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Characterization of Germination and Activation of *Bdellovibrio* Bdellocysts

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A simple method of assaying germination of bdellocysts in liquid medium has been devised. Bdellocysts can be induced to germinate by any of eight L-amino acids or the monovalent cations K^+ and NH_4^+. L-Glutamine was the best individual inducer of germination, although the resulting rate of germination was much slower than in a complex medium. The use of a defined germination medium containing L-glutamine, KCl, and NH_4Cl produced a faster rate of germination than did complex media. Bdellocysts germinated most rapidly at pH 8.0 and at 35°C and required aerobic conditions. Respiration of bdellocysts began to increase at 3 min after the addition of germinants. Germination was inhibited by respiratory-chain inhibitors and by inhibitors of macromolecular synthesis. When bdellocysts were heat shocked at sublethal temperatures for short periods, there was no effect on the rate of germination in the defined germination medium or in the complex medium. However, heat-shocked bdellocysts germinated at a much faster rate in the presence of single inducers of germination when compared to nonshocked bdellocysts.

The bdellovibrios are predacious bacteria that are capable of growth on susceptible prey bacteria. One strain, *Bdellovibrio* sp. strain W (2), produces a resting stage termed a bdellocyst (27, 28). Bdellocysts differ from vegetative bdellovibrios in morphology (2, 15, 28), in their resistance to environmental stresses (27), and in their reduced rate of respiration (27).

Bdellovibrio sp. strain W is obligately predacious, and bdellocysts are produced within their prey. Germination of bdellocysts has been demonstrated in the presence or absence of prey cells in a nutrient medium such as peptone-yeast extract (PYE) (27). Ultrastructural changes occurring during germination have been reported (28). A detailed study of the germination process would increase our understanding of the role of bdellocysts in nature and would enable investigators to compare bdellocyst germination with the germination processes described for other bacterial systems (5, 7, 8, 13, 17, 23).

The purposes of this study were (i) to develop a germination assay that was both simple and accurate as a measure of germination; (ii) to study the germination requirements of bdellocysts; (iii) to determine the effects of a variety of physiological inhibitors on germination; and (iv) to study the effects of heat on germination.

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MATERIALS AND METHODS

Organisms and culture conditions. *Bdellovibrio* sp. strain W (obtained from G. Drews, University of Freiburg, Freiburg, Germany) was used in all experiments. The prey organism employed for growth and encystment of the bdellovibrios was *Rhodospirillum rubrum* Hughes (obtained from G. Sojka, Indiana University, Bloomington, Ind.).

R. rubrum was grown in tryptic soy broth (Difco Laboratories, Detroit, Mich.) to late exponential phase, harvested by centrifugation at 5,000 × *g* for 15 min, washed, and suspended at a concentration of 10^9 to 2 × 10^9 cells per ml in dilute nutrient broth (25) supplemented with 0.002 M $CaCl_2$ and 0.003 M $MgCl_2$. *Bdellovibrio* was grown by inoculating a washed suspension of *R. rubrum* in dilute nutrient broth with a 10% (vol/vol) inoculum from a 12- to 14-h bdellovibrio lysate and incubating at 150 rpm and 30°C in a shaker incubator for 12 to 14 h; lysis of the prey was complete at this time. *Bdellovibrio* cultures were stored as lysates, and *R. rubrum* was maintained on tryptic soy agar (Difco) at 4°C.

Preparation of bdellocysts. For the production of bdellocysts, *R. rubrum* was grown in tryptic soy broth, harvested in the stationary phase of growth by centrifugation at 5,000 × *g* for 15 min, and suspended in 0.05 M potassium phosphate buffer, pH 7.5 (27). Bdellovibrios grown as described earlier were harvested by centrifugation at 12,000 × *g* for 20 min and suspended in 0.05 M potassium phosphate buffer, pH 7.5.

The *R. rubrum* and *Bdellovibrio* (2 × 10^9 cells per ml each) were mixed in 0.05 M potassium phosphate buffer (pH 7.5) and incubated at 30°C in a shaker

incubator at 150 rpm for 20 h (27). The vegetative bdellovibrios in the resulting bdellocyst suspension were removed by the addition of Triton X-100 (Fisher Scientific Co., Pittsburgh, Pa.) at a final concentration of 0.02% (wt/vol), followed by incubation for 30 min at room temperature (22 to 25°C). The Triton X-100 lysed the vegetative bdellovibrios, but had no detectable effect on the bdellocysts, as determined by viability and morphology. The bdellocysts were harvested by centrifugation at 5,000 × *g* for 20 min and washed five times by centrifugation in distilled water. Storage for up to 8 months under these conditions had little, if any, effect on germinability or viability of bdellocysts.

Germination assay. Germination of bdellocysts in liquid medium was assayed as follows: bdellocysts were added to the germination medium to give an initial absorbance at 600 nm of 0.25 to 0.3 (2.2×10^8 to 2.7×10^8 bdellocysts per ml). Germination mixtures were incubated at 35°C in a constant-temperature water bath. One-milliliter samples were taken at intervals, added to 0.01 ml of 10% (wt/vol) Triton X-100 in a 1-ml cuvette, and incubated at room temperature (22 to 25°C) for 5 min. The Triton X-100 lysed germinated bdellocysts, but did not lyse ungerminated bdellocysts. The turbidity as measured by absorbance was then determined by using a Cary 14 recording spectrophotometer (Applied Physics Corp., Monrovia, Calif.) at 600 nm. For this assay, therefore, germination was defined as that point at which bdellocysts became susceptible to lysis by Triton X-100. Triton X-100 was not present in the germination medium.

Bdellocysts were germinated in PYE (1) or in a defined germination medium (pH 8.0) at 35°C. The pH was adjusted with 0.1 M NaOH. The defined medium (GPA medium) consisted of 0.1 M L-glutamine, 0.01 M KCl, and 0.01 M NH_4Cl (pH 8.0). Germination was carried out at 35°C in stationary cultures in a constant-temperature water bath. Samples used to measure absorbance were taken over a 2-h period in most experiments when 85 to 90% of the bdellocysts had germinated.

Oxygen uptake during germination. Oxygen uptake by germinating bdellocysts was determined on 3-ml samples containing 7×10^9 bdellocysts per ml in the defined germination medium at 30°C by using a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, Ohio). Oxygen uptake was monitored until the rate of uptake reached a maximum (about 90 min). Measurements of absorbance and oxygen uptake were performed on parallel cultures.

Heat shock. Heat shock (50°C for 2 to 5 min) was carried out in a constant-temperature circulating water bath. Bdellocysts were suspended in 0.001 M *N*-tris(hydroxymethyl)methylglycine buffer (Tricine; Sigma Chemical Co., St. Louis, Mo.; pH 8.0) to give an absorbance of 0.4 at 600 nm. The suspending medium was preheated to 50°C prior to the addition of the bdellocysts. After heating, the suspensions were rapidly cooled to 25°C in a circulating water bath. Germination inducers were then added, and the suspensions were incubated at 35°C. The rate of germination was followed by periodically measuring the decrease in absorbance of samples to which 0.1% Triton X-100 was added as described above.

Microscopic counts. Direct microscopic counts of prey cells, bdellovibrios, and bdellocysts were made by using a Petroff-Hausser bacterial counting chamber mounted in a Zeiss Universal microscope equipped with phase-contrast optics.

Viability. The decrease in viability of a germinating bdellocyst suspension due to the addition of Triton X-100 was determined by diluting out the Triton X-100 and plating the dilutions by the double-layer agar overlay procedure (4), using PYE agar. Samples taken at the time of addition of germination inducers retained 100% viability.

Chemicals. All amino acids, sugars, vitamins, tricarboxylic acid cycle intermediates, nucleosides, nucleoside monophosphates, and the antibiotics chloramphenicol, streptomycin sulfate, penicillin G, nalidixic acid, and rifampin were obtained from Sigma Chemical Co. Potassium cyanide and sodium azide were obtained from Fisher Scientific Co.

RESULTS

Germination in complex medium. Turbidity changes that occurred in a germinating bdellocyst suspension after addition of Triton X-100 are shown in Fig. 1. Germination was carried out in PYE broth (pH 8.0) at 35°C. The decrease in turbidity illustrated was due to the lysis of the germinated bdellocysts by Triton X-100. Bdellocysts suspended in Tricine buffer did not exhibit a decrease in turbidity over the same time period. The correlation of the decrease in turbidity during germination with the decrease in both viable count and direct microscopic count of bdellocysts due to Triton X-100 is shown in Table 1. The decrease in turbidity as observed with this assay procedure was clearly due to the decrease in the number of bdellocysts in the suspension after treatment with Triton X-100. This assay system was used throughout this study to measure germination of bdellocysts.

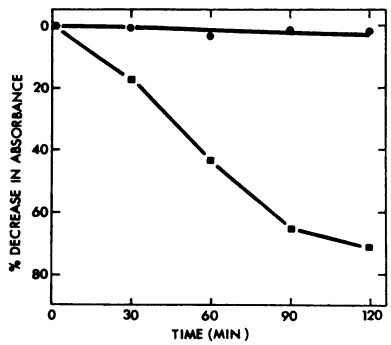

FIG. 1. *Assay of PYE-induced germination of bdellocysts. The decrease in absorbance is due to lysis of germinated cysts by addition of Triton X-100 to samples. Germination was at 35°C in PYE (■) and in 0.001 M Tricine (●), pH 8.0.*

Germination in defined medium. A variety of compounds, singly and in combination, were examined for ability to induce germination of bdellocysts. Complex substrates such as yeast extract and vitamin-free Casamino Acids (Difco) were able to induce germination, but more slowly and less extensively than those induced by PYE. Germination rate is used here as the percent decrease in absorbance per minute.

Since bdellocysts germinated in vitamin-free Casamino Acids, individual amino acids were tested alone and in various combinations for their ability to induce germination. Eight amino acids induced germination, but at a much slower rate than the PYE-induced germination (Table 2). L-Glutamine was the best single inducer of bdellocyst germination tested. Various combinations of amino acids did not enhance the effect of single amino acids.

Although bdellocysts germinated in vitamin-free Casamino Acids and in solutions of individual amino acids, the rate of germination was appreciably lower than that in PYE. Therefore, other compounds present in PYE affected the germination of bdellocysts. A variety of organic compounds were examined for ability to induce germination or to stimulate L-glutamine-induced germination. Compounds tested included various sugars, ribonucleosides, ribonucleoside monophosphates, vitamins, and tricarboxylic acid cycle intermediates. None of these organic compounds induced or enhanced germination under the conditions used.

In addition to the eight amino acids, the monovalent cations K^+ and NH_4^+ induced germination of bdellocysts (Table 2). Other cations, both monovalent (e.g., Na^+, Li^+) and divalent (e.g., Ca^{2+}, Mg^{2+}, Ba^{2+}), did not induce germination under the conditions tested. Several anions (NO_3^-, NO_2^-, Cl^-, PO_4^{2-}, SO_4^{2-}) tested were also incapable of inducing germination of bdellocysts.

TABLE 1. *Relationship of the decrease in absorbancy to the decrease in viability and microscopic count during germination*[a]

Time (min)	Decrease in absorbance (%)[b]	Decrease in viable count (%)[c]	Decrease in microscopic count (%)[d]
0	0	0	0
30	17	18	14
60	45	54	51
90	63	79	75
120	72	90	85

[a] Bdellocysts were suspended in PYE (pH 8.0) at 35°C, and samples were taken at the times indicated and added to Triton X-100 (see text).
[b] Initial absorbance, 0.3.
[c] Initial viable count, 1.75×10^8 bdellocysts per ml.
[d] Initial direct count, 2.5×10^8 bdellocysts per ml.

Figure 2 shows the relative germination rates of bdellocysts induced by L-glutamine, K^+, NH_4^+, alone and in combinations. When combined, these compounds had a synergistic effect. The combination of any two of these compounds increased the germination rate above the sum

TABLE 2. *Extent of germination resulting after incubation of bdellocysts with various inducers of germination*

Inducer[a]	Decrease in absorbance after 120 min (%)[b]
PYE	70
L-Glutamine	35
L-Asparagine	24
Glycine	29
L-Phenylalanine	20
L-Serine	21
L-Valine	21
L-Threonine	22
L-Methionine	20
KCl	19
NH_4Cl	24

[a] Inducer concentrations (with the exception of PYE), 0.1 M. Suspensions were adjusted to pH 8.0 with NaOH and incubated at 35°C.
[b] Initial absorbance, 0.3.

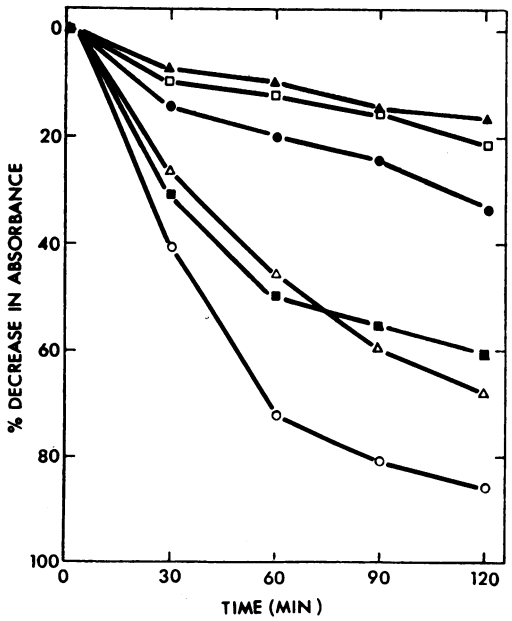

FIG. 2. *Decrease in turbidity of germinating bdellocyst suspensions in defined media. Germination was initiated at 35°C in 0.1 M KCl (▲), 0.1 M NH_4Cl (□), 0.1 M L-glutamine (●), 0.01 M KCl + 0.5 M NH_4Cl (■), 0.1 M L-glutamine + 0.01 M KCl (△), and 0.1 M L-glutamine + 0.01 M KCl + 0.01 M NH_4Cl (GPA medium; ○).*

of those found for the individual compounds. This increased rate of germination was comparable to that for PYE-induced germination. When all three compounds were combined, the resulting rate of germination exceeded that of germination in PYE (Fig. 2). Other combinations of chemicals, with and without the addition of a variety of cations and anions, failed to stimulate the germination rate. The divalent cations Ca^{2+} and Mg^{2+} and the anion NO_2^- inhibited germination at concentrations of 10 mM.

The response of bdellocysts to GPA medium was concentration dependent. The germination rate increased with increasing concentrations of L-glutamine up to 0.1 M, above which there was no further stimulation. The optimum concentration for KCl and NH_4Cl was 0.01 M for both compounds. Increasing the KCl concentration above 0.01 M exerted an inhibitory effect, whereas increasing the NH_4Cl concentration had no detectable effect on the germination rate.

Respiration during germination. The endogenous rate of respiration of bdellocysts before initiation of germination was 0.3 to 0.34 nmol of O_2 per min per 10^9 bdellocysts as previously reported (27). The rate of oxygen uptake began to increase 3 min after the addition of GPA medium to a suspension of bdellocysts (Fig. 3). The rate of respiration continued to increase throughout germination until a maximum rate of 2.49 nmol of O_2 per min per 10^9 cells was reached after 90 min. When bdellocysts were germinated in NH_4^+ and K^+ without L-glutamine, the data on oxygen uptake differed from those for germination in GPA medium (Fig. 3). The rate of oxygen uptake began to increase at the same time (after 3 min) as in the GPA medium, but the subsequent rate of increase was slower, reaching a lower and constant rate of 1.75 nmol of O_2 per min per 10^9 cells after 90 min. This rate of respiration is comparable to the endogenous rate exhibited by vegetative cells of *Bdellovibrio* (27). These results indicate that the germinated bdellocysts are capable of increased respiration (~40%) in the presence of L-glutamine.

Since respiration increased throughout germination, inhibitors of the respiratory chain were examined for their effect on germination of bdellocysts in GPA medium. Figure 4 shows the inhibition of germination by the inhibitors sodium azide and potassium cyanide. These inhibitors almost completely blocked germination at concentrations of 1 mM. These results suggest that bdellocyst germination is an obligately aerobic process. Experiments in which bdellocysts were placed in GPA medium under anaerobic conditions confirmed that germination does not occur anaerobically. When there was a shift from aerobic to anaerobic conditions or when cyanide or azide was added after germination had begun (30 min after addition of germination inducers), germination was shut down almost immediately. Inhibition by anaerobic conditions was reversed when aerobic conditions were restored.

Effect of inhibitors of macromolecular synthesis on germination. Figure 5 shows the effects of various inhibitors of macromolecular

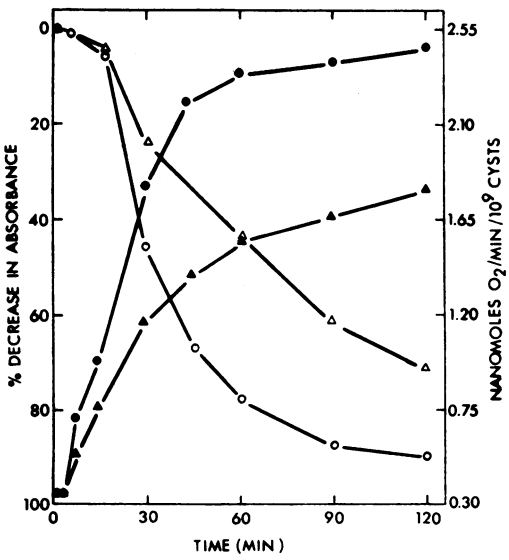

FIG. 3. *Oxygen uptake by bdellocysts germinating in GPA medium (●) and in 0.5 M NH_4Cl + 0.01 M KCl (▲) at 30°C. The rate of germination was monitored by the decrease in absorbance in GPA medium (○) and in 0.5 M NH_4Cl + 0.01 M KCl (△).*

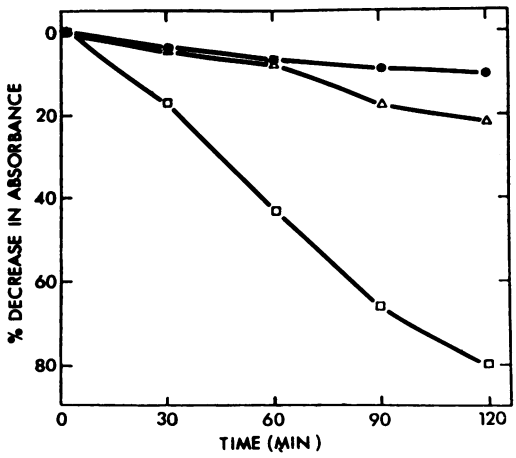

FIG. 4. *Effect of inhibitors of respiration on germination of bdellocysts. Germination was initiated in GPA medium at 35°C to which 1 mM NaN_3 (△) and 1 mM KCN (●) had been added. The control (□) contained no inhibitor.*

synthesis on germination when added simultaneously with GPA medium to a suspension of bdellocysts. Inhibitors of DNA synthesis (nalidixic acid) and peptidoglycan synthesis (penicillin G) had no effect on the rate of germination of bdellocysts. However, as the bdellocysts germinated in the presence of penicillin, stable spheroplasts were produced, indicating that synthesis and/or turnover of peptidoglycan was occurring during or immediately after germination.

Bdellocyst germination was severely inhibited by both chloramphenicol and streptomycin, suggesting a requirement for protein synthesis. The partial inhibition of germination by rifampin during the first 60 min, followed by almost complete inhibition, indicates that RNA synthesis was also required for germination. The bdellocysts may have been initially impermeable to rifampin, resulting in a partial inhibition during the early stages of germination. When bdellocysts were preincubated for 30 min at 35°C in rifampin before the addition of GPA medium, complete inhibition of germination was observed.

When bdellocysts were exposed to germination conditions for 30 min prior to the addition of inhibitors, protein synthesis inhibitors caused an almost immediate cessation of germination. Rifampin also exerted an inhibitory effect after a lag period of 30 min.

Effect of heat shock on germination. After exposure to 50°C for 2 min, neither the rate nor final extent of germination of bdellocysts in PYE was altered significantly. However, as shown in Fig. 6, the rate of L-glutamine-induced germination was altered significantly by the gentle heat shock. Whereas germination of non-heat-shocked bdellocysts in L-glutamine was very slow, heat-shocked bdellocysts germinated at a rate comparable to that of PYE-induced germination. This increase in the rate of germination in L-glutamine was not due to spontaneous germination brought on by the heat shock, because heat-shocked bdellocysts did not germinate in Tricine buffer alone. Heat-shocked bdellocysts also exhibited changes in rates of germination in NH_4Cl or KCl alone, as shown in Fig. 6. Shocked bdellocysts germinated in NH_4Cl and KCl at rates comparable to germination in L-glutamine, and at much faster rates than exhibited by unheated cysts. When all three germination inducers were combined (GPA medium), unheated bdellocysts germinated at the same rate and to the same extent as did heated bdellocysts.

Heat-shocked bdellocysts could also be germinated by a wider range of compounds than could unheated bdellocysts. In addition to the L-amino acids, K^+, and NH_4^+, UMP, TMP, and GMP induced the germination of heat-shocked

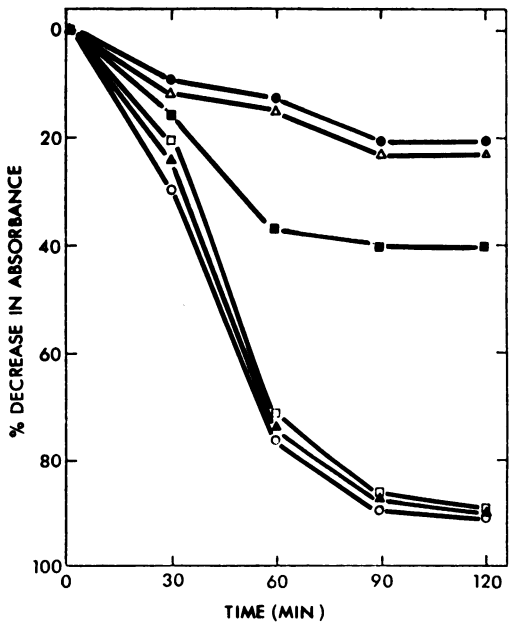

FIG. 5. *Effect of inhibitors of macromolecular synthesis on germination was initiated in GPA medium to which 100 μg of nalidixic acid per ml (○), 100 μg of penicillin G per ml (□), 50 μg of chloramphenicol per ml (△), 50 μg of streptomycin per ml (●), and 30 μg of rifampin per ml (■) had been added. The control (▲) contained no inhibitor.*

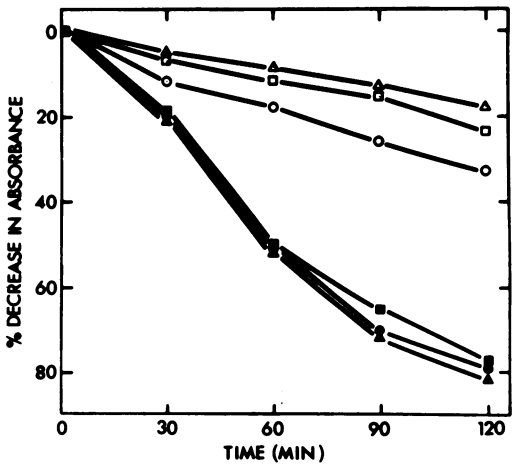

FIG. 6. *Effect of heat activation on the germination of bdellocysts. Bdellocysts were heated at 50°C for 2 min and suspended at 35°C in 0.1 M L-glutamine (●), 0.1 M NH_4Cl (■), and 0.1 M KCl (▲). Controls were parallel suspensions of non-heat-shocked cysts germinated in 0.1 M L-glutamine (○), 0.1 M NH_4Cl (□), and 0.1 M KCl (△).*

cysts at the same rate as did L-glutamine. Heat shock, therefore, appears to alter the bdellocysts in some unknown way, changing their response to individual inducers, but not to a combination of inducers or to complex media. This alteration of bdellocysts by heat will hereafter be termed "activation." We are using the term activation here as defined for spores by Gould and Hurst (10): i.e., "activation results from some treatment which does not itself initiate germination, but afterwards allows spores to germinate more rapidly or more completely or both."

Optimum conditions for heat activation. The optimum temperature and times of heating for bdellocyst activation were determined by heating bdellocyst suspensions in distilled water (pH 8.0) at 40, 45, 50, and 55°C. Samples were removed at intervals and assayed for germinability in 0.1 M L-glutamine. The results of these experiments are shown in Fig. 7. These data show that optimum activation due to heat shock occurred when bdellocysts were heated at 50°C for 2 to 5 min. Heating for longer periods resulted in a small decrease in activation. This decrease was not substantial and was not the result of decreased viability since bdellocysts have been shown to maintain 100% viability when heated to 50°C for up to 30 min (27). Bdellocysts were also activated by heating to 45°C for 20 to 80 min, but to a lesser extent than by heat shock at 50°C for 2 to 5 min.

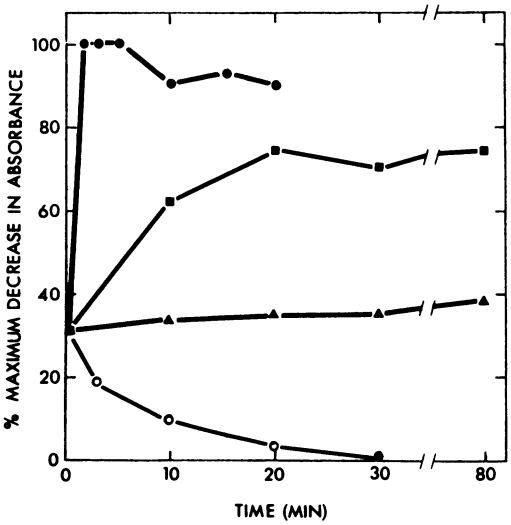

FIG. 7. *Effect of time and temperature of heat activation on the germination of bdellocysts. Bdellocyst suspensions were heated at 40 (▲), 45 (■), 50 (●), and 55°C (○) at the times indicated and germinated in 0.1 M L-glutamine at 35°C. Results are expressed as the percent maximum germination after 90 min. Maximum germination refers to the maximum decrease in absorbance of a germinating suspension after 90 min.*

Heating at temperatures above 50°C decreased germinability of bdellocysts. This decrease was presumably due to a decrease in viability of the cysts. Temperatures below 45°C appeared to have no effect on germinability (Fig. 7).

To determine the effect of pH on heat activation, bdellocysts were heat shocked at 50°C for 2 min in buffer at pH levels from 5.5 to 10.5 (for pH levels from 5.5 to 8.5, 0.02 M tris-(hydromethyl)aminomethane-maleate buffer was used; for pH levels from 8.5 to 10.5, 0.02 M glycine buffer was used). After heat shock, the cysts were harvested by centrifugation and resuspended in 0.1 M L-glutamine (pH 8.0), and germination was assayed. The results of these experiments indicated that heat activation occurred over a pH range of 6.5 to 9.5, with an optimum pH of activation between 8.0 and 9.0.

When bdellocysts were heat shocked at 50°C for 2 min and then incubated at 25°C in distilled water for extended periods (8 h or longer), the effects of activation were lost, and bdellocysts germinated in L-glutamine at a rate comparable to unheated bdellocysts. These "deactivated" bdellocysts, when reheated to 50°C for 2 min, exhibited germination rates similar to activated bdellocysts.

Effect of germination temperature and pH. Germination appeared to be quite sensitive to temperature of incubation. The effect of incubation temperature was identical for unheated and heat-shocked cysts. There was an increase in germination rate accompanying increasing temperatures of up to 35°C, followed by a sharp decrease when this temperature was exceeded.

The effect of pH on the germination of bdellocysts was determined on heated and unheated bdellocysts in 0.1 M L-glutamine and GPA medium, respectively. The pH of the media was adjusted with either HCl or NaOH to give pH values ranging from 5.0 to 9.5. Germination was favored by alkaline pH values, with maximum germination occurring at pH 8.0 for both the heat-shocked and non-heat-shocked cysts.

Effect of bdellocyst age. There appeared to be no loss in germinability of bdellocysts during periods of prolonged storage. Bdellocysts stored as dense suspensions (~2 × 10^{10} bdellocysts per ml) in distilled water at 4°C for up to 8 months germinated in GPA medium at nearly the same rate and to the same extent as did freshly harvested bdellocysts. These storage conditions, however, altered the characteristics of heat activation. Heating at 50°C no longer activated the "aged" bdellocysts to the same extent as it did freshly harvested bdellocysts.

Although activation was decreased, temperatures of 50°C did not have an adverse effect on either viability or germinability as compared with unheated bdellocysts. Heat shocking bdellocyst suspensions at 45°C for 15 to 45 min activated the aged bdellocysts. Aged bdellocysts activated at 45°C germinated at a rate comparable to that of freshly harvested bdellocysts activated at 50°C. It appeared that the optimum temperature for heat activation had shifted from 50 to 45°C.

DISCUSSION

It was essential to develop a rapid and suitable assay of bdellocyst germination to study the germination process. The assay reported here, utilizing Triton X-100 to lyse germinated cells, enabled us to determine the rate and extent of germination of bdellocysts. The results show that the rate and extent of the decrease in turbidity of a germinating suspension after addition of Triton X-100 agree well with the decrease in viable cells in the suspension and the direct microscopic count of bdellocysts. Germination is defined as that point at which bdellocysts become susceptible to lysis by Triton X-100, and the time period preceding sensitivity to Triton X-100 is defined as the lag period. Germination curves generated by this assay therefore describe the distribution of lag times exhibited by the cysts in the suspension.

Nutritive germination inducers for endospores of bacilli and clostridia are usually amino acids or nucleosides (7). Our results indicate that certain amino acids can also serve as inducers of germination of bdellocysts, with L-glutamine being the best single inducer. The rate of germination of bdellocysts in L-glutamine was lower than that in PYE, suggesting that additional factors may enhance the germination rate. A similar situation exists for endospores, in which combinations of compounds will induce a more rapid and complete germination than will a single inducer (9).

Germination of bdellocysts was also promoted by both K^+ and NH_4^+. The germination rate of bdellocysts was potentiated by combining these germinants with one another or with L-glutamine. The influence of cations on the germination rates of some *Bacillus* spores has also been reported (6, 9, 19, 20). The mechanism(s) by which these germination inducers act in bdellocysts is unknown. Perhaps, as suggested for endospores, the increase in ion concentration may serve to release and/or activate degradative enzymes (9) or effect the release of some bound primary endogenous germinant (19). L-Glutamine, however, may act as a utilizable substrate, thereby promoting germination, since in its presence respiration was increased above endogenous levels.

Based on the variety of effective germinants observed, we suggest that bdellocysts are endowed with multiple mechanisms for germination; such is the case for endospores. As suggested for spores (7, 12, 16, 17), this diversity of inducers of germination may reflect either a basic mechanism for germination capable of being activated by several routes, or multiple pathways of germination.

The rate of germination of aerobic spores has been shown to be influenced by the concentrations of the germinating agents (3, 19, 22, 29). Similarly, when L-glutamine, K^+, and NH_4^+ were used singly as inducers, their concentration affected the rate of germination of bdellocysts. In GPA medium, only the increased concentration of L-glutamine appeared to increase the rate of germination.

Oxygen-uptake experiments of germinating bdellocysts revealed that respiration began to increase within a few minutes after the addition of germinants. The rate of uptake increased continually, reaching a maximum rate at about the same time that maximum germination had occurred. Similarly, during endospore and *Azotobacter* cyst germination, there is an abrupt onset of respiration with an increase in the rate during swelling (8, 21). However, once *Bacillus* spore germination has been initiated, it will proceed in the absence of oxygen (7). Bdellocysts appear to require oxygen throughout germination.

Germination of endospores is a degradative process not involving net macromolecular synthesis (7, 26). Cysts of *Azotobacter,* however, seem to require protein and RNA syntheses for germination to occur (21, 24). Likewise, in spores of *Streptomyces,* RNA synthesis occurs immediately upon addition of germinants (13). Based on the data which show that inhibitors of protein and RNA syntheses also inhibit germination, it appears that bdellocysts require synthesis of both RNA and protein to germinate. Inhibition of germination by rifampin and chloramphenicol has been shown for *Streptomyces* spores and *Azotobacter* cysts (13, 24). The DNA synthesis inhibitor nalidixic acid, however, had no effect on bdellocyst germination, indicating either that synthesis of DNA is not required for germination, or that the antibiotic did not penetrate into the bdellocysts. The addition of penicillin did not inhibit germination, but did result in the production of stable spheroplasts, suggesting that peptidoglycan synthesis and/or turnover was occurring during germination. The production of stable spheroplasts in the presence of penicillin has been shown to occur in some bdel-

lovibrios (M. F. Thomashow and S. C. Rittenberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, K115, p. 166).

We conclude that germination of bdellocysts involves degradative processes (e.g., dissolution of the outer layer [28]) and biosynthetic processes as well. This also appears to be the case for *Azotobacter* cysts and *Streptomyces* spores. Further study is necessary to determine whether the syntheses of protein, RNA, and peptidoglycan during germination are turnover or de novo syntheses utilizing exogenous substrates or endogenous storage materials.

Germination of endospores has been referred to as a "trigger" reaction (17), because once begun, germination continues in the presence of inhibitors (11). Bdellocyst germination is not a trigger reaction because inhibitors added after initiation of germination exerted their effect as strongly as when added prior to initiation.

The activation of resting cells by sublethal heating has been demonstrated for endospores (18) and for *Streptomyces* spores (14). However, heat activation has not been demonstrated for *Azotobacter* cysts (24) or microcysts of *Myxococcus* (23). Our results clearly show that heat shocking activates bdellocysts. Like some endospores (18), bdellocysts require heat shock to germinate on some substrates (e.g., ribonucleoside monophosphates), but not on others, such as glutamine. In addition, the activation of both endospores and bdellocysts appears to be reversible. However, the temperatures and heating times required for activation differ for bdellocysts and endospores. This situation may reflect a fundamental difference between the mechanisms of heat activation in the two systems.

Bdellocysts share some of the characteristics found for other and more thoroughly studied microbial resting cells, but they do not appear to possess the characteristics of any one type. For example, the resistance properties of bdellocysts (27) are more similar to those exhibited by *Azotobacter* and *Myxococcus* cysts than to those of endospores. However, the nutritional requirements for germination and heat activation of bdellocysts are characteristics shared with endospores and *Streptomyces* spores, whereas the apparent requirement for macromolecular synthesis during bdellocyst germination is shared with *Azotobacter* and *Myxococcus* cysts and *Streptomyces* spores, but not with endospores.

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