Characterization and Ultrastructure of Mutants of *Escherichia coli* Deficient in α -1,4-Glucan- α -1,4-Glucan 6-Glycosyltransferase (Branching Enzyme)

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SUMMARY

Mutants deficient in branching-enzyme activity (*glgB*) were isolated and classified into four groups. They accumulated different amounts of a linear polysaccharide similar to amylose. The polysaccharides were extracted and purified. Some of their properties were similar, but gel filtration on Agarose fractionated them into three peaks whose size seemed to be related to α -amylase activity. Electron microscopy showed that amylose formed inclusions of varying density and small separated granules; the proportions of these types of aggregates varied from one mutant to another. Amylase deficiency and the *glgC* mutation (altered ADP-glucose pyrophosphorylase) are important factors in amylose accumulation and aggregation.

INTRODUCTION

In previous papers (Damotte, Cattaneo, Sigal & Puig, 1968; Cattaneo *et al.* 1968; Creuzet-Sigal, Latil-Damotte, Cattaneo & Puig, 1972) various mutants of *Escherichia coli* K12 (altered in their ability to synthesize glycogen) were reported and characterized: *glgA* mutants (glycogen synthetase-less), *glgB* mutants (branching enzyme-less) and *glgC* mutants. The *glgC* mutants accumulate up to 48 % of their dry weight in glycogen during the stationary phase of growth. They have an altered ADP-glucose pyrophosphorylase that is much less sensitive to its inhibitors, so the synthesis of glycogen is derepressed in the log phase of growth. The *glgB* mutants synthesize a linear polysaccharide similar to amylose. Although most of them have normal or sufficient levels of ADP-glucose pyrophosphorylase and polyglucoside synthetase, they synthesize smaller amounts of polysaccharide than the parent strain. The lack of branching enzyme activity might explain the slow rate of synthesis but the low amount of polysaccharide could also be the result of the presence of degradative enzymes (such as α -amylase) which would be more active on linear substrates than on branched polysaccharides. This hypothesis seemed to be supported by the isolation of one mutant (3064) of dark-blue phenotype derived from a strain having a very low amylolytic activity. This mutant is able to accumulate up to 20 % of its dry weight in amylose but it is affected not only in branching enzyme activity but also in ADP-glucose pyrophosphorylase by a mutation similar to that of *glgC* mutants. This paper describes several other *glgB* mutants that were studied to elucidate why most of the *glgB* mutants do not accumulate glucan well, and to determine the influence of the level of amylolytic activity and the *glgC* mutation in amylose synthesis. Electron microscopy (Thomas & Cattaneo, 1971) has shown that cells of the dark-blue mutant (3064) contain a single big inclusion of amylose, but in another blue mutant that synthesizes only 5 % (dry wt) of polysaccharide the amylose is heterogeneous

and occurs as small, scattered granules. The ultrastructure of all the new *glgB* mutants was examined.

METHODS

Microbial procedures. Mutations were induced by *N*-methyl-*N'*-nitrosoguanidine (NTG) treatment (Adelberg, Mandel & Chen, 1965) on *Escherichia coli* K12: strains HfrG6 (3058, 3001) and F-356 (Table 1). Mutant colonies grown on nitrogen-limiting solid medium were detected by flooding colonies with iodine solution (0.2 % in 0.4 % KI) (Damotte *et al.* 1968). The cells were grown aerobically at 32 °C on synthetic medium (Segel, Cattaneo & Sigal, 1965). Growth was limited by ammonium chloride or by threonine for mutants auxotrophic for threonine; other factors were in excess. Transduction was done as described by Lennox (1955).

Enzyme extraction and assays. The cells were disrupted in a French pressure cell at 12000 lb/in² in 4 vol. 0.05 M-tris-HCl buffer, pH 8.0, containing 5 mM-reduced glutathione.

ADP-glucose pyrophosphorylase activity was determined by following the synthesis of [³²P]ATP from ADP-glucose and ³²P-labelled pyrophosphate according to the method of Preiss, Shen, Greenberg & Gentner (1966). The polyglucoside synthetase activity was determined by following the transfer of [¹⁴C]glucose from ADP-[¹⁴C]glucose (ICN) to glycogen as described by Preiss & Greenberg (1965). α -Amylase activity was determined by the determination of reducing sugars released from amylose (Calbiochem) or from polysaccharides of mutants (Chambost, Favard & Cattaneo, 1967). The increase in reducing power was followed by the method of Park & Johnson (1949).

Branching enzyme was assayed directly by using as substrate an amylopectin-like polysaccharide which had been isolated (unpublished results) from one dark-brown mutant (201). This polysaccharide, which has long outer chains: $\overline{CL} = 20$, $\overline{ECL} = 14.5$, was a good substrate for branching enzyme. Addition of crude extracts decreases the absorption of the iodine complex ($\lambda_{\max}$, 510 nm) without liberation of reducing sugars in the conditions used for the enzyme assay. The reaction mixture contained 3 mg 201 glycogen/ml 0.1 M-tris-citrate buffer pH 7.5. Incubation was at 32 °C; 0.1 ml samples were taken and put directly into 3 ml iodine solution (sodium citrate 40 mM, iodine 1.3 mM, potassium iodine 21 mM at pH 5.6; Gibson, Brown & Brown, 1971).

Polyglucose polymer was measured after hydrolysis of bacteria (Sigal, Cattaneo & Segel, 1964) by the glucostat method. Protein determinations were carried out as described by Lowry, Rosebrough, Farr & Randall (1951).

Extraction and purification of polysaccharides. Polysaccharides of mutants were extracted with dimethylsulphoxide (DMSO; Merck) by a modification of the method of Whistler & BeMiller (1962): 5 g (dry wt) of bacteria suspended in DMSO were disrupted in a French pressure cell. After centrifugation (480 g) for 20 min the supernatant was poured into 3 l methanol containing 5 g sodium chloride. The pellet was re-extracted with DMSO and after centrifugation, the supernatant was added to the same methanol solution. The precipitate was recovered by centrifugation for 10 min and dissolved in 100 ml DMSO. The solution was centrifuged (500 g) and the polysaccharide precipitated with 3 vol. ethanol. The procedure was repeated twice with diminishing amounts of DMSO. The polysaccharide was then dissolved in 0.1 N-NaOH, deproteinized by the method of Sevag (1934), and then dried with alcohol and ether.

Characterization of polysaccharides. β -Amylolysis and the $\lambda_{\max}$ of the iodine complex were determined as described previously (Sigal *et al.* 1964) except that the polysaccharides were dissolved in 20 or 50 % (v/v) DMSO.

Table 1. *Characteristics of strains used*

Strains	Genotype	Iodine coloration	Origin
3058*	hfr66 <i>his</i>	Red-brown	Pasteur Institute
3001†	hfr66 <i>his</i>	Red-brown	Pasteur Institute
356†	F ⁻ <i>thr leu ade arg pro his thi</i>	Red-brown	Pasteur Institute
618	356 <i>glgC</i>	Dark-brown	NTG mutant of 356
204	3058 <i>glgC</i>	Dark-brown	NTG mutant of 3058
107	3058 <i>glgC</i>	Dark-brown	NTG mutant of 3058
3064	3058 <i>glgB, glgC</i>	Dark-blue	NTG mutant of 3058
197	3058 <i>glgB, glgC</i>	Dark-blue	NTG mutant of 3058
144	3058 <i>glgB, glgC</i> (204)‡	Dark-blue	NTG mutant of 204
200	3058 <i>glgB, glgC</i> (107)‡	Dark-blue	NTG mutant of 107
191	3058 <i>glgB</i>	Dark-blue	NTG mutant of 3058
194	3058 <i>glgB</i>	Dark-blue	NTG mutant of 3058
151	3058 <i>glgB</i>	Dark-blue	NTG mutant of 3058
131	356 <i>glgB, glgC</i> (618)‡	Dark-blue	NTG mutant of 618
134	356 <i>glgB, glgC</i> (618)‡	Dark-blue	NTG mutant of 618
12	356 <i>glgB</i>	Blue	NTG mutant of 356
128	3001 <i>glgB</i>	Blue	NTG mutant of 3001
4	356 <i>glgB</i>	Blue	NTG mutant of 356
138	356 <i>glgB, glgC</i> (618)‡	Dark-blue	NTG mutant of 618

* Low α -amylase activity.† High α -amylase activity.

‡ The strains on which a second treatment by NTG was done are given in parentheses.

Polysaccharides were subjected to gel-filtration chromatography on a column of Agarose (Bio-Gel A-5 M 200–400 mesh) using 20 or 50 % (v/v) DMSO in 1 mM-tris pH 7.0.

Electron microscopy. Bacteria were harvested during the stationary phase of growth. They were fixed 2 h in 2.5 % glutaraldehyde in 0.025 M-cacodylate-HCl buffer pH 6; then they were washed in the pH 6 buffer of Ryter & Kellenberger (1958) and postfixed in 1 % OsO₄ in the same buffer. They were embedded in Araldite. Sections were treated according to Thiéry (1967) to locate polysaccharide by deposition of silver.

Materials. α -Amylase from hog pancreas type I-A and oyster glycogen and β -amylase from sweet potato type I-B were from Sigma, Bio-Gel A-5 M was obtained from Bio-Rad, and deoxyribonuclease was from Koch-Light.

RESULTS

Isolation of mutants

Since amylase-less mutants have so far not been obtained, NTG mutations were induced in strains with normal or low amylolytic activity. One strain of each type carried the *glgC* mutation. The following were used: mutants obtained from wild-type strain with low α -amylase activity (3064, G197, G191, G194, G151); mutants obtained from *glgC* mutants with low α -amylase activity (G200, G144); mutants from *glgC* mutants with normal α -amylase activity (G131, G134); and mutants from wild-type strains with normal α -amylase activity (G12, G128). The characteristics of these mutants are reported in Table 1.

Table 2. *Polysaccharide content and enzyme activities of parent and mutant strains*

Strains	Poly-saccharide (% cell dry wt)*	Poly-glucoside synthetase log phase†	ADPG-pyrophosphorylase‡	Sensitivity of ADPG-pyrophosphorylase to 5-AMP or Pi§	Branching enzyme	α -Amylase¶
3058	18	1.21	0.540	+	0.122	55
3001	20.3	1.30	0.435	+	0.180	310
356	20	1.55	0.500	+	0.210	273
3064 <i>glgBC</i>	22	1.05	0.600	—	—	34
G197 <i>glgBC</i>	23.4	1.22	0.532	—	—	35
G144 <i>glgBC</i>	20.3	1.13	0.415	—	—	28
G200 <i>glgBC</i>	16.7	1.34	0.567	—	—	34.5
G194 <i>glgB</i>	6.9	0.67	0.318	+	—	97.5
G191 <i>glgB</i>	12.4	1.11	0.368	+	—	165
G151 <i>glgB</i>	7.9	0.93	0.618	+	—	18
G131 <i>glgBC</i>	7.9	0.74	0.430	—	—	323
G128 <i>glgB</i>	6.5	1.41	0.527	+	—	313
G12 <i>glgB</i>	5.0	0.47	0.124	+	—	366

* Total cell glucose measured by acid hydrolysis of cells; the value given is the maximum amount accumulated in stationary phase.

† Expressed as $\mu\text{mol } [^{14}\text{C}]\text{glucose incorporated into glycogen/mg protein in 15 min.}$

‡ Expressed as $\mu\text{mol } [^{32}\text{P}]\text{ATP formed/mg protein in 10 min.}$

§ + = Sensitive; — = insensitive.

|| Expressed as $\Delta E_{510 \text{ nm}}/\text{mg protein in 10 min.}$

¶ Expressed as $\mu\text{mol maltose released/mg protein/h.}$

Table 3. *Structure of polysaccharides extracted from blue and dark-blue mutants*

	3064 <i>glgBC</i>	G144 <i>glgBC</i>	G200 <i>glgBC</i>	G131 <i>glgBC</i>	G191 <i>glgB</i>	G12 <i>glgB</i>	G128 <i>glgB</i>
Polysaccharide sedimented at 1500 g* (%)	90.7	64.0	63.5	25.5	38.4	13.5	11.2
β -Amylase limit (PM β)†	89.5	86.5	84.5	85.5	87.0	88.5	90.5
Iodine complex‡ (λ_{max} , nm)	570–580	540	610	550–560	570–580	555–560	570–580

* The extracts were centrifuged at 1500 g for 20 min. The results are expressed as percentages of the polysaccharide in the extracts before centrifugation.

† Percentage conversion to maltose. Reducing sugar determined as maltose by ferricyanide method (Park & Johnson, 1949).

‡ The solution containing 0.01 % polysaccharide, 0.02 % I_2 and 0.2 % KI was read against an I_2 -KI blank.

Accumulation of polysaccharides and enzymic studies

Table 2 shows that the *glgB* mutants accumulate different amounts of polyglucoside (5 to 20 % of their dry weight). Since these variations in the amount of stored polysaccharide might depend on the levels of the enzymes involved in synthesis and degradation of polysaccharide, these activities were measured and compared with those of parent strains.

ADP-glucose pyrophosphorylase. The specific activities of this enzyme are similar to those of the parent strains (Table 2) but some of the mutants possess a structurally-altered ADP-glucose pyrophosphorylase which is not inhibited by 5'-AMP or which is less sensitive to

Fig. 1. Elution patterns of polysaccharides in gel-filtration chromatography on a column of Agarose (Bio-Gel A-5 M 200-400 mesh). Polysaccharide (40 mg) dissolved in 5 ml of 1 mM-tris (pH 7.0) containing 20 or 50% DMSO was applied to a 60 × 2.5 cm column and filtered with the same buffer at a flow rate of 12 ml/h. Fractions (3 ml) were collected and their polysaccharide content determined as follows: to 0.5 ml of each fraction, 5 ml water and 2 drops of iodine solution were added and the extinction was read at 570 nm. Strains 134 and 128 have a high α -amylase activity and strains 200 and 191 have a low α -amylase activity.

Table 4. β -Amyolysis of the different peaks after filtration on agarose

Strains	β -Amyolysis*		
	First peak	Second peak	Third peak
200 <i>glgBC</i>	77	86	95.5
191 <i>glgB</i>	74.5	88	85.5
134 <i>glgBC</i>	53† 65‡	84	95
128 <i>glgB</i>	66	95.5	94.5

* Percentage conversion to maltose.

†, ‡ Refer to the twin peaks found in place of the first peak.

Pi inhibition (strain G200; J. Cattaneo, unpublished results) than wild-type enzyme. These strains synthesize polysaccharide even during the log phase of growth. The mutants 131, 134, 144 and 200 (all *glgBC*) were obtained from *glgC* mutants by a second NTG treatment, but strains 3064 and 197 (also *glgBC*) were induced by a single NTG treatment. It was therefore of interest to see if the latter two strains were double mutants.

Separation of mutations glgB and glgC. A transduction was done with the phage P1Kc grown on the mutant G197 using strain 530 as recipient:

Table 5. Activity of α -amylase of *Escherichia coli* on different substrates

Substrate	Activity* (μ g maltose/ h/ml incubation mixture)
Commercial amylose	560
Polysaccharide of strain 144	440
Polysaccharide of strain 134	290
Glycogen of strain 455	21.5

* The reaction mixture contained: amylose or polysaccharide, 20 mg; NaCl, 0.8 mmol; 0.1 mmol tris-maleate buffer (pH 7.0); 50 μ l purified amylase; water to 4 ml.

Transductants *asd*⁺ were selected, isolated and scored for iodine coloration. Among 150 *asd*⁺ transductants a colony staining dark brown was found; it had branching enzyme activity (co-transduction of *glgB* gene with *asd* gene) and its ADP-glucose pyrophosphorylase was insensitive to AMP (strain 230 *asd*⁺ *glgB*⁺ *glgC*⁻). Mutations *glgB* and *glgC* of strain G197 are two distinct mutations.

Polyglucoside synthetase. The specific activities of this enzyme in log phase of growth were not very different from those of the parent strains (Table 2). However, for a few strains and especially for mutant G12 a lower activity was found. This fact was reported earlier (Creuzet-Sigal *et al.* 1972) and attributed to a peculiar lability of the enzyme during extraction. The addition of glycogen before cell extraction increased the activity. Determinations of polyglucoside synthetase activities of *glgB* mutants (from log or stationary phase) were made on uncentrifuged extracts because a part of the synthetase sediments at a very low speed with the polysaccharide. The specific activity of the polyglucoside synthetase of mutants 3064 and 197 decreased to very weak values during the stationary phase of growth.

Branching enzyme. The determination of branching enzyme activity by following the decrease of absorption of the amylose-iodine complex is not specific and amylolytic activity interferes in the assay. Therefore the method described above was used. Amylase did not interfere because the anions that activate it were absent. Table 2 shows that at 510 nm extinction decreased by 0.100 to 0.200 with extracts of parent strains but no decrease was detected in 2 h with *glgB* mutants.

α -Amylase. An intracellular α -amylase purified and characterized by Chambost *et al.* (1967) is present in *Escherichia coli* K12 but in some strains its activity is very low. α -Amylase activity of the *glgB* mutants was determined in extracts from stationary-phase cells using commercial amylose as substrate (Table 2).

Characterization of polysaccharides

Polysaccharides of mutants were extracted from stationary-phase bacteria and purified as described in Methods. They were insoluble in water but soluble in 0.1 N-NaOH or

Fig. 2. Electron micrographs of strain 3064 (*a, b, c, d*) and strain 197 (*e, f, g, h*). (*a*) Most of the cells contain a single, large, dense, amylose polar inclusion. Single arrows point to rare, less-dense inclusions; double arrows point to small inclusions newly synthesized near the polar membrane. (*b*) One of the very rare cells having numerous small amylose grains close together in most of the cytoplasm. 10% of inclusions (*c*) are less dense than those in (*d*). In (*c*) one inclusion is dense only in its centre. (*e*) Arrows point to the dense inclusions (about half); those in (*f*) are less dense. Dense and less-dense inclusions having a thin, fibrillar structure. The less-dense inclusion (*g*) has a reticular structure. About 5% of the cells have small, separated polysaccharide grains (*h*).

Table 6. Results of electron microscopy and classification of mutants

Strains	Genotypes	α -Amylase activity*	Poly-saccharide (dry wt basis) (%)	Poly-saccharide sedimented at 1500 g (%)	% of amylose-bearing sections	Groups
3064	<i>glgBC</i>	34	22	90.7	90 (d) 10 (s) < 1 (g)	I
197	<i>glgBC</i>	35	23.4	85	47 (d) 47 (s) 5 (g)	
144	<i>glgBC</i>	28	20.3	64	10 (d) 85 (s and reticulate ones) 5 (g)	
200	<i>glgBC</i>	34.5	16.7	63.5	20 (d) 75 (s and reticulate ones) 5 (g)	
151	<i>glgB</i>	18	7.9	—	5 (d without g) 90 (s + g) 5 (g only)	II
194	<i>glgB</i>	97	6.9	—	10 (s or d) 90 (g)	
191	<i>glgB</i>	165	12.4	38.4	10 (s or d) 90 (g)	
131	<i>glgBC</i>	323	7.9	25.5	5 (d, moderate sized) 95 (g)	III
134	<i>glgBC</i>	326	9.0	—	5 (s, moderate sized) 95 (g)	
138	<i>glgBC</i>	325	9.0	—	5-10 (s or d, moderate sized) 90-95 (g)	
499	<i>glgB</i>	300	5.6	—	5-10 (small s or d) 90-95 (g)	IV
12	<i>glgB</i>	366	5.0	13.5	5-10 (small s or d) 90-95 (g)	
128	<i>glgB</i>	313	6.5	11.2	5-10 (small s or d) 90-95 (g)	

* Expressed as μmol maltose released/mg protein/h.

d, Sections with one dense inclusion; s, sections with one less-dense inclusion; g, sections with small, separated grains.

Fig. 3. Electron micrographs of strain 144 (*a, b, c, d*) and strain 200 (*e, f, g, h*). Polar inclusion (*a*) is composed of a dense and a reticulate part. Inclusions (*b*) have different densities. Arrow points to separated grains that accompany a reticulate inclusion. In (*c*), other inclusions are of variable density and in the right bottom corner polysaccharides are granular (5 % of the sections). Most of the inclusions (*d*) are less dense and reticulate but about 10 % are dense (double arrow). Single arrow points to vesicular membrane invaginations that seems to be in contact with amylose. In the rare cell (*e*), a central, dense inclusion separates a region with concentrated polysaccharide grains from a region with separated grains. The three cellular poles (*f*) have granular polysaccharides, or a less-dense inclusion, or a dense inclusion. The three cells (*g*) have separated grains or a not very dense inclusion, or a reticulate inclusion with a dense centre. In an average population (*h*), 20 % of the cells have dense inclusions (at the bottom), 75 % have less-dense or reticulate inclusions, and 5 % have only granular polysaccharides (arrow).

dimethylsulphoxide. The values reported in Table 3 show that β -amylolysis was similar for all the polysaccharides (84.5 to 90.5 %) as was the λ_{max} of the iodine complex (570 to 590 nm). This value corresponds to a degree of polymerization of 61 glucose units (Bailey & Whelan, 1961). However, the polysaccharides did not all sediment at the same speed of centrifugation (Table 3) and were insoluble in water. This suggests a much higher number of glucose units than 61. To see whether these polysaccharides were heterogeneous, gel-filtration chromatography was done on Agarose with 1 mM-tris (pH 7.0) containing 20 or 50 % (v/v) DMSO.

Figure 1 shows that the polysaccharides separated into three peaks whose relative magnitudes differed with the strains. For the polysaccharides of mutant G200 (low α -amylase activity) the first peak was high and the second and third were low; for the polysaccharides of mutants G134 and G128 (with normal α -amylase activity) the second and third peaks were higher than the first. For the polysaccharides of strain G191 all three peaks were of similar size.

The percentage of degradation by β -amylase was highest for the fractions of the third peak (Table 4). Using tris (1 mM; pH 7)-DMSO (1:1, v/v) in 0.1 M-NaCl as solvent, polysaccharides of mutant 144 which, like strain G200, has low α -amylase activity, gave a smaller first peak, no second peak and a larger third peak. Gel-filtration showed they constituted at least three populations of similar structure but differing in their aggregate size.

The polysaccharides may consist of aggregates of small molecules. This hypothesis is supported by the well-known tendency of amyloses to aggregate by hydrogen bonds (corresponding to retrogradation) and by the action of salts on the largest aggregates. β -Amylolysis decreased as the molecules increased in size; this could be explained by the insolubility of substrates which become inaccessible to the enzymes. However, the existence of some α -1,6 linkages, as has been shown in corn amylose (Oka, Shigeta & Sato, 1971), cannot be eliminated.

Polysaccharides of the *glgB* mutants were assayed as substrates for α -amylase purified as described by Chambost *et al.* (1967). Table 5 shows that the polysaccharides of strains G149 and G134 were, respectively, 20 and 15 times more active than glycogen.

Intracellular amylose by electron microscopy

The amylose of all the mutant strains appeared heterogeneous. Sometimes, heterogeneity was seen within single cells. There were large dense inclusions (0.5 to 1.5 μm) which predominated in strain 3064, large less-dense inclusions (sometimes reticulated), and small (20 to 150 nm) separated grains. The proportions of the different amylose types and the total amount of amylose varied between strains. The proportions given in Table 6 are approximate because there were intermediate forms of the different types. The proportions do not correspond to the weights of each sort of amylose because of variation in size and density. At least 100 amylose-bearing sections, of any orientation and size (excluding tangential sections), were analysed for each strain.

Fig. 4. Electron micrographs of strain 151 (*a, b, c, d, e*), strain 194 (*f, g*), and strain 191 (*h, i*). Five per cent of the cells have one dense or less-dense inclusion, without small, separated grains (*a*). In (*b*), one cell contains a dense inclusion and two cells contain separated grains. Ninety per cent of the cells have separated small grains and one polar, less-dense inclusion (*c, d, e*) that seems to be composed of closely-packed grains. Ninety per cent of strains 194 and 191 contain only grains separated or packed together and 10 % contain a large inclusion that is generally dense (*g, h, i*) or less dense (*f*).

The *glgBC* amylase⁻ strain 3064 (Fig. 2*a, b, c, d*) has a remarkably homogeneous amylose: of the sections containing amylose, 90 % possess a single, large, dense inclusion, 10 % a single, large, less-dense inclusion, and less than 1 % have many separate grains that make the cell look like a *glgC* bacterium that has abundant glycogen (Thomas & Cattaneo, 1971).

The *glgBC* amylase⁻ strain 197 was not very different from the 3064 strain, but its amylose was more heterogeneous since 47 % of the bacterial sections had a single, dense inclusion, 47 % possessed one less-dense inclusion and 5 % had small, separated grains (Fig. 2*e, f, g, h*).

In the *glgBC* amylase⁻ strains 144 and 200, the small, separated grains were also rare (5 % of the sections), but there were far more less-dense and reticulate large inclusions (85 and 75 % of the sections) than dense inclusions (10 and 20 % of amylose containing sections) (Fig. 3).

In the *glgB* strain 151 that had low amylase activity, 90 % of the cells contained one less-dense inclusion that seemed to be made of small grains and was accompanied by other separated small grains, 5 % contained only separated grains, and 5 % contained one single dense inclusion (Fig. 4*a, b, c, d, e*). On the other hand, in the *glgB* strains 194 and 191 that were only slightly amylase deficient, 90 % of the cells contained only small, separated grains and 10 % contained large dense or less-dense inclusions (Fig. 4*f, g, h, i*).

In the *glgBC* strains 131, 134 and 138 that had normal amylase activity and little amylose, the amylose was irregularly distributed among the cells: 90 to 95 % of the cells contained separated amylose grains (a few grains generally) and 5 to 10 % contained moderate-sized inclusions (Fig. 5*a, b, c, d, e*).

Lastly, in the *glgB* strains 128, 499 and 12 with normal amylase activity, the amylose had the same distribution, but it was still less abundant and the inclusions (in 5 to 10 % of the cells) were small (Fig. 5*f, g, h*).

In all the mutants, less-dense inclusions were often accompanied by separated grains whereas this was rare with dense inclusions. Table 6 compares the summarized results of electron microscopy with amylase activity, total polysaccharide content and the percentage of polysaccharide that sediments at 1500 g. The percentage of polysaccharide sedimented at 1500 g correlates with the percentage of dense or less-dense (d + s) inclusions.

DISCUSSION

Mutants deficient in branching enzyme activity (*glgB*) accumulate amylose less than their respective parent strains synthesize glycogen, although they have normal levels of ADP-glucose pyrophosphorylase, polyglucoside synthetase and amylase and differ from their parent only by the absence of branching enzyme. This is a confirmation that branching enzyme facilitates polysaccharide synthesis by providing new non-reducing ends as glucosyl acceptors; this has been shown for synthesis *de novo* of glycogen by rabbit phosphorylase (Brown & Brown, 1966) and for glycogen synthesis by polyglucoside synthetase (Walker & Builder, 1971; Fox *et al.* 1973).

Fig. 5. Electron micrographs of strain 134 (*a, b, e*), strain 131 (*c*), strain 138 (*d*), strain 128 (*f*) and strain 499 (*g, h*). In strains 134, 131 and 138, 90 to 95 % of the cells contain only granular, separated amylose, the amounts varying widely from cell to cell (*a*). The other 5 to 10 % contain moderate-sized inclusions, less dense in the 134 strain (*a, b*). The cell of strain 131 (*c*) is very rich in granular, separated amylose. The cell of strain 138 (*d*) contains one moderate-sized dense inclusion, and no grains. The cell of strain 134 (*e*) possesses two small mesosomes on which polysaccharide grains seem to be fixed. The strains with little amylose, 128 (*f*) and 499 (*g* and *h*), have separated granules in 90 to 95 % of the cells, but sometimes make small inclusions (*h*).

The *glgB* mutants have been classified according to their genotypes and the amount of polysaccharide they synthesize (Table 6). In these mutants, the amount of amylose, its intracellular appearance by electron microscopy and its aggregation state in tris-DMSO depend on the amylase level and on the *glgC* mutation.

Mutants of group II (with low α -amylase activity) synthesize more amylose than mutants of group IV (with high levels of amylolytic activity); in the latter mutants the amylose is dispersed and forms only small inclusions in 5 to 10 % of cells, and the first peak obtained by molecular sieve filtration (Fig. 1) is small. The amylose-decreasing and -dispersing action of α -amylase is far more evident when the *glgC* mutation is present (comparison of mutants I and III (Table 6) and of strains 134 and 200 (Fig. 1)).

Comparison of *glgBC* mutants (group III) with *glgB* mutants (group IV) shows that the *glgC* gene is responsible for the greater amount of amylose synthesized, but has no noticeable influence on the aggregation either *in situ* or in the tris-DMSO filtration (strain 134 compared with 128). Comparison of group I mutants (*glgBC*) with mutant 151 (*glgB*) shows that when α -amylase activity is low the *glgC* mutation strongly decreases the dispersed granular appearance of amylose *in situ*. It seems that when amylose synthesis is fast a low α -amylase activity does not have enough time to cut off diffusible primer chains, and that beyond a certain aggregate size of amylose the amylase becomes inefficient because it cannot attack insoluble substrates very well.

The size heterogeneity among amyloses of each strain is surprising but this kind of variability can be explained if we consider that there are two antagonistic self-accentuating tendencies: the aggregation of amylose is favoured by its insolubility, by the adsorption of synthetase on amylose and by the inefficiency of amylase towards an insoluble substrate; on the other hand, amylase easily attacks dispersed amylose and in doing so it liberates new primers that contribute to the dispersion of amylose.

Among the mutants of group I accumulating large amounts of amylose, the specific activity of polyglucoside synthetase decreases during stationary phase only in strains 197 and 3064 which are rich in very dense inclusions of amylose. The synthetase seems to be inactivated or entrapped in the amylose since it does not decrease in strains 144 and 200, the inclusions of which are reticulated.

It is surprising that strain 151, which has less amylase than strain 191, accumulates less amylose, but its amylose inclusions are perhaps more accessible to amylase than those of strain 191.

In conclusion we find that amylase deficiency and the possession of *glgC* mutation are important factors for the accumulation of large amounts of amylose and for the aggregation of this amylose. However, more information is needed about concentration of synthetase and primers in the sites of synthesis, and about the efficiency of catabolic enzymes on native polysaccharides.

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REFERENCES

- ADELBERG, E. A., MANDEL, M. & CHEN, G. C. C. (1965). Optimal conditions for mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in *Escherichia coli* K12. *Biochemical and Biophysical Research Communications* **18**, 788-795.

- BAILEY, J. M. & WHELAN, W. J. (1961). Physical properties of starch. I. Relation between iodine stain and chain length. *Journal of Biological Chemistry* **236**, 969-973.
- BROWN, B. I. & BROWN, D. H. (1966). α -1,4 Glucan: α -1,4-glucan 6 glycosyltransferase from mammalian muscle. In *Methods in Enzymology*, vol. 8, pp. 395-403. Edited by E. F. Neufeld and V. Ginsburg. New York and London: Academic Press.
- CATTANÉO, J., DAMOTTE, M., SIGAL, N., SANCHEZ-MEDINA, F. & PUIG, J. (1968). Genetic studies of *Escherichia coli* K12 mutants with alterations in glycogenesis and properties of an altered adenosine diphosphate glucose pyrophosphorylase. *Biochemical and Biophysical Research Communications* **34**, 694-701.
- CHAMBOST, J. P., FAVARD, A. & CATTANÉO, J. (1967). Purification et propriétés d'une α -amylase endocellulaire d'*Escherichia coli*. *Bulletin de la Société de chimie biologique* **49**, 1231-1246.
- CREUZET-SIGAL, N., LATIL-DAMOTTE, M., CATTANÉO, J. & PUIG, J. (1972). In *Biochemistry of the Glycosidic Linkage*. The Pan-American Association of Biochemical Societies Symposium, vol. 2, pp. 647-680. Edited by R. Piras and H. G. Pontis. New York and London: Academic Press.
- DAMOTTE, M., CATTANÉO, J., SIGAL, N. & PUIG, J. (1968). Mutants of *Escherichia coli* K12 altered in their ability to store glycogen. *Biochemical and Biophysical Research Communications* **32**, 916-920.
- FOX, J., KENNEDY, L. D., HAWKER, J. S., OZBUN, J. L., GREENBERG, E., LAMMEL, C. & PREISS, J. (1973). *De novo* synthesis of bacterial glycogen and plant starch by ADPG: α -glucan 4-glucosyl transferase. *Annals of the New York Academy of Sciences* **210**, 90-103.
- GIBSON, W. B., BROWN, B. I. & BROWN, D. H. (1971). Studies of glycogen branching enzyme preparation and properties of α -1,4-glucan- α -1,4-glucan-6-glycosyltransferase and its action on the characteristic polysaccharide of the liver of children with type IV glycogen storage disease. *Biochemistry* **10**, 4253-4262.
- LENNOX, E. S. (1955). Transduction of linked genetic characters of the host by bacteriophage P1. *Virology* **1**, 190-206.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265-275.
- OKA, S., SHIGETA, S. & SATO, S. (1971). Size distribution of dispersing units in corn amylose. *Agricultural and Biological Chemistry* **35**, 1216-1221.
- PARK, J. T. & JOHNSON, M. S. (1949). Submicrodetermination of glucose. *Journal of Biological Chemistry* **181**, 149-151.
- PREISS, J. & GREENBERG, E. (1965). Biosynthesis of bacterial glycogen. III. The adenosine diphosphate glucose α -4-glucosyltransferase of *Escherichia coli* B. *Biochemistry* **4**, 2328-2334.
- PREISS, J., SHEN, L., GREENBERG, E. & GENTNER, N. (1966). Biosynthesis of bacterial glycogen. IV. Activation and inhibition of the adenosine diphosphate glucose pyrophosphorylase of *Escherichia coli* B. *Biochemistry* **5**, 1833-1845.
- RYTER, A. & KELLENBERGER, E. (1958). Étude au microscope électronique de plasmas contenant de l'acide désoxyribonucléique. I. Les nucléoides des bactéries en croissance active. *Zeitschrift für Naturforschung* **13B**, 597-605.
- SEGEL, I. H., CATTANÉO, J. & SIGAL, N. (1965). The regulation of glycogen synthesis in *Aerobacter aerogenes*. In *Mécanismes de Régulation des Activités cellulaires chez les Micro-organismes*, pp. 337-355. Paris: Colloques Internationaux du C.N.R.S.
- SEVAG, M. G. (1934). Eine neue physikalische Enteiweissungsmethode zur Darstellung biologisch wirksamer Substanzen. *Biochemische Zeitschrift* **273**, 419-429.
- SIGAL, N., CATTANÉO, J. & SEGEL, I. H. (1964). Glycogen accumulation by wild-type and uridine diphosphate glucose pyrophosphorylase-negative strains of *Escherichia coli*. *Archives of Biochemistry and Biophysics* **108**, 440-451.
- THIÉRY, J. P. (1967). Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. *Journal de Microscopie* **6**, 987-1018.
- THOMAS, P. & CATTANÉO, J. (1971). Étude ultrastructurale et chimique des polyglucosides cytoplasmiques synthétisés par des mutants d'*Escherichia coli* K12. *Journal de Microscopie* **12**, 349-362.
- WALKER, G. J. & BUILDER, J. E. (1971). Metabolism of the reserve polysaccharide of *Streptococcus mitis*. Properties of branching enzyme and its effect on the activity of glycogen synthetase. *European Journal of Biochemistry* **20**, 14-21.
- WHISTLER, R. L. & BEMILLER, J. N. (1962). Extraction of glycogen with dimethyl sulfoxide. *Archives of Biochemistry and Biophysics* **98**, 120-123.