Characterization, Biosynthesis, and Regulation of Granulose in Clostridium acetobutylicum

A. L. REYESNACH, N. RAVENSCROFT, S. LONG, D. T. JONES, and D. R. WOODS

Department of Microbiology and Department of Organic Chemistry, University of Cape Town, Rondebosch 7700, South Africa

Received 16 October 1985/Accepted 24 January 1986

Synthesis of granulose was investigated in 15 solvent-producing Clostridium strains. Only one of the strains did not produce granulose. The structure of granulose in Clostridium acetobutylicum P262 consisted of a high-molecular-weight polyglucan containing only (1→4) linked D-glucopyranose units. Biosynthesis of granulose in C. acetobutylicum P262 was dependent on ADPglucose pyrophosphorylase, and granulose synthase and mutants defective in granulose accumulation lacked either one or both enzyme activities. Granulose-positive revertants exhibited both enzyme activities. ADPglucose pyrophosphorylase and granulose synthase were not subject to allosteric control by metabolites. Granulose accumulation and the biosynthetic enzyme activities were initiated immediately before the pH breakpoint and were detected in cells only at the end of the exponential growth phase. Granulose accumulation did not occur under conditions of nitrogen limitation, excess carbon, or excess energy.

The accumulation of glycogen-like reserve polymers is widespread among bacteria and usually occurs under conditions in which growth is limited in the presence of an excess carbon and energy source (9, 28). Glycogen biosynthesis occurs via the ADPglucose pathway in bacteria (28). The key enzymes involved in glycogen synthesis are ADPglucose pyrophosphorylase (EC 2.7.7.27) and glycogen (granulose) synthase (EC 2.4.1.21) which catalyze the following reactions:

\[ \text{ATP} + \text{glucose 1-phosphate} \rightarrow \text{ADPglucose} + \text{PP}_i \]

\[ \text{ADPglucose} + 1.4 \text{glucan} \rightarrow 1.4 \text{glucosyl glucan} + \text{ADP} \]

Among the clostridia, the accumulation of intracellular granules of reserve material is widespread. In most Clostridium species which have been investigated, the reserve material has been shown to be a polyglucan which has been given the generic name granulose (10). The chemical structure of granulose has been determined only in a few species, including Clostridium butyricum (4, 18) and Clostridium botulinum type E (34), in which granulose consisted of a branched glycogenlike polymer composed of linear chains of \((1\rightarrow4)\)-linked D-glucopyranose units showing occasional branching \((1\rightarrow6)\) at linked units. Brown et al. (6) reported the presence of two types of reserve glucans in Clostridium pasteurianum, one an amylopectin showing \((1\rightarrow4)\) linkage and the other a dextran showing \((1\rightarrow6)\) linkage, both of which exhibited only limited branching. However, Darvil et al. (8) reported that an amylopectinlike polysaccharide was the only reserve glucan which occurred in six wild-type strains of C. pasteurianum. Methylation analysis demonstrated that \((1\rightarrow6)\)-linked D-glucose units accounted for less than 20% of the entire glucose content of these organisms.

Granulose accumulation normally occurs at the end of exponential growth before the onset of sporulation and is usually degraded during spore formation. This suggests that the reserve polyglucan can serve as a source of energy and carbon for the formation of spores and their maturation (4, 31, 33).

A relationship between the accumulation of granulose, solvent production, and sporulation in an industrial strain of Clostridium acetobutylicum P262, was reported by Jones et al. (20). In this strain, the accumulation of granulose occurred concomitantly with the shift from an acid-producing metabolism to a solvent-producing metabolism at the end of the exponential growth phase in batch culture. The accumulation of granulose was also linked to the production of a swollenphase-bright clostridial stage, capsule production, and the initiation of a forespore septum. Mutants of the P262 strain were isolated which were unable to accumulate granulose, form a clostridial stage, produce capsules, undergo the shift to the solventogenic phase, or sporulate (20, 22).

The isolation of pleiotrophic cls mutants furnished evidence that the various stationary-phase physiological and morphological events may share common regulatory components. Further evidence for common regulatory components for these stationary-phase events was provided by nutrient studies. Under conditions of nitrogen limitation, the cells failed to undergo the shift to solvent production and did not produce granulose, a clostridial stage, capsules, or endospores (22).

A number of features indicate that the granulose biosynthetic pathway could be a useful system for the study of the regulation of stationary-phase events in C. acetobutylicum. Granulose is easy to assay, mutants are readily isolated, and there seem to be common regulatory components linking granulose and solvent production. We have investigated the nature, biosynthesis, and regulation of granulose in wild-type and mutant strains.

**MATERIAL AND METHODS**

**Bacterial strains, media, and growth conditions.** The C. acetobutylicum, Clostridium beijerinckii, (Clostridium butylicum), and Clostridium saccharoperbutylicum strains used are listed in Table 1. The C. acetobutylicum P262 wild-type strain has been described previously (20–23, 30).

Granulose-negative mutants of C. acetobutylicum P262 were isolated by the method of Robson et al. (31) after
treatment of cells with ethyl methanesulfonate (2.5% [vol/vol]) as described previously (20, 21). The strains were maintained as spore suspensions in sterile distilled water at 4°C (22). The bacteria were propagated anaerobically at 37°C in buffered clostridial basal medium (CBM) (26). The production of granulose and endospores in the different strains of Clostridium was determined after growth in reinforced clostridial medium (RCM) (Biolab), CBM, tryptone yeast glucose medium (TYGM) (19), and the sporulation minimal medium (CAMM) of Long et al. (21). Granulose production and endospore formation were also determined after growth on CBM agar and CBM agar in which glucose was replaced by sucrose (4% [wt/vol]).

The production of granulose, solvents, and endospores by C. acetobutylicum P262 was determined in CAMM in which glucose was replaced by fructose (60 g/liter), galactose (60 g/liter), sucrose (60 g/liter), maltose (60 g/liter), raffinose (30 g/liter), L-arabinose (30 g/liter), or D-xylene (30 g/liter). The effect of different ammonium sources on the production of granulose was determined by linking ammonia phosphate (DAP) replacement with ammonium acetate, ammonium sulfate, and ammonium nitrate at equivalent ammonium concentrations.

### Analytical and assay methods

Total bacterial counts, clostridial stage counts, and spore counts were determined with a Thoma counting chamber (Weber Scientific International, London, England) and a Zeiss photomicroscope fitted with phase- and interference-contrast optics. The presence of granulose was determined by staining with iodine.

Granulose was assayed by the method of Robson et al. (31). Granulose was determined as glucose released after acid hydrolysis by using a Beckman Glucose Analyzer 2 (Beckman Instruments, Inc., Fullerton, Calif.). The granulose was expressed as micrograms of glucose deposited per 10^10 cells.

Glucose consumption during the fermentation was determined by monitoring glucose oxidation of residual glucose in culture filtrates. Acetate, butyrate, acetone, butanol, and ethanol were measured by gas chromatography as described previously (22). Protein was measured by the method of Lowry et al. (24).

### Extraction of granulose

Cells grown in CBM were harvested during the early stationary growth phase, and the pellets were washed and suspended in 25 mM Tris hydrochloride buffer (pH 8.0; 0.5 g [wet weight] per ml). The cells were disrupted by passage through a Yeda Press (Yeda Scientific Instruments, Rehovot, Israel). A preparation of granulose granules was obtained by the centrifuge. The polysaccharide was extracted by the KOH and ethanol procedure of Darvil et al. (8).

### Gel filtration

Lysophosphatidyl cell samples (2 mg) in 1 ml of 1 M NaCl solution were sonicated (10 min), applied to a column (60 by 0.9 cm) of Sepharose 4B, and eluted with 1 M NaCl (14.2 ml/h). The column was calibrated by using dextran standards. Fractions (1.42 ml) were collected and analyzed for carbohydrate by using the phenol-sulfuric acid colorimetric method (7).

### Acid hydrolysis

Polysaccharide samples were hydrolyzed with 2 M trifluoroacetic acid at 100°C for 18 h. After hydrolysis, the acid was removed by codistillation with methanol. Derived alditols were acetylated with sodium acetate and acetic anhydride for 2 h at 100°C.

### Methylation analysis

A lyophilized sample (10 mg) was methylated by a modification (15) of the standard procedure described by Hakomori (14). Two such treatments were followed by a treatment described by Purdie and Irvine (29) to yield a permethylated polysaccharide showing no free hydroxyls in the infrared spectrum. The permethylated polysaccharide (4 mg) was hydrolyzed (2 M trifluoroacetic acid, 100°C for 16 h), and the derived partially methylated alditols were permethylated as described above.

### Thin-layer chromatography

Thin-layer chromatography was done on Kiesel gel 60 F254 (Merck) by using the solvent system CHCl3-MeOH-H2O (10:10:3.5) for sugar analysis of the polysaccharide hydrolysate and by using the solvent system 3% NH3 in n-butanol for the partially methylated hydrolysate.

### Gas-liquid chromatography

Analytical gas-liquid chromatography separations were performed with a Carlo Erba Strumentazione 4200 instrument (S.E.A.L. Instrumentation) fitted with dual flame-ionization detectors. A Superprator-3A electronic integrator (Columbia Scientific Instruments) was used to measure the peak areas. A 3% OV-225 bonded silica-capillary column (J & W Scientific, Inc.) was used to separate the acetylated alditols of methylated sugars.

### Gas-liquid chromatography mass spectrometry

The gas chromatograph was interfaced with a V.G. Micromass 16F mass spectrometer (V.G. Analytical). The mass spectra were recorded at 70 eV, and the position of methoxyl substitution was determined by using the data of Bjornal et al. (5).

### Proton magnetic resonance spectra

Samples (20 mg) were prepared by dissolving in D2O after lyophilizing four times from D2O solutions. The spectrum was recorded on a Bruker WH-90 instrument (Bruker) (90 MHz). A total of 983 scans were accumulated with the probe at 80°C.

### Iodine reaction

The adsorption spectrum of the iodine polysaccharide complex was measured as described by Archibald et al. (2).

### Preparations of cell extracts

Cells were harvested and washed twice at 4°C in Tris hydrochloride buffer (25 mM, pH 8.0) containing 1 mM dithiothreitol. Suspension was carried out under an atmosphere of hydrogen. The bacteria (0.5 g [wet weight] per ml of buffer) were disrupted under nitrogen with a Yeda Press, and the cell lysate was centrifuged at 10,000 x g for 15 min at 4°C. The supernatant was used as the source of ADPglucose pyrophosphorylase. The pellet was suspended in Triton-dithiothreitol buffer and used as the source of granulose synthase. Both crude extracts could be stored at −70°C without loss in enzyme activity.

### Enzyme assays

ADPglucose pyrophosphorylase was as-
served by determining the rate of synthesis of ADP-[14C]glucose from [14C]glucose 1-phosphate and ATP as described by Robson et al. (31). The assay mixture (0.2 ml) contained 0.15 μmol of [14C]glucose 1-phosphate (3 μCi/μmol), 2 μmol of ATP, 4 μmol of MgCl2, 20 μmol of Tris hydrochloride (pH 8.0), 1 U of inorganic pyrophosphatase, and crude enzyme. ADP-glucose pyrophosphorylase activity was expressed as nanomoles of ADP-glucose produced per minute per milligram of protein. Granulose synthase activity was assayed by measuring the rate of incorporation of ADP-[14C]glucose into α-1,4-glucan as described by Robson et al. (31). The assay mixture (0.2 ml) contained 0.2 nmol of ADP-[14C]glucose (0.1 μCi/μmol), 0.05 μg of amylpectin, 5 μmol of Tris hydrochloride (pH 8.0), 1 μmol of MgCl2, and crude enzyme. Granulose synthase activity was expressed as nanomoles of glucose incorporated per minute per milligram of protein.

The effects of the following substances which act as allosteric effectors of some bacterial ADP-glucose pyrophosphorylase and granulose synthase enzymes (28) were determined at a final concentration of 3 mM: acetyl-coenzyme A, ADP, AMP, fructose 1-phosphate, fructose 6-phosphate, fructose 1,6 diphosphate, glucose 6-phosphate, PPy, phosphoenolpyruvate, 3-phosphoglycerate, 6-phosphogluconate, pyridoxal 5'-phosphate, pyruvate, ribose 5-phosphate, NAD+, NADH, NADP+, and NADPH.

RESULTS

Granulose in C. acetobutylicum and related strains. The presence of granulose in the 15 solvent-producing Clostridium strains (Table 1) was determined by direct microscopic examination of iodine-stained cells. In five of the strains (P262, ATCC 10132, ATCC 27021, NCIB 6445, and NCIB 8653), granulose accumulation and phase-bright clostridial forms occurred readily in cells grown in complex liquid media and on CBM agar plates containing either glucose or sucrose. In RCM, TYGM, or CBM, at least 60% of the cells of these strains exhibited substantial accumulation of granulose at the end of the exponential growth phase, and all of these strains sporulated. In TYGM, P262, ATCC 10132, NCIB 6445, and NCIB 8653 produced 5.8 to 8.4 g of solvent per liter, whereas ATCC 2701 produced 2.4 g of solvent per liter. Four of these strains grew well and produced granulose in CAMM, but ATCC 10132 grew very poorly in this minimal medium.

Granulose was not observed in iodine-stained cells of nine Clostridium strains (ATCC 824, ATCC 3625, ATCC 4259, ATCC 8529, NCIB 2951, NCIB 6441, NCIB 6442, NCIB 6443, and NCIB 6444) after growth in any of the liquid media. After growth for 2 to 4 days on CBM agar containing 4% sucrose, seven of these strains accumulated small amounts of granulose, but the ATCC 824 strain produced high levels of granulose. The NCIB 6444 strain did not produce detectable granulose on the agar medium. C. beijerinckii NRRL B593 produced low levels of granulose on CBM 4% sucrose agar and in RC and TYGM liquid media. Solvent production by these 10 strains in TYGM varied between 2.4 and 7.4 g of solvent per liter.

The 10 strains which produced little or no granulose in liquid media showed lower levels of sporulation in liquid media than did the 5 granulose-producing strains. Endospore production by the 10 strains on CBM agar was very variable and ranged from >1% (ATCC 3625) to 70% (ATCC 824).

Characterization of granulose from C. acetobutylicum. Samples of intracellular polysaccharide granules were prepared from stationary-phase granulose-rich cells of C. acetobutylicum P262. The crude polysaccharide was eluted in the void volume of the Sepharose 4B column, indicating that it consisted of components with a molecular mass greater than 6 × 106 daltons (5).

The sample was hydrolyzed (18 h, 100°C, 2 M trifluoroacetic acid) into the constituent sugars. Thin-layer chromatography indicated that glucose was the only sugar present after hydrolysis. This was confirmed by gas-liquid chromatography analysis of the derived alditol acetates.

The nature of the linkage between the glucose residues was investigated by methylation analysis. Full methylation was achieved by using two treatments (described by Hakomori [14]) followed by a subsequent (Ag2O/CH3I) treatment (described by Purdie and Irvine [29]) for 2 days. Methylation was assumed to be complete when the hydroxyl peak was no longer present in the infrared spectrum. The hydrolysate of the permethylated polysaccharide yielded a single spot after thin-layer chromatography and was shown to be 2,3,6-tri-O-methyl-glucose as gas-liquid chromatography and confirmed by gas-liquid chromatography mass spectrometry. No evidence for branch points was found, indicating that the polymer consisted of α (1→4)-linked glucose residues. H-nuclear magnetic resonance of the polysaccharide showed a single peak in the anomeric region at 5.23 ppm with a coupling constant of J1,2 = 3 Hz. These results are typical of glucopyranosyl residues that are α linked. The purified polysaccharide from C. acetobutylicum P262 produced a complex with iodine, with a maximum absorption between 540 and 545 nm.

Biogenesis of granulose in C. acetobutylicum P262. The accumulation of granulose and the activities of ADP-glucose pyrophosphorylase and granulose synthase in crude cell extracts were determined in C. acetobutylicum P262 grown in CAMM. Long et al. (21, 22) showed that in CAMM, the time sequence of the morphological changes associated with the onset of solvent production and sporulation in C. acetobutylicum P262 was sufficiently distinct and constant to facilitate correlative physiological and biochemical studies. In this medium, granulose accumulation was first detected after approximately 25 h, when the cells reached the end of the exponential growth phase (Fig. 1). Initiation of granulose accumulation in a small proportion of the cells occurred a few hours before the pH breakpoint, and at the breakpoint, granulose accumulation was visible in the majority of cells. The pH breakpoint also coincided with the shift to solvent production and an increase in glucose consumption. Maximum granulose deposition (250 to 300 μg of glucose deposited per 106 cells) occurred after approximately 45 h, when over 80% of the available glucose had been consumed and forespore septation had been initiated. At this stage, granulose accumulation within the cells accounted for between 40 and 55% of the dry weight of the cells (determined in four separate experiments).

ADP-glucose pyrophosphorylase and granulose synthase were not detected before 25 h, and the activity profiles of both enzymes coincided with the granulose profile (Fig. 1). The enzyme activities decreased during spore development and maturation when granulose was mobilized within the spore mother cells. Approximately 70 to 80% of the granulose was mobilized during spore development, and no cell-free granulose was detected.

Effect of nutrients on granulose accumulation in C. acetobutylicum P262. The effect of the type and concentration of carbohydrate and ammonia sources on the production of granulose in C. acetobutylicum P262 grown in CAMM was
granulose, fructose, galactose, as residual ADPglucose pyrophosphorylase activity; of investigated. Cultures grown in CAMM containing glucose, fructose, galactose, sucrose, or maltose (60 g/liter) all exhibited a shift to solventogenesis and granulose accumulation during the latter part of the fermentation, and 70 to 95% of the cells formed the clostridial stage. Granulose accumulation was initiated just before the pH breakpoint and reached a concentration of between 190 and 260 μg of granulose per 10^8 cells approximately 15 h after the breakpoint. These cultures all produced between 11 and 15 g of solvent per liter.

The growth of C. acetobutylicum P262 was inhibited by 60 g of raffinose per liter and the pentose sugars L-arabinose and D-xylene. Cultures of CAMM containing 30 g of these sugars per liter produced solvents (5.2 to 6.5 g/liter) and granulose (60 to 80 μg of glucose per 10^8 cells). Similar levels of solvents and granulose were produced by cells grown in 30 g of glucose per liter.

The nature of the nitrogen source (DAP, ammonium acetate, ammonium nitrate, ammonium sulfate), added at equivalent concentrations of ammonia, did not affect the production of solvents (11 to 15 g/liter) or granulose (200 to 300 μg of glucose per 10^8 cells).

Glucose (Fig. 2) and DAP (Fig. 3) concentrations affected solvent and granulose production. Neither solvents nor granulose were detected in C. acetobutylicum P262 cultures containing <10 g of glucose per liter or <0.5 g of DAP per liter. An increase in glucose or DAP concentrations resulted in a proportional increase in granulose and solvent production, and maximum amounts of granulose and solvents occurred at 60 g of glucose per liter and 4 g of DAP per liter.

Characteristics of ADPglucose pyrophosphorylase. ADPglucose pyrophosphorylase was not detected in crude cell extracts of C. acetobutylicum P262 disrupted under anaerobic conditions. When cell disruption was performed under an atmosphere of hydrogen, the crude enzyme extract was not affected by subsequent exposure to aerobic conditions and was stable at ~70°C. Crude extracts of ADPglucose pyrophosphorylase utilized glucose 1-phosphate in the presence of ATP. Plots of enzyme activity against substrate concentration were hyperbolic for both glucose 1-phosphate and ATP, and the K_m values were 0.33 and 3.23 mM, respectively. The optimum pH for ADPglucose pyrophosphorylase was 8.0, and the enzyme was inactivated at pH values below 7.0 and above 11.0. The enzyme showed a marked nucleotide specificity for ATP. Replacement of ATP with equimolar concentrations (10 mM) of UTP, GTP, or CTP resulted in a 68, 94, and 96% decrease,
respectively, in ADPglucose pyrophosphorylase activity. The enzyme was not activated or inhibited by the addition of AMP or ADP at final concentrations of 3 to 50 mM. In addition, 13 other compounds, including those metabolites reported to act as allosteric effectors of the ADPglucose pyrophosphorylase in other bacteria which accumulate polyglucans, were tested as possible effectors of this enzyme (28). None of the following compounds (3 mM) enhanced or inhibited the activity of C. acetobutylicum P262 ADPglucose pyrophosphorylase: acetyl-coenzyme A, fructose 1-phosphate, fructose 6-phosphate, fructose 1,6-diphosphate, glucose 6-phosphate, PPi, phosphoenolpyruvate, 3-phosphoglycerate, 6-phosphogluconate, pyridoxal 5'-phosphate, pyruvate, ribose 5-phosphate, NAD+, NADP+, NADH, and NADPH.

Characteristics of granulose synthase. The preparation of crude enzyme extracts of granulose synthase from C. acetobutylicum P262 was not affected by exposure to aerobic conditions, and 98% of the enzyme activity was associated with the pellet obtained during extraction. There was a linear relationship between granulose synthase activity and ADPglucose at substrate concentrations between 0 and 0.5 mM, and the $K_m$ for ADPglucose was 0.4 mM. Granulose synthase activity was not detected when UDPglucose (1 mM) was substituted as a substrate. Granulose synthase activity was optimal at pH 5.5 and 9.4, was completely inhibited at pH 4.0, and was 80% inhibited at pH 11.0. Glucose 1-phosphate and ATP did not affect the enzyme, but the enzyme was sensitive to ADP and AMP (30 mM), which caused a 50 and 20% decrease, respectively, in enzyme activity. None of the other compounds (3 mM) tested as possible effectors of ADPglucose pyrophosphorylase enhanced or inhibited the activity of granulose synthase.

Characterization of granulose-negative mutants. The isolation of 2 types of C. acetobutylicum P262 mutants which were defective in granulose accumulation and did not form swollen phase-bright clostridial stages was reported by Long et al. (23). One type of granulose-negative mutant produced normal levels of solvents and continued to produce mature endospores, although in most cases at reduced levels. The other type of mutant (cls) did not undergo a shift to the solventogenic phase, and these mutants failed to produce solvents, extracellular capsules, and endospores. Mutants of both phenotypes were assayed for granulose and granulose biosynthetic enzyme activities in crude cell extracts obtained at the end of the exponential growth phase. Three granulose-negative mutants which produced high levels of solvents (14 to 16 g/liter) and sporulated in CAMM were assayed for enzyme activity. Two of these mutants showed no granulose synthase activity but produced normal levels of ADPglucose pyrophosphorylase. The other mutant lacked both enzyme activities. Ten cls mutants were investigated, and they all failed to produce solvents or endospores and lacked both enzyme activities. Spontaneously occurring granulose-positive revertants were isolated from two of the cls mutants. These produced solvents and endospores and contained active granulose biosynthetic enzymes.

**DISCUSSION**

The structure of granulose in C. acetobutylicum P262 was similar to that in C. pasteurianum (6, 8) and consisted of a high-molecular-weight polyglucan containing only α(1→4)-linked β-glucopyranose units. This structure differed from the glycogen-like polymers with α(1→6)-linked branched chains which occur in C. butyricum and C. botulinum (4, 18, 34).

Biosynthesis of granulose in C. acetobutylicum P262 was dependent on ADPglucose pyrophosphorylase and granulose synthase, and mutants defective in granulose accumulation were defective in either one or both enzyme activities. Granulose-positive revertants exhibited both enzyme activities.

In C. acetobutylicum P262, granulose accumulation and the biosynthetic enzyme activities were detected in cells only at the end of the exponential growth phase. These events were associated with the cessation of cell division and the onset of solvent production and spore formation. The absence of granulose enzyme activities in vegetative cells and the onset of enzyme activities associated with the shift to the solventogenic phase suggest that these enzymes are induced or derepressed in stationary phase. The derepression of synthesis of ADPglucose pyrophosphorylase at the end of exponential growth was also reported to occur in C. pasteurianum (27). The absence of activity of both enzymes in all of the cls mutants and the reappearance of activity in the revertants suggest that the enzymes responsible for granulose biosynthesis may be associated in an operon as has been demonstrated to occur in Salmonella typhimurium (32). The synthesis of enzymes involved in the final reactions of solvent production has also been shown to be initiated just before the shift to the solventogenic phase in C. acetobutylicum (1, 16, 17). The isolation of cls mutants defective in both pathways and the resumption of granulose and solvent production in revertants of these mutants suggests that both pathways may share some common regulatory elements.

The mechanism of enzyme regulation of both these pathways is not known, but the shift to solvent and granulose production has been shown to be associated with the accumulation of threshold concentrations of acid end products which are produced during the initial phase of the fermentation (3, 11–13, 22, 25, 35). Limitation of nutrients such as glucose or ammonia does not appear to be directly involved in the initiation of the shift to the solventogenic phase and granulose accumulation (22). In other bacteria which accumulate glycogen, the accumulation of this storage product is favored under conditions of nitrogen limitation and excess carbon and energy (9, 28). However, in C. acetobutylicum P262 cells grown in ammonium-limited media in the presence of excess glucose, granulose accumulation did not occur and solvent production and endospore formation were inhibited.

The C. acetobutylicum P262 ADPglucose pyrophosphorylase was not affected by metabolites or allosteric effectors which control the enzyme activity in other groups of bacteria. The absence of fine control of ADPglucose pyrophosphorylase activity has also been shown in C. pasteurianum (31), but in contrast to C. acetobutylicum P262, the C. pasteurianum enzyme was derepressed by ADP. The granulose synthase of C. acetobutylicum was similar to analogous bacterial enzymes in that its activity was not subjected to allosteric control by metabolites (28). In C. acetobutylicum P262 and C. pasteurianum (31), granulose synthase was inhibited by ADP and AMP.

In C. acetobutylicum P262, the concentration of granulose within the cells was observed to decrease during spore maturation, and granulose mobilization was preceded by a decline in both ADPglucose pyrophosphorylase and granulose synthase activities. This supports the suggestion (4, 31, 33) that granulose may be utilized as a source of carbon and energy during sporulation in Clostridium species.
isolation of granulose mutants which produced solvents and mature spores at a lower frequency indicated that granulose is not essential for sporulation, but it may result in more effective sporulation under appropriate conditions.

The ability to synthesize granulose appears to be widespread among solvent-producing Clostridium strains, and only 1 of the 15 strains investigated did not produce visible granulose under the conditions tested. However, only five of the strains tested showed a significant accumulation of granulose in all of the growth media tested. The remainder accumulated a limited amount of granulose in a small proportion of the cells after prolonged incubation in one of the culture media.

LITERATURE CITED