ISOLATION OF A MUTANT STRAIN OF *CLOSTRIDIUM PASTEURIANUM* DEFECTIVE IN GRANULOSE DEGRADATION

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1. Introduction

Several species of saccharolytic clostridia synthesise granulose, an amylopectin-like α-polyglucosan which is intracellularly deposited in large granules which stain an intense purple colour with iodine [1–6]. *Clostridium pasteurianum* accumulates considerable quantities of granulose at the inception of the stationary phase of its batch culture in glucose-rich medium [3,7], and the polyglucan may thereafter be degraded, as, for example, when the organisms are transferred to a medium devoid of any alternative source of carbon and energy [8]. Mobilisation of the granulose is effected by an α-1,4-polyglucan orthophosphate glucosyl transferase, EC 2.4.1.1 (i.e. granulose phosphorylase) which yields glucose 1-phosphate. This enzyme is constitutively synthesised in *Cl. pasteurianum* but is subject to inhibition by nucleotide sugars (including ADP–glucose and UDP–glucose), which could explain why, in this organism, degradation of granulose does not occur in the presence of exogenous glucose [8].

Though the granulose of *Cl. pasteurianum* may in appropriate circumstances serve as an endogenous reserve material [9], as has been demonstrated in *Cl. botulinum* type E (Minnesota) [10], granulose synthesis itself may serve some useful purpose. The first indication that this could be the case, came from studies with granulose-negative mutant strains of *Cl. pasteurianum* (lacking either ADP–glucose pyrophosphorylase or granulose synthase and hence blocked in granulose synthesis) [11]. Though these organisms sporulated very poorly in glucose-rich minimal medium, they sporulated well when the rate of supply of glucose was so low as to sustain sporulation of the parent, wild-type organism without concurrent production of granulose. It was tentatively suggested [11] that granulose synthesis in *Cl. pasteurianum* can serve as an 'overflow' moderating any rise in the intracellular levels of potentially harmful metabolites (including suppressor(s) of sporulation) – a situation which might well obtain during early stationary phase of glucose-rich cultures.

To distinguish between benefits accruing from the synthesis of granulose and those stemming from its subsequent utilisation, it is necessary to study not only granulose-negative strains, but also mutant strains that are normal in granulose accumulation but defective in its utilisation, and these have hitherto not been available. In this communication we report the isolation of such a mutant, which is defective in granulose degradation as a consequence of gross deficiency in granulose phosphorylase.

2. Experimental

Maintenance and growth of *Cl. pasteurianum* ATCC 6013 have been described [3]. The defined glucose (4%)-ammonium-salts and vitamins medium of Mackey and Morris [3] was used as glucose-rich medium. With the glucose omitted this was designated glucose-free medium, and with 2% lactose in place of glucose, it served as medium devoid of a utilisable carbon source (see Results 3.1). These media and Oxoid Reinforced Clostridial Medium (RCM) were solidified when necessary with 1.5% agar. Culture density was followed spectrophotometrically at

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680 nm, total cell counts were made microscopically using an improved Neubauer counting chamber, and viable counts were made on anaerobically incubated RCM plates. Cell suspensions in 50 mM imidazole buffer pH 6.4 with 0.5 mM dithiothreitol, were disrupted by passage at 20 000 psi through a chilled French pressure cell (Aminco), and a crude preparation of 'native' polyglucan granules was obtained therefrom by differential centrifugation [8]. Granulose was determined as glucose liberated from washed organisms by hydrolysis in 1M H_2SO_4 for 3 hr at 100°C [8]. Glucose was assayed colorimetrically by the standard 'Glucostat' procedure [12], protein was measured by the method of Lowry et al. [13], and radioactivity was determined by scintillation counting [8].

2.1. *Mutagenesis*

A 10 ml sample of an anaerobic culture of *Cl. pasteurianum* in mid-exponential growth in RCN, was aseptically harvested by centrifugation (5000 *g* for 10 min at room temperature) and the organisms resuspended at 37°C in 9 ml of sterile 0.1 M sodium citrate buffer pH 5.5. *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1 ml of a fresh solution containing 1 mg NTG/ml) was added, and the suspension incubated aerobically for 15 min at 37°C. The organisms were recovered by aseptic centrifugation, reinoculated into 25 ml of RCM and the culture allowed to grow to late exponential phase.

2.2. *Mutant selection*

The mutagenised culture was serially diluted in basal medium containing 2% lactose in place of glucose, and 5 ml vols of a suitable dilution were aseptically filtered through Millipore membranes (0.45 μm pore, 37 mm diam.). These were laid on the surface of RCM plates which were incubated anaerobically for 24 hr at 37°C. The membranes were then aseptically transferred to plates of glucose-free medium and anaerobically incubated at 37°C for a further 48 hr. Those few colonies which still stained intensely on exposure to I_2 vapour were inoculated into liquid RCM and the selection procedure was repeated with each of these subcultures.

2.3. *Assay of granulose phosphorylase*

(a) In the degradative direction (phosphorolysis). The inorganic phosphate-dependent production of glucose 1-phosphate from granulose (or amylopectin) was followed spectrophotometrically by measuring the rate of reduction of NADP achieved in the presence of excess phosphoglucomutase (EC 2.7.5.1) and glucose 6-phosphate dehydrogenase (EC 1.1.1.49). The assay was performed in anaerobic cuvettes since extracts of *Cl. pasteurianum* contain appreciable NADPH oxidase activity. The assay mixture contained (in 3 ml final vol): 3 mg of amylopectin, 3 μmol of NADP, 9 nmol of glucose 1,6-diphosphate, 27 μmol of $MgCl_2$, 3 units of glucose 6-phosphate dehydrogenase (Sigma), 3 units of phosphoglucomutase (Sigma), 30 μmol of potassium phosphate buffer pH 6.4 and 150 μmol of imidazole buffer pH 6.4. The cuvette was sealed with a 'Subba Seal' rubber cap, and its contents were sparged for 3 min with O_2-free argon introduced via a hypodermic needle. The reaction was started by the anaerobic addition of crude extract, and the increase in absorbance at 340 nm was followed at 30°C in a Unicam SP 1800 recording spectrophotometer against a blank consisting of reaction mixture minus extract. There was an initial lag of about 3–5 min and the reaction rate was measured after 10 min (when it was reproducibly linear). The partially purified preparations of granule-associated phosphorylase were devoid of NADPH oxidase activity and the assay could be carried out aerobically, using as 'blank' the reaction mixture minus inorganic phosphate.

b) In the synthetic direction. Incorporation into amylopectin of radioactivity from [^{14}C]glucose 1-phosphate was followed by the method of Wang and Essman [14].

3. Results

The mutant selection procedure adopted (2.2) was suggested by the finding that the granulose reserves of wild-type *Cl. pasteurianum* could be entirely exhausted by anaerobic incubation in minimal growth medium lacking any alternative source of carbon. Thus colonies which after growth on glucose-rich medium stained intensely with I_2, lost this character after 48 hr anaerobic incubation on plates of glucose-free medium. On the other hand, colonies of any mutant strain which mobilised its granulose at a

reduced rate should display a strong colour reaction with I_2 even after more prolonged incubation in media devoid of alternative sources of carbon. Since even granulose-negative strains of *Cl. pasteurianum* were able to survive such 'starvation' conditions for quite long periods (Robson and Morris, unpublished work), it was though likely that the mutant strain sought in the present study should also retain sufficient viability over 48 hr incubation to ensure the feasibility of the proposed selection procedure.

Of the several thousand colonies derived from NTG-mutagenised cultures that were 'screened' by this method, seventeen appeared worthy of further investigation, but only one strain (mutant ES 1) retained the desired phenotype through serial subcultivation.

3.1. *Mobilisation of granulose*

The ability of mutant ES 1 to degrade its reserves of granulose was compared with that displayed by the parent, wild-type organism, by transferring granulose-replete cells obtained by growth on glucose minimal medium to the same medium devoid of an utilisable carbon source and then following the progress of granulose loss during continued anaerobic incubation. Freshly autoclaved medium containing 2% lactose in place of glucose, possessed an appropriately low E_h and satisfied the criterion of supplying no utilisable carbon source, since neither *Cl. pasteurianum* ATCC 6013 nor its mutant ES 1 can utilise lactose [8]. Fig. 1 shows that the wild type organism rapidly degraded its granulose, some 80% being consumed in 22 hr at 37°C. In contrast, mutant ES 1 lost very little of its granulose content (< 6%) in the same period.

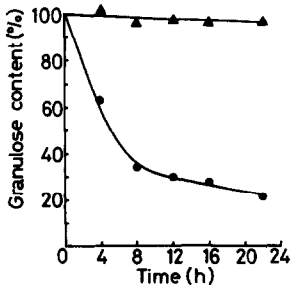

Fig. 1. Degradation of intracellular granulose by parent wild type *Cl. pasteurianum* ATCC 6013 (•) and mutant strain ES 1 (▲) during anaerobic incubation in a utilizable carbon-free medium. Glucose-grown organisms replete with granulose were transferred at 0 hr into a 2% lactose minimal medium and incubated anaerobically at 37°C.

Table 1
Granulose phosphorylase activity in mutant strain ES 1 and in its parent, i.e. wild type *Cl. pasteurianum* ATCC 6013

Source	Specific activity of granulose phosphorylase (nmol/min per mg of protein)	
	Mutant ES 1	Wild type
Crude cell extract	0.116	1.340
Extract of 'native' polyglucan granules	2.030	100.300

Granulose phosphorylase was assayed in the degradative (phosphorolytic) direction [see Experimental, section 2.3a].

3.2. *Granulose phosphorylase activity*

The specific activity of granulose phosphorylase in crude extracts of glucose-grown mutant ES 1 was less than one tenth of that present in extracts of the wild type organism (table 1), irrespective of whether this activity was measured in the degradative or synthetic direction. When the specific activity of the granule-associated granulose phosphorylase was measured, the discrepancy between the mutant and its parent was even more marked (table 1).

3.3. *Accumulation of granulose*

The pattern of granulose deposition displayed by mutant ES 1 (fig. 2) resembled that of normal wild type strains of *Cl. pasteurianum* in that in batch culture in glucose-rich medium the polyglucan was accumulated only at the end of exponential growth and onset of the stationary phase [3,7,8]. Detailed comparative studies of sporulation and concomitant granulose accumulation in mutant ES 1 and its wild type parent are now being undertaken.

4. Discussion

Though the nature of its genetic lesion is not known, mutant strain ES 1 of *Cl. pasteurianum* is critically deficient in granulose phosphorylase activity

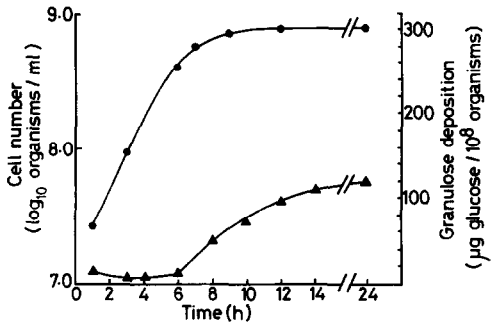

Fig. 2. Accumulation of granulose in mutant strain ES 1 of *Cl. pasteurianum* during anaerobic growth at 37°C in glucose-rich medium. Cell number (●) and granulose content of the organisms (▲) were assayed during late exponential and stationary phases of an anaerobic batch culture of mutant ES 1 on glucose (4%) minimal medium [3].

and is consequently prevented from making normal use of its granulose reserves, though these are still accumulated in the usual manner.

Studies with this novel mutant in which granulose production is effectively divorced from granulose utilisation, should reveal what arcane benefits *Cl. pasteurianum* may derive from its ability to divert substantial amounts of glucose 1-phosphate and ATP to the synthesis of this polyglucan.

Acknowledgements

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