other carbohydrates present in EPS-I by p.c. using the solvent system 10:3:3 (v/v) 1-butanol–pyridine–water; its mobility in this system was found to be quite high (R_{Glc} 2.62), even higher than that of rhamnose (R_{Glc} 2.13; data not shown). Therefore, hydrolyzates of EPS-I were fractionated by preparative p.c. using this solvent in order to obtain crude preparations of the unknown sugar free of all other carbohydrate components.

The unknown compound was obtained in pure form following chromatography of the p.c.-purified material on Dowex 50W-X4 in the calcium form[12], as shown in Fig. 1. The unknown interacted very strongly with the Dowex-immobilized calcium ions and was eluted in a relatively broad peak between 50 and

70 mL. In contrast, L-rhamnose was eluted from this column between 17 and 23 mL (data not shown). Angyal *et al.*[12] showed that elution time from these columns correlates directly with the ability of the polyol to form complexes with the immobilized calcium ions. The most stable polyol–calcium complexes result when three hydroxyl groups are equidistant from the central complexing cation. These conditions are most closely approximated with the sequence of an axial, an equatorial, and an axial hydroxyl group on a six-membered ring. The $^4C_1$ conformer of 6-deoxytalose contains this precise sequence in both anomers, which probably accounts for its relatively late elution from this column.

The spectrum of the chromophore obtained after reaction of the purified unknown with cysteine–sulfuric acid[17] was obtained, and compared to similar spectra obtained both from selected hexose and 6-deoxyhexose standards. As shown in Fig. 2, the chromophore obtained from the standard 6-deoxyhexoses and the unknown had a relatively sharp absorption band, with λ_{max} at 400 nm. The spectrum of the chromophore obtained from glucose and mannose showed a significantly broader absorption band, with λ_{max} at 409 and 407 nm, respectively. An even broader seconary absorption band centered between 500 and 550 nm was also obtained from these two hexoses, but not from the 6-deoxyhexoses nor from the unknown. These results gave preliminary indications that the unknown sugar might be a 6-deoxyhexose.

Comprehensive e.i./c.i. m.s. studies of the alditol acetates prepared from the purified unknown confirmed that the unknown was a 6-deoxyhexose. The $NaBH_4$-reduced unknown gave an M + 1 peak at *m/z* 377; the corresponding $NaBD_4$-reduced compound gave an M + 1 peak at *m/z* 378 (data not shown). The e.i. spectrum of the $NaBH_4$-reduced compound gave primary fragments diagnostic for a 6-deoxyhexose at *m/z* 87 and 159, and also exactly matched the e.i. spectrum of standard rhamnitol pentaacetate. The primary fragments at *m/z* 87 and 159 were

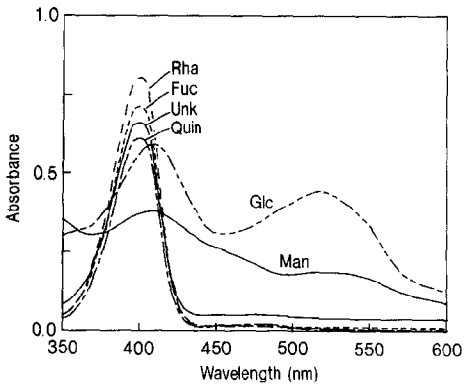

Fig. 2. Absorption spectra of the chromophore obtained from the cysteine–sulfuric acid reaction with L-rhamnose (Rha), L-fucose (Fuc), 6-deoxy-D-glucose (Quin), D-glucose (Glc), D-mannose (Man), and the purified unknown component (Unk) isolated from EPS-I.

TABLE I

COMPARISON OF THE R_{Rha} VALUES OF THE UNKNOWN WITH THE R_{Rha} VALUES OF STANDARD 6-DEOXYHEXOSES

	Sugar	*Solvent systems*[a]					
		IV		*V*		*VI*	
		Obs.[b]	*Pub.*[c]	*Obs.*	*Pub.*	*Obs.*	*Pub.*
1	Rhamnose	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**	**1.00**
2	Fucose	0.73	0.67	0.57	0.68	0.36	0.48
3	6-Deoxyglucose	0.94	0.99	0.80	0.98	0.66	0.78
4	6-Deoxyallose		0.91		1.38		0.78
5	6-Deoxygulose		1.02		0.98		0.78
6	6-Deoxyaltrose		1.13		1.84		0.42
7	6-Deoxyidose		1.28		2.00		0.36
8	6-Deoxytalose		1.37		1.84		0.72
9	Unknown	1.36		1.89		0.78	

[a]Solvent systems were described by Krauss *et al.*[13]. [b]"Obs." refers to the R_{Rha} values obtained in the present study. [c]"Pub." refers to published values[13].

also detected in the e.i. mass spectrum of the $NaBD_4$-reduced material, as expected of a 6-deoxyhexose (data not shown).

The unknown was tentatively identified as 6-deoxytalose on the basis of its migration during p.c. using the deoxy sugar-differentiating solvent systems described by Krauss *et al.*[13]. Three different solvent-systems were used, and, in all cases, the R_{Rha} values of the unknown most closely approximately the published values of 6-deoxytalose. These results are summarized in Table I.

Authentic samples of 6-deoxy-D-talose and 6-deoxy-L-talose were subsequently obtained, in order to confirm the foregoing identification and to assist in configurational analysis. Reduction and acetylation of a small aliquot of authentic 6-deoxy-D-talose yielded an alditol acetate with the same g.l.c. retention-time as the alditol acetate of the unknown (data not shown).

The unknown sugar, 6-deoxy-D-talose, 6-deoxy-L-talose, and other sugars were then converted into mixtures of glycosides by reaction with chiral (−)-2-octanol, as described by Leontein *et al.*[14]. Two furanosides and two pyranosides are usually obtained from an aldehydic sugar after this reaction, and, following acetylation, these can usually be separated by g.l.c. The chromatograms obtained from a D and an L sugar are sufficiently different to allow unambiguous assigning of the absolute configuration of an unknown[14]. In the present case, when analyzed by this method, the purified 6-deoxytalose from EPS-I yielded three peaks having retention times of 9.46, 11.02, and 15.96 min, respectively. The peak at 11.02 min was sometimes partially resolved into two components. A similar analysis of standard 6-deoxy-D-talose yielded peaks having exactly the same retention times (and in the same relative amounts) as those obtained from the isolated unknown. These results

TABLE II

COMPARISON OF THE G.L.C. PEAKS OBTAINED FROM THE UNKNOWN WITH THOSE OBTAINED FROM 6-DEOXY-D-TALOSE AND 6-DEOXY-L-TALOSE FOLLOWING TREATMENT WITH CHIRAL (−)-2-OCTANOL

6-Deoxy-D-talose		*6-Deoxy-L-talose*		*Unknown*	
Ret. time[a] *(min)*	*Rel. area*[b]	*Ret. time (min)*	*Rel. area*	*Ret. time (min)*	*Rel. area*
9.47	24	10.33	23	9.46	21
11.94[c]	73	10.87	56	11.92[c]	77
15.96	3	11.39	17	15.96	2
		16.22	4		

[a]Ret. time, retention time. [b]Rel. area, relative area, determined by flame ionization detector. [c]Usually detected as a partially resolved, double peak.

are summarized in Table II, and prove conclusively that the 6-deoxytalose from EPS-I has the D configuration.

Although L-rhamnose and L-fucose are commonly found as constituents of bacterial polysaccharides, other 6-deoxyhexoses are found only rarely in Nature. 6-Deoxytalose has been found previously in glycolipids isolated from *Mycobacterium avium*[18] and *Mycobacterium marianum*[19], and has also been found in an EPS made by *Bifidobacterium bifidum*[20]. A polysaccharide derived from the cell wall of *Bifidobacterium adolescentis* was also recently reported to contain 6-deoxytalose[21]. It is noteworthy that the genus *Bifidobacterium*, like the genus *Butyrivibrio*, is composed of anaerobic species of gastrointestinal tract origin. The EPS made by an unidentified, Gram-negative soil bacterium termed "GS" also reportedly contains 6-deoxy-D-talose[22]. Only in the last study, and in the present one as well, have the authors determined the absolute configuration of the 6-deoxytalose isolated.

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