APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1989, p. 1380–1385
0099-2240/89/061380-06$02.00/0

Vol. 55, No. 6

Germination of *Clostridium cylindrosporum* Spores on Medium Containing Uric Acid

M. SMITH* AND C. SULLIVAN†

Western Regional Research Center, U.S. Department of Agriculture, 800 Buchanan Street, Berkeley, California 94710

Received 25 October 1988/Accepted 1 March 1989

Clostridium cylindrosporum **spores germinated rapidly under reducing conditions when bicarbonate, uric acid, and calcium were present. Germination rates on 10 mM urate increased with increasing Ca^{2+} (maximum rate at 5 mM Ca^{2+} or greater). Germination rates on urate (limiting Ca^{2+}) increased with increasing urate concentrations to 10 mM urate. At 10 mM Ca^{2+}, germination rates reached a maximum at 1 mM urate and remained constant thereafter. Cations (Na^+, K^+, Li^+, and Mg^{2+}), purines, purine analogs, and EDTA inhibited germination at limiting calcium concentrations but not (except for 10 mM adenine) at 10 mM Ca^{2+}. Methyl viologen or formate did not inhibit germination. Germination was not observed in solutions containing xanthine, hypoxanthine, caffeine, theophylline, 6,8-dihydroxypurine, adenine, allopurinol, formate, glycine, or acetate, even though some of the purines are growth substrates.**

Clostridium cylindrosporum is an obligately purinolytic anaerobe, sporulating efficiently under appropriate conditions (3, 21) and fermenting uric acid (and some purines) to ammonia, carbon dioxide, acetic acid, and formate (3). It was isolated from bird droppings, of which uric acid is an important constituent. Germination of *C. cylindrosporum* spores has not been described previously; knowledge of uric acid-triggered germination is based on experiments with *Bacillus fastidiosus*, a strictly aerobic species that grows solely on uric acid (2, 22). In *B. fastidiosus* spores, uricase (a key enzyme of uric acid catabolism) was implicated in the triggering of spore germination by uric acid (22). Absence of early release of spore cortex fragments and metabolic experiments suggested that aerobic spore germination on urate was a metabolic rather than a purely physical or hydrolytic process. A comparison of anaerobic germination on urate with the reported aerobic germination might be useful in assessing the general importance of key metabolic enzymes in uric acid germination.

In this paper, we describe conditions that give consistently rapid and complete germination of *C. cylindrosporum* spores on uric acid. We show that *C. cylindrosporum* spore germination differed from *B. fastidiosus* spore germination in important respects: calcium was required, and it apparently acted by modifying the spore-triggering response to urate. Although calcium and other ions are known to influence the triggering of spore germination in other species (1, 6, 12–15, 19), previous studies had not indicated a role in initiating spore germination with urate. We also show that a range of purine bases, xanthine dehydrogenase inhibitors, monovalent cations, and EDTA inhibited germination. These inhibitions could be overcome or reversed by increasing the concentration of calcium or urate alone; it was not necessary to increase both calcium and urate concentrations.

MATERIALS AND METHODS

Bacterial strains and culture media. *C. cylindrosporum* HC-1 was obtained from H. A. Barker, University of California, Berkeley. Cultures were grown in a defined medium based on that of Schiefer-Ullrich et al. (23) and contained 10 mM sodium urate, 4 mM K_2HPO_4, 4 mM Na_2HPO_4, 140 μM $MgSO_4$, 100 nM sodium selenite, 21 μM ferric sodium EDTA, 29 μM $CaCl_2$, 20 nM biotin, 44 μM thiamine hydrochloride, 20 mM $KHCO_3$, and 0.0001% (wt/vol) resazurin in glass-distilled water. For some experiments, $MnCl_2$ was added to a concentration of 50 μM (2). Sodium urate was prepared from the free acid (Sigma Chemical Co., St. Louis, Mo.) plus NaOH and contained equimolar concentrations of sodium and urate. The medium was prepared anaerobically in 160-ml serum bottles under 80% N_2–20% CO_2 as described previously (1, 24).

Chemicals and reagents. Ferric sodium EDTA, imidazole, Tris, dipicolinic acid, and purines were purchased from Sigma. Ultrapure sucrose and ultrapure Tris were purchased from Schwartz/Mann, Cambridge, Mass. Reagents were prepared from reagent-grade chemicals when possible and from glass-distilled water. Glassware for reagents and medium preparation was rinsed with 6 N HCl followed by glass-distilled water before use.

Preparation of spores. Cultures for spore preparation were grown in 20-liter carboys containing 10 liters of defined medium prepared as described above. The medium was prepared aerobically without a reducing agent and then plugged with cotton plugs and autoclaved for 1 h. After autoclaving, the carboys were cooled to ambient temperature under a stream of 80% N_2–20% CO_2 and stoppered with sterile black rubber stoppers. The stoppers were then wired in place, and the headspace was flushed with a nitrogen-carbon dioxide mixture through sterile needles inserted through the stoppers. After the headspace had been flushed, sodium bicarbonate and sodium sulfide (reducing agent) were added to final concentrations of 60 and 600 μM, respectively, by injection of concentrated, sterile stock solutions through the stoppers. When the medium became colorless because of reduction of the resazurin, it was inoculated with 5×10^8 spores of heat-shocked (70°C for 20 min) *C. cylindrosporum* and then incubated at 35°C until growth and lysis of sporangia were complete (5 to 7 days).

Spores were harvested with a Pellicon cell harvester (Millipore Corp., Bedford, Mass.) or by continuous-flow centrifugation (12,000 × *g*) with an RC5C centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.). Harvested spores were

* Corresponding author.
† Present address: Department of Chemistry, University of California, Santa Cruz, CA 95064.

washed three times with glass-distilled water and then cleaned by centrifugation (30,000 × *g* for 1 h) through a 60% (wt/vol) solution of ultrapure sucrose in a Sorvall RC5C centrifuge or by mechanical removal of the debris that sedimented at the top of the spore pellet (6). Spores cleaned on sucrose were given an additional three washings in distilled water. Spore suspensions were examined by phase-contrast microscopy for the absence of sporangia, vegetative cells, and germinated spores (less than 10%). The final yield of spores from 20 liters of culture was 2 to 3 g (wet weight). Clean spore suspensions were stored in glass-distilled water at 4°C until used.

Preparation of calcium-soaked spores. Freshly harvested and washed spore suspensions (native spores) were treated with calcium by incubation in an equal volume of 20 mM calcium acetate for 2 h at 60°C followed by incubation overnight at 4°C. The suspensions were then washed three times with glass-distilled water and stored at 4°C until used. For some experiments, spores were soaked in 10 mM calcium acetate at ambient temperature instead of 60°C. Native spores heat treated for 2 h at 60°C without added calcium germinated more rapidly than native spores that had not been heated, but the addition of calcium always stimulated the rate of germination. Spores that had been soaked in a solution of a calcium salt are referred to below as calcium spores.

Germination experiments. Germination experiments were conducted with stoppered 13-mm test tubes containing 10 ml of germination solution, consisting of 20 mM imidazole or Trizma base (neutralized to pH 7.0 with HCl), 6 mM $NaHCO_3$, and 0.2 mM Na_2S plus the indicated concentrations of uric acid or other additions. (As a precaution in some experiments, Schwartz/Mann ultrapure Tris was substituted for Sigma Tris. We observed no differences in our results from the substitution.) All incubations were performed in duplicate, and all experiments were repeated at least once (usually twice) on separate days.

Germination solutions were prepared aerobically, with all ingredients added except sodium bicarbonate and sodium sulfide. The solutions were then sparged for 20 min with nitrogen and transferred to a model A anaerobic glove box (Coy Laboratory Products, Ann Arbor, Mich.) with a 95% N_2–5% H_2 atmosphere, where they were stored until used. For use, solutions were dispensed (10-ml portions) inside the glove box into test tubes, which were stoppered with butyl rubber stoppers and then removed from the glove box. Sodium bicarbonate, sodium sulfide, and spores (final density, 30 to 60 Klett units; no. 54 filter) were added by injection with a needle and syringe. Initial absorbances were read, and the tubes were placed in a 37°C water bath. The tubes reached a temperature within 1°C of the final temperature 2.5 min after the start of incubation. The final pH after the addition of bicarbonate and sulfide was 7.2 to 7.3. Germination was monitored at intervals by loss of absorbance in a Klett-Summerson colorimeter with a green (no. 54) filter. Absorbance measurements with germination solutions containing reduced methyl viologen were done by using a spectrophotometer (model 340; Sequoia-Turner Inc., Mountain View, Calif.) set at a wavelength of 445 nm. Germination of spore suspensions was confirmed by phase-contrast microscopy. The microscopic appearance of germinated spores was generally phase-gray.

RESULTS

Germination with uric acid and requirement for calcium. *C. cylindrosporum* spores germinated optimally in the presence of urate (0.1 to 10 mM), bicarbonate, and calcium salts and under reducing conditions. Urate was an absolute requirement. CO_2 (or bicarbonate) stimulated germination rates (Fig. 1) even in the absence of added reducing agents. Sulfide was normally used as a reducing agent, but 2 mM cysteine hydrochloride (pH 7.0), sodium dithionite, dithiothreitol, thioglycolate, or mercaptoethanol also stimulated germination. Aeration of the germination solutions in the absence of reducing agent prevented germination. Spores presoaked in calcium acetate at ambient temperature or at 60°C (calcium spores) could substitute for native spores to which calcium solutions had been added. Heat treatment without calcium also stimulated germination rates, but did not eliminate stimulation by calcium.

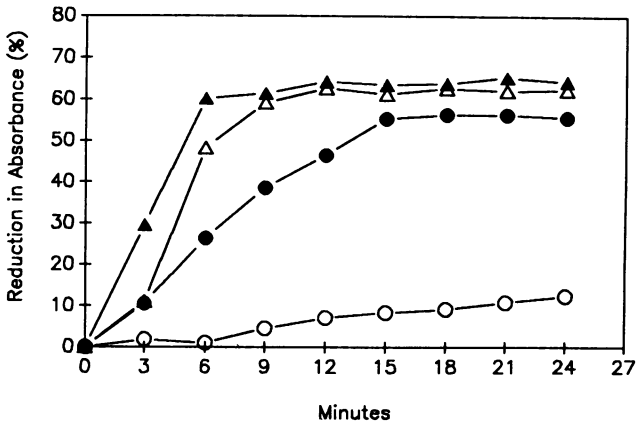

FIG. 1. Effect of sulfide and/or bicarbonate addition on germination. Germination solutions were prepared under nitrogen and contained 20 mM imidazole hydrochloride (pH 7.0) and 10 mM sodium urate. Calcium spores were used as the inoculum. Additions to germination solution were as follows: none (○), 6.0 mM $NaHCO_3$ (●), 0.2 mM Na_2S (△), and 0.2 mM Na_2S plus 6.0 mM $NaHCO_3$ (▲).

The effectiveness of calcium in stimulating germination rates was somewhat reduced in spores produced in culture media containing 50 μM $MnCl_2$ compared with media without added $MnCl_2$. However, manganese did not confer a germination requirement for heat shocking, as it did for *B. fastidiosus* (2), or eliminate stimulation by calcium.

Effect of calcium concentration on germination. Germination rates by native spores in 10 mM urate increased with increasing calcium chloride concentrations to 10 mM $CaCl_2$ (Fig. 2). Control incubations without added calcium salts did not show a loss of absorbance during the incubation period.

Calcium spores (Fig. 3) germinated rapidly in the presence of 0.1 to 10 mM sodium urate and 10 mM $CaCl_2$, with maximum rates at 1 mM urate or above. Germination did not occur within 24 h without sodium urate. We do not know why germination rates were slightly higher at 100 μM urate than at 500 μM urate; the two rates might reflect a negatively cooperative interaction between calcium and urate or a systematic error. When calcium-soaked spores (without added 10 mM $CaCl_2$) were used, germination did not occur at sodium urate concentrations of 500 μM or less even after 24 h. Germination rates were maximum at 5 mM urate or above. For these experiments, sodium concentrations were held constant at 16.5 mM because sodium inhibited germination.

Inhibition of germination by sodium ions. Germination of calcium spores in solutions containing 10 mM urate and buffered with sodium phosphate (5 mM, pH 7.0) was slow. Germination did not occur after 24 h in solutions buffered

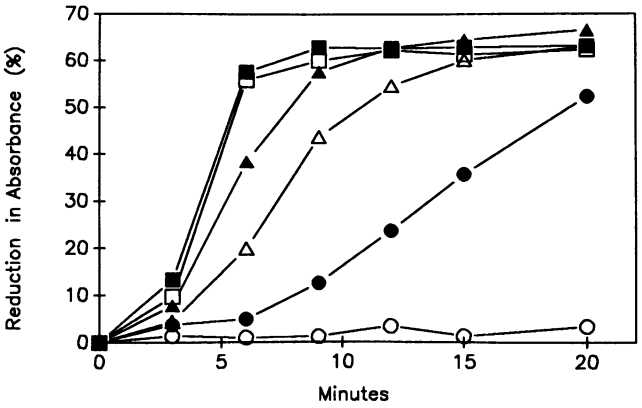

FIG. 2. Calcium requirement for germination. $CaCl_2$ was tested at 0 mM (○), 0.1 mM (●), 0.5 mM (△), 1 mM (▲), 5 mM (□), and 10 mM (■). Germination solution consisted of 10 mM sodium urate, 20 mM imidazole hydrochloride (pH 7.0), 6 mM sodium bicarbonate, and 0.2 mM sodium sulfide plus the indicated concentrations of calcium chloride. Native spores were used as the inoculum.

with 20 mM sodium or potassium phosphate (pH 7.0). However, spores germinated rapidly in solutions buffered over the pH range 6.5 to 9.0 with 20 mM imidazole hydrochloride or 20 mM Trizma hydrochloride buffer. Germination was most rapid at pH 6.5 to 8.0 and slowed slightly at pH 8.5 to 9.0. The absence of germination in phosphate buffer probably resulted at least partly from inhibition by sodium (Fig. 4). NaCl, KCl, LiCl, and $MgCl_2$ (all at 20 mM) inhibited germination in imidazole-buffered germination solutions.

NaCl at concentrations ranging from 0 to 50 mM inhibited the germination of calcium spores (Fig. 4). For this experiment, the uric acid in the germination solution was neutralized with ultrapure Tris base rather than NaOH. The solution was saturated with carbon dioxide; sodium bicarbonate and sodium sulfide were omitted to keep sodium at the

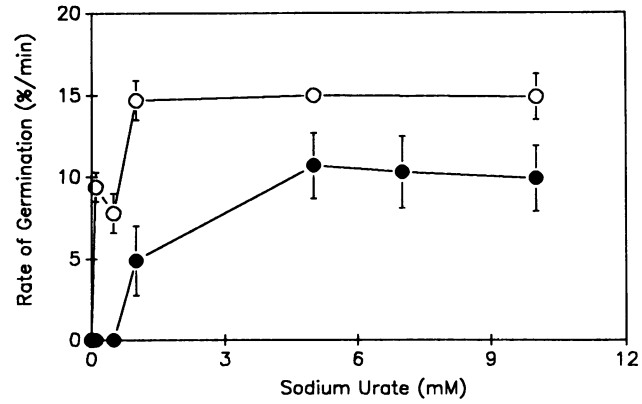

FIG. 3. Germination at various uric acid concentrations. Native spores plus 10 mM $CaCl_2$ (○) and calcium-soaked spores without added $CaCl_2$ (●) were used. Germination solution consisted of 20 mM imidazole hydrochloride (pH 7.0), 6 mM sodium bicarbonate, 0.2 mM sodium sulfide, and 0, 0.1, 0.5, 1, 5, 7, or 10 mM sodium urate. The sodium concentration was kept constant at 16.5 mM by addition of NaCl. Incubation was carried out at 37°C for 15 min, with readings at 3-min intervals. (Native spores without calcium failed to germinate.) The error bars represent standard deviations of four to six determinations.

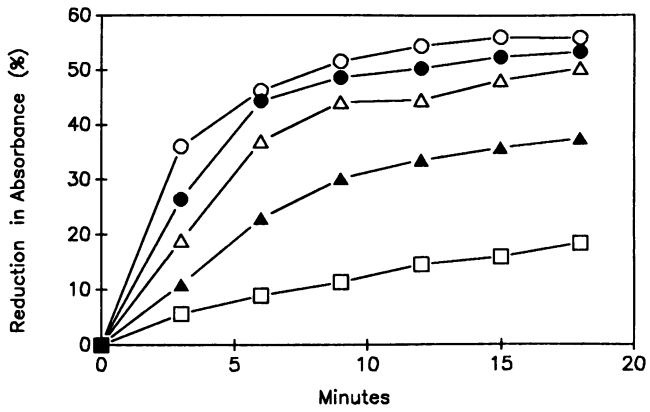

FIG. 4. Inhibition of calcium spores by sodium. NaCl was tested at 0 mM (○), 10 mM (●), 20 mM (△), 30 mM (▲), and 50 mM (□). Germination solution consisted of 5 mM Tris urate (pH 6.5) saturated with CO_2 and without sodium sulfide or sodium bicarbonate.

lowest possible concentration. Little inhibition occurred at 10 mM NaCl, but inhibition increased with increasing NaCl levels to the highest concentration tested. Inhibition of germination rates by sodium in our usual germination solutions (buffered with 20 mM Tris hydrochloride or 20 mM imidazole hydrochloride at pH 7.0 without saturating CO_2 levels) gave results similar to those in Fig. 4.

Inhibition of germination of calcium spores by sodium could be overcome by exogenously adding calcium chloride to the germination solutions (Fig. 5). Without added calcium chloride, 30 to 150 mM NaCl strongly inhibited the rate of germination; inhibition decreased when calcium chloride at 1 mM or above was added.

Germination with purines. Table 1 shows a list of substances tested as possible germination triggers with calcium spores. At least some of these substances were expected to act as germination triggers on the basis of substrate utilization by exponential-phase cultures and cell suspensions (2). Other substances (e.g., formate, glycine, and acetate) are intermediates or end products of uric acid metabolism by vegetative cells. Only uric acid served as a germination trigger among the compounds we tested; germination was

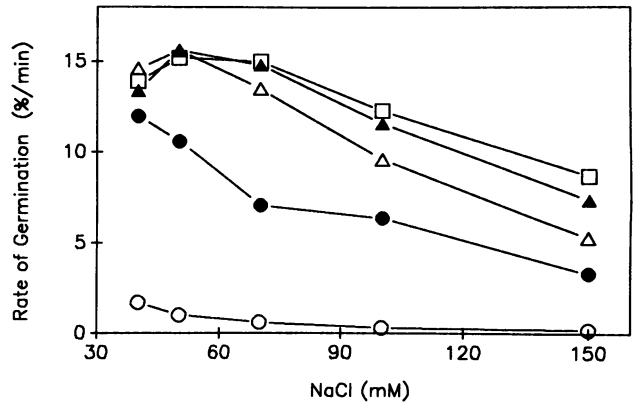

FIG. 5. Reversal by calcium of sodium inhibition of germination. $CaCl_2$ was tested at 0 mM (○), 1 mM (●), 3 mM (△), 5 mM (▲), and 10 mM (□). Germination solution consisted of 10 mM sodium urate, 20 mM imidazole hydrochloride (pH 7.0), 6 mM $NaHCO_3$, and 0.2 mM Na_2S plus calcium chloride and sodium chloride as indicated. A calcium spore inoculum was used.

TABLE 1. Compounds tested as germination triggers[a]

Purine[b]	Concn (mM)	% Reduction in absorbance in 24 h
No additions		4.7
Uric acid	1	63.2
Adenine	10	1.8
Xanthine	1	7.8
Xanthine plus glycine	1 and 10	6.4
Hypoxanthine	5	3.8
Caffeine	10	2.9
Theophylline	10	3.2

[a] Germination solution consisted of 20 mM imidazole hydrochloride (pH 7.0), 6 mM sodium bicarbonate, and 0.2 mM sodium sulfide plus the indicated concentration of purine. Calcium spores were used as the inoculum. Xanthine and hypoxanthine were neutralized with NaOH to dissolve them.

[b] Other substances tested as germination triggers were allopurinol (saturated at 37°C), sodium acetate (10 mM), sodium formate (10 mM), glycine (10 mM), and 6,8-dihydroxypurine (saturated at 37°C). Incubation times at 37°C and percent reduction in absorbance at the end of incubation were as follows: allopurinol (2 h), 5.7%; 6,8-dihydroxypurine (24 h), 5.1%; glycine (19 h), 2.0%; sodium acetate (19 h), 2.8%; sodium formate (19 h), 3.1%.

TABLE 3. Effect of methyl viologen on germination rates[a]

Methyl viologen concn (mM)	Germination rate[b] (% reduction in absorbance/min) with:	
	Oxidized methyl viologen	Reduced methyl viologen
0	9.3	7.2
0.01	10.2	
0.1	13.7	7.0
1	12.0	12.1
10	11.9	

[a] Germination solution consisted of 20 mM imidazole hydrochloride (pH 7.0), 100 μM $CaCl_2$, 10 mM sodium urate, 0.2 mM Na_2S, and 6.0 mM $NaHCO_3$. Oxidized methyl viologen was added to the indicated concentrations. Solutions containing reduced methyl viologen were prepared in the same way, except that 10 mM sodium dithionate was also added to reduce the methyl viologen and germination rates were compared with those of controls containing 10 mM sodium dithionate but without methyl viologen. Calcium-soaked spores were used as the inoculum.

[b] The standard deviation for rate of germination was 0.6% reduction in absorbance per min.

not observed after 24 h with any of the other substances, even if additional calcium chloride (10 mM) was provided.

Although the substances we tested were inert as germination triggers, they were often active as inhibitors of germination with uric acid. All of the purines tested inhibited germination of calcium spores on 1 mM sodium urate (Table 2). These low (1 mM) urate concentrations were used to provide a high level of sensitivity to possible analog inhibitors. Calcium reversed the inhibition; with 10 mM calcium chloride present, only 10 mM adenine continued to inhibit germination. Formate (10 mM) also did not inhibit germination.

Both the oxidized and reduced forms of methyl viologen were tested for their ability to interfere with germination on urate (Table 3) to eliminate possible oxidation-reduction steps in germination. The rate of germination increased slightly with increasing oxidized methyl viologen concentration. Prereduced methyl viologen (in the presence of 10 mM sodium dithionite) also slightly increased the rate of germination relative to controls without methyl viologen but containing 10 mM dithionite.

Inhibition also depended on the concentration of sodium urate (Table 4). With 1 mM sodium urate as the germination trigger, 1 mM adenine, xanthine, hypoxanthine, caffeine, or theophylline inhibited germination by calcium spores. With the exception of 10 mM adenine, germination was not inhibited by purines in 10 mM sodium urate, even without exogenously added calcium. Separate experiments in which calcium-soaked spores were pretreated with 10 mM theophylline or 10 mM adenine before inoculation into a 1 mM urate solution showed that inhibition was reversible; pretreated spores germinated in uric acid without a lag, suggesting that the inhibitors did not owe their inhibitory properties to metabolism of an inactive precursor to an active form. Inhibition of germination on 10 mM urate by 10 mM adenine suggests that adenine was a more potent inhibitor than the other inhibitors tested.

EDTA at 1 mM also inhibited germination, and the inhibition was also overcome by adding calcium. This suggests that inhibition by cations and purine bases resulted from displacement or sequestering of calcium.

TABLE 2. Inhibitors of germination with uric acid and reversal of inhibition by increased calcium levels[a]

Addition	Concn (mM)	% Reduction in absorbance in 15 min with:	
		No added $CaCl_2$	10 mM $CaCl_2$ added
None		58.2	
Adenine	1	3.1	65.1
	10	1.8	2.5
Xanthine	1	3.5	54.7
Hypoxanthine	1	3.1	
	5	4.7	69.0
Theophylline	1	2.4	
	10	1.1	57.6
Caffeine	1	7.5	
	10	4.1	66.1
6,8-Dihydroxypurine	Saturated	27.7	77.4
Oxypurinol	1	1.3	47.7
Allopurinol	1	6.2	45.1
Sodium formate	10	49.8	48.2
EDTA	1	2.8	57.0

[a] Germination solution consisted of 1 mM sodium urate, 20 mM imidazole hydrochloride (pH 7.0), 6 mM sodium bicarbonate, and 0.2 mM sodium sulfide plus the indicated additions. An inoculum of calcium spores was used. Experiments with EDTA were carried out in 10 mM urate instead of 1 mM urate. Incubation was carried out at 37°C.

TABLE 4. Inhibitors of germination with uric acid and reversal of inhibition by increased uric acid levels[a]

Addition	Concn (mM)	% Reduction in absorbance in 1 h with:	
		1 mM sodium urate	10 mM sodium urate
None		62.7	72.5
Adenine	1	3.1	54.4
	10	3.1	2.0
Xanthine	1	10.6	59.9
Hypoxanthine	5	4.3	71.4
Theophylline	10	20.4	61.2
Caffeine	10	3.7	69.8
Allopurinol	1	6.2	46.2

[a] Germination solution consisted of 20 mM imidazole hydrochloride (pH 7.0), 6 mM sodium bicarbonate, and 0.2 mM sodium sulfide plus the indicated concentrations of sodium urate and additions. Calcium spores were used for inoculation.

DISCUSSION

C. cylindrosporum spore germination resembled that of *B. fastidiosus* in that uric acid, but not structurally related purines, triggered germination (21). *C. cylindrosporum* spores differed from *B. fastidiosus* spores in that *C. cylindrosporum* required Ca^{2+} for rapid germination. Other properties exhibited by *C. cylindrosporum*, but not by *B. fastidiosus*, include calcium-reversible inhibition of germination by low cation (Na^+, K^+, Li^+, Mg^{2+}) concentrations, interference by purines with germination on uric acid, and absence of a germination heat shock requirement following production of spores in 50 μM manganese (2).

Inhibition of germination by cations resembled, in some respects, inhibition resulting from acid treatment of *Bacillus* spores (5, 17, 18); acid-treated spores of *B. megaterium* failed to germinate, but germination was restored after the treated spores were soaked in calcium solutions. Although *B. megaterium* spores contain large amounts of calcium (complexed as dipicolinate), the loss of calcium caused by acid treatment was only 6.4% of the total spore calcium, suggesting that only a small fraction of the calcium (the acid-exchangeable fraction) was involved in triggering germination (17). We did not measure the calcium content of our spores, but a similar interpretation can probably be applied to the inhibitions by cations and EDTA and their reversal by calcium in *C. cylindrosporum* spores. The rapidity of inhibition (or reversal of inhibition) suggests that the calcium was readily accessible to and exchangeable with cations in the environment.

Calcium sometimes stimulates germination in spores of other bacterial species after simple exogenous supplementation (1, 15). In *C. perfringens*, calcium alone will initiate germination with heat-activated spores (1). *B. megaterium* spores are stimulated by calcium when germinating on glucose but not alanine, suggesting that calcium may modify the triggering response to selected organic germination enhancers (10, 20). The possibility that calcium affects the conformation of a trigger receptor site has been considered (16, 20).

Spore germination in *B. fastidiosus* was reported to require uricase (22). Analogous germination in *C. cylindrosporum* might require xanthine dehydrogenase. Although we did not assay spores for the presence of xanthine or formate dehydrogenases, we did test spore germination for inhibition by agents known to interfere with xanthine dehydrogenase. Allopurinol and oxypurinol (alloxanthine) are inhibitors of xanthine dehydrogenase and inhibited the germination of *C. cylindrosporum* spores with urate. Xanthine dehydrogenase triggering of germination might depend on an oxidation-reduction signal, but we found no evidence for such a signal based on germination in the presence of formate (as a possible reductant) or methyl viologen. Methyl viologen (at 1 mM or above) stimulated germination slightly, independently of whether it was added as the oxidized or reduced form, suggesting that methyl viologen stimulation did not result from reduction of urate by xanthine dehydrogenase. Viologen dyes are known to participate in reactions with purified xanthine dehydrogenase (4) and formate dehydrogenase (25) from *C. cylindrosporum*.

We observed germination only on uric acid, although all of the purines listed above, EDTA, formate, acetate, and glycine were tested as germination triggers. If xanthine dehydrogenase triggered spore germination, it seems likely that at least some of the purines which serve as growth substrates would act as germination triggers.

The broad range of purines that interfere with uric acid germination resembles the broad substrate specificity of xanthine dehydrogenase; reversal of inhibition by urate suggests that purine-like substances inhibited as urate analogs. Inhibitions by allopurinol, adenine, hypoxanthine, xanthine, 6,8-dihydroxypurine, caffeine, theophylline, EDTA, and sodium were all overcome by increasing the concentration of urate or calcium. The reversal of inhibition by calcium might have resulted from increased affinity of the triggering mechanism to urate because increasing the calcium concentration from 100 μM to 10 mM decreased the concentration of urate required for germination from 1 mM to 100 μM.

An alternate explanation is that the purines inhibited urate-triggered germination by sequestering calcium. Purines are known to form complexes with calcium (8, 9, 11), and calcium seems to be available for exchange with sodium (sodium inhibition) or sequestration by EDTA (EDTA inhibition). Adenine was the most potent of the purine inhibitors tested on the basis of absence of germination in 10 mM adenine when 10 mM urate or 10 mM calcium was available. Its calcium chelation stability constant (1.12×10^{-8}) is approximately 1/30 that of hypoxanthine (3.55×10^{-7}) (9). Adenine is toxic to *C. cylindrosporum* vegetative cells and inhibits growth (7). It was apparently not metabolized to an inhibitory substance by spores, because adenine-pretreated spores germinated without a lag. If germination required calcium bound to urate, competition for calcium by chelators or purines could result in inhibition.

LITERATURE CITED

1. **Ando, Y.** 1981. Germination of *Clostridium perfringens* spores: a proposed role for ions, p. 240–242. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
2. **Aoki, H., and R. A. Slepecky.** 1973. Inducement of a heat-shock requirement for germination and production of increased heat resistance in *Bacillus fastidiosus* spores by manganous ions. J. Bacteriol. **114**:137–143.
3. **Barker, H. A., and J. V. Beck.** 1942. *Clostridium acidi-uridi* and *Clostridium cylindrosporum*, organisms fermenting uric acid and some other purines. J. Bacteriol. **43**:291–304.
4. **Bradshaw, W. H., and H. A. Barker.** 1960. Purification and properties of xanthine dehydrogenase from *Clostridium cylindrosporum*. J. Biol. Chem. **235**:3620–3629.
5. **Charney, V., W. P. Fisher, and C. D. Hegarty.** 1951. Manganese as an essential element for sporulation in the genus *Bacillus*. J. Bacteriol. **62**:145–148.
6. **Douthit, H. A., and R. A. Preston.** 1985. Activation of *Bacillus cereus* spores with calcium, p. 297–307. *In* G. J. Dring, D. J. Ellar, and G. W. Gould (ed.), Fundamental and applied aspects of bacterial spores. Academic Press, Inc., New York.
7. **Durre, P., W. Andersch, and J. R. Andreesen.** 1981. Isolation and characterization of an adenine-utilizing, anaerobic sporeformer, *Clostridium purinolyticum* sp. nov. Int. J. Syst. Bacteriol. **31**:184–194.
8. **Graciunescu, D., and I. Mihalcea.** 1970. Structure moleculaire des complexes du Ca^{+2} et Cu^{+2} avec les composes pyrimidiques (bases puriniques). Rev. R. Acad. Cienc. Exactas Fis. Nat. Madr. **64**:1039–1047.
9. **Kahn, M. M., and C. R. Krishnamoorthy.** 1971. Interaction of metal ions with monosubstituted purines. J. Inorg. Chem. **33**:1417–1425.
10. **Levinson, H. S., and M. T. Hyatt.** 1969. Activation of *Bacillus megaterium* spore germination, p. 267–275. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores IV. American Society for Microbiology, Washington, D.C.
11. **Marzilli, L. G.** 1976. Metal-ion interactions with nucleic acids and nucleic acid derivatives. Prog. Inorg. Chem. **23**:255–378.
12. **Nakatani, Y., M. Imagawa, Y. Takubo, J. Nishihara, and M.**

Kondo. 1985. Germination of the decoated spores of *Bacillus megaterium*. Microbiol. Immunol. **29**:1139–1149.

13. **Nakatani, Y., K. Tani, M. Imagawa, T. Nishihara, and M. Kondo.** 1985. Germinability of coat-lacking spores of *Bacillus megaterium*. Biochem. Biophys. Res. Commun. **128**:728–732.
14. **Nishihara, T., T. Ichikawa, and M. Kondo.** 1981. Role of charged groups of the spore surface in germination, p. 237–239. *In* H. S. Levinson, A. L. Sonenshein, and D. J. Tipper (ed.), Sporulation and germination. American Society for Microbiology, Washington, D.C.
15. **Rode, L. J., and J. W. Foster.** 1962. Ionic germination of spores of *Bacillus megaterium*, Arch. Mikrobiol. **43**:183–200.
16. **Rode, L., and J. W. Foster.** 1965. Gaseous hydrocarbons and the germination of bacterial spores. Proc. Natl. Acad. Sci. USA **53**:31–38.
17. **Rode, L. J., and J. W. Foster.** 1966. Influence of exchangeable ions on germinability of bacterial spores. J. Bacteriol. **91**: 1582–1588.
18. **Rode, L. J., and J. W. Foster.** 1966. Quantitative aspects of exchangeable calcium in spores of *Bacillus megaterium*. J. Bacteriol. **91**:1589–1593.
19. **Rowley, D. B., and H. S. Levinson.** 1967. Changes in spores of *Bacillus megaterium* treated with thioglycollate at low pH and restoration of germinability and heat resistance by cations. J. Bacteriol. **93**:1017–1022.
20. **Sacks, L. E.** 1972. Influence of intra- and extracellular cations on the germination of bacterial spores, p. 437–442. *In* H. O. Halvorson, R. Hanson, and L. L. Campbell (ed.), Spores V. American Society for Microbiology, Washington, D.C.
21. **Sacks, L. E., and M. R. Smith.** 1987. Sporulation of *Clostridium cylindrosporum* HC-1 on a defined low-manganese medium. Appl. Environ. Microbiol. **53**:1696–1698.
22. **Salas, J. A., K. Johnstone, and D. J. Ellar.** 1985. Role of uricase in the triggering of germination of *Bacillus fastidiosus* spores. Biochem. J. **229**:241–249.
23. **Schiefer-Ullrich, H., R. Wagner, P. Dürre, and J. R. Andreesen.** 1984. Comparative studies on physiology and taxonomy of obligately purinolytic clostridia. Arch. Microbiol. **138**:345–353.
24. **Smith, M. R., and J. L. Lequerica.** 1985. *Methanosarcina* mutant unable to produce methane or assimilate carbon from acetate. J. Bacteriol. **164**:618–625.
25. **Wagner, R., and J. R. Andreesen.** 1977. Differentiation between *Clostridium acidiurici* and *Clostridium cylindrosporum* on the basis of specific metal requirements for formate dehydrogenase formation. Arch. Microbiol. **114**:219–224.