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Microscopic and Chemical Studies of a Gelling Polysaccharide from *Lactobacillus hilgardii*

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ABSTRACT

The bacterium Lactobacillus hilgardii *(previously named* L. brevis*), isolated from sugary kefir grains, has been found to produce a gelling extracellular polysaccharide. Methylation analysis and* ^{1}H- *and* ^{13}C-*NMR analysis revealed a dextran-like structure for the polysaccharide. A high proportion of* α*-(1,6)-Glc and small proportions of* α*-(1,3,6)-Glc,* α*-(1,4,6)-Glc and terminal* α*-Glc were detected. The* L. hilgardii *polysaccharide differed from normal dextrans in containing a small proportion of* α*-(1,3)-Glc. The structure of the polysaccharide was found to be independent of harvesting time and unaffected by changes in the growth medium. The development of the gel structure has been followed by transmission electron microscopy.*

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INTRODUCTION

In a previous study a polysaccharide producing strain of *Lactobacillus brevis* was isolated from sugary kefir grains and the polysaccharide identified as a dextran (Pidoux *et al.*, 1988). This strain, which biosynthesises the gel-forming polysaccharide of kefir grains, has recently been re-identified as *L. hilgardii* (Pidoux *et al.*, in press). The conditions for gel production have been examined (Pidoux, 1989). This article reports further chemical analysis of the structure of the polysaccharide produced by *L. hilgardii*. Changes in the composition of the growth medium were found to alter the total polymer yield. In view of this fact, and because other authors (Tsumuraya *et al.*, 1976) have noted that changes in growth medium can alter the structure of microbially produced dextrans, the possible effect of changes in growth medium upon the structure of the *L. hilgardii* polysaccharide was investigated. When the bacteria were grown on agar plates the nature of the gel formed by the extracellular polysaccharide was observed to change over a period of several days. Transmission electron microscopy (TEM) and chemical studies were used to assess changes in the appearance of the gels and to examine possible structural changes in the extracellular polysaccharide.

MATERIALS AND METHODS

Culture of *L. hilgardii* and polysaccharide production

L. hilgardii, previously isolated from sugary kefir grains, was maintained at 4°C on the agar used by Mayeux *et al.* (1962) (MSE) adjusted to pH 6·8, but with the addition of sucrose (100 g litre^{-1}). The composition of the growth medium was 1% (w/v) peptone, 0·5% (w/v) yeast extract, 10% (w/v) sucrose, 1·5% (w/v) agar, 0·1% (w/v) sodium citrate, 0·5% (w/v) glucose, 0·25% (w/v) gelatin and 75 ppm sodium azide. The cultures contained two variants, but only the type producing polysaccharide was used in the present studies. Gels to be examined by means of TEM were formed from cultures on the same agar at 30°C for 2 days or 5–6 days. Cultures at 30°C in modified MSE broth were used to produce dispersed polysaccharide which was harvested at different times. The polysaccharide was isolated in the following manner: the growth medium was centrifuged to remove bacterial cells (25 000 *g*, 1 h) and the polysaccharide precipitated from the supernatant by addition of alcohol (50% v/v); the precipitate was washed in boiling distilled water, redissolved, reprecipitated with ethanol, washed a second time and then freeze-dried.

Cultures of *L. hilgardii* (grown at 30°C) on MSE and MRS broths (De Man *et al.*, 1960) containing sucrose (100 g litre^{-1}) and omitting the peptone (10 g litre^{-1}) were used in order to assess the influence of medium composition upon the yield and structure of the polysaccharide.

Chemical analyses

Total neutral-sugar contents were determined by the method of Dubois *et al.* (1956). Neutral sugars were released by a Seaman hydrolysis and analysed as their alditol acetates by gas chromatography (Selvendran *et al.*, 1979). Inter-sugar linkages were determined by methylation analysis using a modification of the Hakomori method (Jansson *et al.*, 1976). Individual partially methylated alditol acetates were identified by their retention times relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl glucitol and from their mass spectrometric fragmentation patterns (Jansson *et al.*, 1976). Quantitative analysis was achieved using the molar response factors given by Sweet *et al.* (1975).

^{13}C- and ^{1}H-NMR spectra were measured on a Jeol GX-400 spectrometer at operating frequencies of 100·4 and 399·65 MHz, respectively. The polysaccharides were examined in deuterium oxide (pD = 7·0) at 30°C. pD measurements are pH meter readings uncorrected for the isotope effect. Acetone was added to all samples as internal standard and the chemical shifts of the acetone methyl group were taken to be 2·217 ppm (^{1}H) and 31·077 ppm (^{13}C) with respect to tetramethylsilane (TMS).

It was considered possible that *L. hilgardii* might produce a conventional non-gelling dextran plus a separate gelling polysaccharide. Two methods were used to test whether the gelling extracellular slime was a single polysaccharide or a mixture of 'gelling' and 'non-gelling' polysaccharides.

Firstly, possible selective solubility of different components upon addition of ethanol was examined by the method described by Whistler and Sännella (1965). The polysaccharide (50 mg) was dissolved in distilled water (5 ml) and stirred for 1 h. To ensure complete dissolution the pH was raised to 10 by the addition of sodium hydroxide. The solution was then neutralised with acetic acid and any insoluble material removed before ethanol precipitation. Ethanol was added drop by drop with stirring to incipient turbidity or until selected concentrations (20%, 40%, 60%, 80%) were reached. After 5 min the sample was centrifuged and the supernatant retained for further treatment. The precipitates and the final supernatant, after dialysis against water, were freeze-dried.

Secondly, selective solubility at different pH was examined. Phosphate buffers covering the range 7·0–11·5 were prepared. The polymer was dispersed in each buffer (0·1% w/w) and stirred for 1 h. The solution was

centrifuged (18 000 *g*, ½ h), the supernatant removed, and the polymer precipitated with 80% (v/v) ethanol.

Sample preparation for TEM studies

Samples of gel were removed from the MSE agar plates after 2 days (weak gel), 5–6 days (tough gel), or 2 weeks after subculturing in MSE broth. The samples were fixed in 3% (v/v) glutaraldehyde solution buffered to pH 7·2 with 0·1 M cacodylate-HCl for 1 h and then washed in buffer for 2 h. The samples were then fixed in a 1% (w/v) solution of osmium tetroxide in 0·1 M cacodylate-HCl buffer containing 0·15% (w/v) ruthenium red. After dehydration in a series of acetone–water mixtures, culminating in pure acetone, the samples were embedded in an epoxy resin (Araldite-TAAB Laboratories, Aldermaston, Berkshire, UK). Sections were cut using a Reichert Jung Ultracut E ultramicrotome, mounted on uncoated copper grids, stained with lead citrate, and examined using a Hitachi H-600 transmission electron microscope. Material embedded in this way was also used to determine the distribution of periodate-reactive carbohydrate using the periodic acid–thiosemicarbazide–silver proteinate (PA–TSC–Ag) method of Thiéry (1967). Thin sections were mounted on gold grids and oxidised using 1% (v/v) periodic acid for 20 min. Control grids were oxidised in 1% (v/v) hydrogen peroxide before further treatment. After washing in distilled water several times, grids were floated on to a 1% (w/v) solution of thiosemicarbazide for 6 h. The grids were then thoroughly washed in 5% (v/v) aqueous acetic acid and distilled water and stained with 1% (w/v) silver proteinate in the dark for 0·5 h. They were then washed with water and examined by TEM without further staining.

RESULTS

Analysis of the *L. hilgardii* polysaccharide preparation revealed > 99% carbohydrate of which > 96% was found to be glucose. Results of the methylation analysis of the sample are shown in Table 1. Terminal-Glc, (1,6)-Glc and (1,3,6)-Glc are common characteristic linkages found in dextrans, and (1,4,6)-Glc has also been observed in certain dextran structures (Sidebotham, 1974). The unusual feature of the *L. hilgardii* sample is the presence of (1,3)-Glc linkages. The anomeric configuration of the linkages can be determined by NMR. The peaks in the ^{1}H-NMR spectrum are broad and only the coupling constant (2·75 Hz) belonging

to the principal resonance (1,6)-Glc could be measured. This confirms the presence of α-(1,6)-Glc (Perlin, 1982). The ^{13}C-NMR signals can be assigned by reference to the literature (Colson *et al.*, 1974, 1979; Usui *et al.*, 1973), and this assignment is shown in Table 2. All the linkages were found to have the α-configuration, and the ^{13}C-NMR spectrum confirmed the presence of a small amount of α-(1,4,6)-Glc. The presence of β-(1,3)-Glc can be definitely excluded, because this linkage would yield chemical shifts of C-1 (104·7) and C-4 (88·0) (Colson *et al.*, 1979).

Progressive precipitation with ethanol led to the separation of the polymer into two fractions. The first fraction (7·3 mg) precipitated at 45% (v/v) ethanol and the second (42·7 mg) at >80% (v/v) ethanol. Methylation analysis of both fractions revealed the same linkages, suggesting that the precipitation was selecting different molecular weight fractions of the polysaccharide. No separation was obtained by varying the sample pH. The polysaccharide was found to be either completely soluble or completely insoluble at different pH values.

TABLE 1
Linkage Analysis of *L. hilgardii* Polysaccharide (Grown in MSE Broth, 30°C, 48 h)

Terminal-Glc	*(1,3)-Glc*	*(1,6)-Glc*	*(1,3,6)-Glc*	*(1,4,6)-Glc*
7·8%	6·3%	77·6%	7·6%	0·7%

TABLE 2
^{13}C-NMR Data for *L. hilgardii* Polysaccharide

Chemical shift (ppm)	*Atom*	*Linkage*
101·62	C-1	α-(1,3); α-(1,3,6)
100·65	C-1	α-(1,4,6)
98·56	C-1	α-(1,6); α-terminal
83·71	C-3	3-linked
82·65	C-4	4-linked
74·45	C-3	α-(1,6)
72·25	C-2	α-(1,6)
70·96	C-4	α-(1,6)
70·41	C-5	α-(1,6)
66·29	C-6	α-(1,6)
61·38	C-6	non-substituted

The composition of the growth medium was found to affect the total polymer yield. As expected, the yield obtained in MRS–sucrose broth (29·6 mg ml^{-1}) was higher than that obtained in MSE–sucrose broth (27 mg ml^{-1}) or in MSE–sucrose broth without peptone (22 mg ml^{-1}). It is well established that meat extract and minerals, present as Mg^{2+}, Mn^{2+} and PO_4^{2-} in MRS broth or peptone, increase the growth of lactic acid bacteria (Kandler & Weiss, 1986) and, consequently, the amount of polysaccharide produced. Results of the methylation analysis of the polymers produced in the different growth media are presented in Table 3. The data reveal no significant change in composition and, in particular, no significant change in the (1,3)-Glc content with changes in growth medium. The appearance of (1,3,4,6)-Glc and (1,2,4,6)-Glc are likely to be artefacts resulting from under-methylation. The presence of these compounds is not matched by a corresponding increase in terminal-Glc but corresponds to the reduction in (1,3)-Glc and (1,6)-Glc.

The effect of harvesting time on the composition of the *L. hilgardii* polysaccharide preparation was also investigated. The results of the methylation analysis of the fractions obtained after centrifugation are presented in Table 4. The composition of the polysaccharide was found to be independent of harvesting time and there was no obvious difference in structure between the soluble (supernatant) and insoluble (aggregate) fractions. In particular, the amount of (1,3)-Glc was found to be independent of harvesting time. The detected (1,3,4,6)-Glc and (1,2,4,6)-Glc are again likely to be artefacts arising from under-methylation. The total polymer yield was found to increase from 8·8 mg

TABLE 3
Linkage Analysis of *L. hilgardii* Polysaccharide Prepared under Different Growth Conditions, Determined by Methylation Analysis

Linkage	*Media*[a]		
	MSE	*MSE without peptone*	*MRS without peptone*
Terminal-Glc	7·6	6·2	8·0
(1,3)-Glc	6·7	7·6	7·6
(1,6)-Glc	60·5	63·4	60·3
(1,3,6)-Glc	10·2	10·3	9·2
(1,4,6)-Glc	4·1	3·9	4·0
(1,3,4,6)-Glc	8·4	6·7	6·2
(1,2,4,6)-Glc	2·5	1·9	4·7

[a]Media (broths with 10% sucrose): cultured at 30°C, 48 h.

ml^{-1} after 21 h to 11·7 mg ml^{-1} after 45 h, and then to decrease slightly, becoming 9·3 mg ml^{-1} after 69 h and 9·7 mg ml^{-1} after 93 h. The observed decrease in yield probably results from hydrolysis.

After 2 days, a weak gel started to form on the surface of MSE agar plates. TEM studies of ruthenium red treated gel showed that it consisted of globular, filamentous and fibrillar components arranged in a complex 3D network (Fig. 1). All of these structural elements were composed of periodate oxidisable carbohydrate, as demonstrated by the PA-TSC-Ag staining (Fig. 2). Control sections showed some slight granular staining of the bacterial cell walls, but the capsular material was found to be completely negative (Fig. 3). After 5–6 days growth on MSE medium a tougher gel was formed. The internal structure of this gel showed an expansion of the fibrillar structures (Fig. 4). If this material was subsequently cultured in MSE broth, hard granular material was formed, which consisted of very dense, highly cross-linked networks of periodate-reactive carbohydrate (Fig. 5) containing the immobilised bacterial cells (Fig. 6).

DISCUSSION

The polysaccharide derived from *L. hilgardii* was found to be a single polysaccharide and not a mixture of a non-gelling dextran and a gelling

TABLE 4
Linkage Analysis of Fractions of *L. hilgardii* Polysaccharides as a Function of Harvesting Time, after Growth in MSE Broth

Linkage	*Harvesting time*							
	21 h		*45 h*		*69 h*		*93 h*	
	A	*B*	*A*	*B*	*A*	*B*	*A*	*B*
Terminal-Glc	7·7	8·4	9·7	9·3	10·3	8·3	9·4	10·4
(1,3)-Glc	6·6	7·2	9·8	8·6	8·9	9·2	8·5	8·7
(1,6)-Glc	70·7	67·9	63·5	65·7	66·6	62·4	60·8	68·2
(1,3,6)-Glc	7·6	8·5	8·8	8·5	6·9	10·1	9·5	7·5
(1,4,6)-Glc	2·7	2·9	3·0	2·9	2·2	3·8	3·8	2·4
(1,3,4,6)-Glc	3·7	4·0	4·0	3·9	3·8	4·8	5·9	2·3
(1,2,4,6)-Glc	1·0	1·1	1·2	1·1	1·3	1·4	2·1	0·5

Samples were examined after centrifugation (25 000 *g*, 1 h, 20°C); A = aggregate of polysaccharides, B = soluble polymer in supernatant.

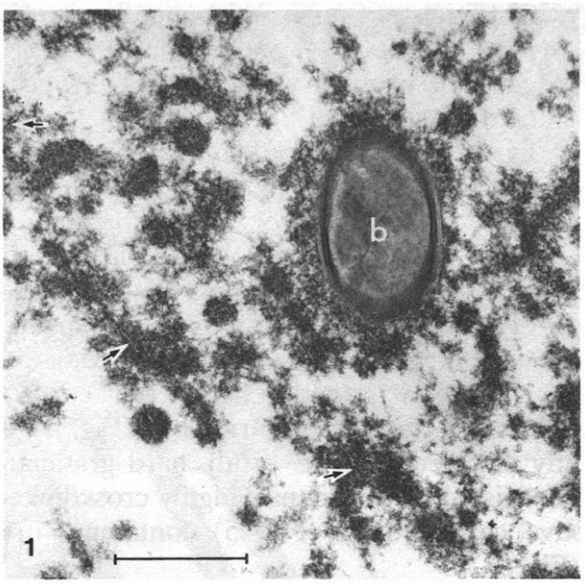

Fig. 1. *L. hilgardii* polysaccharide gel examined by TEM; gel produced after 2 days growth on MSE agar, showing fibrillar material traversing globular component in places (arrows) and more dispersed filaments; stained with ruthenium red and lead citrate; b = bacterial cell; bar marker = 0·5 μm.

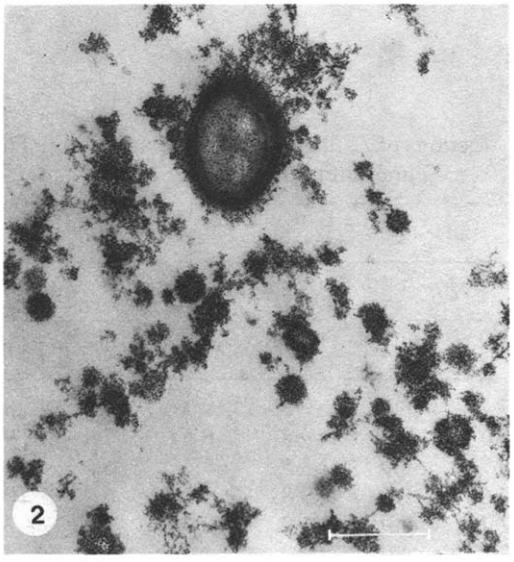

Fig. 2. *L. hilgardii* polysaccharide gel examined by TEM; gel produced after 2 days growth on MSE agar but stained by PA–TSC–Ag for the presence of periodate-oxidisable carbohydrate; the globular and fibrillar components of the gel contain periodate-oxidisable carbohydrate; bar marker = 0·5 μm.

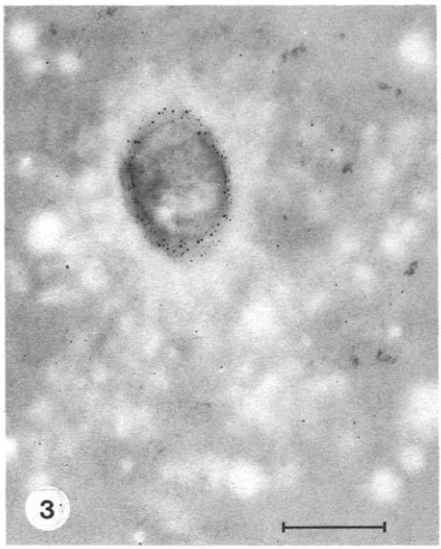

Fig. 3. Control samples treated with 1% (v/v) hydrogen peroxide instead of 1% (v/v) periodic acid; no further staining was carried out; bar marker = 0·5 μm.

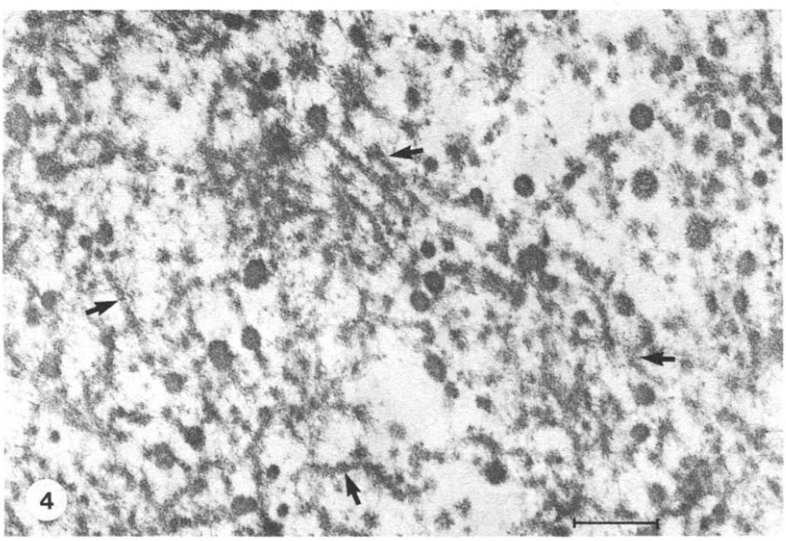

Fig. 4. *L. hilgardii* polysaccharide gel examined by TEM: gel produced after 6 days growth on MSE agar showing a dense network of globular and fibrillar carbohydrate; filamentous material has condensed on the fibrils (arrows); stained with ruthenium red and lead citrate; bar marker = 0·5 μm.

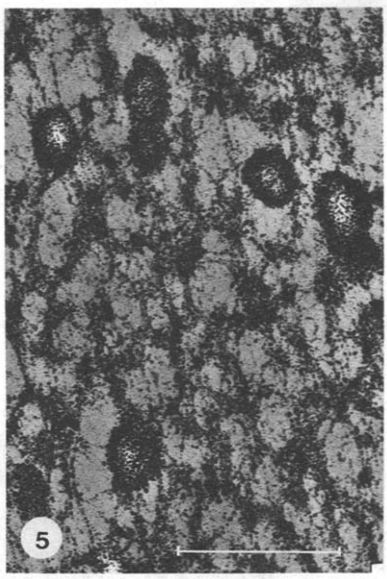

Fig. 5. *L. hilgardii* polysaccharide gel examined by TEM; gel obtained after 2 weeks by subculture in MSE broth; hard grains of polysaccharide stained with PA–TSC–Ag and showing extensive network of carbohydrate; note, the centre of the globular carbohydrate is unstained and free of periodate-oxidisable material; numerous filaments are stained; bar marker = 0·5 μm.

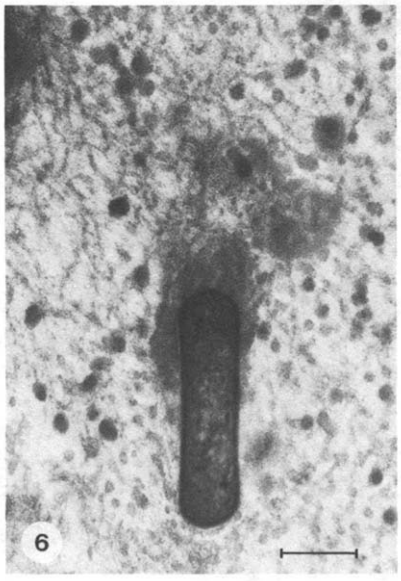

Fig. 6. Sample preparation as for Fig. 5; picture illustrates a bacillus, partially surrounded by a capsule, embedded in the dense polysaccharide network of a hard granule; globular and fibrillar components of the grain corresponding to those in Fig. 5 can also be seen; stained with ruthenium red plus lead citrate; bar marker = 0·5 μm.

polysaccharide. The polysaccharide shows characteristics of a normal dextran structure (α-(1,6)-Glc, α-(1,3,6)-Glc, terminal-α-Glc and α-(1,4,6)-Glc), but contains additional α-(1,3)-Glc. The presence of α-(1,3)-Glc in dextrans is known to render them insoluble in water (Sidebotham, 1974). Where such linkages lead to gelation of dextrans they are believed to occur in short blocks. Marchessault and Deslandes (1981) have shown that linear α-(1,3)-Glc regions will form 'ribbon-like' conformations similar to cellulose. Cross-linking of these ribbon-like regions by hydrogen bonding would act as the junction points of the gel. The low level of α-(1,3)-Glc present in the *L. hilgardii* polysaccharide probably accounts for the high concentration ($>4\%$) required for gelation.

When *L. hilgardii* is grown on MSE agar plates the nature of the gel formed appears to change over a period of days. Gel formation cannot be simply correlated with polymer concentration because, although the total polymer yield reached a maximum after 2 days, the gel structure was still shown to be evolving, even after 4 days. The gelling dextran of *L. hilgardii* presents several similarities with the pictures of capsular dextrans of *Leuconostoc mesenteroides* as described by Brooker (1979): the existence of globular and filamentous forms of carbohydrate stained with Thiéry (1967) polysaccharide histochemistry (Fig. 5) and fibrillar forms staining only on their surface (fig. 2). The fibrils seemed to form an increasingly dense network as the gel hardened (Fig. 4); the filamentous forms appeared to cover the fibrils, creating many small spaces (Figs 4 and 5). Since the centres of the globules contained very little periodate-reactive carbohydrate, and as the fibrils seemed to traverse the globules near their axis (Fig. 2), we suggest that the fibrils could consist of carbohydrates containing periodate-unreactive glycosidic linkages like highly branched dextrans. It could be possible that the fibrils originated from the globular dextran as described by Brooker (1979) for *Leuconostoc mesenteroides* NCDO 523 and created a network by rearrangement, since no significant change in chemical structure was observed with time. The capsules around some bacilli (Fig. 6) seemed to consist of packed filamentous and fibrillar material.

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