Germination of Azotobacter vinelandii Cysts: 
Sequence of Macromolecular Synthesis and 
Nitrogen Fixation

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Azotobacter vinelandii cysts undergo conversion to vegetative cells in Burk's nitrogen-free medium utilizing glucose, sucrose, or acetate. In 1% glucose, this overall process was complete in 8 hr and consisted of a germination and an outgrowth phase. Respiration, ribonucleic acid, and protein synthesis began soon after the addition of the germinant, and these processes proceeded at rates characteristic of the germination. The rates of respiration and synthesis increased sharply between 4 and 5 hr, the beginning of the outgrowth, at which time deoxyribonucleic acid synthesis and nitrogen fixation began. Respiration, macromolecular synthesis and nitrogen fixation continued at high rates until the emergence of vegetative cells from the cyst coats.

Encystment of Azotobacter vinelandii occurs with a low frequency after growth on media containing glucose. However, encystment can be induced in cells after an initial growth period in Burk's N-free medium by replacing the substrate, glucose, with either B-hydroxybutyrate (BHB) or crotonate as the carbon source (4). The cysts which then form are metabolically dormant cells, considerably more resistant to deleterious physical and chemical agents than the parent vegetative cells (5, 14). Under suitable conditions of pH, temperature, and exogenous substrate availability, cysts germinate and reinitiate the vegetative phase of their life cycle. We have been concerned with this differentiation process in A. vinelandii and with the mechanism of metabolic dormancy which is operative in cysts. Because fundamental similarities exist between encystment in Azotobacteriaceae and sporulation in the Bacillaceae, we have initiated a comparative study of the two processes in an effort to discern the general principles controlling differentiation in the prokaryotes. In this report we describe the sequence of some macromolecular syntheses and N fixation which occur during germination and which precede the emergence of vegetative cells from cysts.

MATERIALS AND METHODS
Organism. Cysts of A. vinelandii ATCC 12837 were prepared in a two-step procedure. Cells were grown in 100 ml of Burk's N-free buffer (12) with 1% glucose as the carbon source. At late exponential phase, they were removed from the medium by centrifugation, washed once, and suspended in 4 ml of Burk's buffer. Portions of this suspension (0.2 ml) were spread onto thick plates of Burk's buffer containing 1.5% agar and 0.2% BHB (Sigma). The plates were inverted and incubated for 5 days at 30°C. Approximately 95% encystment was obtained by this procedure, and the cysts could be stored on the plates at 4°C without germinating. Suspensions of cysts for germination experiments were prepared by scraping the growth from the surface of plates into Burk's buffer and washing once in fresh buffer. Sufficient washed cysts were added to germination media to achieve a final concentration of 10⁸ cysts per ml.

Germination. Germination was initiated by adding an appropriate carbon source to cysts suspended in N-free buffer at 30°C. Wyss et al. (14) have described germination of Azotobacter cysts as a "process in which the exine ruptures and a large vegetative cell is liberated." The same criterion of germination was utilized in these studies, and the time required for the process was determined by sampling cyst suspensions at regular intervals and examining them by phase-contrast microscopy. Alternatively, observations were made of turbidities of cyst suspensions during the course of germination. A suspension of nongerminated cysts, 10⁵/ml, had a turbidity corresponding to an optical density (OD) of 0.80.

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Respiration. Respiration rates, oxygen uptake, and CO₂ evolution were measured at 30°C using a Warburg manometer apparatus (11). A cyst suspension (2.40 × 10⁷ cysts per ml in Burk’s buffer) was placed in the flask with 0.10 ml of 25% glucose in the sidearm, and 0.2 ml of 20% KOH was added to the center well. When using the Warburg indirect method for determination of CO₂ evolution, 0.3 ml of 3 N H₂SO₄ was used to terminate the reaction and liberate dissolved CO₂. The reactions were started after thermal equilibrium by tipping the glucose into the cyst suspension. The endogenous control was a cyst suspension without added glucose. The rate of CO₂ release from ¹⁴C-labeled glucose during germination was monitored in the following manner. To a 300-ml stoppered Erlenmeyer flask were added: 29 ml of Burk’s buffer containing 3 × 10⁴ washed cysts, 0.6 ml of 50% glucose, and 5 µCi of either glucose-¹⁴C or glucose-6-¹⁴C (6.9 mCi/m mole and 3.88 mCi/m mole, respectively, New England Nuclear Corp.). Carbon dioxide was trapped on a piece of fluted filter paper (3 by 5 cm) which was saturated with 10% KOH (0.35 ml) and supported above the cyst suspension in a small vial. At appropriate intervals, the vials were removed, and the papers were dried and counted for 10 min in 5 ml of toluene-based scintillation fluid (1) using a Packard Tri-Carb scintillation counter.

Macromolecular syntheses. The synthesis of protein, ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) during germination was determined by a modification of the method of Roodyn (6). The radioactive tracer for protein synthesis was 5 µCi of ¹³C-leucine (8.3 mCi/m mole, Calbiochem) plus 5 µmoles of cold leucine added to 30 ml of Burk’s buffer containing 1% glucose. Duplicate 0.5-ml samples were taken at 30-min intervals for 8 hr, and 0.5 ml of cold 10% trichloroacetic acid was added to stop uptake. After 30 min of cooling in ice, the samples were washed onto prewetted membrane filters (0.45 µm pore size, Gelman, Ann Arbor, Mich.) and washed three times with 5 ml of 5% cold trichloroacetic acid containing 5 µmoles of leucine per ml. Filters were dried in scintillation vials, 5 ml of toluene-based scintillation fluid was added, and 10-min counts were made in a Packard Tri-Carb scintillation counter. The control was a 30-ml cyst suspension without glucose containing 20 mg of chloramphenicol. The procedure for RNA synthesis utilized 5 µCi of ¹³C-uracil (216 mCi/m mole, New England Nuclear Corp.) plus 5 µmoles of cold uracil added to 30 ml of Burk’s buffer containing 1% glucose and 20 µg of cytidine per ml to minimize uracil incorporation into DNA (2). The control was a nongerminating cyst suspension.

DNA synthesis was monitored by using ¹³C-uracil at a concentration of 0.5 µCi/ml. One-milliliter samples were taken at 30-min intervals, and 0.1 ml of 5.5 N NaOH was added to effect the hydrolysis of RNA in 23 hr at 37°C. After cooling, 0.1 ml of 6 N HCl was added to neutralize each sample and then 1.2 ml of 10% trichloroacetic acid to precipitate the DNA. This material was cooled for 30 min in an ice bath, washed onto prewetted membrane filters, and washed three times with 5 ml of cold 5% trichloroacetic acid containing 5 µmoles of uracil per ml. The filter discs were dried in scintillation vials, toluene-based scintillation fluid was added, and they were counted for 10 min in a Tri-Carb scintillation counter.

Nitrogen fixation. The time course for the initiation of nitrogenase activity was determined by using a modification of the acetylene reduction technique of Stewart (9) which results in the formation of ethylene. Ten milliliters of a cyst suspension were put into a 100-ml serum bottle fitted with a septum; air was replaced by flushing with a gas mixture of 22% O₂, 0.04% CO₂, and 78% argon (Matheson), and 5 ml of acetylene was added with a syringe. The culture was incubated in a rotary water bath at 30°C, and, at various times, 50 µlitters of the gas in equilibrium with the cyst suspension was injected into a Varian aerograph (model 600D) gas chromatograph fitted with a 3-foot (0.914-m) Porapak R column. The column temperature was 45°C, and the flow rate of the carrier N₂ was 25 cm³/min. The activity of the nitrogenase system was determined by the reduction of acetylene and the appearance of ethylene. The standard was a gas mixture of 47 µlitters of ethylene per liter of N₂.

RESULTS

Germination. Germination studies were performed in Burk’s N-free buffer with a variety of compounds as germination substrates. The time for this process was marked from the addition of germinant to the emergence of cells from the cyst exine. Microscopically, the first evidence of germination was the gradual loss of their refractivity when cysts were viewed with phase-contrast optics. This was a slow process lasting 4 to 6 hr in glucose-containing media, during which time the central body enlarged and occupied the volume which was taken up by the intine. The exine then fractured and a "peanut-shaped" vegetative cell emerged leaving a characteristic "horseshoe-shaped" exine. These events have also been observed by Wyss et al. (14) by means of thin sectioning and electron microscopy.

During germination, the turbidity (OD) of cyst suspensions decreased slowly and as a linear function of time. The basal level was reached concomitantly with the complete loss of optical refractivity observed in phase microscopy. In 1% glucose solutions, cyst suspensions had initial OD values of 0.80 which then decreased to approximately 0.60 after 5.5 hr at 30°C. The optical densities rose at 6.5 hr and were equal to the initial turbidities by 8 hr. A variety of compounds was tested as germination substrates by using the turbidimetric procedure and microscopy. The results of these studies are listed in Table 1, where it is
apparent that the most effective germinants were glucose, sucrose, or sodium acetate. Because 1% glucose produced rapid germination of cysts and is used routinely in growth medium for *A. vinelandii*, this concentration of the carbon source was used for the study of the physiological changes during germination.

The effects of heat and cold shock on the initiation and rate of germination were tested. Heat shock for 10 min at 55°C inhibited about 25% of the cysts from germinating, but heating at the same temperature for 20 min or longer was lethal for more than 95% of the cysts. Freezing or extended incubation at 0°C neither enhanced nor inhibited the initiation or rate of germination.

**Respiration.** The oxygen uptake and CO₂ evolution from germinating cysts began very soon after the addition of glucose, and their rates increased to constant values at about 4 hr (Fig. 1). Streptomycin sulfate at 330 μg/ml blocked the initial increase in the respiration rate and cyst germination. The latter was judged by microscopy examination. The inhibitory effects of chloramphenicol, 33 μg/ml, and dinitrophenol, 3.3 × 10⁻⁴ M, on the respiration of germinating cysts were identical in magnitude to that produced by streptomycin. In these cells, the initial O₂ uptake and CO₂ release rates were maintained for 5 hr and then they decreased to 0 by 8 hr. No detectable oxygen uptake occurred in cyst suspensions without glucose and germination did not occur in the absence of oxygen.

In Fig. 1 (inset) are shown the rates of CO₂ evolution from glucose-1-¹⁴C and glucose-6-¹⁴C during cyst germination. The evolution of CO₂ from carbon 1 of glucose began immediately and was linear during the 30-min observation period.

**Macromolecular syntheses and N fixation.** The synthesis of protein and RNA began upon the addition of glucose to cysts suspended in Burk's buffer (Fig. 2). The rate of ¹⁴C-leucine incorporation was constant for 5 hr and then increased threefold and remained at the elevated rate until germination was complete. The pattern of RNA synthesis (¹⁴C-uracil uptake) was similar during germination; RNA synthesis occurred at a low rate up to 3.5 hr, increased sevenfold by the 5th hr, and remained at this elevated rate.

These data taken at 1-hr intervals suggested that both RNA and protein synthesis began upon the initiation of germination by glucose. A more precise timing of these events was obtained by measuring the extent of uracil and leucine incorporation over short time intervals early in germination. Cysts, glucose, and ¹⁴C-

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**TABLE 1. Germination substrates for Azotobacter vinelandii**

<table>
<thead>
<tr>
<th>Group</th>
<th>Germination time (hr)</th>
<th>Germination substrates</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>8-9</td>
<td>1% Glucose, 1% Sodium acetate, 0.5% Sucrose, 0.5% Glucose</td>
</tr>
<tr>
<td>II</td>
<td>9-11</td>
<td>0.5% Glucose + 0.25% NH₄⁺, 0.5% Fructose</td>
</tr>
<tr>
<td>III</td>
<td>11</td>
<td>1% Sorbitol, 1% Maltose, 0.5% Glycerol</td>
</tr>
<tr>
<td>IV</td>
<td>Germination does not occur</td>
<td>Burk's buffer, 1% Succinate, 1% NaCl, 0.25% NH₄⁺, 0.5% Ribose, 1% Trehalose, 1% L-Arabinose, 1% Raffinose, 1% Inulin, 1% Starch, 1% Casamino Acids</td>
</tr>
</tbody>
</table>

*The basal medium was Burk's N-free buffer.  
*The time period is from the initiation of germination to the completion of outgrowth. The latter event is defined as that time at which cysts rupture and release vegetative cells.  
*Each substrate was tested individually at the stated concentration.
uracil were mixed and sampled at 3- to 5-min intervals for 1 hr. A culture to which 20 μg of rifampin per ml was also added served as a control because this concentration of antibiotic was known to inhibit RNA polymerase in both cells and germinating cysts. Uracil incorporation occurred after a 20-min uracil absorption period (Fig. 3A). When cysts were preincubated with uracil, incorporation of the pyrimidine began immediately upon the addition of glucose (Fig. 3B). Rifampin blocked both RNA synthesis and subsequent cyst germina
tion.

The data presented in Fig. 4 show that leucine incorporation occurred very soon after the addition of glucose to cysts. Also presented is the extent of leucine incorporation by cysts in the presence of 20 μg of rifampin per ml which was added either 30 min prior to or at the time of addition to cysts of glucose and 14C-leuc
cine.

Both DNA synthesis and N fixation were initiated at approximately 5 hr after glucose addition (Fig. 5). The rates of these processes were constant until the exines ruptured and neither occurred in cysts in the absence of glucose. N fixation was completely inhibited by the incorporation of 0.25% NH₄Cl into the germination medium.

DISCUSSION

Conversions from the cryptobiotic state to active vegetative growth occur in several prokaryotic genera, but the phenomenon which has been most studied is the germination of bacterial spores (3, 10). The research described in this report was performed to determine the extent of similarities between germination in Azotobacter cysts and Bacillus spores. It should be emphasized that the operational definition of germination employed in these studies included both the germination and outgrowth processes as they are defined for the transition of bacterial endospores to vegetative
The initiation of DNA synthesis signals the beginning of outgrowth after spore germination (8). By applying the same parameter to the cyst-to-cell transition, it was possible to divide the overall process into "germination" and "outgrowth" and to compare the developmental events in cysts and spores. A parallel was observed, particularly with respect to their sequences of macromolecular syntheses.

A. vinelandii cysts germinated in Burk's N-free buffer plus glucose and required aerobic conditions and an exogenous carbon source to initiate and promote the process. Their poly-β-hydroxybutyrate reserves apparently could not be mobilized to initiate germination. Neither heat shock of cysts nor cold shock or freezing enhanced their germination rate, and temperatures at the lower limits for spore activation were lethal for Azotobacter cysts. Turbidimetric measurements were unsatisfactory for monitoring the time course of cyst germination.

The overall germination time for cysts in 1% glucose was 8 ± 0.25 hr, suggesting that the process is reasonably synchronous. It occurred in two phases, each having characteristic rates of oxygen uptake and RNA and protein syntheses which corresponded rather well to germination and subsequent outgrowth (8). During the germination, cysts underwent a shift-up or derepression of their metabolic processes which was reflected in the increasing respiration rate. Key enzymes of glucose metabolism which were "lost" during encystment (V. M. Hitchins and H. L. Sadow, Bacteriol. Proc. p. 39 1971) were presumably resynthesized during this period. The blocking of protein synthesis or adenosine 5' triphosphate (ATP) generation also blocked increases in the respiration rate and cyst germination. Both RNA and protein syntheses began very soon after the addition of the germinant, and thus cysts contained either appropriate enzymes and pools of nucleic acid precursors and amino acids or the capability for a rapid turnover of RNA and protein during germination. The ability of germinating cysts to incorporate some 14C-leucine into protein, despite pretreatment with rifampin suggests that stable messenger RNA may exist in dormant cysts. No uracil incorporation into RNA occurred at the concentration of rifampin which was employed.

The early RNA synthesis in cysts is reminiscent of that which occurs during the germination of Bacillus megaterium spores (8). In that process, "new" RNA formed at the expense of previously existing RNA and was detectable 1 min after the addition of the germinant. The ATP which was required was generated from a depot of 3-phospho-d-glyceric acid (7). The nature and quantity of energy yielding low-molecular-weight compounds in cysts is unknown.

The beginning of outgrowth of cysts at 5 hr was indicated by the initiation of both N fixation and DNA synthesis. Increases to new characteristic values also occurred in the cysts' rates of respiration and RNA and protein syntheses. The termination of this phase at 8 hr occurred with the emergence of typical peanut-shaped vegetative cells (14). This morphology is characteristic of A. vinelandii cells which are in the process of cell division.

The extended time interval between the initiation of cyst germination and DNA synthesis is similar to that which occurs with endospores, differing only in time scale (8, 13). Spores utilize endogenous N sources in their germination until just before outgrowth and DNA synthesis begin. The coincidence of N fixation and the initiation of DNA synthesis in germinating Azotobacter cysts suggests that the same may be true for this organism.

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LITERATURE CITED

