THE NUTRITIONAL REQUIREMENTS FOR GERMINATION AND OUTGROWTH OF SPORES AND VEGETATIVE CELL GROWTH OF SOME AEROBIC SPORE FORMING BACTERIA[1, 2]

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During the last 10 years the subject of bacterial spore germination has been intensively investigated. In most of these studies it has been found that bacterial spores are capable of germination under conditions which do not permit the germinated form to survive (Schmidt, 1955; Stedman, 1956). Hills (1949) pointed out that a distinction must be drawn between the requirements for germination and those for growth. He felt that the requirements for germination are more exacting than those for growth, since adenosine stimulated germination but not growth of *Bacillus anthracis*. Pulvertaft and Haynes (1951) in studies on the effect of adenosine on spore germination of *Bacillus cereus* and *Bacillus subtilis* concluded that newly germinated bacilli were more exacting in their growth requirements than bacilli developing from vegetative forms.

Amaha and Sakaguchi (1952) studied the nutritional requirements of vegetative cells and spores of *Bacillus natto*, *B. subtilis*, *Bacillus megaterium* I and II, *Bacillus mycoides*, and *B. cereus*. They found that the vegetative cells of all strains and the spores of *B. subtilis* and *B. mycoides* did not require any of the eighteen amino acids tested. However, *B. natto* spores required isoleucine, and *B. cereus* spores required valine. *B. megaterium* I spores required alanine and threonine, and the spores of *B. megaterium* II required phenylalanine.

This paper is concerned with a comparative study of the nutritional requirements for spore germination and outgrowth and vegetative cell growth of *Bacillus stearothermophilus*, *Bacillus coagulans*, and *B. cereus* var. *terminalis*. In determining requirements we have used the terms germination and outgrowth to indicate that spore inocula showed either growth or no growth. When using heat lability, darkening under a phase contrast microscope, and reduction in optical density at 630 mμ as criteria of germination it was found that the requirements were for outgrowth of the germinated form rather than for germination. A preliminary report of this work has appeared (O'Brien and Campbell, 1956).

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MATERIALS AND METHODS

Cultures. *B. stearothermophilus* strain 1518 and *B. coagulans* strain 43P were obtained through the courtesy of Mr. C. W. Bohrer of the National Canners Association, Washington, D. C. *B. cereus* var. *terminalis* was kindly supplied by Dr. H. Orin Halvorson. Stock cultures were maintained on nutrient agar (Difco) slants.

Materials. Micro Fernbach flasks (Kimble Glass Co.) of 25-ml capacity were used throughout the study. Prior to use the flasks were washed in detergent, rinsed well in tap and distilled water, and placed in a hot air oven at 200 C for 12 to 18 hr. Pipettes were cleaned by soaking in potassium dichromate cleaning solution, rinsed thoroughly in tap and distilled water, and allowed to air dry. Distilled water was used throughout the investigation. Vitamins and amino acids were purchased from commercial sources. All other chemicals used were of reagent grade.

Media. The complete chemically defined medium employed was that of Campbell and Williams (1953*a*), except that the glucose concentration was increased two-fold and the cystine was omitted. This modified medium supported good growth of the organisms through several serial transfers. For determination of nutritional requirements of the organisms, individual amino acids and vitamins were omitted from the complete medium. The media were prepared in screw cap bottles, adjusted to pH 7.2, and autoclaved

at 116 C for 10 min. Heat sterilized medium was found to support as good growth as sintered glass filtered medium.

Preparation of inocula. (1) Spore inocula: Spore suspensions of *B. stearothermophilus* were prepared by inoculation of Roux bottles containing nutrient agar. Incubation was at 55 C for 7 days. *B. coagulans* spore suspensions were prepared by inoculation of large petri plates containing proteose peptone acid agar (as modified by Frank, 1955). Incubation was at 55 C for 2 wk. Spore suspensions of *B. cereus* var. *terminalis* were prepared in a similar manner except that medium G, of Church *et al.* (1954), solidified with 1.5 per cent agar, was used as the sporulation medium. Incubation was at 30 C for 48 hr. After the appropriate incubation period the growth was harvested with sterile distilled water. The spores were washed 5 times by centrifugation, resuspended in sterile distilled water, and stored at 5 C in screw cap bottles containing about 1 inch of glass beads.

(2) Vegetative cell inocula: Vegetative cell inocula were prepared from the respective spore suspensions by inoculation of nutrient agar plates. After incubation for 12 hr at 55 C for *B. stearothermophilus* and *B. coagulans* and at 30 C for *B. cereus* var. *terminalis* growth was harvested from the agar surface with sterile distilled water. The vegetative cells were washed and stored as described for spores. The optical density of both spore and vegetative cell suspensions was adjusted to 0.40 at 525 mμ (corresponding to 0.17 mg dry weight of cell material per ml) with a Bausch and Lomb "spectronic 20" colorimeter.

Determination of nutritional requirements. Micro Fernbach flasks were inoculated with 0.01-ml aliquots of spore or vegetative cell suspension and 5.0 ml of the desired medium added aseptically. Spore cultures were heated at 100 C for 10 min to heat activate the spores, and to kill any vegetative cells present. Incubation times and temperatures varied with each organism. For *B. stearothermophilus* 24 hr at 55 C was used; for *B. coagulans*, 48 hr at 55 C was used. At 35 C, 48 hr were required for vegetative cells and 96 hr for spores. *B. cereus* var. *terminalis* cultures were incubated for 48 hr at 35 C.

Growth response was determined by optical density measurement at 420 mμ with the spectronic 20 colorimeter. All flasks were reconstituted to 5.0 ml with distilled water before optical density readings were made. The complete medium was used as a control. When the optical density reading in the deletion media was less than one-third of that of the complete medium the culture was considered negative with respect to growth.

RESULTS AND DISCUSSION

The results presented are typical of those obtained in at least six and in most cases ten separate experiments. The minimal nutritional requirements of the spores and vegetative cells of *B. stearothermophilus* are shown in table 1. It can be seen that spores required leucine and nicotinic acid for growth, but that vegetative cells did not.

Table 2 shows the requirements for *B. coagulans*. Since this organism grows readily at 35 and 55 C, requirements were determined at both temperatures. Spore requirements were more complex than those for vegetative cells. Only methionine, biotin, folic acid and thiamin were required for vegetative cell growth at both temperatures. In addition to these requirements spores also required glutamic acid, histidine, isoleucine, valine, and leucine at 55 C, while leucine was not required by spores at 35 C. The influence of temperature on the nutritional requirements of microorganisms has been reported by Campbell and Williams (1953*a*, *b*), Campbell (1954), Baker *et al.* (1955) and others.

Proom and Knight (1955) did not find a folic acid requirement for vegetative cell growth with

TABLE 1

Nutritional requirements for spore germination and outgrowth and vegetative cell growth of Bacillus stearothermophilus strain 1518 at 55 C

Medium	Spore Germination and Outgrowth	Vegetative Cells
Complete minimal	0.75*	0.80
Less arginine	0.00	0.15
Less histidine	0.00	0.00
Less isoleucine	0.00	0.10
Less leucine	0.00	0.70
Less methionine	0.00	0.00
Less valine	0.00	0.00
Less biotin	0.19	0.14
Less nicotinic acid	0.00	1.00
Less thiamin	0.06	0.18

* Optical density at 420 mμ.

any one of 16 strains of *B. coagulans* investigated. However, the medium used in their experiments contained serine, and this amino acid has been shown to spare or supplant the folic acid requirement for some microorganisms. Since the medium employed in this study did not contain serine, the possibility existed that the folic acid requirement of our strain of *B. coagulans* could be replaced by serine. This did not prove to be the case with either spores or vegetative cells at 35 or 55 C, nor could serine replace the folic acid requirement of our strain of *B. coagulans* when tested at 45 C (the temperature employed by Proom and Knight).

A difference in nutritional requirements was also found for the spores and vegetative cells of *B. cereus* var. *terminalis*. Data presented in table 3 show that leucine was essential for spore but not vegetative cell growth.

Adenosine and L-alanine have been shown to be required to begin or to stimulate spore germination of some organisms (see the reviews of Schmidt, 1955, and Stedman, 1956). Growth of *B. stearothermophilus* spores and vegetative cells was not affected by the addition of 12 μmoles per ml of L-alanine to the respective minimal media. However, addition of 24 μmoles per ml of adenosine completely inhibited spore outgrowth and vegetative cell growth. Growth of *B. coagulans* spores and vegetative cells was inhibited by L-alanine and not affected by adenosine at 55 C; at 35 C, however, both L-alanine and adenosine inhibited spore and vegetative cell growth. Spore and vegetative cell growth of *B. cereus* var. *terminalis* was only slightly inhibited by adenosine and not affected by alanine. The finding that adenosine inhibits spore outgrowth and vegetative cell growth of *B. stearothermophilus* and *B. coagulans* is similar to that reported by Pulvertaft and Haynes (1951) for *B. cereus*. They reported that while adenosine was essential for rapid spore germination of this organism it completely inhibited the growth of vegetative cells in concentrations higher than 0.006 per cent.

TABLE 2

Nutritional requirements for spore germination and outgrowth and vegetative cell growth of Bacillus coagulans strain 43 P at 35 and 55 C

Medium	Spore Germination and Outgrowth		Vegetative Cells	
	35 C	55 C	35 C	55 C
Complete minimal	0.66*	0.82	0.80	0.68
Less glutamic acid	0.15	0.20	0.76	0.43
Less histidine	0.12	0.05	0.76	0.65
Less isoleucine	0.04	0.22	0.54	0.45
Less leucine	0.58	0.26	0.80	0.59
Less methionine	0.18	0.25	0.20	0.13
Less valine	0.01	0.09	0.92	0.58
Less biotin	0.23	0.25	0.21	0.23
Less folic acid	0.13	0.07	0.14	0.13
Less thiamin	0.07	0.07	0.10	0.02

* Optical density at 420 mμ.

TABLE 3

Nutritional requirements for spore germination and outgrowth and vegetative cell growth of Bacillus cereus var. *terminalis at 35 C*

Medium	Spore Germination and Outgrowth	Vegetative Cells
Complete minimal	0.80*	0.75
Less isoleucine	0.15	0.19
Less leucine	0.18	0.56
Less methionine	0.28	0.20
Less valine	0.01	0.20

* Optical density at 420 mμ.

Germination experiments were carried out with spores of *B. stearothermophilus* in a glucose mineral salt medium which would not support outgrowth and in the minimal medium necessary for spore outgrowth. Germination, as evidenced by decrease in optical density at 630 mμ and loss of heat resistance, occurred readily in both media. This shows that the nutritional requirements for spores of this organism are for outgrowth of the germinated form rather than for germination.

Germination studies were also performed with *B. cereus* var. *terminalis* spores in distilled water, the minimal outgrowth medium, and in nutrient broth (Difco). Germination, as evidenced by a loss of refractility under a dark phase contrast microscope and a decrease in optical density at 630 mμ, occurred readily in the minimal medium and in nutrient broth but not in distilled water. The addition of L-alanine and adenosine greatly accelerated the rate of germination in the minimal medium, although only a small fraction of the germinated spores gave rise to vegetative cells. Spores lost refractility and enlarged very rapidly in nutrient broth, although here again many of the germinated spores did not give rise to vege-

tative cells. The experiment in nutrient broth was discontinued after 8 hr since most of the vegetative cells present had begun to sporulate, indicating that there was little likelihood of further development of the germinated spores.

The data presented show that the requirements for spore outgrowth are more complex than those for vegetative cell growth. The phase of outgrowth (i. e., emergence, elongation, or division) involved is not known at the present time.

SUMMARY

This paper reports the nutritional requirements for spore germination and outgrowth and vegetative cell growth of *Bacillus stearothermophilus*, *Bacillus coagulans*, and *Bacillus cereus* var. *terminalis*.

The minimal requirements for germination and outgrowth of spores of *B. stearothermophilus* at 55 C were isoleucine, leucine, valine, methionine, histidine, arginine, thiamin, nicotinic acid, and biotin. The vegetative cell requirements were identical, except that leucine and nicotinic acid were not required.

The requirements for spore germination and outgrowth of *B. coagulans* also differed from those of the vegetative cells. Methionine, thiamin, biotin, and folic acid were essential for vegetative cell growth at 35 and 55 C. In addition to these compounds spores at 55 C required glutamic acid, histidine, isoleucine, leucine, and valine. Leucine was not essential for spores at 35 C.

The spores of *B. cereus* var. *terminalis* required isoleucine, leucine, valine, and methionine at 35 C but the vegetative cells did not require leucine. It is evident that the nutritional requirements for spore germination and outgrowth are more complex than those for vegetative cell growth. Germination experiments revealed that the requirements for spores were for outgrowth of the germinated form rather than for germination.

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