Polysaccharides Synthesized by Aerobic Mesophilic Spore-forming Bacteria

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(Received 5 August 1948)

In the classification proposed by Smith, Gordon & Clark (1946) the genus *Bacillus* has been divided into three groups depending principally on morphological differences in the size and shape of the spores and the shape of the sporangia. Their classification and nomenclature has been used throughout this paper.

The bacilli of group I are known to synthesize fructosans from sucrose; those obtained from *B. subtilis*, *B. megatherium* and *B. pumilus* (*mesentericus*) have been intensively studied (Harrison, Tarr & Hibbert, 1930; Challinor, Haworth & Hirst, 1934; Lyne, Peat & Stacey, 1940; Evans & Hibbert, 1946) and all have been shown to be levans of the same general structure containing D-fructose as the sole constituent sugar.

The same attention has not been paid to the polysaccharides produced by the bacilli of group II. *B. polymyxa* has been shown to produce a levan from sucrose, and an unidentified polysaccharide which is not a levan from other sugars (Hestrin, Avineri-Shapiro & Aschner, 1943). Kleczkowska, Norman & Snieszko (1940) isolated a new capsulated bacillus from cultivated soil which they named *B. krzemieniewski*. In some strains well-defined capsules were present, while in others the mucilage was dispersed throughout the medium. Kleczkowski & Wierzchowski (1940) reported the separation of the capsular polysaccharide, and solely on the optical rotation of the total hydrolysate and the isolation of mannose phenylhydrazones, deduced that the polysaccharide was a mannan composed of L-mannose units. The sugar L-mannose has not been known to occur previously, and it has been commented (Evans & Hibbert, 1946) that such a result requires confirmation. *B. krzemieniewski* is now considered by Smith *et al.* (1946), pending the study and isolation of more strains, as a mucoid stage of growth of *B. circulans*. It is classified as *B. circulans* 760 in their collection. Group III bacilli do not appear to produce exocellular polysaccharides.

It was not our intention to make an intensive structural analysis of any of the polysaccharides produced by the bacilli, but rather, by studying the nature of the constituent sugars, to discover if any correlation exists between the groups of the above and the type of polysaccharide synthesized in presence of various sugars.

EXPERIMENTAL

Cultures. We are grateful to Dr Ruth E. Gordon, the Curator of the American Type Culture Collection, for her kindness in sending us the following strains of polysaccharide-producing bacilli from their collection.

Group I			
	Strain no.		Strain no.
<i>B. megatherium</i>	697	<i>B. subtilis</i>	1248
<i>B. pumilus</i>	355	<i>B. cereus</i>	793
Group II			
<i>B. polymyxa</i>	354	<i>B. circulans</i>	294
<i>B. brevis</i>	799	<i>B. circulans</i>	295
<i>B. alvei</i>	683	<i>B. circulans</i>	
		<i>B. macerans</i>	396
		intermediate	

A culture of *B. circulans* strain no. 760 (*B. krzemieniewski*) was also sent. The culture, as received, was a mixture of the smooth and mucoid stages. These were separated on nutrient agar and designated for convenience *Km* (mucoid stage) and *Ks* (smooth stage). Both were tested for gum production on medium II of Cooper & Preston (1937). *Km* produced the typical colonies depicted by Kleczkowska *et al.* (1940), resembling glass beads in appearance, firm but resilient, and adhering strongly to the medium. *Ks* also produced gummy colonies on this medium, but the colonies were not firm, and the growth soon coalesced to form a gummy mass on the surface of the plate. The mucoid stage was unstable and slowly reverted to the smooth stage either on frequent subculture on Cooper & Preston's medium or after prolonged incubation or storage on this medium. It seems probable that *Km*, which is capsulated, is the same as strain A of Kleczkowska *et al.* (1940), while *Ks* corresponds to their strain B. These American cultures, Dr Gordon informs us, have been in storage for 6–10 years. In addition to the above, the following cultures of *B. polymyxa* were available for study: *B. polymyxa* N.C.T.C., nos. 1380, 4745, 4744, and a strain of *B. polymyxa* isolated in Prof. Kluyver's laboratory (obtained through the kindness of Dr Swaby of Rothamsted). Culture no. 4744 was that used by Hestrin *et al.* (1943).

Polysaccharide production. For the preparation of sufficient polysaccharide for analysis of the constituent sugars, cultures of each organism were grown on the surface of agar in four 9 cm. Petri dishes. The dishes, each containing 15–20 ml. of Cooper & Preston's (1937) basic medium II with 2% of the various sugars, sterilized separately, were heavily inoculated and incubated at 25° for 2–7 days, depending on the organism. The surface growth was removed by means of

a sterile bent glass rod after the addition of a minimal quantity of sterile distilled water to facilitate harvesting. With *Km*, because of the toughness of the colonies and their adhesion to the agar surface, it was often necessary to leave the distilled water in contact with the surface growth for 24 hr. in the incubator. This was usually sufficient to detach the colonies, which could then be removed without any danger of scraping up pieces of agar. Frequent tests subsequently showed that with reasonable care no contamination from the agar would be encountered. Polysaccharide synthesis by each organism was tested on sucrose, D-fructose, D-glucose, D-galactose, and L-arabinose. With each bacillus, polysaccharides containing identical sugar constituents were produced from the last four sugars, although in different yields. For brevity, description will thus be limited to the polysaccharides produced on sucrose and monosaccharides.

Partial purification of polysaccharides. The growth, washed off with distilled water from four Petri dishes, was precipitated by dropping into 4 vol. of ethanol containing 2% glacial acetic acid and stirring vigorously. After standing for 1 hr. the precipitate was filtered on a sintered-glass funnel and washed with ethanol of increasing strength, and then with ether. When dried in this manner the crude gums were generally in the form of fine white powders which could be redissolved in distilled water. Impurities could be spun down, and the polysaccharide reprecipitated with ethanol-acetic acid and dried as before. Where the mucilages were not readily dissolved in water, it was sometimes necessary to redissolve in 0.1 N-NaOH. The products generally contained a considerable quantity of nitrogenous material derived from the bacteria. While the yield of this gum varied with the bacillus and the substrate sugar, in general c. 50–150 mg. were obtained from the four Petri dishes.

Hydrolysis of the polysaccharides. Two hydrolyses were carried out on each product: (a) c. 20 mg. gum were hydrolyzed with 0.5 ml. 0.1 N-H₂SO₄ for 30 min. in a sealed ampoule in a water bath at 100°; (b) c. 20 mg. gum were hydrolyzed with 0.5 ml. N-H₂SO₄ for 4 hr. as above. After the completion, the seals were broken and the hydrolysate neutralized to Congo red with solid BaCO₃. The Ba salts were then centrifuged down, still in the ampoule, and the supernatant liquid used for determination of sugars. The short hydrolysis in 0.1 N-H₂SO₄ serves to show the presence of furanose sugars such as D-fructose which would be almost totally destroyed in the stronger hydrolysis. When fructose was found to be present, the remainder of the gum was generally hydrolyzed with 0.5% oxalic acid for 1 hr. on a water bath and the fructose-free residue, if present, was precipitated with ethanol, washed and dried as before. The residue could then be hydrolyzed and analyzed without interference from fructose and its decomposition products.

Identification of constituent sugars. The sugars present were identified by the paper chromatographic technique (Partridge, 1946, 1948; Forsyth, 1948). From the *R_F* values of the constituent sugars using *n*-butanol, phenol, and *s*-collidine as solvents, by comparison with the position of standard sugars on the papers and by spraying with resorcinol and naphthoresorcinol (Forsyth, 1948), it was possible to identify all the sugars which were encountered. Uronic acids were detected both by the naphthoresorcinol test on the gum itself, and by spraying chromatograms of the hydrolysates with the reagent. In some cases quantitative estimations of sugars were made, after extraction from chromatograms, by the method of Flood, Hirst & Jones (1947).

RESULTS

Polysaccharides of Bacillus cereus 793, Bacillus subtilis 1248, Bacillus pumilus 355

When grown on sucrose these bacilli produced copious yields of gum, but no synthesis took place on a monosaccharide substrate. On hydrolysis with 0.1 N-sulphuric acid or 0.5% oxalic acid, no appreciable residue of ethanol-insoluble material remained, and only one spot was obtained on paper chromatograms, which was readily identified as fructose from its *R_F* values (*n*-butanol 0.10; phenol 0.51; *s*-collidine 0.42) and its colour reaction on spraying with naphthoresorcinol. That D-fructose was the reducing sugar present was readily confirmed in each case by measuring the optical rotation and the reducing sugar value of a hydrolysate of the mother sample of the gum.

Polysaccharide of Bacillus megatherium 697

This bacillus produced polysaccharides on both the sucrose and monosaccharide media. The gum from the sucrose substrate, on hydrolysis with the 0.1 N-sulphuric acid gave only D-fructose, identified as before. In the more concentrated N-sulphuric acid, however, the fructose was mainly destroyed giving only a faint spot, whereas another sugar was present which proved to be glucose. After hydrolysis in oxalic acid c. 40% of ethanol-insoluble residue still remained, which on further hydrolysis with N-sulphuric acid gave a hydrolysate free from fructose, but containing the other sugar as well as uronide material. This sugar was shown by spraying with resorcinol to be an aldose and was readily identified as glucose from its *R_F* values (*n*-butanol 0.07; phenol 0.39; *s*-collidine 0.39). This was confirmed by the preparation of glucosazone from the hydrolysate of the fructose-free residual polysaccharide. The gum from the monosaccharide media contained no fructose, giving only glucose and uronic material on hydrolysis. The presence of uronides was confirmed by the naphthoresorcinol test. It is probable that the gum produced on sucrose media is a mixture of levan and the glucose-uronic polysaccharide, which is synthesized alone on the monosaccharide media.

When grown on the liquid media of Tarr & Hibbert (1931) with the addition of 2% yeast extract and with sucrose as substrate sugar, only levan was produced, no glucose being detected, and the gum was completely hydrolyzed with oxalic acid. This phenomenon has been more closely investigated with *B. polymyxa*.

Polysaccharide of Bacillus polymyxa

All the strains of *B. polymyxa* investigated gave the same results. When grown on monosaccharide media a fructose-free polysaccharide was obtained,

which on hydrolysis with *N*-sulphuric acid, but not with 0.1*N*-sulphuric acid, gave a hydrolysate shown by chromatography to contain two sugars. One of these was glucose and the other was identified as mannose from its R_F factors (*n*-butanol 0.10; phenol 0.45; *s*-collidine 0.46) and resorcinol spray, which showed it to be an aldose. This was confirmed by the preparation of mannose phenylhydrazone from a hydrolysate of the remainder of the gum. Uronides were also present.

When grown on sucrose medium a gum was obtained, which on hydrolysis with dilute acid gave fructose, identified as before. On hydrolysis with stronger acid a more complex sugar mixture was formed. With *n*-butanol two spots were present with R_F factors corresponding to glucose and fructose, but with phenol and *s*-collidine three spots were apparent. Apart from the glucose and fructose, mannose was also identified. Mannose and fructose have the same R_F factor in butanol. After removal of the fructose with oxalic acid, the residual polysaccharide gave the same sugars on hydrolysis as were produced from the polysaccharide formed on the monosaccharide substrate. As in the case of *B. megatherium*, a much larger proportion of levan was formed when *B. polymyxa* was grown on Tarr & Hibbert's liquid medium.

Dependence of levan production on culture media

As we have seen, both *B. megatherium* and *B. polymyxa* produce, from sucrose on Tarr & Hibbert's (1931) liquid medium, mainly levan, whereas on Cooper and Preston's (1937) solid media a considerable proportion of other polysaccharides, which are the sole products from a monosaccharide substrate, is produced. This difference may be due to one, or more than one, of the following factors: (1) aeration differences between solid and liquid culture; (2) differences in sucrose concentration (2% in Cooper & Preston's media and 10% in that of Tarr & Hibbert); (3) intrinsic differences in the basic media such as phosphate concentration.

Accordingly, *B. polymyxa* was grown under a variety of conditions, and levan estimated as reducing sugar after hydrolysis of the gums with oxalic acid. The residual ethanol-insoluble polysaccharide was also estimated by isolation and weighing. The results are shown in Table 1. The residual polysaccharide, even when only present in traces, gave glucose and mannose on hydrolysis. It is apparent from Table 1 that the aeration factor plays no part in levan production. Indeed a similar mixture of polysaccharides is produced when *B. polymyxa* is grown anaerobically. High concentrations of both sucrose and phosphate appear to be necessary for optimal levan production.

Table 1. *Levan production by Bacillus polymyxa*

Basic media	Type of culture	Sucrose (%)	PO ₄ (g./l.)	Levan (% of total gum)	Residual gum as % of total gum
Cooper & Preston (1937)	Liquid	2	0.11	44	43
	Solid	2	0.11	39	39
	Solid	10	0.11	37	41
	Solid	10	0.53	69	13
Tarr & Hibbert (1931)	Liquid	10	0.53	86	7
	Solid	10	0.53	70	4
	Solid	2	0.53	37	40
	Solid	10	0.11	57	25

Polysaccharides of Bacillus brevis 799, Bacillus alvei 683

These strains produced polysaccharides containing the same sugars on both sucrose and monosaccharide substrates. No levan was formed. On hydrolysis with *N*-sulphuric acid, glucose and a uronide fraction were the only sugars identified.

Polysaccharides of Bacillus circulans 294, Bacillus circulans, Bacillus macerans intermediate 396

These bacilli, like the last two and all subsequently to be discussed, produced polysaccharides containing the same sugars on both sucrose and monosaccharide substrates and did not synthesize levan. Hydrolysis of the gum with *N*-sulphuric acid gave two sugars which were identified as before from their R_F factors and resorcinol spray as glucose and mannose. Uronides were also present.

Polysaccharide of Bacillus circulans 295

Hydrolysis of the gum produced by this bacillus with *N*-sulphuric acid gave a mixture of three sugars as well as uronic residues. Two of the sugars were readily identified as before as glucose and mannose. The third sugar was shown to be a pentose by resorcinol spray and was identified as xylose from its R_F factors (*n*-butanol 0.13; phenol 0.44; *s*-collidine 0.50). Comparison was made on chromatograms with the other three pentoses, arabinose, ribose, lyxose, in order to confirm that the sugar was xylose. The presence of xylose was further confirmed by the preparation of the dimethyl acetal of dibenzylidene xylose (Breddy & Jones, 1945; Wise & Ratcliff, 1947) from the sugar syrup prepared from a hydrolysate of the remainder of the gum.

Polysaccharides of Bacillus circulans 760 (Bacillus krzemieniewski)

These have been most carefully studied since it has been claimed (Kleczkowski & Wierchowski, 1940) that a mannan composed of L-mannose units is the main polysaccharide synthesized.

Ks (smooth form). The strain was grown on a range of sugars and the crude gum isolated as for the other bacilli previously discussed. A rough estimate of the production of polysaccharide by the strain on different sugars may be gained from a comparison of the yields of this partly purified product shown in Table 2. The gums contained no levan, and on hydrolysis gave glucose and mannose, identified chromatographically as before.

Table 2. *Yields of gum by Bacillus circulans 760 (smooth form) on different sugars*

	mg./plate		mg./plate
Sucrose	108	D-Galactose	24
Mannitol	62	Lactose	24
D-Glucose	58	L-Arabinose	14
D-Fructose	46	L-Rhamnose	4

Km (mucoid form). Similar analysis was made on the polysaccharide synthesized by the well-capsulated form of the organism and the same constituent sugars were found to be present. To exclude the possibility that this polysaccharide, which is of a more complex nature than a simple mannan, is synthesized because of different culture conditions, both *Ks* and *Km* were grown on the media used and the conditions prescribed by Kleczkowski & Wierzechowski (1940). Again both glucose and mannose were detected in the hydrolysates.

For further investigation larger amounts of gum were prepared from *Ks*, and further purified in a similar manner to that described by Kleczkowski & Wierzechowski (1940): 5 g. of the gum was heated under reflux with 300 ml. N-KOH for 3 hr. on a water bath at 100°, and the hot solution filtered through glass wool and then centrifuged. On cooling a very viscous gel formed. The alkaline solution was poured into 4 vol. of ethanol containing acetic acid, washed with ethanol, then ether, and dried. When purified by electrodialysis, as described by Kleczkowski & Wierzechowski, a low yield of purified product was obtained. Nevertheless, this method was used with a portion of gum to obtain sufficient material for sugar analysis. On hydrolysis of the electrodialyzed polysaccharide, glucose and mannose were formed as before. The bulk of the material was further purified in good yield by forming the copper complex by the addition of CuSO₄ to an alkaline solution of the gum. The complex was washed with hot water, and then decomposed with ethanol containing HCl and washed free from Cu⁺⁺ and Cl⁻ ions with ethanol. After drying with ether, the regenerated gum was redissolved in a small volume of 0.1 N-NaOH and precipitated by a large excess of glacial acetic acid. After drying with ethanol and ether the product (1.45 g.) was a fine white powder, difficultly soluble in water to give an opalescent, very viscous solution. It was readily soluble in dilute alkali and was almost free from ash and N. After electrodialysis it gave an equivalent weight of 598 on titration with 0.1 N-NaOH using phenolphthalein as indicator. The equivalent weight was not significantly altered on fractional precipitation of the polysaccharide with ethanol. Determination of uronic anhydride

by CO₂ evolution on refluxing with 12% HCl gave a value of 28.9% (calculated from equivalent weight, 29.8%). This uronic acid would give rise to c. 6.3% furfural (found, 6.1%) which confirms the absence of pentoses. Glucose, mannose, and a uronic acid were again shown to be present by paper chromatography.

The sugars on the paper chromatograms were determined by the method of Flood *et al.* (1947): glucose/mannose ratio (Exp. 1) 1.53; (Exp. 2) 1.45. Using a known weight of xylose as an internal standard, the actual percentages of glucosan and mannan were determined (Table 3). Mannose was also estimated as the phenylhydrazone by the method of Jones, Hirst & Woods (1947).

Table 3. *Composition of purified gum from mucoid form of Bacillus circulans 760*

	Glucosan (%)	Mannan (%)	Uronic anhydride (%)	Equiv. wt.
Theoretical	41.8	27.9	30.3	581
Found	40.5	25.6	28.9	598
		25.8*		

* As phenylhydrazone.

Hydrolyses with 0.2% oxalic acid for 4 hr. or with 0.1 N-H₂SO₄ for 30 min. at 90° were not effective in splitting off reducing sugars, suggesting the absence of furanose sugars: 1.0 g. polysaccharide was refluxed with 50 ml. N-H₂SO₄ for 3 hr., and the change in rotation determined (1 hr., $[\alpha]_D^{20} + 29.5^\circ$; 2 hr., $+ 33.3^\circ$; 3 hr., $+ 33.3^\circ$). While this rotation is of the right order for a mixture of D-uronic acids, D-glucose and (?)mannose, such as are produced, it is in direct contrast to that of Kleczkowski & Wierzechowski, who obtained a negative rotation. The hydrolysate was neutralized with BaCO₃, centrifuged and concentrated. On addition of methanol no barium aldobionate fraction separated. The solution was concentrated to a syrup and taken up in 10 ml. water and mannose phenylhydrazone prepared, m.p. 192°. The hydrazone was then decomposed by boiling in water with a slight excess of benzaldehyde, and, after filtration and removal of the excess benzaldehyde with ether, the regenerated mannose was shown by optical rotation, $[\alpha]_D^{20} = + 14^\circ$, to be D-mannose. That mannose was the sugar regenerated was confirmed by paper chromatography.

DISCUSSION

The results obtained are tabulated in Table 4. It is apparent that levan formation is confined to group I bacilli, with the exception of *Bacillus polymyxa*, and the synthesis of other polysaccharides to group II bacilli, with the exception of *B. megatherium*. *B. polymyxa* and *B. megatherium* might be considered as intermediate since they produce varying proportions of both levan and other polysaccharides from sucrose depending on the culture conditions. *B. circulans* strains produce polysaccharides containing glucose, mannose and a uronic acid. The nature of the polysaccharide is independent of the substrate sugar. It is, of course, by no means proved that these complex polysaccharides are not merely

mixtures of simple dextrans, mannans and polyuronic acids, but attempts to fractionate them by fractional ethanol precipitation have proved unsuccessful. We have not been able to confirm Kleczkowski & Wierzchowski's (1940) claim that *B. krzemieniewski* produces a mannan composed of L-mannose units. The strains available synthesized complex polymers of glucose, mannose and uronic acid in the approximate ratio 3:2:2. The mannose was D-mannose and not the unnatural L-form. *B. circulans* 295 gum is unique in that it contains the pentose, xylose.

Table 4. *Constituent sugars of the mucilaginous polysaccharides synthesized by the genus Bacillus*

Bacilli		Levan formation on sucrose	Other polysac- charides synthesized on sucrose and monosaccharides
Group	Species		
I	<i>cereus</i>	793	+ —
	<i>pumilus</i>	355	
	<i>subtilis</i>	1248	
	<i>megatherium</i>	697	+ Glucose-uronic
II	<i>polymyxa</i>	354	+ } Glucose-mannose- uronic
	<i>circulans</i>	760	
	<i>circulans</i>	294	
	<i>circulans-macerans</i>	396	
	<i>brevis</i>	799	- Glucose-uronic
	<i>alvei</i>	683	
	<i>circulans</i>	295	- Glucose-mannose- xylose-uronic

It may be of some interest that when a crude ethanol precipitate of a culture of these bacilli is hydrolyzed, ribose, presumably from the nucleic

acids, is readily identified among the polysaccharide sugars.

SUMMARY

1. The constituent sugars of polysaccharides synthesized by the genus *Bacillus* from sucrose and monosaccharide sugars have been identified. Strains of *B. cereus*, *B. pumilus*, *B. subtilis*, *B. megatherium*, *B. polymyxa*, *B. circulans*, *B. circulans-macerans*, *B. brevis*; and *B. alvei* have been studied.

2. There appears to be a relation between type of polysaccharide synthesized and the group classification as proposed by Smith *et al.* (1946). Levan synthesis from sucrose is confined to group I bacilli and *B. polymyxa*. On the other hand, the synthesis of other polysaccharides from sucrose and monosaccharides is confined to group II bacilli and *B. megatherium*.

3. *B. polymyxa* and *B. megatherium* are intermediate in that they synthesize both levan and other polysaccharides from sucrose depending on the culture medium.

4. Group II bacilli synthesize from a wide variety of sugars polysaccharides containing (a) glucose and a uronic acid; (b) glucose, mannose, and a uronic acid; (c) glucose, mannose, xylose, and a uronic acid. The type of polysaccharide is characteristic of the bacillus and not of the substrate sugar.

5. We have not been able to confirm Kleczkowski & Wierzchowski's (1940) claim that *B. krzemieniewski* produces a mannan composed of L-mannose units. The strains available synthesized a complex polymer of glucose (3 parts), mannose (2 parts) and uronic acid (2 parts). The mannose was the 'natural' D-mannose.

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