Encystment of *Azotobacter vinelandii* in liquid culture

L. H. STEVENSON AND M. D. SOCOLOFSKY

Department of Biology, University of South Carolina, Columbia, S. C. 29208, U.S.A., and Department of Microbiology, Louisiana State University, Baton Rouge, La. 70803, U.S.A.

STEVENSON, L. H. and SOCOLOFSKY, M. D. 1972. Encystment of *Azotobacter vinelandii* in liquid culture. Antonie van Leeuwenhoek **38**: 605–616.

Cyst formation in liquid cultures of *Azotobacter vinelandii* could be raised to 90% by including 0.6% $CaCO_3$ in the Burk's basal salts solution. The cysts were resistant to desiccation and possessed exine and intine capsular components when observed by electron microscopy of ultrathin sections.

Cells grown in liquid medium containing no $CaCO_3$, but in which the pH was controlled by addition of 0.1 M KOH, were not resistant to desiccation. The capsular material was loosely aggregated and not organized into the typical coat structure. The culture supernatant fluid became viscous and could be precipitated with $CaCl_2$, yielding a thick, polysaccharide-like gel.

Calcium carbonate in the liquid encystment medium served both as a means of acid neutralization and as a source of calcium ions. Calcium ions appear to be structural units necessary for coordination of the coat components into the rigid cyst structure. Other metal ions examined could not substitute effectively for calcium in the encysting system.

INTRODUCTION

The cyst of *Azotobacter* is a modified vegetative cell, and consists of a central body surrounded by a multilayered coat. The coat is responsible for the resistance exhibited by the intact cyst, since without this structure the central body is as vulnerable as the vegetative cell to deleterious agents (Parker and Socolofsky, 1966). The rupture of the coat, which occurs when cysts are treated with various chelating agents, suggests that metal ions are involved in maintaining the integrity of this structure (Socolofsky and Wyss, 1961).

The development of the cyst has been studied on a solid medium with various carbon substrates (Socolofsky and Wyss, 1961, 1962; Stevenson and Socolofsky, 1966). Since a liquid medium would present a more suitable system, various

attempts have been made to induce cyst production in liquid media. Layne and Johnson (1964*a*, *b*) described resistant forms of *Azotobacter* produced in liquid culture by the omission of one or more minerals from the medium. However, their resistant forms lacked the characteristic coat structure and attempts to duplicate their work have been unsuccessful (Parker and Socolofsky, 1966). Romanow (1965) reported cyst formation by *A.chroococcum* in a liquid medium, but offered neither quantitative nor cytological evidence. Lin and Sadoff (1968) reported cyst development in liquid cultures supplemented with various derivatives of *n*-butyl alcohol, however, they provided no information concerning the resistance properties of these cells.

The purpose of this communication is to report the utilization of a liquid medium which supports the encystment of *A. vinelandii* and to describe various environmental factors associated with cyst development.

MATERIALS AND METHODS

Organism and culture medium. The organism used in this study was *Azotobacter vinelandii*, ATCC 12837. The cells were grown in 50 ml of a modified Burk's nitrogen-free salts solution (Wilson and Knight, 1952) in 250-ml Erlenmeyer flasks at 33 C with shaking. Because of its greater solubility, $CaCl_2$ was substituted for $CaSO_4$ which is normally employed. One percent glucose was used as the carbon source. The modified Burk's salts solution and aqueous solutions of $CaCl_2$ and glucose were sterilized by autoclaving at 121 C for 15 min and mixed aseptically. Measured amounts of $CaCO_3$ were hot-air-sterilized at 175 C for 3 hr and added to the basal medium in the appropriate experiments.

Viable cell counts. Viable cell counts were made by the smear plate technique on the basal medium supplemented with 2% agar.

Determination of encystment. The extent of encystment was determined by the desiccation technique of Socolofsky and Wyss (1962). A cyst was considered to be that form of the organism which could survive exposure to predetermined desiccation conditions for a period of 4 days (Stevenson and Socolofsky, 1966).

Acidity control. Control of pH was effected by adding 0.1 M KOH at suitable intervals.

Electron microscopy. The cells were collected by centrifugation, washed, fixed with 2% unbuffered $KMnO_4$ for 1 hr, dehydrated, and embedded in Maraglas resin by the procedure of Freeman and Spurlock (1962). Sections were poststained with 2% unbuffered $KMnO_4$. Carbon replicas were prepared by the method of Bradley and Williams (1957). The replicas were shadowed with germanium.

Viscosity measurement. Culture supernatant materials were clarified by

centrifugation at $70000 \times g$ for 2 hr. The viscosities of the supernatant fluids were recorded as the efflux times with a Cannon-Fenske number 200 viscometer in a 33 C waterbath.

RESULTS

The encystment medium. The pH of a liquid culture of *A. vinelandii* decreased from an initial value of 7.2 to about 5 during the late growth stages. The cells in the culture rapidly lost viability although the optical density remained unaffected. Hence, care had to be taken to maintain a lightly alkaline pH during the period required for cyst formation.

Three methods were tested for counteracting the fall of the pH with the following results: (1) raising the phosphate concentration reduced growth and the cells developed abnormalities such as giant cells, chains, and filaments; (2) periodically adding sufficient 0.1 M KOH to keep the pH at 7.0 to 7.5 maintained the viability of the cells, but these did not encyst and the medium became very viscous; (3) adding 0.6% $CaCO_3$ to the basal medium maintained the viability of the cells and the culture fluid remained slightly alkaline – phase contrast microscopy indicated that the cells encysted.

Electron microscopy of cells grown in liquid culture. Sections of 4-day-old cells grown in liquid culture with $CaCO_3$ exhibited the typical cyst structures of the exine, intine, and central body (Fig. 1a). Figure 1b shows 4-day-old cells grown in a KOH-controlled culture. The cells have shortened and resemble the central bodies of the cyst; however, no organized coats were observed. Some loose material, presumably unorganized coat components, was present in the surrounding matrix.

The appearance of the cyst cultivated in a liquid medium was also examined utilizing the carbon replica technique (Fig. 2). The coat surface was irregular and appears to be composed of layers of material. A carbon replica of a 4-day-old cell grown in a KOH-neutralized culture is seen in Fig. 3a. The cell is small and smooth with no indication of extracellular coat material. The surface is similar to that of central bodies released on ethylenediaminetetraacetic acid (EDTA)-rupture of intact cysts (Fig. 3b).

Vegetative cells and cysts of *Azotobacter* respond differently to a variety of chemical and physical treatments (Socolofsky and Wyss, 1961, 1962). The 4-day-old cells grown in a liquid medium supplemented with $CaCO_3$ were analogous to solid grown cysts in respect to their reaction to EDTA and resistance to desiccation and sonication. In contrast, the 4-day-old cells produced in a KOH-controlled culture exhibited reactions similar to vegetative cells and central

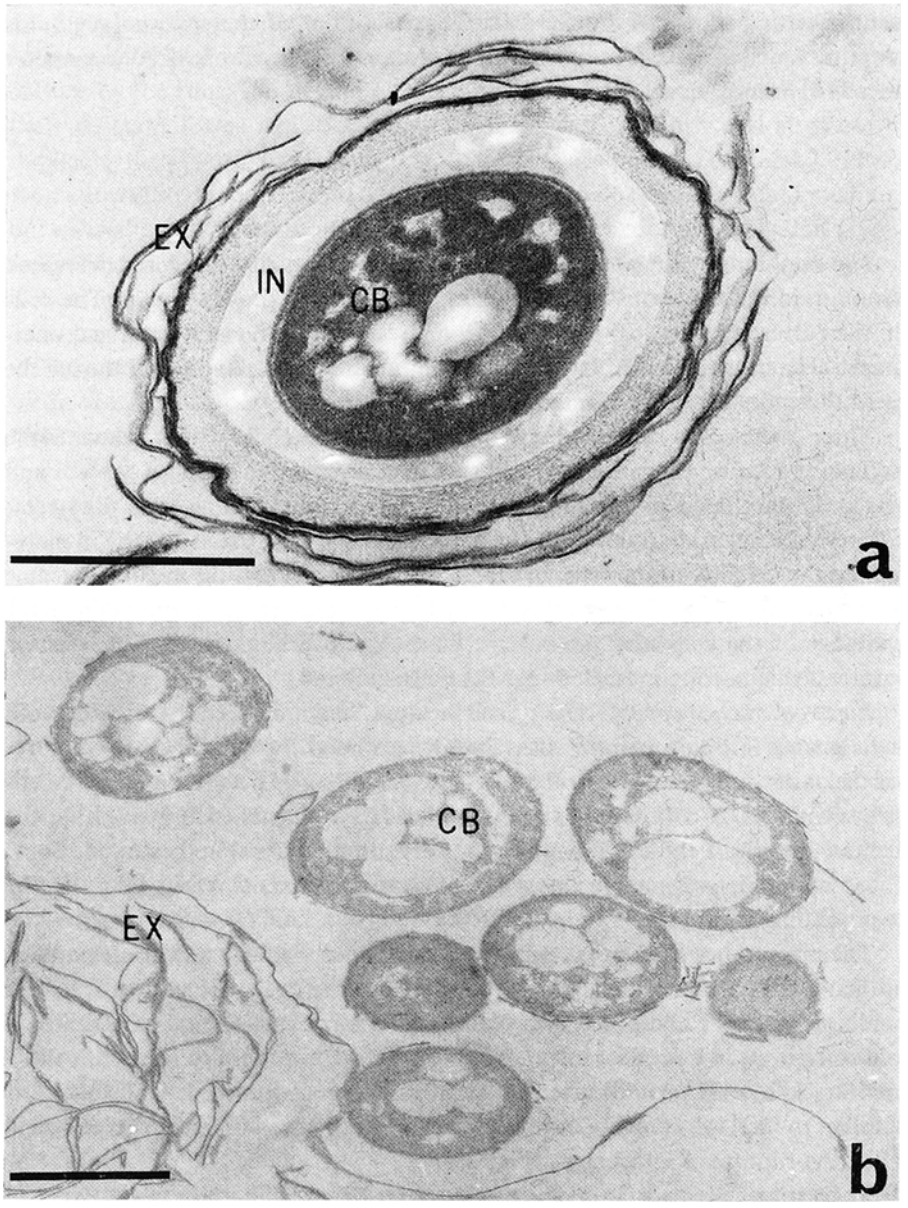


Fig. 1. Electron micrographs of ultra-thin sections of 4-day-old *Azotobacter vinelandii* 12837 cells grown in Burk's nitrogen-free salts solution with 1% glucose. (a) Encysted cell grown in a medium supplemented with 0.6% $CaCO_3$. (b) Non-encysted cells taken from a culture in which the pH was controlled by the periodic addition of 0.1 M KOH.

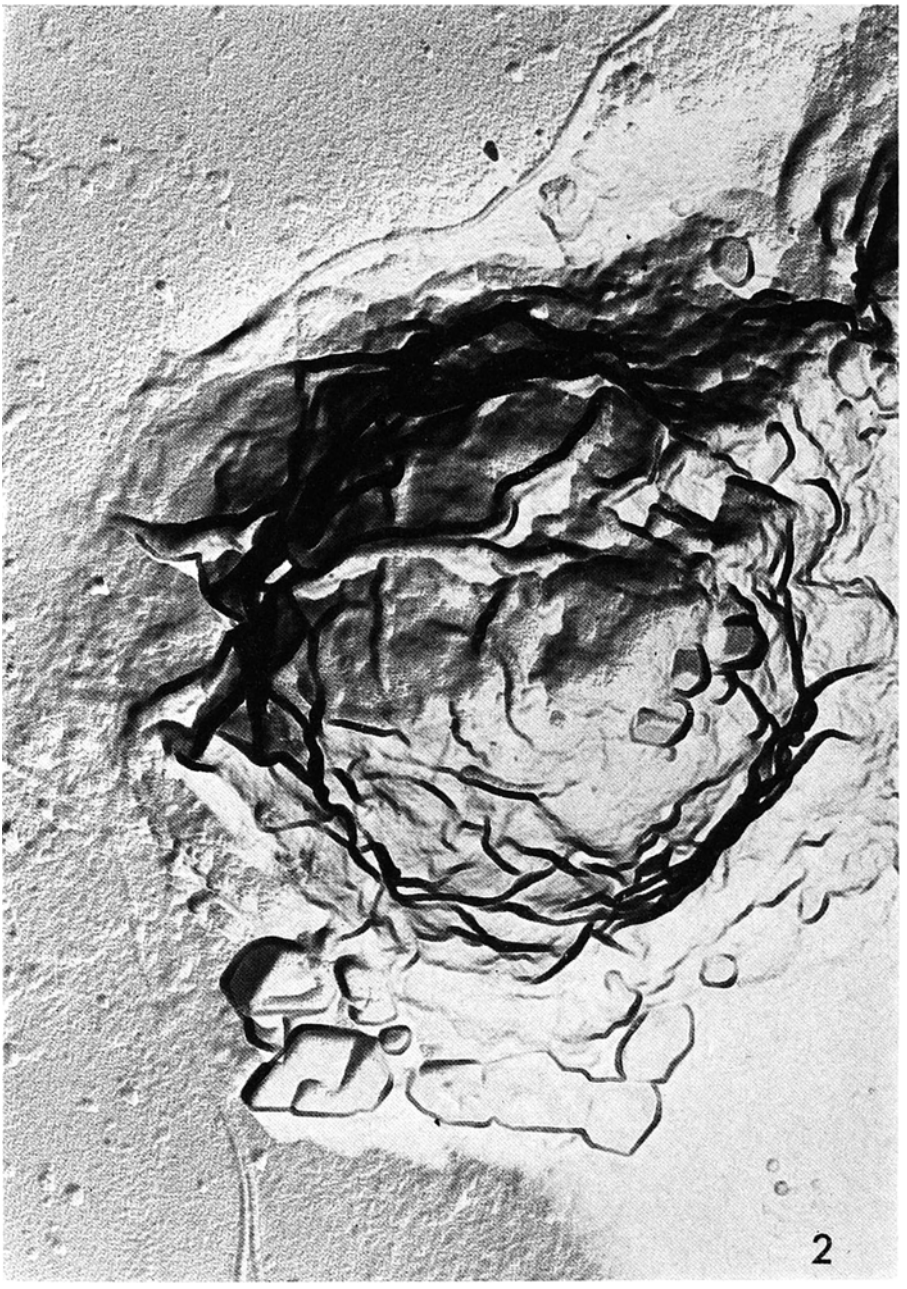


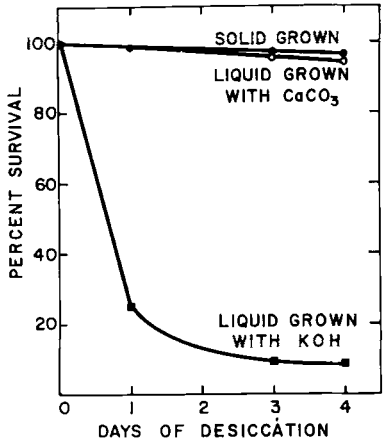
Fig. 2. Carbon replica of an encysted cell of *Azotobacter vinelandii* 12837 grown in a liquid medium with supplemental $CaCO_3$.

Fig. 3. Carbon replicas of *Azotobacter vinelandii* 12837 cells grown under various environmental conditions. (a) Non-encysted cell taken from a culture in which the pH was controlled by the periodic addition of 0.1 M KOH. (b) An EDTA-ruptured cyst which exhibits a collapsed exine coat (EX) and an ejected central body (CB).

bodies (Parker and Socolofsky, 1966) when subjected to the same treatments. The desiccation resistance of 4-day-old cells grown under varying cultural conditions is illustrated by the curves presented in Fig. 4. The cysts cultivated on agar plates and the cells grown in a liquid medium supplemented with $CaCO_3$ show almost complete encystment as judged by their resistance to desiccation. Less than 10% of the cells produced in the KOH-controlled culture were viable after desiccation.

Role of calcium ions. The lack of cyst formation in the KOH-controlled cultures indicated that $CaCO_3$ performed a function in addition to that of acid neutralization. Several experiments were completed in order to investigate its role. When the organism was cultivated with varying amounts of $CaCO_3$, there was a sharp increase in the extent of encystment in the range of 0 to 0.2% $CaCO_3$. With more $CaCO_3$, only a gradual increase in encystment was noted.

The effect of calcium ions on the viscous material remaining after growth in a liquid medium without $CaCO_3$ was examined. A thick polysaccharide-like material separated from a watery residue when 1 ml of a 1 M $CaCl_2$ solution was added to 20 ml of clarified supernatant. After dehydration of the gel with absolute alcohol, a fibrous white material was recovered. The gel could be dissolved by adding 5% EDTA. Preliminary chemical analysis has indicated that the precipitated material is a high-molecular-weight polysaccharide.



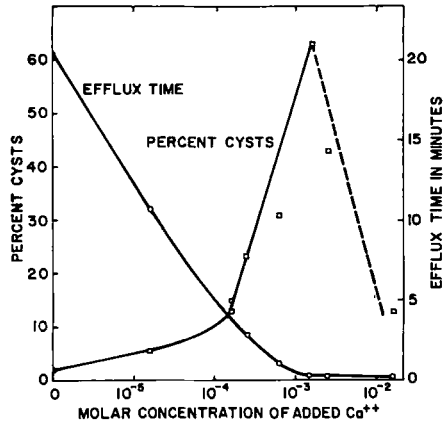


Fig. 4. Effect of desiccation on 4-day-old cells of *Azotobacter vinelandi* 12837 grown under different cultural conditions.

Fig. 5. Relationship between the efflux time of culture supernatants and the extent of encystment when cultures of *Azotobacter vinelandii* 12837 were produced in a liquid medium supplemented with varying concentrations of $CaCl_2$ at the end of the log growth phase. The percent encystment was determined by desiccation resistance and the efflux times of the culture supernatants were measured with a viscometer at 33 C. Both parameters were measured after 4 days of growth.

The effect of calcium ion concentration on cyst-coat formation was studied since Ca^{++} was involved in the aggregation of the polysaccharide-like material. In these experiments 1 ml of the appropriate dilution of $CaCl_2$ was added to the growth medium at the beginning of the stationary phase when there were less than 0.1 % cysts. One ml of distilled water was added to the control culture. The culture pH was controlled by addition of KOH. The results, illustrated in Fig. 5, reveal a decrease in the viscosity of the supernatant of 4-day-old cultures and an increase in the percentage of cysts produced with increasing concentrations of $CaCl_2$. Maximum encystment was observed at 2×10^{-3} M $CaCl_2$. A drastic reduction at higher concentrations was due to severe precipitation of minerals which altered the medium to such an extent that development was hindered.

Role of other metal ions. Several other metal ions were examined for their ability to precipitate the supernatant material (Table 1). Mn^{++}, Ni^{++}, Cu^{++},

Table 1. Effect of cations on cyst formation and on the supernatant polysaccharide-like material produced by *Azotobacter vinelandii* 12837 cells in late log phase growing in Burk's salts solution without the addition of $CaCO_3$

Cation	Salt utilized	Range of molar concentrations examined	Effect when added to: polysaccharide-like material in late log phase supernatant fluids	Effect when added to: late log phase culture growing in Burk's salts solution
Ca^{++}	$CaCl_2$	10^{-1} to 10^{-}	precipitation (10^{-1} to 10^{-4})[1]	cyst development and loss of culture viscosity (10^{-2} to 10^{-5}
Na^{+}	NaCl	10^{-1} to 10^{-}	no effect	no effect
Li^{+}	$LiCl_2$	10^{-1} to 10^{-}	no effect	no effect
Mg^{++}	$MgSO_4$	10^{-1} to 10^{-}	no effect	loss of culture viscosity, no encystment (10^{-2} to 10^{-})
Mn^{++}	$MnCl_2$	10^{-1} to 10^{-}	precipitation (10^{-1} to 10^{-2})	loss of culture viscosity, no encystment (10^{-2} to 10^{-4})
Ni^{++}	$NiSO_4$	10^{-1} to 10^{-9}	precipitation (10^{-1} to 10^{-5})	toxic (10^{-1} to 10^{-5})
	$NiCl_2$	10^{-1} to 10^{-9}	precipitation (10^{-1} to 10^{-5})	toxic (10^{-1} to 10^{-5})
Cu^{++}	$CuSO_4$	10^{-1} to 10^{-9}	precipitation (10^{-1} to 10^{-4})	toxic (10^{-1} to 10^{-5})
Zn^{++}	$ZnSO_4$	10^{-1} to 10^{-9}	precipitation (10^{-1} to 10^{-4})	toxic (10^{-1} to 10^{-5})

[1]Range of molar concentrations which produced indicated results.

and Zn^{++} precipitated the polysaccharide-like material from viscous supernatants, but Na^{+}, Li^{+}, or Mg^{++} did not. However, at concentrations of 10^{-5} M and greater, Ni^{++}, Cu^{++}, and Zn^{++} proved too toxic for use as a substitute for Ca^{++} in the encysting system and concentrations of 10^{-6} M and below had no effect on either the viscosity or cyst development. Mg^{++} and Mn^{++} concentrations of 10^{-2} M caused a drop in the viscosity of the culture medium; however, there was no increase in the extent of cyst formation.

DISCUSSION

The results reported here do not confirm the observations of Layne and Johnson (1964*a*, *b*). These investigators reported that the formation of resistant *Azotobacter* cells was induced by the omission of certain ions, including Ca^{++}, from the basal salts medium. We have observed, on the contrary, that the medium must be supplemented with additional Ca^{++} for encystment to occur. The cells produced in this study by cultivation in a liquid medium with periodic adjustment of the pH did resemble those described by Layne and Johnson since they lacked organized coat components. However, they were not resistant to various deleterious agents and we consider them to be cytologically and physiologically analogous to the central bodies isolated and described by Parker and Socolofsky (1966). The cysts cultivated in the liquid medium cannot be distinguished from cysts produced on a solid substrate on the basis of their cytological lytic, and resistance properties, and they meet the criterion of a cyst proposed by Stevenson and Socolofsky (1966).

The practice of incorporating $CaCO_3$ into bacterial growth media is ubiquitous and the importance of the carbonate in maintaining cell viability in acid producing cultures is well known. Cohen and Johnstone (1963) proposed that $CaCO_3$ in the medium used to cultivate *Azotobacter* served either to neutralize the acidic products formed or to inhibit the ability of the cell to synthesize acidic slime. We propose that the $CaCO_3$ used in the encystment medium serves the dual function of acid neutralization and calcium ion source. The neutralization function is very important because the acid conditions produced are detrimental to the cells. It is apparent that the calcium ions fulfill an important role in facilitating the organization of the polysaccharide-like material into a rigid cyst coat surrounding the cell. If an adequate supply of ions is not available, abortive encystment ensues resulting in the loss of the coat material into the surrounding medium with a resultant increase in the viscosity of the solution. This observation may explain the production of viscous polymer by *Azotobacter* observed by Lin and Sadoff (1968). They reported that the addition

of β-hydroxybutyric acid to encysting cultures promoted disorganization of the exine and precluded the formation of mature cysts. Their findings, however, may have resulted from a lack of sufficient Ca^{++} in the medium used in their experiments.

We are unable to account for the apparent disparity between the amount of supplemental Ca^{++} required to effect encystment utilizing a solid medium and that required in a liquid medium. However, the concentration of Ca^{++} present in solid substrates may be more than realized since Chan, Basavanand and Liivak (1970) have reported that Bacto-Agar alone contains calcium ions concentrations sufficient to support the growth of *Azotobacter*.

A correlation of the information reported in this communication with other published research affords a coherent understanding of the formation and germination of the *Azotobacter* cyst. A diagrammatic sketch of these processes is shown in Fig. 6. The dormant cyst is depicted at the bottom of the diagram. Under appropriate conditions the cyst will undergo a germination process. During this phase the central body of the cyst enlarges and the outer capsular components are gradually torn apart (Tchan, Birch-Andersen and Jensen, 1962; Wyss, Neumann and Socolofsky, 1961) releasing the young vegetative cell which is sensitive to various deleterious agents (Socolofsky and Wyss, 1962). Active multiplication starts about 10 hr after transfer to a fresh medium. The initial step in cyst development involves the accumulation of intracellular

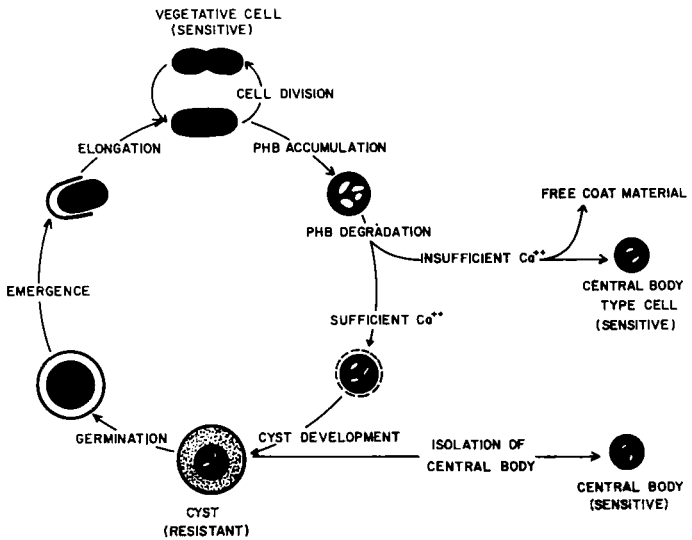


Fig. 6. Diagrammatic sketch of the formation and germination of *Azotobacter* cysts.

deposits of poly-β-hydroxybutyric acid (Stevenson and Socolofsky, 1966). This is followed by an endogenous utilization of the polymer as a carbon and energy source for the encystment process. During the polymer degradation phase there is a concurrent production of cyst coat components. If sufficient calcium ions are not available in the environment for the coordination of the coat material into a rigid cyst coat, an abortive encystment takes place and the coat material is lost into the surrounding medium. This results in an increase in the viscosity of the culture supernatant and the production of small vegetative cells that are believed to be analogous to the central bodies isolated by Parker and Socolofsky (1966). On the other hand, if sufficient calcium ions are available, the coat materials are coordinated around the periphery of the developing central body. This leads to the formation of mature cysts which display the cytological, lytic, and resistance properties reported to be characteristic of *Azotobacter* cyst.

This investigation was supported by Public Health Service research grants AI-02924 and AI-05551 and by Public Health Service predoctoral fellowship 5-F1-GM-29, 322-02.

Received 25 *April* 1972

REFERENCES

BRADLEY, D. E. and Williams, D. J. 1957. An electron microscope study of the spores of some species of the genus *Bacillus* using carbon replicas. – J. Gen. Microbiol. **17**:75–79.

CHAN, E. C. S., BASAVANAND, P. and LIIVAK, T. 1970. The growth inhibition of *Azotobacter chroococcum* by *Pseudomonas* sp. – Can. J. Microbiol. **16**:9–16.

COHEN, G. H. and JOHNSTONE, D. B. 1963. Acid production by *Azotobacter vinelandii*. – Nature **198**:211.

FREEMAN, J. A. and SPURLOCK, B. O. 1962. A new epoxy embedment for electron microscopy. – J. Cell Biol. **13**:437–443.

LAYNE, J. S. and JOHNSON, E. J. 1964*a*. Natural factors involved in the induction of cyst formation in *Azotobacter*. – J. Bacteriol. **87**:684–689.

LAYNE, J. S. and JOHNSON, E. J. 1964*b*. Resistant properties of *Azotobacter* cysts induced in response to mineral deficiencies. – J. Bacteriol. **88**:956–959.

LIN, L. P. and SADOFF, H. L. 1968. Encystment and polymer production by *Azotobacter vinelandii* in the presence of β-hydroxbutyrate. – J. Bacteriol. **95**:2336–2343.

PARKER, L. T. and SOCOLOFSKY, M. D. 1966. Central body of the *Azotobacter* cyst. – J. Bacteriol. **91**:297–303.

ROMANOW, I. 1965. Recherches sur la synthèse de l'acide indole-3-acétique par les *Azotobacter*. – Ann. Inst. Pasteur **109**, Suppl. au no. 3 – Ann. Inst. Pasteur **109**, Suppl. au no. 3:280–292.

SOCOLOFSKY, M. D. and Wyss, O. 1961. Cysts of *Azotobacter*. – J. Bacteriol. **81**:946–954.

SOCOLOFSKY, M. D. and Wyss, O. 1962. Resistance of the *Azotobacter* cyst. – J. Bacteriol. **84**:119–124.

STEVENSON, L. H. and SOCOLOFSKY, M. D. 1966. Cyst formation and poly-β-hydroxybutyric acid accumulation in *Azotobacter*. – J. Bacteriol. **91**:304–310.

TCHAN, Y. T., BIRCH-ANDERSEN, A. and Jensen, H. L. 1962. The ultrastructure of vegetative cells and cysts of *Azotobacter chroococcum*. – Arch. Mikrobiol. **43**:50–66.

WILSON, P. W. and KNIGHT, S. G. 1952. Experiments in bacterial physiology. – Burgess Publishing Co., Minneapolis.

WYSS, O., Neumann, M. G. and Socolofsky, M. D. 1961. Development and germination of the *Azotobacter* cyst. – J. Biophys. Biochem. Cytol. **10**:555–565.