4-*O*-(1-Carboxyethyl)-L-rhamnose, a second unique acidic sugar found in an extracellular polysaccharide from *Butyrivibrio fibrisolvens* strain 49

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Butyrivibrio fibrisolvens strain 49 excretes a polysaccharide that contains D-glucose, D-galactose, 4-*O*-(1-carboxyethyl)-D-galactose, and an acidic component of previously unknown structure. We report here the identity of the unknown as 4-*O*-(1-carboxyethyl)-L-rhamnose. The structure of this previously unknown compound was deduced from (1) comprehensive electron-impact and chemical-ionization mass-spectroscopic studies of differentially labelled derivatives prepared from the unknown, (2) ¹³C-n.m.r. and ¹H-n.m.r. studies of purified neutral sugars derived from the unknown and (3) chemical degradation experiments.

INTRODUCTION

Butyrivibrio fibrisolvens is a strictly anaerobic bacterial species commonly isolated from the gastrointestinal tract of herbivorous mammals. Stack [1] recently reported that most strains of *B. fibrisolvens* produce significant amounts of extracellular polysaccharides (EPSs), the composition of which was used to infer the degree of relatedness among these strains. This analysis was greatly facilitated by the presence of several unique or rare monosaccharides that were used as taxonomic markers, and the results suggested that additional species of *Butyrivibrio* need to be defined. Subsequent genetic studies of Mannarelli [2] have generally confirmed the taxonomic groupings constructed from EPS compositional data.

The unusual sugars identified in the EPSs made by *B. fibrisolvens* consist of both neutral and acidic types. For example, L-altrose was first reported in nature as a major component of the EPS made by strain CF3 [3], and was then subsequently found in the EPS from many additional strains [1,4]. Of the two different EPSs made by strain X6C61, one contained 6-deoxy-D-talose [5] whereas the other contained L-iduronic acid [6]. We recently reported the structure of a unique acidic sugar found in the EPS from *B. fibrisolvens* strain 49, and roughly half of the 37 other isolates initially studied [1], as 4-*O*-(1-carboxyethyl)-D-galactose [7]. In this present study we report the structure of a second unique acidic sugar discovered in the EPS of *B. fibrisolvens* strain 49 as 4-*O*-(1-carboxyethyl)-L-rhamnose.

MATERIALS AND METHODS

Microbiological methods and EPS purification

Cultures of *B. fibrisolvens* strain 49 (M. P. Bryant, Department of Animal Sciences, University of Illinois, Urbana, IL, U.S.A.) were grown anaerobically to stationary phase at 37 °C on the defined medium of Cotta & Hespell [8]. The EPS produced was purified from culture supernatants by phenol extraction and dialysis as previously described [3]. As appropriate, portions of purified EPS were reacted with 1-(3-dimethylaminopropyl)-3-ethylcarbodi-imide and reduced with either NaBH₄ or NaB²H₄ to yield carboxy reduced EPS preparations [9].

Purification of the neutral sugar (NS-B) derived from the unknown acidic sugar (AS-B)

Carboxy reduced EPS preparations (50–300 mg) were hydrolysed at 20 mg/ml in 2 M-trifluoroacetic acid (at 100 °C for 1 h), dried down under a stream of N₂ and redissolved in 0.6 ml of water. Partial purification of NS-B was achieved as previously described [7], after chromatography on a column (1.5 cm × 97 cm) of Dowex 50W-X4 (Ca²⁺ form) prepared as described by Angyal *et al.* [10]. Fractions containing NS-B from this column were contaminated with D-glucose (As shown by g.l.c. of prepared alditol acetates), which was separated and removed with the use of preparative paper chromatography. Sheets of Whatman no. 1 paper (23 cm × 57 cm) streaked with 50–100 mg of the NS-B/D-glucose mixture were developed in the descending mode with butan-1-ol/pyridine/water (10:3:3, by vol.) for 16 h. The resolved carbohydrates were located on a thin strip cut from the dried chromatogram by using the alkaline AgNO₃ dip [11], and NS-B was then eluted with water from the corresponding sections of the sheet. NS-B was then re-chromatographed on a small column (1 cm × 28 cm) of Dowex 50W-X4 (Ca²⁺ form) (as above) with water as the eluent to remove non-carbohydrate material that was eluted from the paper. The column eluate was monitored with a refractive-index detector, and the fractions containing only NS-B were pooled and freeze-dried. The purity of column fractions and individual purified preparations was checked by g.l.c. analysis of alditol acetate derivatives as described elsewhere [3]. Similar preparations of NS-B isolated from 1-(3-dimethylamino)-3-ethylcarbodi-imide-activated NaB²H₄-reduced EPS were designated as d₂-NS-B, to reflect the two deuterium atoms incorporated into AS-B.

Chemical degradation of NS-B and identification of the resultant hexose

Purified NS-B (0.2–0.5 mg) was dealkylated by using the ether-cleaving reagent BBr₃ by means of the Hough & Theobald procedure [12] as modified by Stack *et al.* [7]. The dealkylated NS-B obtained was reduced and acetylated, and analysed by g.l.c. and g.l.c.-m.s. to identify the resultant hexose. The absolute configuration of this hexose was determined by g.l.c. analysis of

Abbreviations used: EPS, extracellular polysaccharide; c.i.m.s. and e.i.m.s., chemical-ionization and electron-impact mass spectroscopy respectively.

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its acetylated diastereomeric glycosides prepared from (–)-octan-2-ol by a modification [6] of the procedure of Leontein *et al.* [13]. Results were compared with those obtained from standard L-rhamnose that had reacted with either (–)-octan-2-ol or (+)-octan-2-ol, because D-rhamnose is not commercially available.

Spectroscopic methods

^{13}C -n.m.r. and ^1H -n.m.r. spectra of NS-B and d_2 -NS-B in $^2\text{H}_2\text{O}$ were obtained with a Bruker WM-300 WB spectrometer equipped with a 5 mm dual probe. A distortionless-enhanced-via-polarization-transfer experiment [14] aided the assignment of ^{13}C resonance signals. Chemical-ionization (c.i.) mass spectra of various samples were obtained with a Finnigan 4535 TSQ GLC/MS instrument as described previously [7], and electron-impact (e.i.) mass spectra were generated by using a Hewlett-Packard MSD detector linked in tandem to a Hewlett-Packard model 5890 gas chromatograph.

Miscellaneous methods and procedures

The optical rotation of purified NS-B (following mutarotation) was determined on a Perkin-Elmer model 241 polarimeter. Various EPS samples, column fractions or other preparations were analysed by g.l.c. as previously described [3] after conversion into the corresponding alditol acetate derivatives [15].

RESULTS

Stack [1] has previously reported that the EPS made by *B. fibrisolvens* strain 49 contained two neutral sugars (D-glucose and D-galactose) and two acidic sugars of unknown structure. The structure of one of these (previously designated as AS-A) was subsequently identified as 4-*O*-(1-carboxyethyl)-D-galactose [7]. This compound was detected by g.l.c. both as an acetylated lactone (Fig. 1, peak A-2) and as a fully reduced alditol acetate (peak A-1) after hydrolysis, reduction and acetylation [7] of wild-type EPS. The acidic sugar AS-B, the object of the present study, gave four different g.l.c. peaks (peaks B-1, B-2, B-3 and B-4), as indicated in Fig. 1. However, g.l.c. analysis of the alditol acetates prepared from carboxy-reduced EPS preparations was characterized by the absence of peaks B-1, B-2, B-3 and A-2, and corresponding increases were observed in the relative intensities of peaks B-4 and A-1 [7].

M.s. studies of these g.l.c.-separated alditol acetates were undertaken in attempts to characterize preliminarily the compounds represented by peaks B-1, B-2, B-3 and B-4. C.i.m.s. showed that the three compounds corresponding to peaks B-1, B-2 and B-3 all had very stable molecular ions at m/z 347, with very little subsequent fragmentation (except for the loss of acetic acid to provide m/z 287; result not shown). E.i.m.s. of these three compounds did not yield informative fragmentation data. The absence of these peaks from the chromatograms of carboxy-reduced EPS preparations, along with the corresponding increase in peak B-4, suggested that all three might represent different acetylated lactones derived from AS-B. The c.i. mass spectrum of the compound represented by peak B-4 showed three principal peaks, namely a molecular ion at m/z 435, a base peak at m/z 375 ($M\text{H}^+ - 60$) and a fragment at m/z 101. The gain of 88 atomic mass units noted for the molecular ion of peak B-4 over that of peaks B-1, B-2 and B-3 was consistent with the above lactone hypothesis. The e.i. mass spectrum of the compound represented by peak B-4 also showed a prominent fragment at m/z 101, as shown in Fig. 2. The presence of m/z -101 fragments in both the c.i. and e.i. mass spectra of B-4 suggested that the

Fig. 1. G.l.c. separation of the alditol acetates prepared from the purified EPS of *B. fibrisolvens* strain 49

Galactose (Gal), glucose (Glc) and inositol (Ins, internal standard) are indicated. Peaks labelled A-1 and A-2 are both derived from 4-*O*-(1-carboxyethyl)-D-galactose, as reported previously [7]. Peaks labelled B-1, B-2, B-3, and B-4 are all derived from the unknown acidic sugar AS-B.

Fig. 2. E.i.m.s. of the compound corresponding to peak B-4

An equivalent spectrum was obtained from the alditol acetate prepared from purified NS-B.

original acidic sugar AS-B was likely to be a 1-carboxyethyl-substituted compound, as we have noted similar observations for 4-*O*-(1-carboxyethyl)-D-galactose [7].

Partial purification of NS-B was achieved by column chromatography on Dowex 50W-X4 (Ca^{2+} form) as previously described [7]. However, the NS-B was contaminated with D-glucose, necessitating the removal of the latter by preparative paper chromatography (the R_{Glc} of NS-B was 3.0 in butan-1-ol/pyridine/water, 10:3:3, by vol.). The eluted NS-B, after re-chromatography, was essentially free of all contaminating carbohydrate material (as judged by alditol acetate analysis) and yielded a single g.l.c. peak with a retention time and mass spectrum identical with those of the compound corresponding to peak B-4. The specific rotation of several purified preparations of NS-B ranged from $+3.7^\circ$ to $+4.2^\circ$.

Treatment of purified NS-B with BBr_3 , followed by reduction and acetylation, gave a compound with the same g.l.c. retention time as rhamnitol penta-acetate. C.i.m.s. and e.i.m.s. confirmed that the product was the alditol acetate of a 6-deoxyhexose (results not shown).

G.l.c. analyses of the acetylated diastereomeric glycosides prepared from the dealkylated product and (–)-octan-2-ol were used to confirm the identification of the 6-deoxyhexose and to deduce its configuration. The retention times of the peaks corresponding to the acetylated glycosides derived from standard L-rhamnose and (–)-octan-2-ol were at 4.87, 5.90 and 6.21 min. Similarly treated L-rhamnose and (+)-octan-2-ol (the D-rhamnose 'standard') gave g.l.c. peaks at 4.76, 5.96 and 6.43 min. The dealkylated product and (–)-octan-2-ol yielded three peaks with exactly the same retention times as those mentioned above for L-rhamnose and (–)-octan-2-ol. A base peak at m/z 273 was

Table 1. Relative intensities of diagnostic fragments obtained from e.i.m.s. analyses of four unique deuterium-labelled acetylated alditors derived from the unknown sugar (base peak equals 10000)

See Fig. 3 for assignment and the text for designations.

<i>m/z</i>	Derived from NS-B		Derived from <i>d</i> ₂ -NS-B	
	NS-B- <i>h</i>	NS-B- <i>d</i>	<i>d</i> ₂ -NS-B- <i>h</i>	<i>d</i> ₂ -NS-B- <i>d</i>
101	10000	9346*	133	—
103	240	160	10000	8570*
145	114	36	97	31
146	10	70	13	70
217	966	908	34	39
219	15	16	855	729
317	1103	—	1118	—
318	214	1267	194	1051
319	33	179	44	166
320	—	36	—	38
347	349	—	—	—
348	56	341	—	—
349	12	57	307	—
350	—	9	63	254

* Base peak for NS-B-*d* and *d*₂-NS-B-*d* was at *m/z* 43.

present in the c.i. mass spectra of all of the above peaks, corresponding to the loss of octan-2-ol from each of the expected triacetylated molecular ions ($MH^+ - 130$). It should be noted that two additional peaks, with retention times of 5.02 and 6.10 min, were also observed in the analysis of the dealkylated product. The c.i. mass spectra of these latter two compounds were both characterized by two pairs of fragments at *m/z* 233 and 235 and *m/z* 293 and 295, highly suggestive of brominated compounds. Although the identification of these compounds was not pursued further, they may represent 2,3,4-tri-*O*-acetylated α - and β -rhamnopyranosyl bromide ($MH^+ - 60 = 293$ and 295). The brominated rhamnose could represent an additional product of the reaction between NS-B and BBr_3 .

The linkage position of the alkyl group to the L-rhamnose moiety was determined by comprehensive m.s. analyses of deuterated derivatives prepared from NS-B. The aldehydic groups in separate aliquots of purified NS-B and *d*₂-NS-B were reduced with $NaBH_4$ or NaB^2H_4 , yielding four unique alditors. These pairs were defined as NS-B-*h* and NS-B-*d* and as *d*₂-NS-B-*h* and *d*₂-NS-B-*d* respectively in order to designate the hydrogen or deuterium incorporated into the carboxylic and aldehydic carbon atoms respectively. C.i.m.s. showed the molecular ions of these four compounds respectively increasing stepwise from *m/z* 435 to *m/z* 436 to *m/z* 437 to *m/z* 438, with intensities generally about 50% of the base peaks (*m/z* 375, 376, 377 and 378). E.i.m.s. data of these four acetylated alditors are summarized in Table 1, and the assignments of diagnostic fragments are shown in Fig. 3. The *m/z*-101 fragment quantitatively shifted to *m/z* 103 for both preparations derived from *d*₂-NS-B, clearly establishing the incorporation of the two deuterium atoms into the acidic alkyl group substituent following carbodi-imide activation and reduction with NaB^2H_4 . The complete absence of *m/z* 101 from both *d*₂-NS-B-*h* and *d*₂-NS-B-*d* confirmed that all of the unknown sugar in the original EPS must be present in the acidic form. The other shifts in the fragments observed in Table 1 can only be reconciled by linking the original 1-carboxyethyl group through the 4-position of L-rhamnose, as is shown in Fig. 3.

Additional support for the proposed structure was provided by ¹³C-n.m.r. spectroscopy of purified NS-B, *d*₂-NS-B and

Fig. 3. Structures of (a) 4-*O*-(1-carboxyethyl)-L-rhamnose, showing the numbering of carbon atoms as assigned in Table 2, and (b) the fully reduced alditor acetate derived from the above, showing diagnostic primary fragments observed in the e.i.m.s. analysis of the four differentially deuterated alditor acetates

Each fragment has four *m/z* values listed vertically the bold-faced value at the top of each column corresponding to the predicted one for NS-B-*h*. Each successive value is the *m/z* of the same fragment predicted for NS-B-*d*, *d*₂-NS-B-*h* and *d*₂-NS-B-*d* respectively. These predictions are in complete agreement with the experimental values shown in Table 1. The corresponding molecular ions detected in separate c.i.m.s. analyses are also listed for these same compounds.

L-rhamnose. The spectra of NS-B and *d*₂-NS-B are shown in Figs. 4(b) and 4(a) respectively. The methylene resonance at 65.4 p.p.m. is absent from the spectrum of *d*₂-NS-B, allowing the assignment of this signal to the hydroxymethyl carbon, C-3' (the carboxy-group carbon atom of AS-B; see Fig. 3). A secondary deuterium isotope effect caused a slight (0.1 p.p.m.) upfield shift for the methine carbon atom in *d*₂-NS-B compared with the same resonance in NS-B, allowing this signal to be assigned to C-1'. Although the ring carbon atoms appeared as pairs of resonances because of the presence of two anomers, the C-6 methyl and alkyl-group substituent carbon atoms each gave only a single resonance. Complete assignment of the ¹³C chemical shifts for NS-B are made in Table 2, and are compared with the analogous ¹³C shifts determined for L-rhamnose. The only significant difference is the large downfield shift (6.9 p.p.m.) observed in the C-4 resonance of NS-B compared with that of L-rhamnose,

Fig. 4. ^{13}C -n.m.r. spectra of (a) d_2 -NS-B and (b) NS-B

Note the absence of the ^{13}C resonance at 65.4 p.p.m.

Table 2. Comparison of the ^{13}C chemical shifts of NS-B [4-*O*-(1-hydroxypropyl)-L-rhamnose] with those of L-rhamnose

Carbon atom	^{13}C chemical shift (p.p.m.)			
	L-Rhamnose		4- <i>O</i> -(1-Hydroxypropyl)-L-rhamnose	
	α	β	α	β
Hexose				
1	94.5	94.1	94.5	94.1
2	71.4	71.9	71.8	72.1
3	70.5	73.3	70.4	73.2
4	72.8	72.3	79.7	79.2
5	68.9	72.6	68.4	72.3
6	17.4	17.4	17.7	17.7
Side chain*				
1'	—	—	78.2	78.2
2'	—	—	16.9	16.9
3'	—	—	65.4	65.4

* Numbering of side-chain carbons is as follows: C-1' corresponds to $-\text{CH}$, C-2' to $-\text{CH}_3$, and C-3' to $-\text{CH}_2\text{OH}$. See also Fig. 3.

suggesting that the alkyl group is linked through the oxygen atom attached to C-4.

^1H -n.m.r. data were consistent with the observed ^{13}C -n.m.r. data. Anomeric proton resonances (and coupling constants) were almost identical for L-rhamnose [δ 4.96 p.p.m. ($J = 1.8$ Hz) and 4.72 p.p.m. ($J = 1.1$ Hz)], NS-B [δ 4.95 p.p.m. ($J = 1.8$ Hz) and 4.70 p.p.m. ($J = 1.1$ Hz)] and d_2 -NS-B [δ 4.95 p.p.m. ($J = 1.9$ Hz) and 4.70 p.p.m. ($J = 1.1$ Hz)]. The C-3' methylene proton resonance signals observed at δ 3.45 p.p.m. for NS-B were absent with d_2 -NS-B (results not shown). The presence of C-1' methyl proton signals was clearly observable in the ^1H -n.m.r. spectra of NS-B and d_2 -NS-B at δ 1.06 p.p.m. (α) and δ 1.05 p.p.m. (β). The observation that the C-1' ^1H resonances depended on the anomeric configuration is in contrast with the ^{13}C data, where only a single resonance was observed for both anomeric forms.

DISCUSSION

The EPS made by *B. fibrisolvens* strain 49 contains two unusual acidic sugars not previously reported in Nature, in addition to D-glucose and D-galactose. The structure of one of these acidic sugars, previously designated AS-A, was established as 4-*O*-(1-carboxyethyl)-D-galactose in an earlier paper [7]. This

compound was detected in the EPS made by about half of the *B. fibrisolvens* strains examined previously [1], and was used as a primary biochemical marker for sorting these isolates into taxonomic groups that have been subsequently confirmed by using comprehensive genetic methodologies [2].

The identity of the other acidic sugar contained in this EPS, previously designated as AS-B [7], is established here as 4-*O*-(1-carboxyethyl)-L-rhamnose. This compound was also previously reported to be a constituent of the EPS made by strain H17c (where it was designated as Unk-1), but it is not apparently present in any other *B. fibrisolvens* strains [1]. The presence of this sugar was previously used as a biochemical marker to distinguish isolates within the group IV taxonomic cluster of *B. fibrisolvens* proposed by Stack [1].

Analysis of the EPS made by *B. fibrisolvens* strain 49 by using the alditol acetate procedure yielded numerous compounds detectable by g.l.c. that have now been shown to originate from non-neutral sugar components. For example, 4-*O*-(1-carboxyethyl)-L-rhamnose yielded three different acetylated lactones of undetermined structure (peaks B-1, B-2 and B-3 in Fig. 1) and a fully reduced alditol acetate of its corresponding neutral sugar (peak B-4) generated as an artifact by the action of NaBH_4 on one or more of these lactones. Interestingly, all three acetylated lactones derived from 4-*O*-(1-carboxyethyl)-L-rhamnose had shorter g.l.c. retention times than the fully reduced alditol acetate, in contrast with what we have previously observed for 4-*O*-(1-carboxyethyl)-D-galactose [7]. The reason for this is not readily apparent. Preparations of carboxy reduced EPS analysed by the alditol acetate procedure did not show peaks B-1, B-2 or B-3, but had a relatively higher content of the compound represented by peak B-4. The reduction of these lactones and acetylation of the two resultant hydroxy groups accounts for the observed increase of 88 Da in the molecular mass of peak B-4 ($M = 434$) over peaks B-1, B-2 and B-3 ($M = 346$). That none of the unknown was present as the neutral sugar in the original EPS was confirmed by the absence of an m/z -101 fragment in the e.i. spectra of alditol acetates prepared from d_2 -NS-B.

The structural elucidation of 4-*O*-(1-carboxyethyl)-L-rhamnose was accomplished by using a variety of physicochemical techniques. After purification, identification of L-rhamnose after treatment of NS-B with the ether-cleaving reagent BBr_3 left only the linkage of the original 1-carboxyethyl substituent to be determined. The greater yields of diagnostic primary fragments obtained during e.i.m.s. coupled to the asymmetry of the alditol of a 6-deoxyhexose {compared with what we observed previously for 4-*O*-(1-carboxyethyl)-D-galactose [7]} allowed us readily to assign the linkage to L-rhamnose through the 4-position. ^{13}C -n.m.r. data presented in Table 2 provided additional support for the structure presented in Fig. 3.

Muramic acid [3-*O*-(1-carboxyethyl)-D-glucosamine] is the only 1-carboxyethyl sugar commonly encountered in Nature. Kenne & Lindberg [16] have summarized additional examples of more rarely encountered 1-carboxyethyl sugars, such as 3-*O*-(1-carboxyethyl)-L-rhamnose, 3-*O*- and 4-*O*-(1-carboxyethyl)-D-glucose and 4-*O*-(1-carboxyethyl)-D-mannose. A recent report by Parolis *et al.* [17] described the structural elucidation of 4-*O*-(1-carboxyethyl)-D-glucuronic acid in *Klebsiella*, and undoubtedly the list of new such sugars will continue to grow.

Although the function(s) of this or other EPS made by *B. fibrisolvens* remain(s) unknown, it has been hypothesized [3] that the presence of these and other unusual monosaccharide constituents might confer some measure of resistance to hydrolysis from the many glycosidases and glycanases present in the gastrointestinal tract. The precise structures of some of these interesting polysaccharides made by *B. fibrisolvens* remain to be investigated.

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