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Structural elucidation of the viscous exopolysaccharide produced by *Lactobacillus helveticus* Lb161

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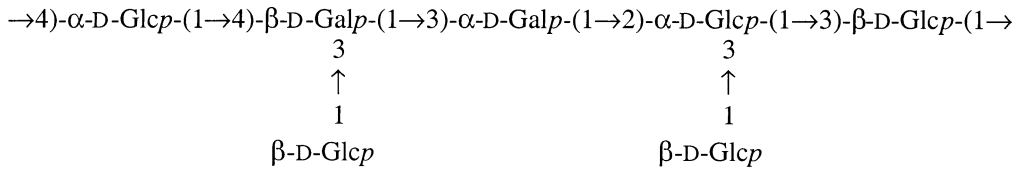
Abstract

A viscous extracellular polysaccharide produced by *Lactobacillus helveticus* Lb161 isolated from raw milk has been investigated. Sugar and methylation analysis, and ^{1}H and ^{13}C NMR spectroscopy revealed that the polysaccharide is composed of a heptasaccharide repeating unit. The sequence of sugar residues was determined by use of two-dimensional nuclear Overhauser effect spectroscopy and heteronuclear multiple bond connectivity experiments. The structure of the repeating unit of the exopolysaccharide from *L. helveticus* Lb161 is as follows:



The polysaccharide contains approximately 0.6 equivalents of *O*-acetyl group per repeating unit (not located).

Keywords: *Lactobacillus helveticus*; Exopolysaccharide; Viscous; NMR

1. Introduction

Lactic acid bacteria produce exopolysaccharides, which may have suitable rheological properties for the food industry [1]. The polysaccharides produced by these food-grade microorganisms are an important source of natural alternatives to commercial thickeners and stabilizers [2]. These polymers have also been shown to have advantageous biological functions, being beneficial to human health [3,4].

Structural studies on the exopolysaccharides produced by lactic acid bacteria have appeared in the last decade [2]. Robijn et al. [5] showed that the exopolysaccharide produced by *Lactobacillus helveticus* 776 had hexasac-

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charide repeating units containing D-glucose and D-galactose. Yamamoto et al. [6] reported that the exopolysaccharide produced by *L. helveticus* TY1-2 consisted of heptasaccharide repeating units with D-glucopyranosyl, D-galactopyranosyl, and 2-acetamido-2-deoxy-D-glucopyranosyl residues. Recently, Stingele et al. [7] showed that the exopolysaccharide produced by *L. helveticus* Lh59 had an identical primary structure to the one produced by *L. helveticus* TN-4 [8], a presumed spontaneous mutant of the strain TY1-2. The repeating unit of the TN-4 polymer is composed of a tetetrasaccharide backbone with a lactosyl side-chain. The molar ratio of D-glucose and D-galactose is 1:1.

We have previously reported the structure of an exopolysaccharide produced by a strain of *L. helveticus*, presently named *L. helveticus* Äki 4, isolated from cheese milk [9]. The polysaccharide consists of hexasaccharide repeating units with D-glucose and D-galactose in a 1:2 molar ratio. Here we report the structure of a viscous exopolysaccharide produced by *L. helveticus* Lb161 grown in skim milk.

2. Results and discussion

L. helveticus Lb161 was grown in skim milk, and the exopolysaccharide was isolated from the culture medium by ethanol precipitation, filtration, dialysis, and lyophilization. The exopolysaccharide eluted in the void volume on a Bio-Gel P-30 size-exclusion column, indicating that it was of high molecular mass.

A hydrolysate of the exopolysaccharide from *L. helveticus* Lb161 contained glucose and galactose in a 5:2 ratio. The absolute configuration analysis showed that the sugars had the D configuration. Methylation analysis of the polysaccharide revealed 2,3,4,6-tetra-*O*-methylglucose, 2,4,6-tri-*O*-methylglucose, 2,3,6-tri-*O*-methylglucose, 2,4,6-tri-*O*-methylgalactose, 2,6-di-*O*-methylgalactose, and 4,6-di-*O*-methylglucose in the ratio 19:11:12:11:13:13. The native preparation of Lb161 contains 0.6 equivalents of *O*-acetyl group (δ 2.17). However, it was rapidly hydrolysed, resulting in an O-deacetylated preparation hereafter referred to as the polysaccharide. From the ^{1}H NMR spectrum (Fig. 1) it was possible to identify seven reso-

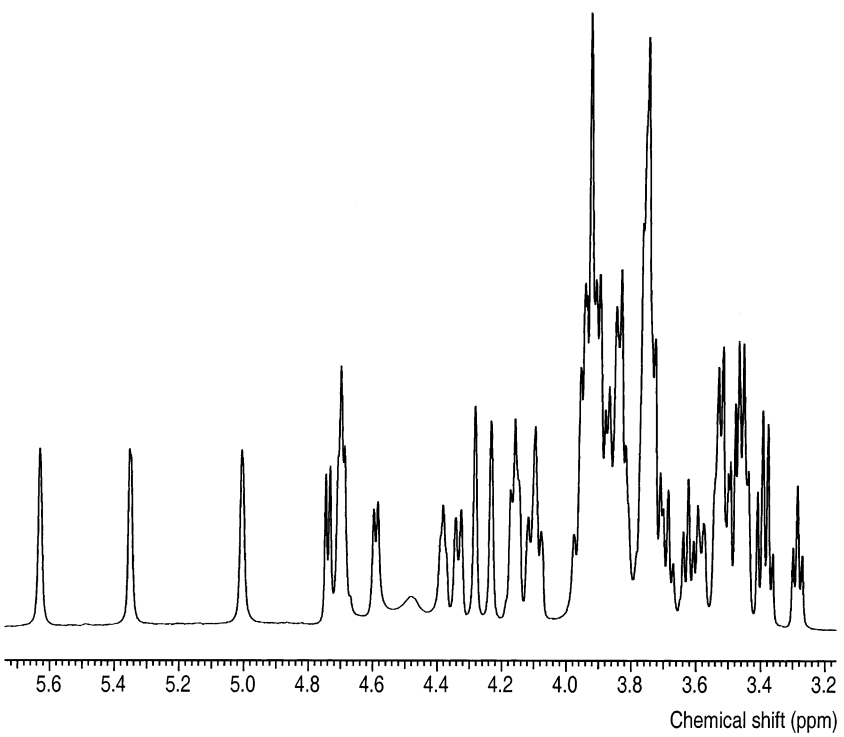


Fig. 1. Part of the ^{1}H NMR spectrum at 600 MHz of the *L. helveticus* Lb161 exopolysaccharide.

Table 1
Chemical shift (ppm) of the ^{1}H and ^{13}C NMR signals of the exopolysaccharide from *L. helveticus* Lb161

Sugar residue	$^{1}H/^{13}C$					
	1	2	3	4	5	6
→2,3)-α-D-Glc*p*-(1→	5.63 (~3) [a]	3.91	4.16	3.62	4.11	3.84
A	96.4 [173] [b]	73.1	80.1	69.1	72.3	61.2
→3)-α-D-Gal*p*-(1→	5.35 (3.8)	4.08	4.16	4.23	4.38	~3.75
B	95.1 [175]	68.0	80.1	70.2	71.4	62.0
→4)-α-D-Glc*p*-(1→	5.00 (3.6)	3.58	3.92	3.74	4.33	3.88, 3.95
C	99.9 [173]	72.4	72.1	79.5	71.1	60.5
β-D-Glc*p*-(1→	4.74 (7.8)	3.37	3.48	3.44	3.53	3.75, 3.94
D	103.5 [167]	74.1	77.4	70.5	76.7	~61.6
→3,4)-β-D-Gal*p*-(1→	4.70 (7.3)	3.87	3.89	4.28	3.83	~3.82, 3.90
E	105.4 [~168]	71.8	81.5	76.6	76.1	~60.7
β-D-Glc*p*-(1→	4.69 (7.8)	3.28	3.51	3.39	3.44	~3.74, 3.93
F	105.2 [~166]	74.5	76.7	70.7	76.8	~61.7
→3)-β-D-Glc*p*-(1→	4.59 (7.7)	3.46	3.71	3.68	3.53	~3.76, 3.93
G	103.6 [~168]	72.9	82.9	70.8	76.7	~61.4

[a] $J_{H-1,H-2}$ values are given in Hz in parentheses.
[b] $J_{C-1,H-1}$ values are given in Hz in brackets.

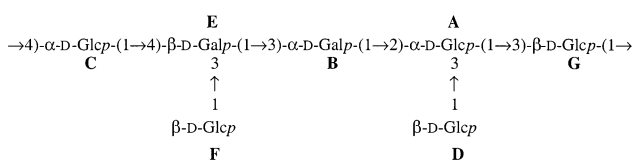
nances in the region for anomeric protons at δ 5.63, 5.35, 5.00, 4.74, 4.70, 4.69, and 4.59. The ^{1}H detected ^{13}C decoupled $^{1}H,^{13}C$ HSQC spectrum contained, inter alia, seven resonances in the region for anomeric signals. The above sugar and methylation analyses together with NMR spectra show that the polysaccharide is composed of a heptasaccharide repeating unit. From the methylation analysis and NMR spectra, it is evident that the sugars are pyranoid.

The chemical shifts for the protons and carbons, together with coupling constants for anomeric protons and carbons, are given in Table 1. The assignments of the spin system for each sugar residue were performed using homo- and heteronuclear two-dimensional techniques. Homonuclear COSY gave the chemical shifts of the H-2, H-3, and H-4 signals for residues **A**–**C** and **F**. The H-2 and H-3 signals for residues **D** and **G**, H-2 for residue **E**, H-5 for residues **A**, **C**, and **F**, and the H-6 signals for residues **A** and **F** could also be assigned with the COSY experiment. Using $^{1}H,^{1}H$ TOCSY experiments, H-4–H-6 resonances for residue **D**, H-3 and H-4 for residue **E**, as well as H-4 and H-5 for residue **G** were assigned. Intra-residue NOE correlations used in the assignment of spin systems were, inter alia, H-1 in **A** to its H-2, H-1 in **B** to its H-2, H-1 in **C** to its H-2, H-1 in **D** to its H-3 and H-5, H-1 in **E** to its H-5, H-1 in **F** to its H-3 and H-5, and H-1 in **G** to H-5. From the $^{1}H,^{13}C$ HSQC spectrum the carbon chemical shifts corresponding to the protons for residues **A**–**F** could be assigned. Further assignments to corroborate these results were deduced from intra-residue correlations employing an HSQC–TOCSY experiment. These correlations were, inter alia, C-3 in **D** to its H-1, H-2, H-4 and H-5, H-2 in **F** to its C-3 and C-4, C-1 in **F** to its H-2 and H-3, C-2 in **G** to its H-1, H-3 and H-4 and C-3 in **G** to its H-2 and H-4. To assign the H-6 and C-6 resonances COSY, TOCSY, HSQC and HSQC–TOCSY experimental results were used.

The identification of a spin system with a specific sugar residue and substitution pattern, as determined from the methylation analysis, was carried out as follows. For residues **A**, **B**, and **C** the values of $J_{H-1,H-2}$ were ~3, 3.8, and 3.6 Hz, respectively, showing α configuration and for residues **D**–**G** the $J_{H-1,H-2}$ values were around 8 Hz, showing β configuration. In the TOCSY experiment, the magnetization transferred only to H-4 for residues **B** and **E** due to small couplings between H-4 and H-5, and residues **B** and **E** could therefore be assigned to the Gal*p* residues. The remaining residues

A, **C**, **D**, **F**, and **G** could then be assigned to the Glc*p* residues in accordance with sugar and methylation analysis. The downfield chemical shifts for the C-3 signals, δ 80.1 and δ 82.9, of residue **B** and **G**, respectively, compared with α-D-Gal*p* and α-D-Glc*p* [10], respectively, demonstrated that these were 3-substituted in accordance with methylation analysis. Residue **C** was assigned to the 4-substituted glucose supported by the downfield chemical shift of the C-4 signal, δ 79.5, compared with α-D-Glc*p*. The chemical shifts of residues **D** and **F** compared with those for terminal β-D-Glc*p* show that these residues are terminal. Residue **E**, having downfield chemical shifts for C-3 (δ 81.5) and C-4 (δ 76.6), demonstrated that this was the 3,4-disubstituted galactose in accordance with methylation analysis. Finally, the chemical shifts for C-2 and C-3, δ 73.1 and δ 80.1, respectively, of residue **A** are in good agreement with the chemical shifts from the trisaccharide, α-D-Gal*p*-(1→2)[β-D-Gal*p*-(1→3)]α-D-Glc*p*OMe [11], revealing that residue **A** is the 2,3-disubstituted glucosyl residue and indicating the anomeric configurations of the sugar residues substituting residue **A**.

In order to determine the sequence of the sugar residues, $^{1}H,^{13}C$ HMBC and $^{1}H,^{1}H$ NOESY experiments were performed. In the HMBC experiment there are, in addition to correlations within a sugar residue, inter-residue correlations from the anomeric protons as well as the anomeric carbons, over the glycosidic linkage to carbons and protons on the adjacent residue. Inter-residue HMBC and NOE correlations are compiled in Table 2. From the results, it was concluded that the neutral exopolysaccharide from *L. helveticus* Lb161 is composed of a heptasaccharide repeating unit with structure **1**. The exopolysaccharide contains approximately 0.6 equivalents *O*-acetyl group per repeating unit.



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The NOESY spectrum shows that residues **E** and **D** both have an inter-residue correlation between the anomeric proton and a signal at δ 4.16 corresponding to H-3 of residues **A** and **B** (Table 1). Even using high magnetic field strength (800 MHz for ^{1}H), sufficient separation of the H-3 signals from residues **A** and **B** was not attainable for straightforward interpretation. The carbon chemical shifts for C-3 also showed complete overlap and the sequence was deduced as follows. In a

Table 2
Inter-glycosidic correlations from the anomeric atoms observed in $^{1}H,^{1}H$ NOESY and $^{1}H,^{13}C$ HMBC spectra of the exopolysaccharide from *L. helveticus* Lb161

Sugar residue		δ_H or δ_C			Connectivity
		Anomeric atom	NOE to	HMBC to	
→2,3)-α-D-Glc*p*-(1→	**A**	5.63	3.71		**A**, H-1 to **G**, H-3
		96.4		3.71	**A**, C-1 to **G**, H-3
→3)-α-D-Gal*p*-(1→	**B**	5.35	3.91		**B**, H-1 to **A**, H-2
→4)-α-D-Glc*p*-(1→	**C**	5.00	4.28		**C**, H-1 to **E**, H-4
				76.6	**C**, H-1 to **E**, C-4
		99.9		4.28	**C**, C-1 to **E**, H-4
β-D-Glc*p*-(1→	**D**	4.74	4.16		**D**, H-1 to **A**, H-3
				80.1	**D**, H-1 to **A**, C-3
		103.5		4.16	**D**, C-1 to **A**, H-3
→3,4)-β-D-Gal*p*-(1→	**E**	4.70	4.16		**E**, H-1 to **B**, H-3
β-D-Glc*p*-(1→	**F**	4.69	3.89		**F**, H-1 to **E**, H-3
				81.5	**F**, H-1 to **E**, C-3
→3)-β-D-Glc*p*-(1→	**G**	4.59	3.74		**G**, H-1 to **C**, H-4
				79.5	**G**, H-1 to **C**, C-4
		103.6		3.74	**G**, C-1 to **C**, H-4

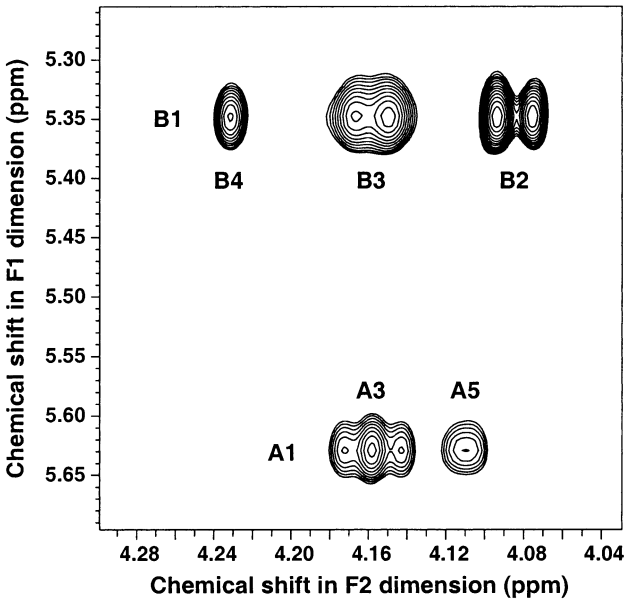

Fig. 2. Part of a ^{1}H,^{1}H TOCSY spectrum of the *L. helveticus* Lb161 exopolysaccharide at 600 MHz using a mixing time of 90 ms. Cross-peaks are annotated.

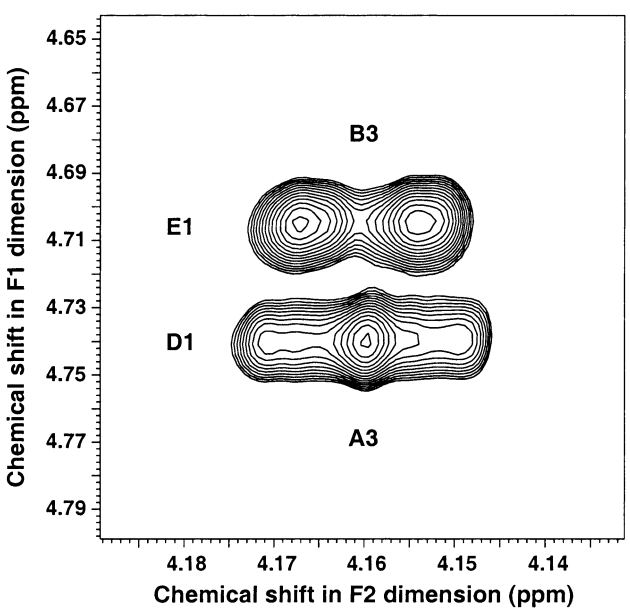

Fig. 3. Part of a ^{1}H,^{1}H NOESY spectrum of the *L. helveticus* Lb161 exopolysaccharide at 800 MHz using a mixing time of 100 ms. Cross-peaks are observed for anomeric protons of residues **E** and **D** to H-3 of residues **B** and **A**, respectively.

TOCSY spectrum (Fig. 2) the cross-peak for the H-3 signal of residue **B** shows a typical spin–spin coupling pattern as expected for a galactosyl residue, with a large ($\sim$ 10 Hz) and a small coupling constant. On the other hand, the H-3 signal of residue **A** has two large couplings ($\sim$ 10 Hz) typical for a glucosyl residue. In the NOESY spectrum (Fig. 3) the coupling pattern of the cross-peaks from the anomeric protons of residues **E** and **D** and the signals at δ 4.16 corresponds to the coupling pattern of H-3 in **B** and H-3 in **A** in the TOCSY spectrum, respectively. Thus, this proves residue **B** to be substituted by residue **E**, and **A** by **D**.

Previously, we have reported a neutral exopolysaccharide named *L. helveticus* Äki 4 [9], which contained a single branched hexasaccharide repeating unit with only D-glucose and D-galactose. *L. kefiranofaciens* K_1 [12], *L. helveticus* 766 [5] and *L. helveticus* TN-4 [8], a mutant of *L. helveticus* TY1-2 [6], also contain only glucose and galactose, but the latter two with one galactofuranosyl residue in the repeating unit. Anionic exopolysaccharides with a phosphodiester or a phosphate group have also been reported [13,14]. Rheological studies reveal that there is considerable variation among the different exopolysaccharides from lactic acid bacteria [15]. The connection between structure and viscosity of the exopolysaccharides requires further structural analysis combined with rheological studies.

3. Experimental

Growth of the organism.—*L. helveticus* Lb161 was obtained from Valio's Culture Collection, Valio Ltd., Research and Development Service, Helsinki, Finland. The organism was isolated from raw milk and maintained at -80 °C in glass beads. It was subcultured twice in MRS broth [16] at 37 °C before use. The growth medium used for the production of exopolysaccharides consisted of 10% skim milk powder (Valio) in water, heat treated at 121 °C for 15 min. The growth of the organism was carried out at 37 °C for 20 h with a 1% inoculum.

Isolation of the exopolysaccharide.—After bacterial growth, trichloroacetic acid (E. Merck, Darmstadt, Germany) was added to the culture (1 L) to a final concentration of 4% (w/v), and stirred for 2 h. Cells and precipitated proteins were removed by centrifugation (35 min, 22,000*g*, 4 °C). The supernatant was collected and filtered through an AcroCap filter (0.2 μm, Gelman Sciences, MI, USA). To precipitate the exopolysaccharide, cold

EtOH was gradually added to the filtered supernatant from one, two, and three volumes of the supernatant with intermediate centrifugation. Most of the exopolysaccharide precipitated at two volumes. The material was washed, and dissolved in water obtained from an Alpha-Q Reagent Grade Water Purification System (Millipore Co., Milford, MA, USA). The aqueous solution of the exopolysaccharide was again filtered through an Acrocap filter and lyophilized (0.26 g) on a DURA-DRY freeze-dryer (FTS Systems Inc., Stone Ridgeny, NY, USA). Part of the material was extensively dialysed against water overnight at 4 °C, and the exopolysaccharide solution was again lyophilized (5 mg). The uniformity of the exopolysaccharide material was further checked by gel permeation chromatography using a column (75 × 1.5 cm) of Bio-Gel P-30 polyacrylamide gel (exclusion limit 40,000 Da, 100–200 mesh, Bio-Rad Laboratories, Richmond, CA, USA). A sample (1 mg) was loaded onto the column and eluted with 0.05 M NH_4OAc (Riedel-deHaën AG, Seelze, Germany) with UV monitoring at 280 nm (Econo UV monitor, Model EM1, Richmond, CA, USA). The presence of sugar in the fractions was tested qualitatively with a Molish test [17] .

GLC/GLC–MS.—Alditol acetates and partially methylated alditol acetates were separated on an HP-5 fused silica column (0.20 mm × 25 m) using a temperature program of 180 °C for 1 min, followed by 3 °C min^{-1} to 250 °C. Hydrogen was used as carrier gas. The column was fitted to a Hewlett–Packard model 5890 series II gas chromatograph (Hewlett–Packard, Palo Alto, CA, USA) equipped with a flame ionization detector. GLC–MS analysis was performed on a Hewlett–Packard model 5970 mass spectrometer equipped with an HP-5MS fused silica column (0.20 mm × 25 m). A temperature program of 170 °C for 3 min, followed by 3 °C min^{-1} to 250 °C was used with helium as carrier gas.

NMR spectroscopy.—NMR spectra of a polysaccharide solution in D_2O (3 mg in 0.7 mL) were recorded at neutral pD and 55 °C using Varian Inova 600 or 800 MHz instruments. Chemical shifts are reported in ppm relative to sodium 4,4-dimethyl-4-sila-[2,2,3,3-$^2H_4$]pentanoate (TSP), δ_H 0.00 or 1,4-dioxane, δ_C 67.4 as external references. Data processing was performed using standard Varian VNMR software. ^1H,^1H correlated spectroscopy (COSY) [18], total correlation spectroscopy (TOCSY) [19], gradient selected heteronuclear single quantum coherence (gHSQC) [20], gradient selected heteronuclear multiple-bond correlation (gHMBC) [20] and ^1H, ^{13}C HSQC–TOCSY [21,22] experiments were used to assign signals and performed according to standard pulse sequences. For interresidue correlations, two-dimensional nuclear Overhauser effect spectroscopy (NOESY) [23] experiments with mixing times of 75, 100, and 150 ms, and HMBC experiments with 45 and 90 ms delays for the evolution of long-range couplings were used.

Sugar analysis.—The samples were hydrolysed with 2 M trifluoroacetic acid at 120 °C for 2 h. After reduction with $NaBH_4$ and acetylation, the samples were analysed by GLC. The absolute configuration of the sugars present in the exopolysaccharide from *L. helveticus* Lb161 was determined essentially as devised by Leontein et al. [24], but with (+)-2-butanol [25].

Methylation analysis.—The analysis was performed according to Hakomori [26] using sodium methylsulfinylmethanide and MeI in Me_2SO. The methylated compounds were recovered by use of Sep-Pak C_{18} cartridges (Millipore) [27]. The purified methylated sample was then hydrolysed (2 M trifluoroacetic acid, 120 °C, 2 h), reduced, and acetylated. The partially methylated alditol acetates were analysed by GLC–MS.

Acknowledgements

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