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Formation of an extracellular energy reserve by *Cellulomonas flavigena* strain KU

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SUMMARY

Conditions of growth are described which lead to the formation of a dense capsule about *Cellulomonas flavigena* and provide data which suggest that, although accumulated as an extracellular structure, it may function as an energy reserve. The capsule is formed when the bacteria are cultured in a minimal medium containing an excess of one of several carbohydrates. The bacterial cells which are encapsulated are also densely aggregated. The capsule is not formed and the cells are not aggregated when the bacteria are cultured in complex growth media. The transfer of aggregated cells to a medium devoid of carbon and energy source results in disappearance of the capsule and disaggregation of the cells.

INTRODUCTION

Many bacteria are able to accumulate endogenous energy reserves when they are cultured in media containing an excess of a carbon and energy source and often the process is enhanced if the growth medium has growth limiting amounts of nitrogen, phosphate, or some other required metabolite (5,6,12,16,19,20]. Glycogen, starch, volutin, and/or polyhydroxybutyrate may accumulate as energy reserves in a variety or bacteria [18,21]. The growth of *Cellulomonas flavigena* strain KU under conditions which are conducive to the formation of energy reserves leads to the formation of a capsule and aggregation of the cells. The relationship of such aggregation and capsule formation to energy storage is examined herein.

MATERIALS AND METHODS

Bacteria

The bacterium used in these experiments was originally isolated from leaf litter. Its characteristics closely resemble those of *Cellulomonas flavigena*

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[10,15]. We have designated it as *C. flavigena* strain KU [1].

Culture media

The minimal salts medium, designated as CM9, contained (g/l): KH_2PO_4, 3.18; Na_2HPO_4, 5.20; $MgSO_4$, 0.12; NH_4Cl, 0.20; yeast extract (Difco), 0.50. Five milliliters of heavy metal solution which contained, mg per liter of H_2O: disodium EDTA, 500; $FeSO_4 \cdot 7H_2O$, 200; $ZnSO_4 \cdot 7H_2O$, 10; $MnCl_2 \cdot 4H_2O$, 3; H_3BO_3 30; $CoCl_2 \cdot H_2O$, 20; $CuCl_2 \cdot 2H_2O$, 1; $NiCl_2 \cdot 6H_2O$, 2; and $Na_2MoO_4 \cdot 2H_2O$, 3 [17], was added to the minimal medium and the pH was adjusted to 6.8. The heavy metal solution was added to insure the availability of metals which commonly are used as cofactors. Solutions of the carbohydrates that were used as energy source were sterilized separately by autoclaving them.

Non-encapsulated cells were produced by growing the bacteria in complex, rich media, such as tryptone broth or nutrient broth.

All cultures were incubated at 30°C, with aeration by shaking at 250 rpm, in baffled Erlenmeyer flasks. The volumes of cultures were not in excess of 0.25 of the rated capacity of the flasks used.

Measurement of cell mass

The dry weight of washed bacteria was determined by incubating them at 60°C in tared vials until a constant weight was reached.

Hydrolysis of bacteria

Dried cells were resuspended, at a concentration of 1 mg/ml of 2 N HCl, in capped tubes, and held for 8 h in a block heater at 100°C.

Estimation of reducing sugar content

The total content of reducing sugars of washed, dry cells was determined by the phenol-sulfuric acid assay, using glucose as a standard [9]. The amount of glucose in acid hydrolyzates of such cells was determined by the glucose oxidase assay (Sigma Diagnostics Procedure No. 510).

Electron microscopy

Bacteria were prepared for transmission electron microscopy according to the procedure of Ryter and Kellenberger [7,14]. The bacteria were prefixed by 30 min of incubation, with shaking, at room temperature, in a 0.2% (w/v) solution of osmium tetroxide (OsO_4) in the Ryter-Kellenberger veronal-acetate buffer. They were then washed with cold Ryter-Kellenberger buffer and fixed overnight, at room temperature, with 1% OsO_4. After washing the fixed cells with buffer they were stained for 2 h, at room temperature, in 0.5% (w/v) solution of uranyl acetate in Ryter-Kellenberger buffer. Then, after washing the cell pellet with distilled H_2O, it was enrobed in 2% agar. After dehydration through a graded acetone series the samples were infiltrated with Araldite 502 embedding resin (Polysciences, Inc. Warrington, PA) [11] and placed in beem capsules filled with fresh resin. After polymerization of the resin the samples were sectioned by a Sorval MT 5000 ultramicrotome, using a diamond knife. The sections were picked up on uncoated specimen screens and then stained with lead citrate [13] and uranyl acetate. Micrographs were obtained with a Philips 300 microscope operating at 60 kV.

RESULTS

Growth yield and reducing sugar content

The growth of *C. flavigena* strain KU in tryptone broth was compared with growth in minimal salts media, each containing one of a number of different carbon and energy sources. In these experiments the biomass yield and the reducing sugar content of cells in the stationary phase of growth were compared. The yields of biomass, expressed as mg of dry cells/ml, in CM9-sugar or CM9-glycerol cultures ranged from 12.8 to 10.6 times that of tryptone broth cultures (Table 1). Much of the difference in biomass apparently is accounted for by the very high reducing sugar content of cells grown in synthetic media. In these experiments the total reducing sugar content was indicated by a phenol-sulfuric assay of hydrolyzates and the glucose oxidase assay was used to measure the amount of glucose released upon acid hydrolysis of the biomass.

Except for the tryptone broth culture, cells in all

Table 1

Carbon and energy sources tested for ability to support polysaccharide synthesis

Growth substrate	Yield (mg dry cells/ml culture)	Reducing sugar content (% of dry weight) measured by	
		Phenol sulfuric method	Glucose oxidase method
1% Glucose	6.81	94.1	87.2
1% Xylose	6.44	93.9	82.3
1% Glycerol	6.34	93.5	85.1
1% Maltrin 200*	6.12	90.0	85.2
1% Tryptone	0.53	12.6	–

Growth media: minimal salts containing indicated carbon and energy source and 0.005 M NH_4Cl.
* Maltrin 200 is classified as a corn syrup solid or malto-dextrin. It is a product from Grain Processing Corporation, Muscatine, Iowa.
Cultures were incubated at 30°C for 72 h, with shaking at 250 rpm.

of the cultures depicted in Table 1 were aggregated. Aggregation began within 22 h and was complete in the stationary phase.

Figs. 1 and 2 are comparisons, by phase contrast and electron microscopy, respectively, of aggregated and non-aggregated cells. Virtually all of the cells in tryptone cultures existed as free floating cells. Electron micrographs of cross sections of aggregat-

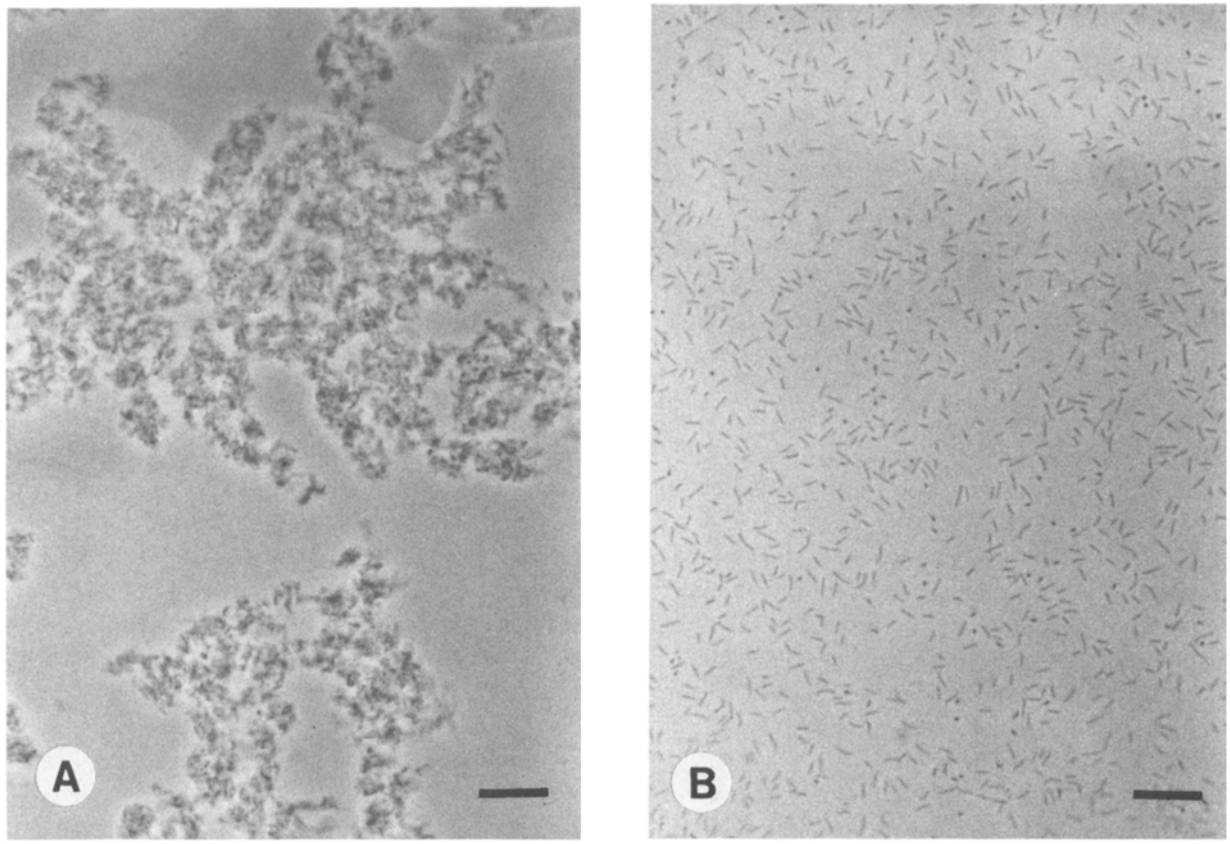

Fig. 1. Phase contrast micrographs of the isolate: A, aggregated cells grown in CM9 medium with excess carbon source and growth limiting nitrogen (bar = 25 μm); B, non-aggregated cells grown in tryptone broth (bar = 12.5 μm).

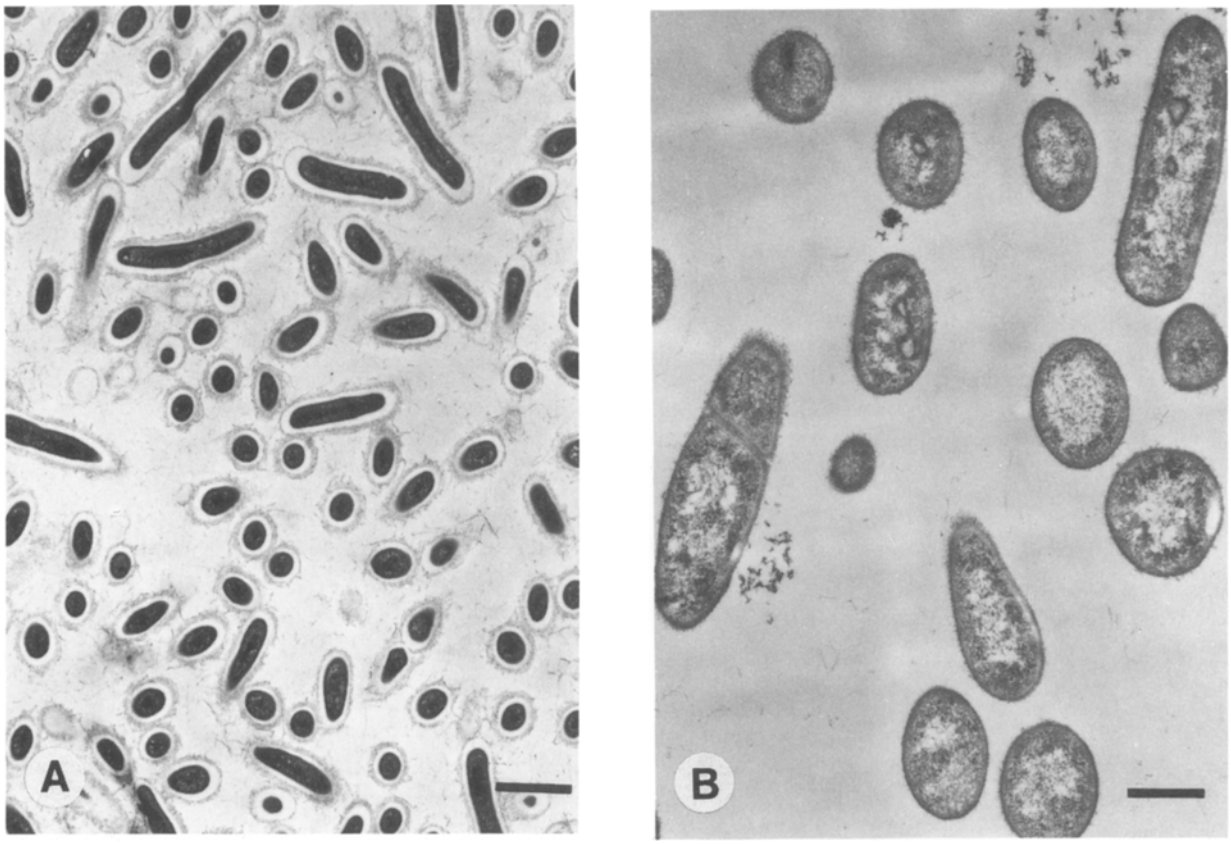

Fig. 2. Electron micrographs of thin sections of the isolate: A, aggregated cells, grown in CM9 medium (bar = 1 μm); B, non-aggregated cells grown in tryptone broth (bar = 0.3 μm).

ed cells indicate the presence of a large extracellular capsule and non-aggregated cells were completely devoid of such a structure.

Decrease in endogenous reducing sugar content

Growth conditions leading to the accumulation of a high reducing sugar content, i.e. accumulation in the late stages of cultures in media containing an excess of carbohydrate and growth limiting amounts of nitrogen, suggested that it might be the manifestation of an energy reserve. To further test such an interpretation cells which had become aggregated and encapsulated were washed and transferred to a medium devoid of a carbon and energy source. During the initial 18 h of incubation in such a medium the reducing sugar content of the bacteria decreased by 450 μg per mg of dry cells (Table 2). No reducing sugar was detected in supernatant medium of the cultures during that time, indicating that loss was not due to simple hydrolysis. Furthermore, the decline in the reducing sugar content was accompanied by the loss of cell aggregation (Fig. 3) and disappearance of the capsule (Fig. 4). Examination by phase contrast microscopy (Fig. 3) indicated an increase in the number of free cells that correlated with time of incubation in substrate-free medium and corresponded to the decrease in reducing sugar content (Table 2). However, because of technical problems attendant to cell aggregation, neither measurement of light absorbance nor dilution and plating could be used to determine the actual number of cells in the culture at the time of their transfer to glucose-deficient medium. Furthermore, assays for changes in DNA and/or protein content also could not be performed reliably because aggregated cells are very difficult to fraction-

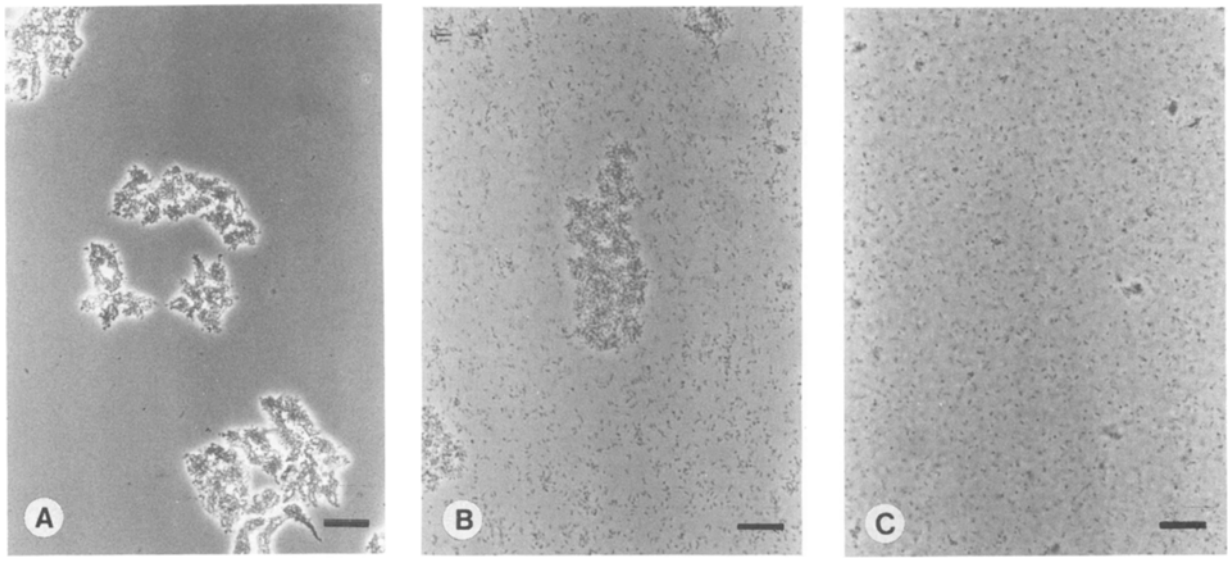

Fig. 3. Phase contrast micrographs of cells in a starvation experiment. Aggregated cells were washed and resuspended in fresh CM9 medium containing a nitrogen source in excess but lacking a carbon and energy source: A, immediately after resuspension (bar = 25 μm); B, after 12 h incubation (bar = 12.5 μm); C, after 24 h incubation (bar = 12.5 μm).

ate. For example, treatment as harsh as that of refluxing cells suspended in 2 N sulfuric acid did not disaggregate them. Attempts to lyse aggregated cells with lysozyme were not successful.

Although the initial number of aggregated cells transferred to glucose-deficient medium in the above experiment (Table 2) could not be determined it was demonstrated in a separate experiment that cells remain viable during such treatment. Washed, aggregated cells were resuspended at a concentration of 8.05 mg (wet weight)/ml of glucose-deficient CM9 medium. The culture was then incubated as in the experiment summarized in Table 2. After 18 h of incubation, when the cells no longer

Table 2

Starvation experiment using *C. flavigena* strain KU

Hours incubation in a medium without exogenous carbon	Reducing sugar content		Dry weight	
	μg/mg of dry cells	% of original	mg/ml culture	% of original
0	581.5	100.0	1.41	100.0
18	131.1	22.5	1.19	84.4
40	139.9	24.1	1.14	80.1
64	126.7	21.8	1.16	82.3

Cells were grown in CM9 medium containing 0.75% glucose and incubated at 30°C with shaking at 250 rpm until maximum aggregation was achieved. The aggregated cells were harvested by centrifugation (15 000 × *g*, 20 min) and washed with sterile saline. The cells were then transfered to fresh CM9 medium which contained an excess of nitrogen (0.018 M NH_4Cl) but lacked a carbon and energy source, and the cultures were incubated as described above. Reducing sugar content and dry weight determinations were performed as described in the Materials and Methods.

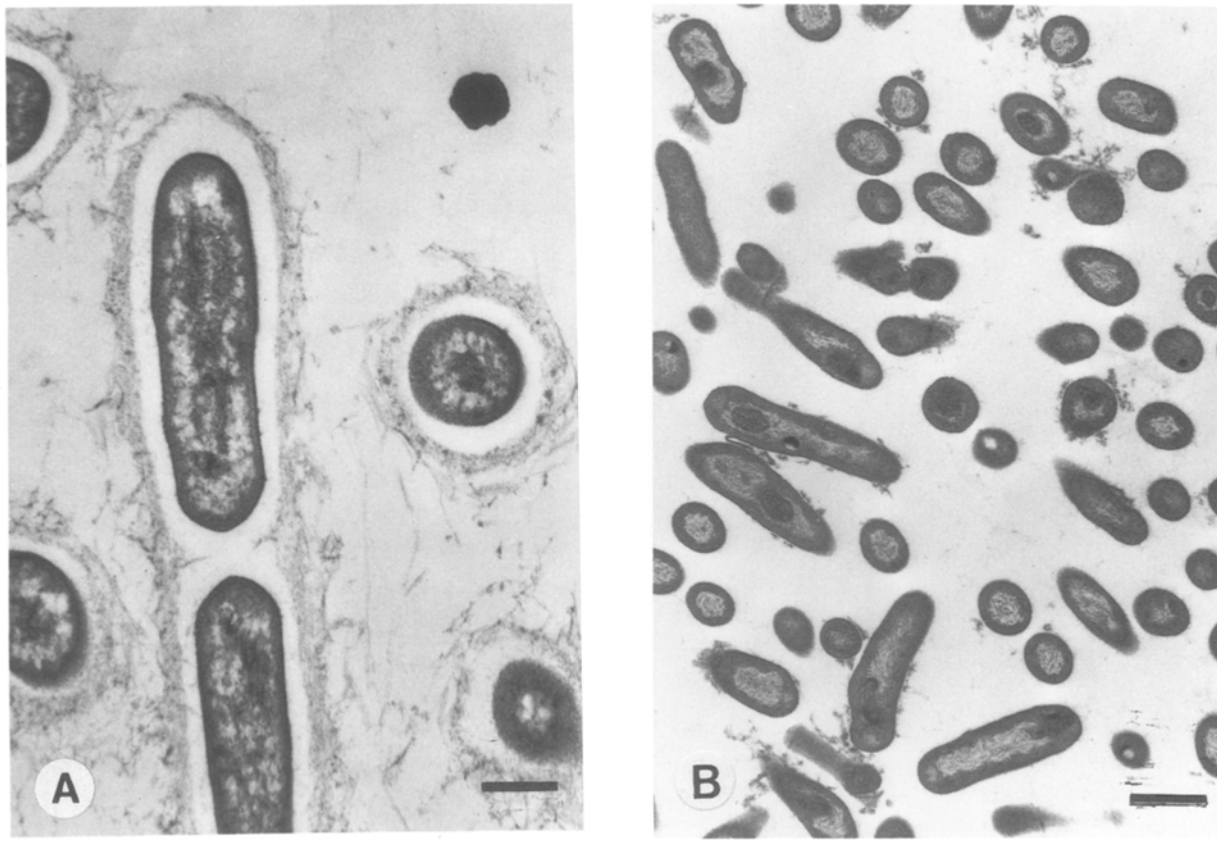

Fig. 4. Electron micrographs of the isolate used in a starvation experiment. Culture conditions same as in Fig. 3. A; aggregated cells immediately after resuspension (bar = 0.2 μm); B, after 24 h incubation (bar = 0.4 μm).

were aggregated, quadruplicate samples of the culture were removed for dilution and plating. Such samples contained an average of 9.05×10^9 viable cells/ml. After 40 h of incubation the number had increased to 1.09×10^{10} cells/ml, and at 60 h it was 1.07×10^{10}/ml.

Growth with a polyglucan extracted from aggregated cells as energy source

Although an increase in cell numbers at the expense of the consumption of the capsule could not be determined absolutely, a polyglucan extracted from aggregated cells was capable of supporting growth. Fig. 5 indicates that CM9 agar medium supports growth when it contains a water-insoluble polyglucan as the only carbon and energy source. This polyglucan can be extracted from *Cellulomonas flavigena* only if the cells have been grown under conditions which lead to aggregation and encapsulation of the cells. Zones of clearing which surround the colonies indicate that the growing organisms synthesize and excrete enzymes capable of hydrolyzing the polyglucan. The details of the production, extraction, and the chemical characterization of this product have been described [2].

DISCUSSION

A compound may be considered as an energy reserve if it meets the following conditions: (i) it is synthesized and accumulated intracellularly under conditions in which a carbon and energy source is in excess; (ii) it is utilized when an exogenous energy

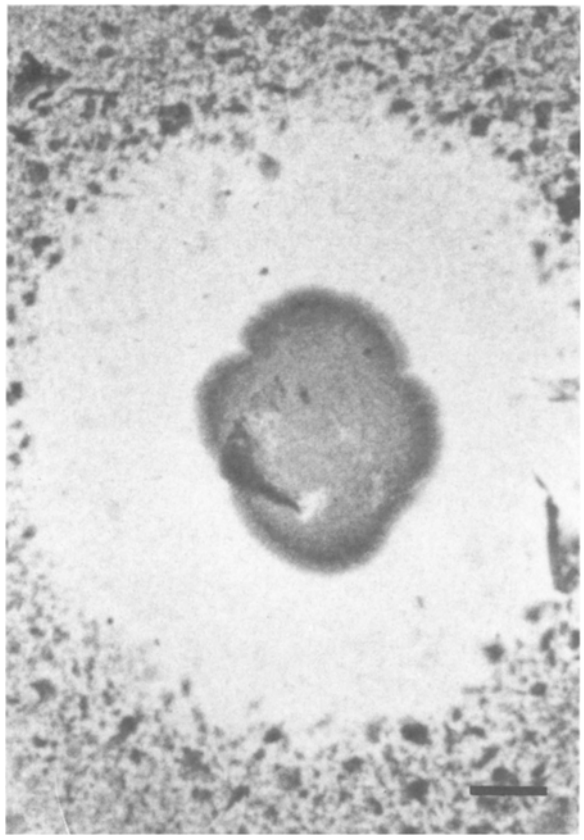

Fig. 5. Colony of the isolate growing and producing a zone of clearing on CM9 agar containing aniline blue-stained polyglucan as the sole carbon source (bar = 0.5 cm).

source no longer is available; and most importantly, (iii) the proposed storage compound is degraded yielding energy that is utilized by the cell, enabling it to fare better in that environment [11,18]. The experiments described here suggest that even though it accumulates extracellularly, the capsule produced by *C. flavigena* may also be utilized as an energy reserve.

The capsule is produced when *C. flavigena* strain KU is grown under conditions of nitrogen limitation and carbohydrate excess. Encapsulated cells are aggregated and have a high reducing sugar content. In contrast, when grown in a medium which does not contain a carbohydrate excess, *C. flavigena* strain KU does not become encapsulated and aggregated, and its reducing sugar content is relatively low.

The transfer of encapsulated cells to a medium containing nitrogen but lacking an exogenous carbon and energy source leads to the disappearance of their capsule (Fig. 4) and to their disaggregation (Fig. 3). From Table 2 it can be calculated that the reducing sugar content of the bacteria decreased by 450 μg/mg of dried cells (78%) during the initial 18 h of incubation in the glucose-deficient medium. That amount, if expressed as the reducing sugar lost from the cells in 1 ml of culture, corresponds to 664 μg. If that loss was a consequence of simple hydrolysis of a cellular polysaccharide and release of the sugars into the medium, or if that amount of reducing sugar was used for maintenance energy, there should be a commensurate decrease in biomass. An increase in the reducing sugar content of cell-free supernatant medium was not observed, and Table 2 shows that the actual decrease in the weight of cells in 1 ml of culture was only 220 μg. This implies that during the initial 18 h after transfer of the aggregated cells, a time during which the reducing sugar content of the biomass in 1 ml of culture decreased by 664 μg, the non-sugar component of that biomass actually increased by 444 μg/ml. The initial aggregation of the cells precluded a direct demonstration of an increase in the number of viable cells during that time. However, in a similar experiment it was demonstrated that there was an increase in the number of viable cells between 18 h, when cells no longer were aggregated, and 40 h, and that after 60 h of incubation most of the cells were still viable.

The temporal correlation between the decrease in the reducing sugar content of cells with their loss of aggregation and capsules as well as the change in biomass suggests that the capsules may at least in part function as an energy reserve. This is further supported by the demonstration (Fig. 5) that a water insoluble polyglucan which can be extracted from aggregated and encapsulated cells can serve as an energy source for growth and that soluble enzymes capable of hydrolysing it are produced. Such a polyglucan cannot be extracted from non-aggregated, nonencapsulated cells [2].

Energy reserves most commonly are accumulated intracellularly [12], presumably because they are there protected from predation by other organisms.

A further advantage offered by intracellular location is that energy need not be provided for export of the energy reserve polymer during its accumulation. The disadvantage of having an extracellular energy reserve that is exposed to the environment, however, may be outweighed if the reserve polymer is recalcitrant to hydrolysis by other organisms and/or if it provides other useful functions. However, even though bacterial capsules have been studied for many years relatively little is known about their function [8]. Capsules often are synthesized by bacteria growing in their natural habitats and they represent one form of the bacterial glycocalyx, a polysaccharide and/or glycoprotein component of the bacterial cell surface [3,4]. In contrast to the glycocalyx, which frequently is lost upon culture in vitro [3,4], the trait for the synthesis of capsule has not been lost from *C. flavigena* strain KU even after many generations of growth in media unfavorable for its synthesis, or after prolonged growth in chemostat cultures.

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