DISSOCIATION OF SPORE GERMINATION FROM OUTGROWTH BY USE OF AUXOTROPHIC MUTANTS OF *BACILLUS SUBTILIS*

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The development of a bacterial spore into a mature vegetative cell is a complex phenomenon, during which the organism passes through several definite phases. The first of these is termed "germination," in which the spores lose their heat resistance, take up stains, decrease in optical density, increase in respiratory activity, and darken under phase contrast microscopy. The subsequent phases of development into a vegetative cell have been described as "outgrowth" (Campbell, 1957) which includes swelling, emergence, elongation, and cell division (Mandels *et al.*, 1956).

The nutritional requirements for germination, outgrowth, and vegetative growth generally differ for any one organism. Germination has the simplest requirements i. e., L-alanine, adenosine, or glucose; or combinations of these (Stedman, 1956). Outgrowth usually requires an energy source, various additional amino acids, and, in some cases, vitamins (O'Brien and Campbell, 1957; Amaha and Sakaguchi, 1952). The requirements for vegetative growth are usually simpler than those for outgrowth.

Spores of the Marburg strain of *Bacillus subtilis* rapidly develop into dividing vegetative cells in a medium containing glucose, alanine, asparagine, glutamic acid, and mineral salts (Demain, 1958). Of the amino acids, only alanine is required for germination. It seemed of interest to determine the effect of imposing further nutritional deficiencies on the development of spore to vegetative cell. The present paper shows that nutritional auxotrophs, requiring an amino acid, a vitamin, or a pyrimidine, are capable of undergoing germination but not outgrowth or vegetative growth.

MATERIALS AND METHODS

Cultures. The parent organism was the Marburg strain of *B. subtilis* (ATCC 6051). The mutants 5 (uracil-less), 210 (phenylalanine-less) and 365 (nicotinic acid-less) were isolated by delayed enrichment (Lederberg and Tatum, 1945; Guthrie and Saperstein, 1949) after ultraviolet irradiation of wild-type vegetative cells. Washed spore suspensions were prepared and stored as previously described (Demain, 1958).

Medium. The liquid medium used was the "spore minimal medium" (Demain, 1958) without agar (SM medium). It contained glucose, L-glutamic acid, L-asparagine, L-alanine, and mineral salts. Glass-distilled water was used. The volume of medium was 10 ml in each 20 by 175 mm colorimeter tube.

Germination and outgrowth. Germination was followed by the increase in per cent light transmission (decrease in turbidity) after inoculation of the SM medium with the spore suspension. Unless otherwise noted, the volume of spore suspension was 1 ml. Various crops of spores were used in the present work. Therefore, absolute values for light transmission and viable counts are not strictly comparable in the various experiments to be described. Incubation was at 37 C on a shaking machine imparting a rotary motion of 220 rpm. Light transmission was measured in the Lumetron colorimeter (660 mμ filter) which was set so that uninoculated medium showed 100 per cent transmission. Outgrowth was followed by the decrease in light transmission after the germination phase had terminated.

Viable counts. To avert spreading, counts were made in duplicate by the three layer "sandwich" technique, as previously described (Demain, 1958). Nutrient agar (Difco) was used in all experiments except that shown in table 1 where a chemically defined medium composed of glucose, amino acid mix, uracil, washed agar, and mineral salts was employed.

Heat resistance. Heating was carried out in a water bath at 80 C for a period of 20 min. Sam-

ples were cooled immediately after removal from the bath.

RESULTS

Germination and outgrowth studies on uracil-less mutant 5. When washed spores of the wild type and of uracil-less mutant 5 were inoculated into tubes of SM medium, the changes shown in figure 1 were observed. Both types germinate rapidly but only the wild type undergoes outgrowth after germination. If uracil is present in the medium, mutant 5 is now capable of outgrowth (figure 2). Microscopic examination showed that during the germination phase, the spores became stainable with 1 per cent crystal violet. In the absence of uracil, the majority of the cells at the end of the experiment were stained but had not emerged from the spore coat. In its presence, the spores had enlarged, emerged, and begun to divide.

Confirmation of the above findings was accomplished by the use of viable counts. Table 1 shows that mutant 5 does not increase in count during and after germination in the absence of uracil. In addition, the data show that germinated spores retain their viability. Further work has indicated that the viability is not decreased for at least 12 hr following germination.

Plating experiments were also used to demonstrate the loss of heat stability during germination. In table 2, the results of two experiments are given. In experiment 1, 0.1 ml of washed spores of both the wild type and mutant 5 were inoculated into tubes of SM medium and were immediately plated on nutrient agar. The tubes were then placed on the shaker for 7 hr, after which counts were again made before and after a heat treatment. It is evident that both the vegetative cells of the wild type and the mutant 5 germinated spores were rendered heat-labile. In experiment 2, 0.1-ml samples of mutant 5 spores were inoculated into a tube of SM medium and a tube containing only the mineral salts component. After 4 hr of shaking, the tube contents were plated out before and after heating. Only the spores incubated in the medium allowing germination were killed by the heat.

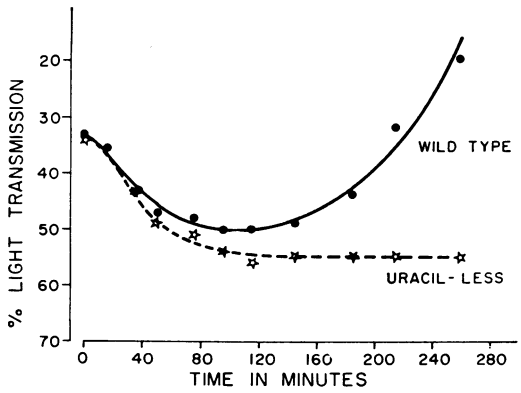

Figure 1. Germination and outgrowth experiment with spores of *Bacillus subtilis* and its uracil-less mutant 5.

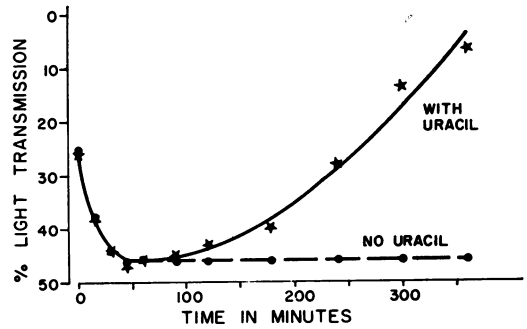

Figure 2. Germination and outgrowth experiment with spores of uracil-less mutant 5 in the presence and absence of 10 μg uracil per ml.

TABLE 1

*Viable counts of mutant 5 spores germinating in the absence of uracil**

Time	Light Transmission	Viable Count per ml
hr	%	$\times 10^8$
0	13	1.5
1	25	1.8
2	32	1.4
3	29	—
4	28	—
5	27	1.3
6	28	—

* Plating medium consisted of glucose, amino acid mix, uracil, and mineral salts.

Germination of phenylalanine-less mutant 210 and nicotinic acid-less mutant 365. Mutants 210 and 365, which require phenylalanine and nicotinic acid, respectively, also germinate but do not grow in the spore minimal medium. This is shown in table 3.

Lysozyme sensitivity of germinated spores. It is a well-known fact that vegetative cells of *B.*

TABLE 2

Loss of heat stability of mutant 5 spores during incubation in spore minimal (SM) medium

Expt	Time	Medium	Mutant 5			Wild Type		
			Light transmission	Viable count per ml		Light transmission	Viable count per ml	
				Preheat	Postheat		Preheat	Postheat
	hr		%	× 10^6	× 10^6	%	× 10^6	× 10^6
1	0	SM	86	6.4	—	90	7.4	—
	7	SM	92	5.1	0.045	18	28	0.28
2	4	SM	—	17	0.54	—	—	—
	4	Salts	—	17	18	—	—	—

subtilis are susceptible to the lytic action of lysozyme (Salton, 1957). On the other hand, Strange and Dark (1956) observed that this enzyme does not attack resting spores although isolated spore coats are acted upon. It was thus considered of interest to test the action of lysozyme on intact germinated spores since they are intermediate between the resistant spore and the susceptible vegetative cell. Figure 3 shows that mutant 5 germinated spores as well as vegetative cells are lysed by the enzyme but spores are completely resistant.

Stability of germinated spores in absence of their growth factors on agar plates. The stability of germinated spores of the mutants was tested by plating approximately 50 to 150 spores onto a series of plates containing SM medium plus 2.5 per cent washed agar. Wild-type spores were also included as a control and were the only spores capable of forming a significant number of visible colonies without further supplements. Each day, for 3 days, duplicate plates were removed from the incubator, counted, flooded with a layer of SM agar containing 0.3 per cent yeast extract to supply the necessary growth factor, reincubated, and counted again after 3 days of secondary incubation. The results in table 4 show that germinated spores remain viable in SM agar for at least 3 days.

Stability of germinated spores in liquid media simpler than SM medium. Demain (1958) showed that wild-type vegetative cells rapidly die in water or saline. K_2HPO_4 (0.2 M) could stabilize cells for 30 min whereas the entire mineral salts mixture allowed no killing for at least 75 min. Spores, on the other hand, were perfectly stable even in water. For this reason, phosphate has been used throughout this work as diluent for plating, and the salts mixture has been the menstruum of choice for washing and centrifugation procedures. Since germinated spores are intermediates in the conversion of the resistant spore to the labile vegetative cells, studies were conducted on the stability of germinated spores in media simpler than SM medium.

In the first experiment, 0.1-ml samples of washed mutant 210 (phenylalanine-less) spores were inoculated into tubes of SM medium and SM medium containing 0.039 per cent DL-phenylalanine. These were shaken for 9¼ hr to provide germinated spores and vegetative cells, respectively. Both types were washed once with salts mixture and plated immediately on nutrient agar. Each suspension was then incubated in salts mixture and phosphate at room temperature for 1 hr without agitation and then replated.

TABLE 3

Germination of phenylalanine-less mutant 210 and nicotinic acid-less mutant 365 in absence of their growth factors

Time	Light Transmission	
	Mutant 210	Mutant 365
hr	%	%
0	19	17
0.83	22	19
1.75	25	24
2.33	26	26
4.33	27	29
5.33	26	28
6.33	26	29
7.50	27	30

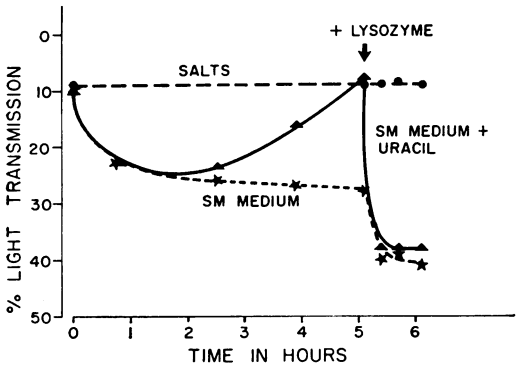

Figure 3. Effect of lysozyme on spores, germinated spores, and vegetative cells of uracil-less mutant 5. Washed mutant 5 spores (1.5 ml) were placed in salts, in spore minimal (*SM*) medium and in SM medium plus uracil to produce spores, germinated spores, and vegetative cells, respectively. After shaking for 5.1 hr, lysozyme (Nutritional Biochemicals Corporation) was added to each tube at 18 μg per ml.

TABLE 4

Stability of germinated spores of mutants in spore minimal agar in absence of growth factor

Culture	YE Layer*	Days of Primary Incubation			
		0	1	2	3
		Viable count per plate			
Wild type	Before	—	126	114	126
	After	123	134	123	127
Mutant 5	Before	—	0	0	2
	After	131	138	142	138
Mutant 210	Before	—	0	8	1
	After	49	44	46	50
Mutant 365	Before	—	0	—	3
	After	123	110	—	123

* Spore minimal agar containing 0.3 per cent yeast extract (Difco).

The results showed that the germinated spores were completely stable in both media, whereas the vegetative cells suffered a 94 per cent kill in salts mixture and a 97 per cent kill in phosphate.

A second experiment was conducted using conditions under which the cell types were subjected to a greater degree of stress than encountered either in the first experiment or in the previously published studies with wild-type vegetative cells. Here, spores, germinated spores, and vegetative cells of uracil-less mutant 5 were

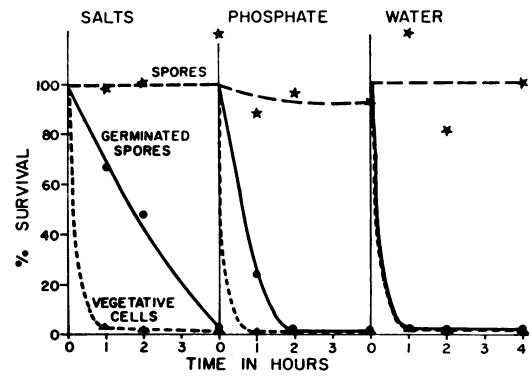

Figure 4. Comparative stabilities of spores, germinated spores, and vegetative cells of uracil-less mutant 5 in salts mixture of spore minimal medium, in 0.02 M K_2HPO_4 and in glass-distilled water.

produced in the usual manner. After washing *twice* in salts mixture and plating,[1] samples were diluted into salts mixture, phosphate, and glass-distilled water at a concentration of about 10^4 cells per ml. The tubes were incubated *on the shaker at 37 C* and plated at 1, 2, and 4 hr. The results, plotted in figure 4, show that germinated spores lie midway in stability toward salts mixture between the stable spore and the labile vegetative cell. In phosphate, the greater stability of the germinated spore over the vegetative cell is somewhat diminished, whereas in water, both types are rapidly killed.

DISCUSSION

The above experiments show that the imposition of a nutritional requirement for uracil, phenylalanine, or nicotinic acid via mutation does not affect the ability of spores of *B. subtilis* to germinate but completely inhibits outgrowth to the vegetative state. Viable, lysozyme- and heat-sensitive germinated spores can thus be produced from 97 to 99 per cent of the resting spore population by incubation in a simple medium containing glucose, alanine, asparagine, glutamic acid, and mineral salts (SM medium). These cells remain viable for at least 12 hr after germination and, on SM agar plates, they have been shown to retain viability for at least 3 days in the absence of their specific growth factor.

[1] Counts after heating showed that the germinated spore and the vegetative cell populations contained only 1 to 2 per cent ungerminated spores.

The finding that germinated spores are more resistant to killing by incubation in mineral salts mixture or phosphate than vegetative cells is in accord with the results of other investigators using different killing agents. These agents include sodium chloride (Riemann, 1957), ethylene oxide (Church *et al.*, 1956), and ultraviolet irradiation (Stuy, 1956). It should be noted, however, that Stuy (1956) found that germinated spores of *B. cereus* were more susceptible to X-irradiation than were vegetative cells. The fact that the germinated forms were produced in defined medium, and the vegetative cells were prepared in a crude medium, may be partly responsible for this observation.

Previous work has suggested that outgrowth is a phase characterized by active synthesis of macromolecules as opposed to the degradative nature of germination (Halvorson and Church, 1957). Up to the present work, the requirements for outgrowth in addition to energy source have been shown to include amino acids (Amaha and Sakaguchi, 1952), vitamins (O'Brien and Campbell, 1957), sulfur (Hyatt and Levinson, 1957), phosphate, and oxygen (Hyatt and Levinson, 1959). These same nutrients are not required for germination by the specific species involved. The necessity for protein synthesis during outgrowth is indicated by the fact that amino acid analogues do not inhibit germination but do inhibit outgrowth. This effect can be reversed by the homologous amino acids (Nakada *et al.*, 1956). The requirement for uracil observed in the present work suggests that ribonucleic acid synthesis is also a vital part of the outgrowth process. This observation is in accord with the data of Fitz-James (1955).

SUMMARY

Inoculation of resting spores of uracil-less, phenylalanine-less, and nicotinic acid-less *Bacillus subtilis* mutants into a defined medium (glucose, alanine, asparagine, glutamic acid, and mineral salts) leads to the production of viable, germinated spores which do not undergo outgrowth. In the same medium, wild-type spores germinate and grow into vegetative cells. The mutant germinated spores are labile to both heat and lysozyme. They retain their viability for at least 12 hr after germination in this medium. On agar plates containing the same medium, germinated spores remain viable in the absence of their growth factor for at least 3 days.

The germinated spores were compared to resting spores and vegetative cells as to resistance to killing caused by incubation in mineral salts mixture, in phosphate, and in water. Spores were completely resistant to all three treatments. In salts mixture and in phosphate, germinated spores were killed but at a lower rate than vegetative cells. In water, both type cells rapidly lost viability.

REFERENCES

AMAHA, M. AND SAKAGUCHI, K. 1952 Nutritional requirements of vegetative cells and spores of aerobic spore-forming bacilli. J. Agr. Chem. Soc. Japan, **26**, 353–359.

CAMPBELL, L. L., JR. 1957 Bacterial spore germination—definitions and methods of study. In *Spores*, pp. 33–38. Edited by H. Orin Halvorson. AIBS, Washington, D. C.

CHURCH, B. D., HALVORSON, H., RAMSEY, D. S., AND HARTMAN, R. S. 1956 Population heterogeneity in the resistance of aerobic spores to ethylene oxide. J. Bacteriol, **72**, 242–247.

DEMAIN, A. L. 1958 Minimal media for quantitative studies with *Bacillus subtilis*. J. Bacteriol., **75**, 517–522.

FITZ-JAMES, P. C. 1955 The phosphorus fraction of *Bacillus cereus* and *Bacillus megaterium*. II. A correlation of the chemical with the cytological changes occurring during spore germination. Can. J. Microbiol., **1**, 525–548.

GUTHRIE, R. AND SAPERSTEIN, J. 1949 Studies of plate methods for isolation of *Bacillus subtilis* strains with induced growth requirements. Bacteriol. Proc., **1949**, 12.

HALVORSON, H. AND CHURCH, B. 1957 Biochemistry of spores of aerobic bacilli with special reference to germination. Bacteriol. Revs., **21**, 112–131.

HYATT, M. T. AND LEVINSON, H. S. 1957 Sulfur requirement for postgerminative development of *Bacillus megaterium* spores. J. Bacteriol., **74**, 87–93.

HYATT, M. T. AND LEVINSON, H. S. 1959 Utilization of phosphates in the postgerminative development of spores of *Bacillus megaterium*. J. Bacteriol., **77**, 487–496.

LEDERBERG, J. AND TATUM, E. L. 1945 Detection of biochemical mutants of microorganisms. J. Biol. Chem., **165**, 381–382.

MANDELS, G. R., LEVINSON, H. S., AND HYATT, M. T. 1956 Analysis of respiration during germination and enlargement of spores of

Bacillus megaterium and of the fungus *Myrothecium verrucaria*. J. Gen. Physiol., **39,** 301–309.

NAKADA, D., MATSUSHIRO, A., AND MIWATANI, T. 1956 Studies on the development of aerobic sporeforming bacteria. I. Incorporation of amino acids in the development of *Bacillus cereus*. Med. J. Osaka Univ., **6,** 1047–1060.

O'BRIEN, R. T. AND CAMPBELL, L. L., JR. 1957 The nutritional requirements for germination and outgrowth of spores and vegetative cell growth of some aerobic spore forming bacteria. J. Bacteriol., **73,** 522–525.

RIEMANN, H. 1957 Some observations on the germination of *Clostridium* spores and the subsequent delay before the commencement of vegetative growth. J. Appl. Bacteriol., **20,** 404–412.

SALTON, M. R. J. 1957 The properties of lysozyme and its action on microorganisms. Bacteriol. Revs., **21,** 82–99.

STEDMAN, R. L. 1956 Biochemical aspects of bacterial endospore formation and germination. Am. J. Pharm., **128,** 84–97.

STRANGE, R. E. AND DARK, R. A. 1956 The composition of the spore coats of *Bacillus megatherium*, *B. subtilis* and *B. cereus*. Biochem. J., **62,** 459–465.

STUY, J. H. 1956 Studies on the mechanism of radiation inactivation of microorganisms. III. Inactivation of germinating spores of *Bacillus cereus*. Biochim. et Biophys. Acta, **22,** 241–246.