Intracellular Polysaccharide of *Bacteroides fragilis*

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Formation of iodophilic polysaccharide (IPS) from glucose was demonstrated in 27 strains of *Bacteroides fragilis*. Synthesis was dependent on the glucose concentration of the medium, the pH and the growth phase. When glucose was in short supply the cellular polysaccharide was degraded rapidly at pH 4.5 to 6.5 and fatty acids accumulated in the medium. Storage of IPS was not responsible for the low carbon recoveries observed in fermentation balance studies. In electron micrographs of thin sections, the IPS was observed as cytoplasmic granules dispersed throughout the whole cell. After extraction and purification the IPS was characterized as a glycogen.

INTRODUCTION

Gas chromatographic analysis of fatty acids has greatly facilitated the identification and classification of the genus *Bacteroides*. In saccharolytic strains, the main products are succinic, acetic, lactic and propionic acids. Quantitative gas chromatography permits detailed studies of fermentation balances. In our studies of these balances in growing cultures of *B. fragilis* strains, we found that C recoveries ranged from 73 to 100 %, whereas residual glucose could never be demonstrated. The low C recovery might be explained by the formation of some other products, not detected by gas chromatographic analysis, or by the synthesis of polysaccharides from glucose. The latter process is well known in streptococci (Crowley & Jevons, 1955; Gibbons & Socransky, 1962), diphtheroids (Gibbons & Socransky, 1962) and fusobacteria (Gibbons & Socransky, 1962; Van Houte, 1967).

In the present work, we have studied the synthesis of iodophilic polysaccharide (IPS) by *B. fragilis*, the localization of the IPS and its nature and physical properties.

METHODS

Organisms. *Bacteroides fragilis* NCTC 9343, 9344, 10583, *B. fragilis* subsp. *thetaiotaomicron* NCTC 10582 and 23 strains of *B. fragilis* isolated from stool or pus [subsp. *fragilis* (9 strains), subsp. *distasonis* (7), subsp. *vulgatus* (5), subsp. *ovatus* (1) and subsp. *thetaiotaomicron* (1)] were used. All strains were identified by the procedures outlined by Holdeman & Moore (1972); gas chromatographic analysis of fatty acids was performed according to Lindner & Marcelis (1978). All strains were tested for IPS production.

Cultural methods. Viande-Leuvre medium (Beerens & Fievez, 1971) with 2 mg haemin l⁻¹ (HVL) and synthetic medium (SM) according to Macy *et al.* (1975) were used as basal media. HVL medium with 1.5 % (w/v) Difco Bacto-agar was used as a solid medium. All media were used freshly poured. The cultures were incubated anaerobically in jars under an atmosphere of H₂/CO₂ (9:1, v/v).

Growth was assayed in terms of the absorbance at 660 nm (*A*₆₆₀) measured using a Klett–Summerson photoelectric colorimeter (filter no. 66).

Dry weight was determined by filtration according to Stouthamer (1969). The membrane filters used (Sartorius, Göttingen, West Germany) had a pore size of 0.2 µm.

Determination of fermentation balances. At various times during growth, 10 ml samples were taken from a culture in HVL with 27 mM-glucose, and centrifuged. Fatty acids in the supernatant solution were

determined quantitatively by gas chromatography. The residual glucose was determined enzymically according to Huggett & Nixon (1957) by means of a test combination containing glucose oxidase and peroxidase (Boehringer) or with hexokinase (Schmidt, 1961). Carbon recoveries were calculated according to Dawes *et al.* (1971). No gas production was observed.

Determination of IPS. Bacteria from 10 ml samples of cultures grown in HVL for 24 to 36 h were washed once with saline [0.85 % (w/v) NaCl] and resuspended in saline to an A_{660} of 80 to 100 Klett units. To 10 ml of this suspension 2 ml of a solution of 0.2 % (w/v) I_2 in 2 % (w/v) KI was added (Van Houte, 1967). The absorbance of the IPS-iodine complex (E_1) was measured at 540 nm (filter no. 54); the absorbance of the same suspension without iodine was used as a blank (E_0). The photoelectric colorimeter was adjusted with 10 ml water and 2 ml iodine solution. The amount of IPS was expressed as Brown Value (BV) = $[(E_1 - E_0)/E_0] \times 100$.

Preparation of cell suspensions. Bacteria from a 250 ml culture in SM with 14 mm-glucose incubated for 24 h at 37 °C were harvested by centrifuging (1000 g, 30 min), washed twice in sterile saline and resuspended in 30 ml saline.

Determination of the effect of pH on IPS content. Portions (1 ml) of cell suspension were added in triplicate to tubes containing 5 ml 0.06 M-Sørensen phosphate buffer (pH range 4 to 8) supplemented with 14 mm-glucose. After 3, 6, 20 or 48 h at 37 °C, the pH, residual glucose and the amount of IPS were measured.

Preparation of cell-free extracts. Bacteria were frozen and passed through a French press according to Hughes *et al.* (1971). Disrupted material was centrifuged for 30 min at 36000 g. The IPS contents of both the wall fraction and the supernatant solution were determined.

Isolation and purification of IPS. The cell-free extract obtained from bacteria grown in 3.6 l SM with 56 mm-glucose was incubated with 1 mg lysozyme ml⁻¹ (Fluka, Germany) at 37 °C for 1 h to remove contaminating wall residues. Subsequent extraction followed the procedures of Gibbons & Kapsimalis (1963). The polysaccharide was dialysed against water at 4 °C for 4 d and lyophilized.

Analytical methods. The reaction of iodine with polysaccharide was studied by adding 1 ml of the iodine solution to 5 ml of a solution containing 1 mg polysaccharide. The absorbance of the iodine-polysaccharide complex was monitored between 250 and 750 nm using a Perkin Elmer 124 spectrophotometer. Water and the same iodine solution were used as blanks.

Standard curves were prepared relating the concentration of iodine-polysaccharide complexes to absorbance at 540 nm measured using a Klett-Summerson photoelectric colorimeter (filter no. 54).

The optical rotation of a solution containing 26.5 mg polysaccharide in 10 ml 1 M-NaOH was measured on a Bendix Ericsson Automatic Polarimeter type 143A using the sodium D line at 589 nm.

The molecular weight of the polysaccharide was determined using a 10 ml Sephadex 4B column and 0.85 % (w/v) NaCl as eluant. Fractions of 0.45 ml were collected and tested for neutral sugars by the phenol/H₂SO₄ method (Dubois *et al.*, 1956) and for iodine-staining power. Molecular weight standards were from the following sources: apotransferrin (Boehringer), polyvinylpyrrolidone-DNP₃₅ (kindly supplied by Dr H. Snippe, Department of Immunology, Laboratory of Microbiology, Utrecht, The Netherlands), rabbit liver glycogen (Boehringer), dextran HM VII (Poviet Production, Amsterdam, The Netherlands), blue dextran 2000 (Pharmacia).

Acid hydrolysis of polysaccharide. Samples were hydrolysed with 1 M-HCl at 100 °C for 0.5 to 8 h. The HCl was neutralized with 2 M-NaOH.

Enzymic hydrolysis of polysaccharide. For hydrolysis of 1,4- α and 1,6- α bonds, samples were treated with amyloglucosidase. For hydrolysis of 1,4- α bonds, samples were treated with β -amylase followed by maltase to degrade disaccharides. The glucose contents of the digests were determined by means of a Biochemica test combination (hexokinase method). Enzymes were obtained from Boehringer.

For detection of carbohydrates, thin-layer chromatography of hydrolysates and digests was carried out according to Lato *et al.* (1969). Chromatographic standards were: D-glucose, D-fructose, D-rhamnose, maltose, glyceraldehyde and rabbit liver glycogen. Amino acids were detected by ninhydrin (1 %, w/v, in acetone).

Electron microscopy. Cells cultivated in SM with 56 mm-glucose (24 h at 37 °C) or 14 mm-glucose (48 h at 37 °C) were centrifuged at 1000 g for 10 min. The pellets were fixed in a solution containing 1 % (w/v) glutaraldehyde, 1 % (w/v) formaldehyde and 1 % (w/v) sucrose in 0.1 M-phosphate buffer, pH 7.4, at room temperature for 45 min. This was followed by postfixation in Palade-buffered 1 % (w/v) OsO₄, pH 7.4, for 1 h. The pellets were then embedded in Epon 812. Ultrathin sections were either stained with lead citrate for morphological examination or treated with periodic acid, thiocarbohydrazide and silver proteinate for the demonstration of polysaccharides according to Thiéry (1967). As a control for the latter method, periodic acid oxidation was omitted. Sections were examined in a Philips 301 electron microscope.

Table 1. Fermentation balances in a growing culture of *B. fragilis* subsp. *fragilis* C₁₆₁ in SM with 28 mM-glucose

Time of incubation (h)	Dry weight (mg l ⁻¹)	Fatty acids (mM)					Residual glucose (mM)	C recovery (%)
		Acetic	Propionic	Butyric	Lactic	Succinic		
12	340	4.4	0.9	0.5	2	5	1.1	32
25	570	1.4	—	—	—	10	0.2	35
50	530	12.5	0.6	0.3	8	17	0.06	82
70	530	12.0	0.6	0.3	10	19	0.06	88

—, Not detectable.

Fig. 1

Fig. 2

Fig. 1. Growth of *B. fragilis* subsp. *fragilis* C₁₆₁ expressed as Klett units of absorbance (○) and the corresponding values of IPS content of the cells in BV units (●), pH (□) and glucose concentration (■) in the medium.

Fig. 2. The IPS content of cell suspensions of *B. fragilis* subsp. *fragilis* C₁₆₁ as a function of the final pH after incubation at 37 °C for 3 h (●), 6 h (○), 20 h (■) or 48 h (□). In general the final pH was 0.5 lower than the initial pH. The shape of the 20 h curve was confirmed in other experiments. No IPS could be detected at time zero.

RESULTS

IPS production was demonstrated in all strains of *B. fragilis* examined, the Brown Values (BV) varying from 15 to 150. The observed BV was dependent on the phase of growth (Fig. 1). IPS synthesis started during the exponential phase and continued until the maximum growth yield was reached after 30 h. In the first 20 h of the stationary phase the amount of IPS decreased to 25 % of the maximum present at the end of the exponential phase; in the next 20 h it was totally consumed.

The glucose concentration decreased to 1.1 mM after 10 h. After 25 h growth, residual glucose could no longer be detected; the amount of fatty acids produced then increased sharply (Table 1) and at the same time the pH fell to 5.9.

Fermentation balances, calculated at different stages of growth of strain C₁₆₁, are shown in Table 1. After 25 h growth, the dry weight value was high but the C recovery was low (35 %). After 70 h growth, the dry weight was similar but the C recovery was more satisfactory (88 %). Analogous experiments resulted in C recoveries of 98 % for strain BB₄₉ and 100 % for F₂₄.

Influence of pH on IPS synthesis and degradation

The effect of pH on cellular polysaccharide content was studied using cell suspensions incubated in phosphate buffer, pH 4.5 to 8, with 14 mM-glucose (Fig. 2). At pH 4 to 4.5, IPS was synthesized in small quantities (BV 50) and was not degraded. The supernatant solution contained residual glucose. At pH values between 4.5 and 6.5, IPS was rapidly synthesized in the first 6 h of incubation, up to BV 130. After 6 h no residual glucose could be detected. By 20 h, degradation became apparent and the polysaccharide content was lower, and had fallen further by 48 h. The rate of IPS production and degradation increased with the pH. At higher pH (≥ 7) the synthesis was rapid up to BV 130, but degradation was delayed. However, after 48 h IPS degradation was complete.

Influence of glucose concentration on IPS synthesis

The amount of IPS production in cell suspensions was directly correlated with the glucose concentration of the medium. At concentrations below 1.7 mM no IPS was synthesized. At higher concentrations the rate of IPS synthesis increased reaching a maximum with 5.5 mM-glucose in the medium.

Localization of the IPS

After disruption of bacteria, IPS was found in the cytoplasmic fraction but not in the wall fraction. The intracellular localization of IPS was confirmed by electron microscopy. In thin sections of cells having a high IPS content (BV 140), a regular dispersion of many electron-dense polysaccharide particles distributed over the whole bacterial cell was seen (Fig. 3*a, b*). In starved cells without IPS (BV 0), only a few particles of polysaccharide were apparent (Fig. 3*c, d*).

Nature of the IPS

The 3.6 l culture incubated for 48 h at 37 °C yielded 230 mg purified and lyophilized polysaccharide.

The absorbance of the iodine-polysaccharide complex showed a maximum at 490 nm. Commercial rabbit liver glycogen produced a brown colour with an absorption maximum at 495 nm when treated in the same manner. The iodine-staining power of the commercial glycogen was about twice that of the IPS, as calculated from the standard curves relating IPS and commercial glycogen concentration to absorbance.

Development of thin-layer chromatograms of the acid hydrolysates and enzymic digests of the IPS revealed a single compound with an R_F value (0.15) equal to that of glucose. No peptide bonds or free amino acids could be detected, as no absorbance occurred at 250 to 280 nm and ninhydrin reactions were negative.

The calculated mol. wt was 304000 ± 21000 and the optical rotation of the IPS was $+167.7^\circ$.

DISCUSSION

IPS synthesis by anaerobic bacteria was demonstrated in fusobacteria and leptotrichia isolated from dental plaque (Van Houte, 1967). This study shows that IPS synthesis from glucose or other carbohydrates is also a common characteristic of *B. fragilis*. The *B. fragilis* strains could rapidly catabolize the accumulated IPS if the carbohydrate in the medium was limited. The time interval between the disappearance of glucose and the decrease in pH or the formation of fatty acids suggests that the main formation of these acids is not due to fermentation of the initial glucose but to conversion of stored IPS. However, the low C recovery after 24 h growth cannot be explained in terms of the storage of IPS as the dry weight reached at that time would contribute to the C recovery the equivalent of only 12 to 13 %. Formation of intermediates not detected by gas chromatography or

Fig. 3. Electron micrographs of *B. fragilis* subsp. *fragilis* BB₂: (a, b) cells containing IPS (BV 140) showing dispersed granules of electron-dense material; (c, d) cells without IPS (BV 0).

synthesis of extracellular products serving as substrates may be the cause of the deficiencies in the fermentation balances after 25 h growth.

Degradation of IPS was dependent on pH in the same manner as demonstrated in streptococci (Van Houte, 1967). However, in contrast to streptococci, no degradation occurred below pH 4.5. The IPS produced by *B. fragilis* is located intracellularly, as it is in streptococci (Gibbons & Kapsimalis, 1963). In electron micrographs, the IPS of *B. fragilis* was observed as cytoplasmic granules, as in *E. coli* (Cedergren & Holme, 1959), *Streptococcus mutans* (DiPersio *et al.*, 1974) and *Nocardia asteroides* (DiPersio & Deal, 1974).

The chemical properties of this IPS indicate that it is a glycogen. The similarity in absorbance, with a $\lambda_{\max}$ at 490 nm for IPS and at 495 nm for rabbit liver glycogen, suggests IPS may have a similar structure. The $\lambda_{\max}$ value of the glycogen of *B. fragilis* conforms to that of the glycogen from streptococci (Van Houte, 1967). The iodine-staining power of IPS differs from that of rabbit liver glycogen; this may be related to differences in the average distances between branch points in the interior of the molecules (Van de Vies, 1954). Digestion of the polymer was complete after treatment with amyloglucosidase, which hydrolyses 1,4- α and 1,6- α bonds, but not after treatment with β -amylase, an enzyme that hydrolyses 1,4- α bonds. This indicates that the polymer is an 1,4- α -glucan joined by 1,6- α branch points. As molecular weights of glycogens range from 270 000 to 100 000 000, this glycogen of *B. fragilis* (mol. wt 304 000) must be considered as rather small.

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