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The Extracellular Polysaccharide of a Methylotrophic Culture

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Abstract

The constituent sugars of the extracellular polysaccharide produced by an enrichment culture, growing on methane as sole carbon source, were identified as glucose, galactose, mannose, fucose and rhamnose, in the approximate molar ratios of 1·00 : 0·36 : 0·19 : 0·31 : 0·16. When the culture was grown on methanol as sole carbon source, only glucose, galactose and mannose, in the approximate molar ratios of 1·00 : 0·67 : 0·42, were identified as components of the extracellular polysaccharide.

The data support the concept that a methanol-utilizer, incapable of methane-utilization, produced a polysaccharide which was partially deoxygenated by a methane-utilizer when the two organisms were present in a mixed culture growing on methane as sole carbon source. An alternate possibility, that each polysaccharide was produced by a different organism, is discussed.

Introduction

The extracellular polysaccharides of bacteria listed by Salton (1960) range from the relatively simple homopolysaccharides of the glucan, mannan, levan, etc. types to the heteropolysaccharides which contain a mixture of two or more of the following monosaccharide forms: sugars, uronic acids, amino sugars, *N*-acetylamino sugars and deoxy sugars. The deoxy sugars are generally fucose (6-deoxy-galactose) and/or rhamnose (6-deoxy-mannose).

Only a few reports are available in the literature on the production of polysaccharide by microorganisms using liquid or gaseous hydrocarbons as their carbon source. Slime formation by *Pseudomonas methanica* utilizing methane was observed by Söhngen in 1910 and about half a century later slime production by *Pseudomonas methanica* during growth on methane was again reported (Dworkin and Foster 1956). More recently, Wyss and Moreland (1968) grew *Methylococcus capsulatus* on methane and on methanol and isolated the capsular material from this obligate 'one carbon'-utilizing bacterium by a sonication method. They identified the sugar components as rhamnose, arabinose and glucosamine in the molar ratios 1·08 : 2·24 : 1·00. The polysaccharide excreted by our methane-utilizing enrichment culture comprised glucose, galactose, mannose, fucose and rhamnose. Transfer of the culture from methane to methanol resulted in loss of its ability to utilize methane as sole carbon source, presumably as a result of selective pressure imposed by change in the carbon source. Selection was accompanied by the disappearance of deoxy sugars from the extracellular polysaccharide, which was otherwise little altered in its sugar composition. Thus, this preliminary study provided data which suggested that interactive effects of mixed cultures can affect extracellular polysaccharide

composition. To the best of our knowledge it is the first report of its kind and hopefully will inspire further, more definitive, studies.

Methods

Growth Conditions

A methylotrophic enrichment culture was obtained from the natural environment by the conventional technique of enrichment followed by repeated alternation of single-colony transfer from plate to broth and inoculum transfer from broth to plate. It appeared to be identical to the *Pseudomonas methanica* described by Dworkin and Foster (1956).

Ten 1-litre culture flasks, each containing 100 ml of sterile inorganic salts medium, pH 6·9 (Medium L of Leadbetter and Foster 1958), were inoculated with the culture to give an initial concentration of 0·1 mg dry weight of cells/ml. The flasks were then sealed with rubber bungs fitted with inlet and outlet ports attached to sterile cotton wool filters. A methane–air mixture (1 : 2, v/v) was prepared by mixing 99·9% pure methane (Matheson Gas Products, California, U.S.A.) with filtered compressed air by a water displacement method. The flasks were flushed and filled with the methane–air mixture, the ports clamped and the culture incubated at 30°C on a reciprocal shaker (Paton Industries Pty Ltd, South Australia) at 100 cycles/min. The gas mixture was replaced every 10 h.

Alternatively, methanol was used instead of methane as the sole carbon source. When this was so, analytical grade methanol was sterilized by filtration through a Millipore Solvinert filter and added at the desired level (0·5%) to the sterile bulk medium. The inlet and outlet gas ports had previously been plugged with cotton wool and, to ensure air transfer, were not clamped. In all other respects, inoculation and growth conditions were identical to those described for methane culture.

Isolation and Purification of Extracellular Polysaccharide

Polysaccharide production was monitored daily by assay of the carbohydrate (method of Dubois *et al.* 1956) in the dialysed cell-free culture fluid. Production was considered to be complete when similar values were obtained on two consecutive days.

When polysaccharide accumulation was complete (14 days in methane culture and 5 days in methanol culture) the contents of each flask were centrifuged at 23 500 *g* in a Sorvall RC2-B centrifuge, the cells discarded and the supernatants of the 10 flasks combined. The combined supernatants were reduced to 250 ml by vacuum evaporation (Büchi Rotary Evaporator) at 40°C and treated with 500 ml of 95% (v/v) ethanol. The suspension of polysaccharide was centrifuged and the precipitate washed once with 75% (v/v) ethanol.

The precipitate was dispersed in 20 ml of distilled water, after which 40 ml of slightly acidified 95% (v/v) ethanol (one drop of concentrated HCl per litre) was added. After standing for 6–8 h, the dispersion was centrifuged, the precipitate redispersed in 20 ml of distilled water and the total dispersion dialysed at 5°C for 24 h against slightly acidified distilled water (pH 4·0). The dialysed suspension was freeze-dried to give a final product which was a white, fibrous, cotton-like material.

Determination of the Sugar Composition of the Polysaccharide

The component sugars of the polysaccharide were released from the freeze-dried material by complete acid hydrolysis in 72% H_2SO_4 according to the method of Saeman *et al.* (1945). Neutralized hydrolysates were then examined by one-dimensional and two-dimensional descending paper chromatography on Whatman No. 1 and on 3MM paper in the solvent systems described by Partridge (1948), Bayly *et al.* (1951) and Hough and Jones (1962). The sugars were located by the visualizing systems of Partridge (1949), Hough *et al.* (1950), Trevelyan *et al.* (1950) and Zentner (1968).

The proportions of monosaccharides were estimated as follows: a strip was cut from a developed chromatogram and the monosaccharides located by visualization with a dipping reagent; the corresponding areas were cut from the remainder of the chromatogram, eluted with 5 ml of water, treated with phenol and water as described by Herbert *et al.* (1971) and the absorbance read at 488 nm in the case of glucose, 487 nm in the case of galactose and mannose and 480 nm in the case of fucose and rhamnose; standard solutions of the five monosaccharides were treated by the same procedure and used for conversion of absorbance values to concentration values.

The hydrolysates were also examined by gas–liquid chromatography (g.l.c.) after conversion of the component monosaccharides to their alditol acetates. The methods of preparation of the alditol acetate derivatives and g.l.c. analysis of the mixtures obtained were essentially those described by Sawardeker *et al.* (1965).

Methods for Determining Polysaccharide Homogeneity

Complete dissolution of freeze-dried polysaccharide in water could not be achieved and hence tests for homogeneity were made on the crude cell-free culture fluid after it had been dialysed and concentrated to a solids content of approximately 0·3% (w/v). The homogeneity of the concentrated dialysate was tested by fractional precipitation with ethanol as described by Whistler and Sannella (1965), by electrophoresis on glass fibre paper (Whatman GF/A) as described by Northcote (1965), and by gel filtration with Sephadex G200.

Results

Homogeneity of the Polysaccharides

Some evidence for homogeneity of the polysaccharides was obtained by gradient addition of absolute ethanol to the concentrated, dialysed cell-free culture fluids (0·3% w/v solids) of methane and methanol cultures. In the case of the polysaccharide from methane culture, little precipitate was produced between 0 and 60% (v/v) ethanol, but when the ethanol concentration was increased to 65% (v/v) about 90% of the polysaccharide precipitated as a fibrous precipitate. When the precipitate was removed and the ethanol concentration increased to 72% (v/v) or greater, no further precipitation was observed, even after standing overnight. [The remaining 10% presumably represented the solubility of the polysaccharide in ethanol concentrations of 72% (v/v) or greater. If other polysaccharides were present in the remaining 10% (approximately) they were not separated by electrophoresis from that which precipitated at 65% (v/v) ethanol.] Electrophoresis of the polysaccharide from methane culture was carried out after 3 ml of concentrated dialysed cell-free culture fluid (0·2% w/v solids) had been dialysed overnight against borate buffer. The borate-charged polysaccharide moved as a single band towards the anode. (Electrophoresis was not effective if the concentration of the polysaccharide was more than 0·25%, as the solution was then very viscous.)

The bulk of the polysaccharide from methanol culture precipitated between 55 and 60% (v/v) ethanol.

The polysaccharides from both methane and methanol culture were eluted as single peaks immediately following collection of the void volumes from Sephadex G200 gel filtration columns.

Complete confidence in the homogeneity of the polysaccharides would require a more rigorous physicochemical examination than that reported above.

Component Sugars of the Polysaccharides

As a result of preliminary experiments it was found that the most suitable conditions for complete hydrolysis of the polysaccharides involved treatment with 72% H_2SO_4. Paper chromatography and g.l.c. of the hydrolysates revealed the presence of compounds indistinguishable from galactose, glucose, mannose, fucose and rhamnose in the polysaccharide isolated from methane culture, and galactose, glucose and mannose in the polysaccharide from methanol culture. The approximate stoichiometries of the sugar components, calculated from quantitative analysis of

paper chromatograms, are shown in the following tabulation:

Culture	Glucose	Galactose	Mannose	Fucose	Rhamnose
Methane	1·00	0·36	0·19	0·31	0·16
Methanol	1·00	0·67	0·42	0·00	0·00

Some equivocal evidence was obtained for the presence of approximately 4% nitrogen in the polysaccharide from methane culture.

Effect of Prolonged Growth on Methanol

After the culture had been grown on methanol for 10–15 successive subcultures over a period of more than 30 weeks, the growth rate and total cell mass production had decreased and there was a longer lag phase. Moreover, the culture was no longer able to grow on methane as sole carbon source. The fact that cultures which had been grown on methane failed to grow on that carbon source after they had been grown successively on methanol indicates a selective pressure, whereby a mixed culture changed in species dominance to one or more methanol-tolerant species, while methane-utilizing species was/were lost from the mixture.

Discussion

According to the analytical procedures employed, the enrichment culture, growing on methane as sole carbon source, produced an extracellular heteropolysaccharide containing glucose, galactose, mannose, rhamnose and fucose. The ratios of glucose : galactose : mannose : fucose : rhamnose of 1·00 : 0·36 : 0·19 : 0·31 : 0·16 approximate a composition of 6 : 2 : 1 : 2 : 1, i.e. glucose was present in approximately the same molar ratio as the sum of other sugars. When methanol was the carbon source for growth the molar ratios were glucose : galactose : mannose = 1·00 : 0·67 : 0·42, or approximately 6 : 4 : 2; again, glucose was present in approximately the same molar ratio as the sum of the other sugars.

The most notable feature therefore was the lack of fucose and rhamnose in the polysaccharide obtained from methanol cultures. Fucose is the 6-deoxy-derivative of galactose, and rhamnose the 6-deoxy-derivative of mannose. If a lack of conversion of sugar to deoxy sugar in methane culture was imagined, then we could add the fucose component to galactose and the rhamnose component to mannose and obtain a glucose : galactose : mannose ratio of 1·00 : 0·67 : 0·35. Such ratios would then be in close agreement with the ratios of the components of the polysaccharide produced when methanol was used as the growth substrate (i.e. glucose : galactose : mannose = 1·00 : 0·67 : 0·42).

Failure to grow on methane after successive culture on methanol suggests that the latter carbon source had imposed a selective pressure on the culture. This would imply that, in spite of rigorous purification and careful maintenance of the enrichment culture, it was in fact a mixed culture of two methylotrophs, only one of which was capable of methane utilization. Many methane-utilizers are inhibited by methanol unless it is supplied in the vapour phase (Anthony 1975), and it seems that viability of our methane-utilizer was lost by successive subculture on liquid phase methanol. (Irreversible adaptation by loss of genetic material, for example a plasmid coding for the enzyme responsible for oxidation of methane to methanol, is a less feasible possibility.) The isolation of symbiotic methane- and methanol-utilizers, and the difficulty of separating such organisms is well documented (see review by Quayle

1972), the presence of the latter serving as methanol-scavengers and thereby enhancing the growth of methanol-sensitive methane-utilizing species when methane is the sole carbon source.

The chemical relationship between the two polysaccharides suggests they were initially produced by one organism. If so, then, since the evidence points to loss of the methane-utilizer after transfer to methanol, the methanol-utilizer would be the organism responsible for polysaccharide production. Extension of this concept would suggest that, in mixed culture on methane, the methane-utilizer partially deoxygenated the polysaccharide. This would result in conservation of oxygen atoms, a possible advantage considering that the first metabolic reaction in methane assimilation requires molecular oxygen for conversion of methane to methanol (see Quayle 1972). The possibility cannot, however, be discounted that an organism, or organism mixture, quite distinct from that which developed on methanol produced the polysaccharide isolated from methane culture. Such a concept would have very interesting taxonomic implications in the light of the chemical similarity of the two polysaccharides.

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