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CARBOHYDRATE RESEARCH

Note

Structural analysis of the exopolysaccharide produced by *Pediococcus damnosus* 2.6

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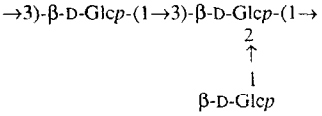
Abstract

The exopolysaccharide produced by a ropy strain of *Pediococcus damnosus* (2.6) in a semi-defined medium was found to be an homopolymer composed of D-glucose. On the basis of monosaccharide and methylation analysis, ^{1}H, ^{13}C, 1D and 2D NMR experiments the polysaccharide was shown to consist of repeating units with the following structure.



Keywords: *Pediococcus damnosus*; Exopolysaccharide; Repeating unit

1. Introduction

It has been reported that some species of lactic bacteria produce exopolysaccharides (EPSs), which are capsular polysaccharides attached to the cell wall or viscous polysaccharides secreted into the extracellular environment [1]. In ciders, the polysaccharide production by strains of lactic acid bacteria has been described in relation to the alteration named oilness or ropiness [2] that also occurs in wines [3] and beers [4]. This alteration is characterized by the production of a polysaccharide substance, which thickens the

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consistency of the beverage. In the Basque Country, cider ropiness is a widely found disorder and constitutes a source of economic loss. Recently, Dueñas et al. and Fernandez et al. [5,6] have isolated EPS-producing strains of *Lactobacillus* sp. and *Pediococcus* sp. from ropy Basque Country ciders. Llauberes et al. [7] described the structure of an exocellular β-D-glucan produced by a *Pediococcus* sp. strain isolated from ropy wine. Here we report the structure determination of the exopolysaccharide produced by a ropy strain of *Pediococcus damnosus* (2.6). This knowledge is important for the selection of specific enzymes for the elimination of cider viscosity.

2. Experimental

Bacteria and culture conditions.—*Pediococcus damnosus* 2.6 was isolated from a ropy Basque Country cider [6]. The taxonomical position of this strain was determined by study of morphological, physiological and biochemical characteristics according to Bergey's Manual of Systematic Bacteriology [8] and by hybridization with DNA probes following previously described techniques [9]. *Pediococcus damnosus* 2.6 was routinely maintained in MRS broth (pH 4.8) containing ethanol (6%) [10]. The semidefined medium (SMD) that was used for culturing EPS production had the following composition: Glucose 20 g/L, casaminoacids 5 g/L, bacto yeast nitrogen base (Difco) 6.7 g/L, $MnSO_4 \cdot H_2O$ 0.05 g/L, K_2HPO_4 2 g/L, NaAcO 5 g/L, adenine 0.005 g/L, guanine 0.005 g/L, xanthine 0.005 g/L, uracil 0.005 g/L, and L-malic acid 4 g/L. The pH of the SMD medium was 4.8. The glucose and bacto yeast nitrogen base were sterilized by passing through a 0.2 mm sterile filter and added after autoclaving.

For inocula, cells harvested from 48 h cultures in MRS medium with ethanol were washed and resuspended in phosphate-buffered saline with peptone. *Pediococcus damnosus* 2.6 was cultured in semidefined medium and incubated for 7 days (nonshaken, nonaerated) at 28 °C. The flasks were inoculated to give an initial viable count of approximately 10^8 CFU/mL ($OD_{600} = 0.2$).

Isolation and purification of the exopolysaccharide.—Cells were removed by centrifuging for 30 min at 20,000 *g*. The clear supernatant was collected, and the EPS was precipitated by adding 3 volumes of cold ethanol, followed by storage overnight at 4 °C. The precipitate was recovered by centrifugation at 4,500 *g* for 20 min at 4 °C and dissolved in distilled water. The polysaccharide was purified by precipitation with ethanol three times and the final precipitate was dissolved in and dialyzed against distilled water for 2 to 3 days (changed twice each day) and lyophilized.

Sugar analysis.—Monosaccharides were determined as their trimethylsilylated methyl glycosides [11]. The polysaccharide was treated with 0.625 M methanolic HCl at 80 °C for 16 h and then silylated with 1:1 pyridine-BSTFA for 16 h at 80 °C. Isobutanol was added to the mixture and then dried under a stream of nitrogen. The TMS derivatives were analyzed by GLC–MS performed with a Kratos MS80RFA instrument fitted with a CP-Sil5-CB WCOT column (25 m × 0.32 mm i.d.). The temperature programme was isothermal at 140 °C for 2 min followed by an 8 °C/min gradient up to 250 °C.

Determination of D *and* L *- configuration.*—The polysaccharide was methanolized with 0.625 M methanolic HCl, treated with 0.625 M (+)-2-butanolic-HCl under the same conditions and then thrimethylsilylated. The trimethylsilylated 2-butyl glycosides were analyzed by GLC–MS [12] as above. The temperature programme was isothermal at 130° followed by a 2 °C/min gradient up to 250 °C

Methylation analysis.—The polysaccharide was methylated twice by the method of Ciucanu and Kerek [13]. The product was further purified by reversed-phase chromatography on a Sep-Pak C_{18} cartridge [14] and hydrolysed with 2 M TFA acid. The products were then reduced and acetylated by using the method described by Blakeney [15]. The partially methylated alditol acetates were analyzed by GLC–MS and the temperature programme was isothermal at 100 °C for 1 min followed by a 5 °C/min gradient up to 250 °C.

NMR Spectroscopy.—The sample was deuterium exchanged several times by freeze drying from D_2O and then examined as solutions (3 mg/mL) in 99.98% D_2O. Spectra were recorded at 333 K on a Bruker AMX500 spectrometer operating at 500.13 MHz (^1H) and 125.75 MHz (^{13}C). Chemical shifts are given in ppm, using the HDO signal (4.23 ppm) (^1H) and external dimethyl sulfoxide (39.5 ppm) (^{13}C) as references.

The 2D homonuclear proton double-quantum filtered correlation experiment (DQF-COSY) [16] was performed in the phase-sensitive mode using the Bruker standard pulse sequence. A data matrix of 512 × 1K points was used to digitize a spectral width of 4500 Hz; 32 scans were used per increment with a delay between scans of 1 s. Prior to Fourier transfor-

mation, zero-filling was used in F_1 to expand the data to 1K × 1K. The 2D ROESY experiment [17] was carried out in the phase-sensitive mode, with a mixing time of 300 ms. The rf carrier was set at 6.0 ppm in order to minimize spurious Hartmann–Hahn effects. A data matrix of 256 × 1K points was used to digitize a spectral width of 6000 Hz; 32 scans were acquired per increment. Squared sine-bell functions were applied in both dimensions and zero-filling was used to expand the data to 1K × 1K. The 2D heteronuclear one-bond proton–carbon correlation experiment [18] was registered in the ^{1}H-detection mode via multiple-quantum coherence (HMQC). A data matrix of 512 × 1K points was used to digitize a spectral width of 3500 and 26,000 Hz in F_2 and F_1; 64 scans were used per increment with a delay between scans of 1 s and a delay corresponding to a J value of 160 Hz. A BIRD pulse was used to

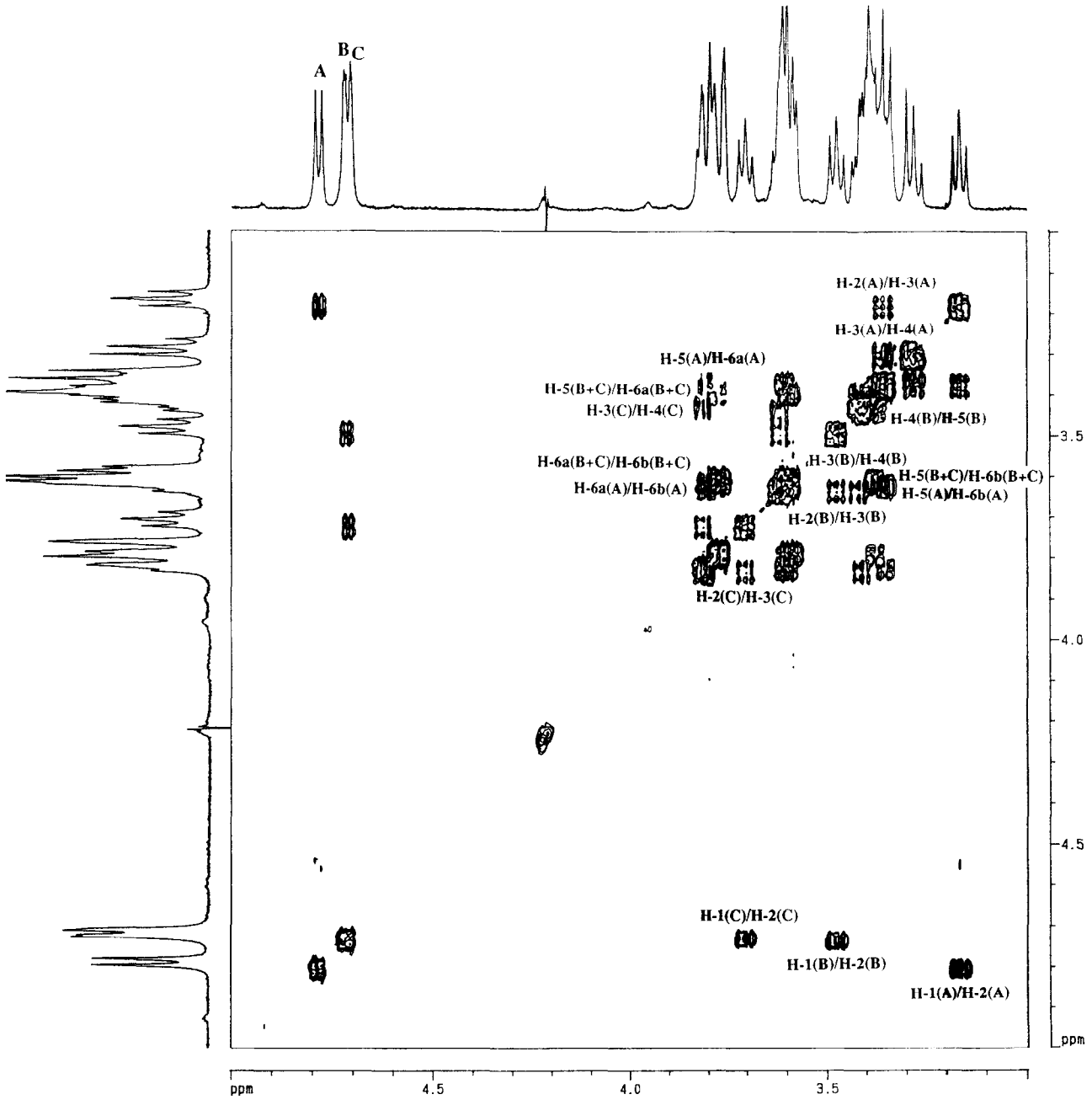


Fig. 1. 500 MHz ^{1}H NMR spectrum of the exopolysaccharide from *Pediococcus damnosus* 2.6 recorded in D_2O at 60 °C and the 500 MHz 2D homonuclear proton double-band filtered correlation spectrum (DBF-COSY), obtained by double band selective excitation of the 3–6 ppm region.

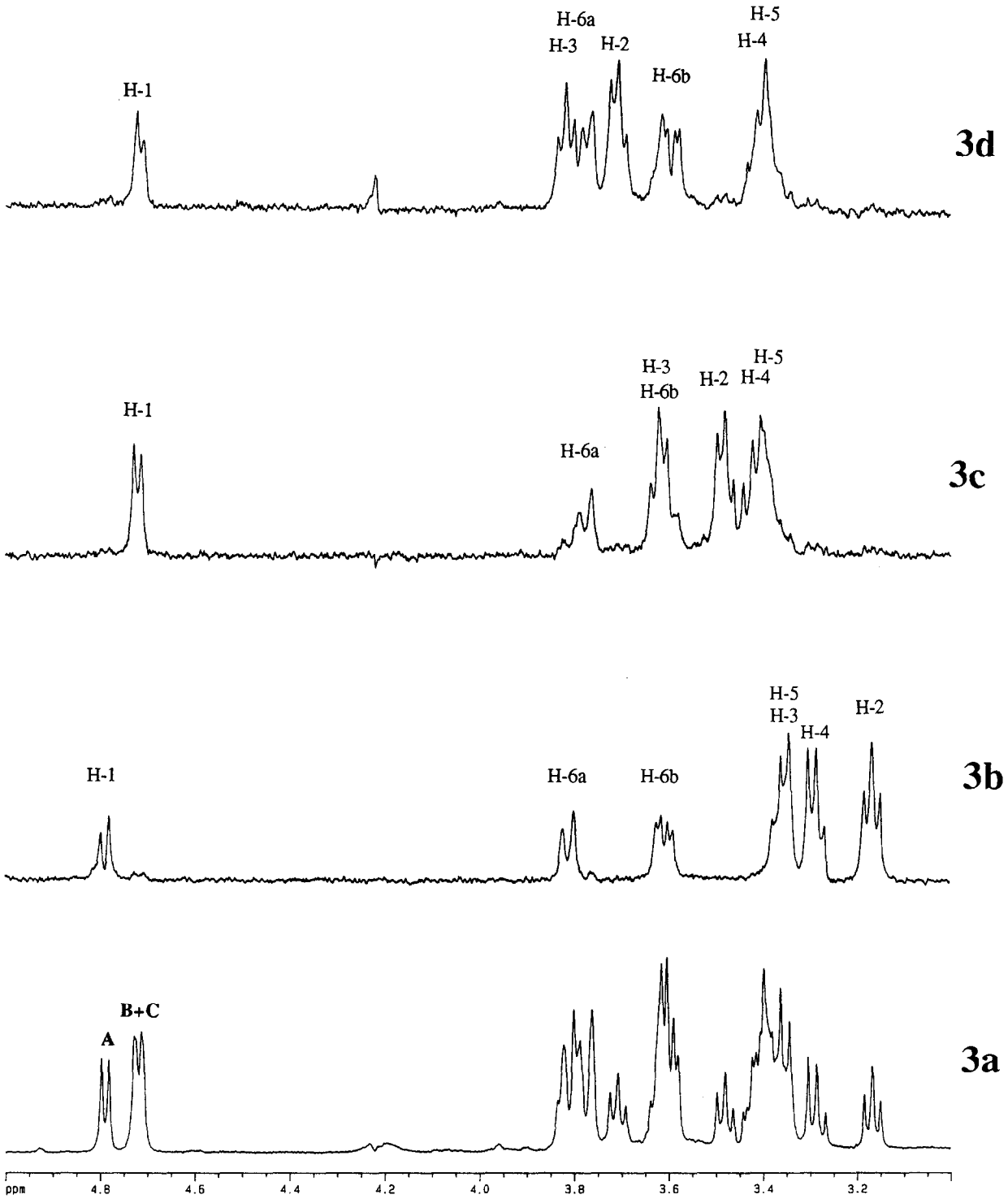


Fig. 2. (a) 500 MHz ^{1}H NMR spectrum of the EPS from *Pediococcus damnosus* 2.6. (b), (c) and (d) 1D TOCSY subspectra obtained by selective inversion by DANTE-Z pulse train of triplets at 3.19 ppm (b), 3.51 ppm (c), and 3.73 ppm (d). The isotropic mixing period was 120 ms in all the cases.

minimize the signals from protons bonded to ^{12}C. ^{13}C decoupling was achieved by the GARP scheme. Squared cosine-bell functions were applied in both dimensions.

Selective excitation 1D experiments were carried out by application of the DANTE-Z pulse train (n = 300, $\tau = 100$ μs, $\theta = 0.3°$) [19]. This train was also concatenated to a TOCSY sequence (isotropic mixing

time of 120 ms, $\pi/2$ pulse width of 49 μs) [20] to yield the 1D-TOCSY subspectra. The number of accumulated scans was 512 which corresponded to a total acquisition time of 30 minutes. The 2D homonuclear proton double-band filtered selective correlation experiment (DBF-COSY) [21] was collected by application of two BURP pulses based on the DANTE-Z scheme [22] ($n = 10$, $\tau = 50$ μs, $\theta = 10°$). A data matrix of $512 \times 1K$ points was used to resolve a spectral width of 1500 Hz; 32 scans were used per increment. The HDO signal was presaturated by a low-power pulse (transmitter attenuation, 65 dB) of 1 s.

3. Results and discussion

GLC–MS of the TMS ethers of EPS methylglycosides showed that only glucose was present. Analysis of the trimethylsilylated (+)-2-butyl glycosides derivatives by GLC showed that the glucose had the D-configuration. Methylation analysis showed the presence of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol derived from terminal glucose, 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methylglucitol from 3-linked glucose in the main chain, and 1,2,3,5-tetra-*O*-acetyl-4,6-di-*O*-methylglucitol from 2,3-linked glucose in the main chain bearing the side chain, in a ca. (1:1:1) molar ratio.

The chemical shifts for the ^{1}H and ^{13}C resonances of the polysaccharide were assigned from COSY, TOCSY and HMQC experiments (Tables 1 and 2). The three anomeric protons observed in the 1D ^{1}H NMR spectrum (Fig. 1) were labelled A–C from low to high field. From δ and ^3J values, all of them were identified as belonging to β-glucopyranose residues. From double quanta filtered (DQF) and double band filtered (DBF) COSY (Fig. 1) experiments, these anomeric protons were correlated with their respective H-2 protons, which appeared as isolated triplets at 3.73, 3.51 and 3.19 ppm. The triplet at 3.19 was identified as H-2 in unit A. In the same way, the three H-3 protons were located inside the three groups of signals at 3.85–3.73 ppm, 3.63–3.55 ppm and 3.48–3.36 ppm, making further correlation analysis difficult.

In order to completely assign the 1D ^{1}H NMR spectrum, selective excitation 1D experiments were carried out. Fig. 2b–d show the 1D-TOCSY spectra from selective inversion of the previously identified H-2 signals, via a DANTE-Z pulse train, followed by a convenient isotropic mixing time (120 ms). These subspectra allowed the observation of all protons from each unit separately. Combination of these subspectra with the information from DBF-COSY, enabled the assignation of all the individual protons within the polysaccharide chain (Table 1). Chemical shifts for the ^{13}C resonances were assigned from the HMQC experiment (Table 2). Correlation of the three different spin systems identified in the ^{1}H and ^{13}C NMR experiments with the three different β-D-glucopyranose residues identified by methylation analysis, was made as follows: signals for C-2 at 79.4 ppm and its H-2 at 3.73 ppm appeared shifted downfield for unit C, with respect to the same nucleus in units A and B. So, unit C should be substituted in position 2, and therefore it could be identified as the 2,3-linked β-D-glucopyranose unit, the branched point of the main chain, and the only residue with such a type of substitution. On the other hand, the C-3 signal at 76.1 ppm and H-3 at 3.39 ppm appeared at a higher field in unit A than those for unit B and C, meaning that position 3 in unit A was unsubstituted. This fact allowed the identification of the unit A as the terminal β-D-glucopyranose unit in the side chain, the only one without substitution in this position. Finally, unit B was correlated with 3-linked β-D-glucopyranose in the main chain non-substituted by the side chain. A H-1(A)/H-2(C) cross-peak was observed in the ROESY experiment which confirmed the above correlations.

Table 1
^{1}H NMR chemical shifts (δ, ppm) and coupling constants (J, Hz) for the EPS of *Pediococcus damnosus*

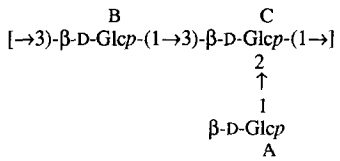
Unit	H-1	H-2	H-3	H-4	H-5	H-6a	H-6b
A	4.82	3.19	3.39	3.31	3.39	3.84	3.64
B	4.74	3.51	3.65	3.45	3.41	3.81	3.63
C	4.74	3.73	3.84	3.44	3.41	3.81	3.63
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{5,6b}$	$J_{6a,b}$	
A	7.9	8.7	ca. 9.0	9.3	5.5	−11.6	
B	7.9	8.6	ca. 9.0	9.8	–	−11.7	
C	7.9	8.2	8.5	ca. 10.0	5.3	ca. −11.6	

Table 2
^{13}C NMR chemical shifts (δ, ppm) for the EPS of *Pediococcus damnosus*

Unit	C-1	C-2	C-3	C-4	C-5	C-6
A	101.7	73.5	76.1	69.2	75.2 [b]	60.3 [c]
B	101.3 [a]	72.1	85.1	67.6	74.8 [b]	60.1 [c]
C	100.9 [a]	79.4	82.9	67.6	75.0 [b]	60.1 [c]

[a,b,c] Assignment could be interchanged.

On the basis of the above chemical and spectroscopic data, the polysaccharide secreted by *Pediococcus damnosus* 2.6 has the following trisaccharide repeating unit:



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