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LIPID-LINKED SACCHARIDES FORMED DURING PULLULAN BIOSYNTHESIS IN *Aureobasidium pullulans*

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ABSTRACT

Radioactive glycolipids were extracted from cells of *Aureobasidium pullulans* pulsed with D-[^{14}C]glucose. Labelled, alkali-stable lipids were resolved into one neutral and two acidic fractions. The neutral fraction was stable to mild hydrolysis with acid, whereas the acidic fractions could be hydrolysed, yielding D-glucose and a series of oligosaccharides having mobilities corresponding to those of isomaltose, panose, and isopanose. Amyloglucosidase (EC 3.2.1.3) catalysed the hydrolysis of 60% of the liberated radioactive oligosaccharides to D-glucose, indicating the presence of (1→4)-α- and (1→6)-α-D-glucosidic bonds. Since these lipid-linked saccharides are produced during pullulan biosynthesis in *A. pullulans*, it is proposed that they are intermediates in the biosynthetic pathway of that extracellular polysaccharide. A mechanism incorporating these glycolipids into a possible scheme of polysaccharide assembly is presented.

INTRODUCTION

Polyprenyl-linked oligosaccharides are formed as intermediates in the biosynthesis of many oligo- and poly-saccharides occurring in both prokaryotic and eukaryotic cells, *e.g.*, the "inner core" region of *N*-glycosylic glycoproteins in yeast[1] and mammalian systems[2,3], and in the repeating units of such bacterial polymers as peptidoglycan[4–7], *Salmonella* O-antigenic side-chains[8–12], and *Enterobacter aerogenes* exopolysaccharide[13,14].

Pullulan, an extracellular neutral glucan elaborated by the yeast-like fungus *Aureobasidium pullulans*, is composed of a repeating-unit structure which may be described as a poly(α-maltotriose), the terminal residues of the trisaccharides being linked by (1→6)-α bonds[15]. Internal maltotetraosyl units have also been reported to be present in pullulan[16,17]. Whereas the physiological conditions for the growth of *A. pullulans* leading to a major diversion of assimilated carbon sources to the production of pullulan have been described[18], very little is known about the molecular mechanism of assembly of the polysaccharide, except that uridine 5′-(D-gluco-

pyranosyl pyrophosphate) (UDPG) in the presence of ATP functions as a D-glucosyl donor in cell-free preparations[19].

It is reasonable to propose that the repeating unit of pullulan is assembled on a lipid before being passed through the hydrophobic membrane to a polymerisation site on the external face of the plasmalemma. In this laboratory, attempts at the *in vitro* synthesis of pullulan from UDPG have been unsuccessful. An alternative strategy, in which the supposed intermediates of the pullulan biosynthetic pathway, having been labelled with [^{14}C]-isotope, were extracted and characterised, proved more rewarding[20].

EXPERIMENTAL

Micro-organism and culture conditions. — *Aureobasidium pullulans* (ATCC 9348) was grown in liquid culture as described previously[21], but with the substitution of yeast extract (Oxoid, 0.04%) for yeast nitrogen base.

Radioactivity measurements. — Radioactive label was determined by standard techniques of liquid scintillation spectrometry in a Packard Tri-Carb instrument. A scintillation fluid comprising 2,5-diphenyloxazole (0.5% w/v) and 1,4-bis(5-phenyloxazol-2-yl)benzene (0.01%) in toluene was used throughout, except for scrapings from t.l.c. plates which were immersed in a scintillation fluid comprising naphthalene (15%), 2,5-diphenyloxazole (1.0%), and 1,4-bis(5-phenyloxazol-2-yl)-benzene (0.03%) in 1,4-dioxane.

Formation of radioactive lipid and extracellular polymer from D-[^{14}C]*glucose by cells of* A. pullulans *growing in the log phase.* — Washed cells from an *A. pullulans* culture (200 mL) grown for 48 h at 25° on a gyratory shaker at 200 r.p.m. were suspended in uptake medium[22] (30 mL) and shaken at 25° for 10 min to equilibrate the cells. At zero time, uniformly labelled D-[^{14}C]glucose (222 kBq, 0.48 μmol, Radiochemical Centre, Amersham) was added, and immediately an aliquot (5 mL) of the suspension was removed and centrifuged for 5 min at 2,400*g*. The supernatant fluid was decanted, and a portion (200 μL) was spotted onto a square (2 × 2 cm) of Whatman No. 17 paper which was then extracted in 66% ethanol[23]. In this way, the labelled glucose was separated from the labelled polymer, since the latter had been precipitated into the paper and resisted extraction into the aqueous ethanol. The pelleted cells were made up to 4 mL with distilled water. Aliquots (2 mL) were filtered through cellulose acetate filters (Millipore, 0.8 μm), washed thrice with water (5 mL), and dried under a heat lamp. One filter was extracted with chloroform–methanol (2:1, 10 mL) for 1 h and the extract evaporated to dryness. The residue was partitioned between chloroform–methanol (2:1, 2 mL) and water (0.4 mL). After removing the aqueous phase, the lower phase was washed according to Folch[24] and dispensed into scintillation vials where it was dried, and then scintillation fluid was added. The remaining filter was dried, immersed in scintillation fluid, and counted. This provided a measure of isotope incorporation into the cell.

The above procedure was repeated by taking further aliquots (5 mL) of the

cell suspension at recorded times over the subsequent 80 min. Pullulan in the extracellular medium was determined with pullulanase (EC 3.2.1.41) as described by Catley[25].

Preparation of lipid extract from cells of A. pullulans. — Cells (200 mL) grown for 48 h, and shown to be actively elaborating pullulan, were suspended in uptake medium[22] (30 mL) containing uniformly labelled D-[^{14}C]glucose (925 kBq) and shaken at 25° for 4 h. The labelled cells were collected on a Whatman GF/C filter, washed three times with ice-cold, distilled water (30 mL), and finally suspended in water to give a thick paste. The frozen cells were lysed by one passage through an Eaton press[26] and the lysate was freeze-dried. The freeze-dried material was extracted twice with chloroform–methanol (2:1) at room temperature for 1 h and centrifuged after each extraction. The supernatant fluids were combined and the residual pellet was further extracted twice with chloroform–methanol–water (10:10:3) at room temperature for 1 h followed by centrifugation. The combined extracts were evaporated to dryness under reduced pressue at 40°, to give the crude lipid.

Alkaline hydrolysis of crude lipid. — A solution of the crude lipid in 50% 1-propanol containing 0.1M NaOH (10 mL) was heated at 37° for 25 min, followed by partitioning against chloroform–methanol (2:1, 50 mL) to remove alkali. The upper phase was removed, and the interface was washed according to Folch[24]. The solution of alkali-stable lipids was evaporated to dryness under reduced pressure, and a solution of the residue in chloroform–methanol–water (10:10:3) was stored at −20° under nitrogen.

Chromatography of the alkali-stable lipids on DEAE-Cellulose. — Alkali-stable lipid was applied to a column (1.5 × 30 cm) of DEAE-Cellulose (acetate form) prepared as described by Behrens and Tabora[27] and equilibrated in chloroform–methanol–water (10:10:3). After the column had been washed with one bed volume of solvent to elute neutral lipids, the acidic lipids were eluted with a gradient of ammonium formate (0→60mM, 160 mL). Aliquots of fractions were dispensed into scintillation vials and dried, and scintillation fluid was added.

Mild, acid hydrolysis of alkali-stable lipid. — Alkali-stable lipid was dried under reduced pressure, dissolved in 50% 1-propanol containing 0.1M HCl (0.5 mL), and heated at 100° for 15 min. HCl was removed by repeated evaporation under reduced pressure, with addition of small amounts of methanol to aid its removal as an azeotrope.

Treatment with amyloglucosidase of the glycosyl residues released by mild, acid hydrolysis of alkali-stable lipid. — Alkali-stable lipids were treated with acid as described above and the hydrolysate was partitioned between chloroform–methanol (2:1, 1 mL) and water (0.2 mL). The upper phase was evaporated to dryness under reduced pressure. *Aspergillus niger* amyloglucosidase (EC 3.2.1.3; Boehringer Mannheim Ltd; 117 nKat) and acetate buffer (pH 4.0, 50 μmol) were added to give a final volume of 0.5 mL. The digest was incubated at 37° for 24 h under toluene and the reaction was stopped by heating at 100°. After deionisation (Zerolit DM-F), the digest was examined by paper chromatography. Using the same conditions, amylo-

glucosidase catalysed the hydrolysis of rabbit-liver glycogen, pullulan, and isomaltose, but not (1→3)-β-D-glucan, as judged by reductiometric assay[28].

Partial, acid hydrolysis of pullulan to produce chromatographic standards. — Pullulan (50 mg) in 0.05M H_2SO_4 (5 mL) was heated at 100° for 30 min and the acid was then neutralised with $BaCO_3$. After centrifugation, the supernatant fluid was decanted and the pellet was washed once with water (5 mL). The supernatant fluids were combined, concentrated under reduced pressure, and examined by paper chromatography, which revealed glucose, isomaltose (R_{Glc} 0.45), panose (6^2-α-D-glucosylmaltose, R_{Glc} 0.28), and isopanose (6^1-α-maltosyl-D-glucose R_{Glc} 0.19) as the principal products. The partial, acid hydrolysate was used as a chromatographic standard in the analysis of the glycosyl residues released by mild, acid hydrolysis from the glycolipids.

Paper and thin-layer chromatography. — Paper chromatography was performed on Whatman 3MM paper with ethyl acetate–pyridine–water (10:4:3, solvent *A*). Reducing sugars were located with alkaline silver nitrate[29]. Labelled material was located by cutting the chromatogram into 1-cm strips, which were then immersed in scintillation fluid.

T.l.c. of radioactive lipids was performed on silica gel (G-60 plates, Merck) with chloroform–methanol–water (60:25:4; solvent *B*) and chloroform–methanol–formic acid–water (140:32:16:1; solvent *C*). Lipid was stained with iodine vapour, and phosphate-containing lipids were located with a modified Hanes–Isherwood spray[30]. Radioactive lipids were located by scraping 0.5-cm portions of the plate into scintillation vials followed by the addition of scintillation fluid and subsequent measurement of radioactivity.

RESULTS AND DISCUSSION

Concomitant incorporation of radioactive label into lipid and extracellular polymer was observed with cells grown on D-[^{14}C]glucose as carbon source (Fig. 1). The lipid appears to become rapidly saturated with label during incorporation of D-[^{14}C]glucose into extracellular polymer and probably represents the small pool of intermediate glycolipids undergoing rapid turnover. Treatment of the extracellular polymer with pullulanase produced radioactive maltotriose as the major product, indicating that pullulan was being synthesised.

Cells actively elaborating pullulan were exposed to D-[^{14}C]glucose for 4 h, and the radioactive lipids were extracted with chloroform–methanol (2:1) and chloroform–methanol–water (10:10:3). The extracted, lipophilic material was hydrolysed with alkali, to give alkali-stable lipids which should be similar to the polyprenyl-linked saccharides that have been extracted from *Saccharomyces cerevisiae*[31] and mammalian liver[27] using similar procedures. Alkaline hydrolysis is sufficient to cleave acyl glycerides[2], but polyprenyl phosphates of the dolichol type are stable to 0.1M alkali at 37° due to the saturated α-isoprene unit[32].

Chromatography on DEAE-cellulose fractionated the alkali-stable glycolipids

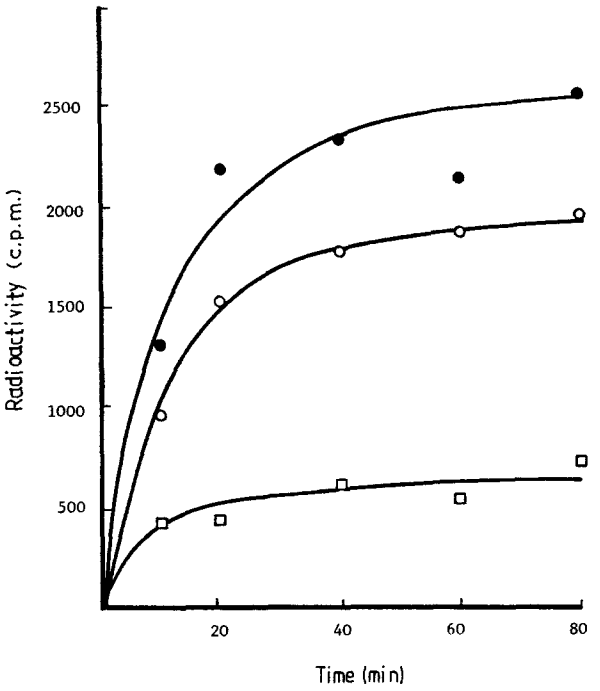

Fig. 1. Incorporation of ^{14}C-isotope into cells (—●—); extracellular polymer, pullulan (—○—); and lipid (—□—).

into 3 major components (Fig. 2). Neutral lipid (fraction 1) was eluted from the column with solvent alone and comprised 70% of the radioactive label. The two acidic fractions (2 and 3), comprising 12 and 18%, respectively, of the radioactive label, were eluted from the column with an ammonium formate gradient. The concentrations of ammonium formate required for elution indicate that pyrophosphate bonds are present in these two fractions. Mono- and pyro-phosphate containing derivatives of dolichol can be separated on DEAE-cellulose[27,33]; monophosphates are eluted with 3mM ammonium formate, whereas pyrophosphate derivatives require[33] concentrations of ~9mM.

The neutral fraction 1 was stable to mild, acid hydrolysis; in t.l.c. (solvent *B*), it had R_F 0.95. A similar, neutral lipid isolated from *Saccharomyces cerevisiae* was identified as a steryl glucoside[34]. A neutral glycolipid from soya bean was characterised as a steryl glucoside by its stability to acid, and had R_F 0.95 in t.l.c. with a system[35] (chloroform–methanol–water 65:25:4) similar to solvent *B*. There is also the possibility that the neutral fraction is a triglyceride which is metabolically close to glucose and may have been labelled in the glyceride moiety.

Examination of Fig. 2 shows that the acidic fractions 2 and 3 overlap. T.l.c. (solvent *C*) indicated that, whereas fraction 2 remained at the origin and was stained positively for phosphate, fraction 3 was resolved into two components, one of which contained phosphate and remained at the origin while the other migrated with the

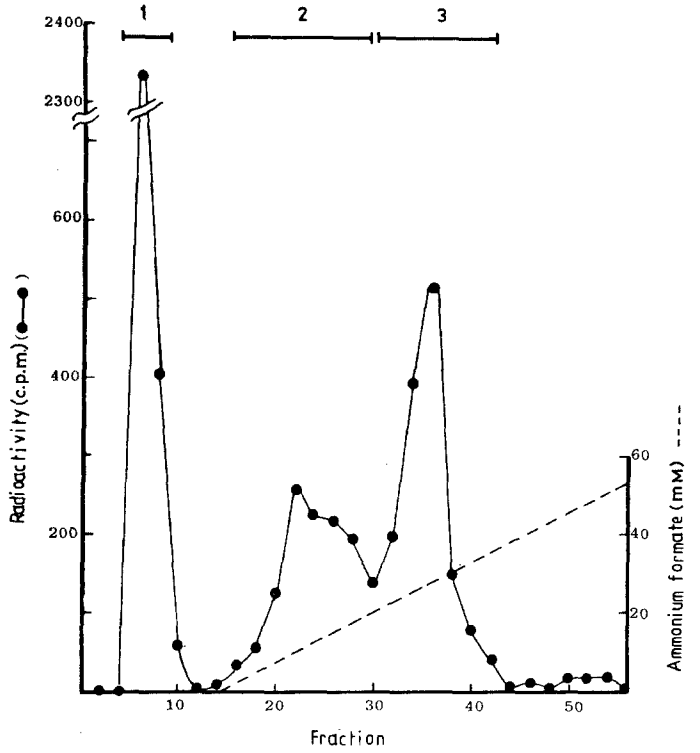

Fig. 2. Ion-exchange chromatography of ^{14}C-labelled, alkali-stable lipids using DEAE-cellulose.

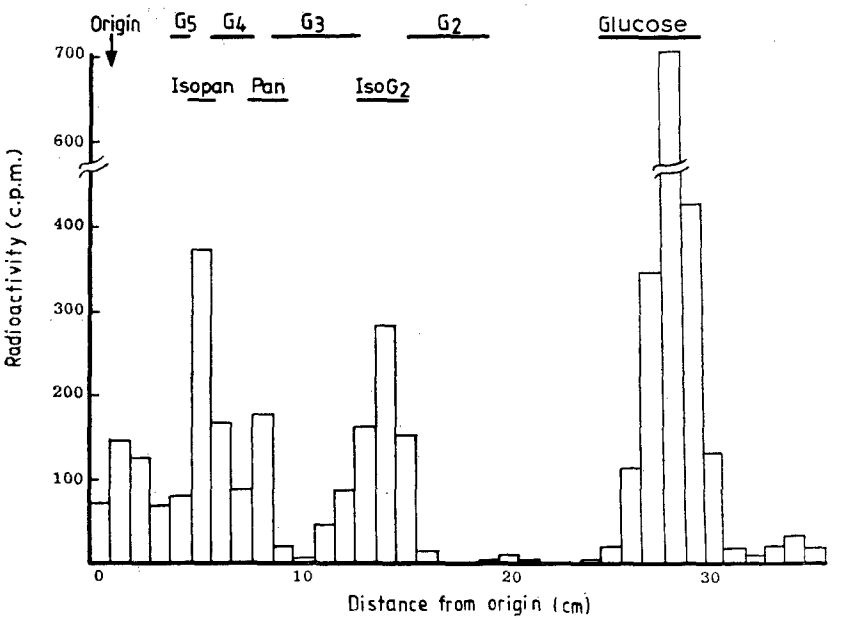

Fig. 3. Paper chromatography of ^{14}C-labelled sugars released by mild, acid hydrolysis of fraction 2 (Fig. 2). The mobilities of standard sugars are indicated by bars: G_2–G_5 are maltose–maltopentaose; $IsoG_2$, Isopan, and Pan are isomaltose, isopanose, and panose, respectively.

solvent front and did not contain phosphate. Fraction 3 may contain the trailing edge of fraction 2, together with another charged lipid species that does not contain phosphate.

Mild, acid hydrolysis of fraction 2 liberated water-soluble products that were separated by paper chromatography (solvent *A*) (Fig. 3). Glucose was the major product together with a series of radioactive peaks having R_{Glc} 0.46, 0.28, 0.19, and 0.04. The mobilities of the first three peaks suggested that they were isomaltose (R_{Glc} 0.45), panose (6^2-α-D-glucosylmaltose, R_{Glc} 0.28), and isopanose (6^1-α-maltosyl-D-glucose, R_{Glc} 0.19). Fractionation of oligosaccharides released by mild, acid hydrolysis of fraction 3 revealed a similar pattern, with the major products being isomaltose and isopanose. The conditions of mild, acid hydrolysis were chosen such that the cleavage of the bond between phosphate and the adjacent glucosyl residue was complete before appreciable hydrolysis of glucosyl bonds in the oligosaccharide moiety had commenced. However, the differential susceptibility of these two bonds to acid-catalysed hydrolysis is not large, and it is possible that some of the labelled di- and tri-saccharide moieties (Fig. 3) released may be derived from larger, lipid-linked oligosaccharides.

The glucosyl nature of the released oligosaccharides was confirmed by treatment with amyloglucosidase, a fungal enzyme derived from *Aspergillus niger* that is capable of hydrolysing both the (1→4)-α- and (1→6)-α-D-glucosidic linkages in isomaltose, panose, and isopanose[36]. The chromatographic pattern of the products of mild, acid hydrolysis that had been digested with amyloglucosidase (Fig. 4) was

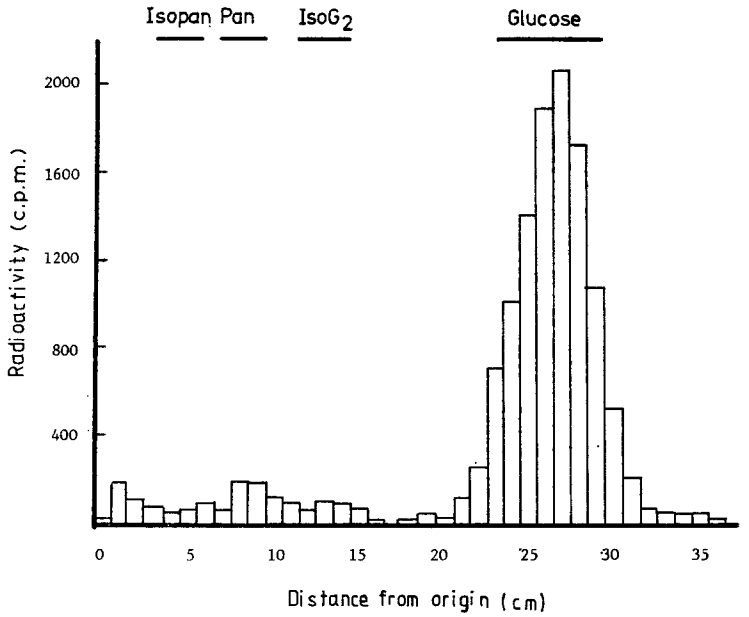

Fig. 4. Paper chromatography of ^{14}C-labelled sugars released by mild, acid hydrolysis of fraction 2 (Fig. 2) and subsequently treated with amyloglucosidase. For key, see Fig. 3.

TABLE I

DISTRIBUTION OF RADIOACTIVE LABEL PRESENT IN GLUCOSE AND OLIGOSACCHARIDES[a] RESOLVED BY PAPER CHROMATOGRAPHY (Figs. 3 and 4) BEFORE AND AFTER TREATMENT WITH AMYLOGLUCOSIDASE

Fraction	*Radioactivity (c.p.m.)*		
	Before amyloglucosidase	*After amyloglucosidase*	*Change after amyloglucosidase*
Glucose	8,858	11,228	+2,370
Oligosaccharides[b]	4,046	1,679	−2,367

[a]Prepared by mild, acid hydrolysis of alkali-stable glycolipids. [b]Those sugars having a molecular size equal to or greater than isomaltose.

compared with that prior to digestion (Fig. 3), and the amounts of radioactive label present as glucose and oligosaccharide were determined (Table I). Thus, 60% of the label associated with oligosaccharide before digestion appears in glucose afterwards, indicating that the major linkages present are (1→4)-α- and (1→6)-α-D-glucosidic bonds.

The results indicate that glucose, isomaltose, panose, and isopanose linked to lipid by pyrophosphate bonds are synthesised during pullulan biosynthesis by *A. pullulans*. There is no conclusive evidence that these lipid intermediates are participants in the assembly of pullulan and yet, if they are not, it is difficult to see of what they are the precursors. Glycogen, which is similar to pullulan in that its constituent D-glucosyl residues are linked by (1→4)-α and (1→6)-α bonds, and a pool of which may exist externally to the plasma membrane, has never been shown to require lipid intermediates in its biosynthesis. Other cell-wall constituents of *A. pullulans* do not contain (1→4)-α- or (1→6)-α-D-glucosyl residues. These observations, together with the concurrent production of both pullulan and the characterised glycolipids, strongly suggest that the latter are intermediates in the assembly of this extracellular polysaccharide; although glucosyl residues are temporarily associated with lipid intermediates in the assembly of mannoproteins in *Saccharomyces cerevisiae*[37], they are part of larger oligosaccharide moieties than those described here.

Both the panosyl and isopanosyl residues can be regarded as repeating units of pullulan. There are two mechanisms by which panose and isopanose could be synthesised attached to lipid. The first, described in Fig. 5a, involves direct transfer of a D-glucosyl residue from a nucleotide donor (UDPG). Although this mechanism can account for the synthesis of isopanose, it cannot be invoked for panose, since lipid-linked maltose would have to be formed; no maltose has been detected in mild, acid hydrolysates (Fig. 3). An alternative scheme is illustrated in Figs. 5b and 5c. Lipid pyrophosphate D-glucose is formed by transfer of D-glucopyranosyl phosphate from UDPG to lipid phosphate (Fig. 5a). A further D-glucosyl residue is transferred with the formation of a (1→6)-α-D-glucosyl bond to produce lipid-linked isomaltose.

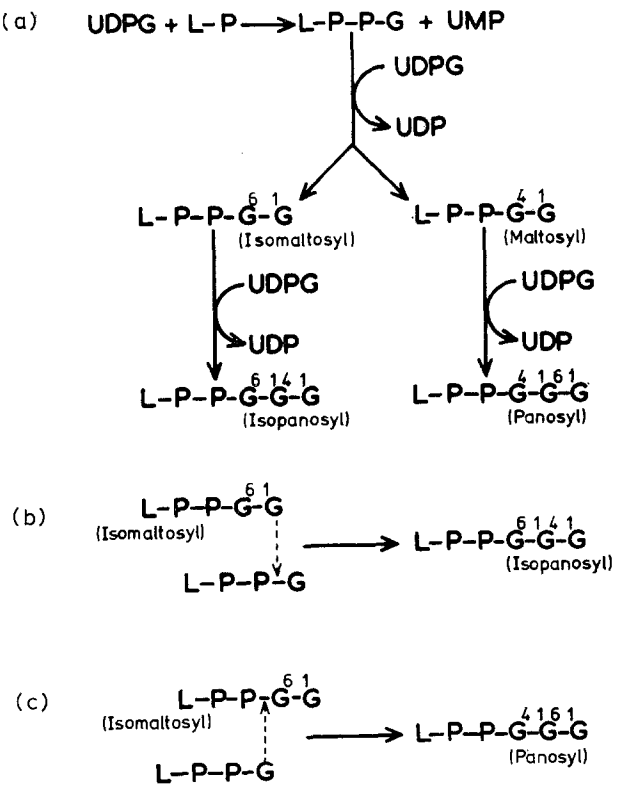

Fig. 5. Proposed scheme for the synthesis of lipid–pyrophosphate–oligosaccharide intermediates thought to participate in pullulan biosynthesis.

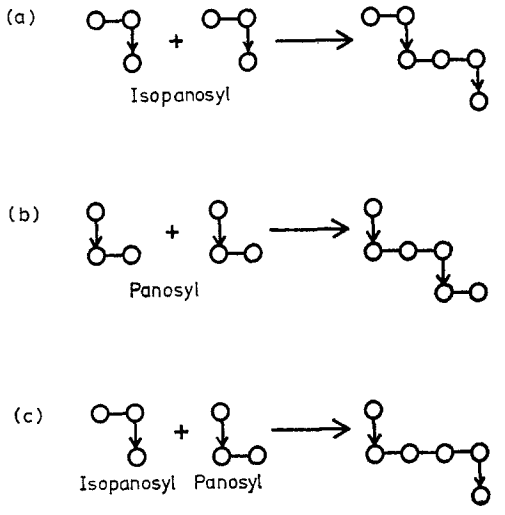

Fig. 6. Proposed scheme for the assembly of activated oligosaccharides into pullulan.

Lipid-linked trisaccharides panose and isopanose would then be formed by transfer reactions between lipid-linked D-glucose and lipid-linked isomaltose by way of insertion mechanisms. Thus (1→6) bonds are formed by glucosyl transfer from a nucleotide donor, whereas (1→4) bonds arise through transfer from lipid-linked precursors.

Figs. 6a and 6b show how the maltotriosyl repeating-unit could be incorporated into the macromolecule by the polymerisation of either panosyl or isopanosyl moieties. Maltotetraosyl units[17] could arise through the combination of panosyl and isopanosyl residues (Fig. 6c), possibly yielding a lipid-linked hexasaccharide. A radioactive component having the mobility of a hexasaccharide is present on the chromatogram of the mild, acid hydrolysate (Fig. 3). Equally, there is the possibility that panosyl and isopanosyl residues may be transferred sequentially and directly from their lipid donors to the pullulan molecule at the time of tetrasaccharide incorporation.

The above mechanism is similar to those described for the biosynthesis of Salmonella O-antigenic side-chains[10,38] and *Enterobacter aerogenes* exopolysaccharide synthesis[13,14], in which the repeating units are synthesised by sequential glycosyl transfer from sugar nucleotide donors. The preformed oligosaccharides are then transferred to the reducing end of a growing chain, resulting in the insertion of the repeating unit between the reducing end of the chain and the activating group, lipid pyrophosphate.

These results extend the observations of Taguchi *et al.*[19], who isolated lipid pyrophosphate–D-glucose from acetone-dried cells incubated with [^{14}C]sucrose. However, they did not show the concomitant incorporation of radioactive label into pullulan and lipid. The accumulation of glycolipids in *A. pullulans* cells cultured at pH 2.2 has also been reported[39]. Under these conditions, very little pullulan is synthesised[40], and it was suggested that the precursor to pullulan may be accumulating in such cultures[39]. Glucolipids were extracted which, when hydrolysed, yielded glucose and gluco-oligosaccharides. However, these were not characterised further.

Cells of *A. pullulans*, actively elaborating extracellular pullulan, have been shown to contain lipid-linked saccharides that are labelled in their glycosyl moieties when the cells are incubated with D-[^{14}C]glucose. A scheme is proposed in which these lipid-linked saccharides function as intermediates of pullulan biosynthesis.

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