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Nutritional Requirements for Germination, Outgrowth, and Vegetative Growth of Putrefactive Anaerobe 3679 in a Chemically Defined Medium[1]

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ABSTRACT

FUJIOKA, ROGER S. (University of Hawaii, Honolulu), AND HILMER A. FRANK. Nutritional requirements for germination, outgrowth, and vegetative growth of putrefactive anaerobe 3679 in a chemically defined medium. J. Bacteriol. 92:1515–1520. 1966.—A chemically defined medium was used to study the nutritional requirements for germination, outgrowth, and vegetative growth of putrefactive anaerobe 3679. Vegetative growth required arginine, cysteine, phenylalanine, threonine, valine, tryptophan, K_2HPO_4, and Na^+; was markedly stimulated by isoleucine, tyrosine, nicotinic acid, and *p*-aminobenzoic acid; and was stimulated slightly by alanine, biotin, pyridoxamine, glucose, and salts ($MgSO_4$, $FeSO_4$, $MnSO_4$). Growth occurred over an initial *p*H range of 6.0 to 8.2, and at incubation temperatures ranging from 20 to 45 C. No autolysis occurred during vegetative growth, although loss of motility and cell settling were observed upon prolonged incubation. Vegetative growth was inhibited completely by aerobic conditions. Completion of spore germination was inhibited at *p*H 4.8, outgrowth was inhibited at *p*H 8.5 and higher, and germination was inhibited at 9.0 and higher. Slow germination, but no outgrowth, was observed at 8 C; at 47 and 52 C, spore inocula germinated only partially. Under aerobic conditions, all inoculated spores germinated completely, and one-half of these also emerged. Alanine was considered the primary germinant in the chemically defined medium, and arginine and mineral salts ($MgSO_4$, $FeSO_4$, $MnSO_4$, and NaCl), as secondary germinants. During outgrowth of germinated spores, cysteine and K^+ were required for emergence, Na^+ for elongation, and arginine and valine for division of the elongated cell.

Studies concerning the nutritional requirements for different stages in the life cycle of spore-forming bacteria have been limited mainly to several species of *Bacillus*. The requirements for germination are generally regarded as the simplest for the various growth stages (5, 6), whereas requirements for vegetative growth were reported to be simpler than those for outgrowth (3, 8). Hyatt and Levinson (4) reported that outgrowth phases (swelling, emergence, elongation, and cell division) of *B. megaterium* also appeared to have specific requirements.

This paper reports the nutritional requirements determined for germination, outgrowth, and vegetative growth of an anaerobic sporeformer in a chemically defined medium. The effects of initial *p*H, incubation temperature, and aerobic conditions on various growth stages are also discussed.

MATERIALS AND METHODS

Organism. Putrefactive anaerobe (PA) 3679h, employed previously (9, 10), was used. This organism is probably closely related to *Clostridium sporogenes*, but its taxonomic position has never been established clearly.

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Materials. Pyrex and Kimax glassware was washed with a strong detergent, rinsed thoroughly in tap and distilled water, and dried in a hot-air oven. Pipettes and test tubes were cleaned by overnight soaking in a sulfuric-nitric acid mixture (95:5) before washing and drying. Amino acids and vitamins were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio, and other chemicals (reagent grade) were obtained from commercial sources. All solutions were made with glass-distilled, deionized water.

Media and incubation. Preliminary studies were conducted in a modification of the chemically defined medium of Campbell and Frank (2), adjusted to *p*H 7.5 with NaOH. By deleting unnecessary components and by altering concentrations of others, a simplified medium was obtained (Table 1) for studying the nutritional requirements of germination, outgrowth, and vegetative growth in PA 3679h. A series of test media was made by omitting singly the different components of this complete chemically defined medium. The nutritional role of the deleted component was evaluated by comparing growth response at 35 C in the test medium with that observed in the complete medium.

All media were sterilized by passage through a 0.45-μ membrane filter (Millipore Filter Corp., Bedford, Mass.), and 5-ml amounts were transferred aseptically to sterile screw-cap test tubes. To check for contamination, the final uninoculated 5 ml was incubated aerobically at 35 C. Except during aerobic experiments, inoculated media were incubated anaerobically, as described previously (9).

The influence of the following factors on growth stages of spore and vegetative cell inocula also were studied: initial *p*H, from 4.8 to 10.0; incubation temperature, between 8 and 52 C; and aerobic incubation at 35 C.

Vegetative cell inoculations. Vegetative cells were obtained by inoculating spores into the complete chemically defined medium, subculturing serially at least twice through the same medium, and using log-phase growth of the final culture as the inoculum. A 5-ml amount of each medium was heated to 95 C for 5 min, cooled rapidly to room temperature, and inoculated with 0.05 ml of vegetative cells. Vegetative growth was measured as absorbancy at 600 mμ with a Bausch & Lomb Spectronic-20 colorimeter.

Spore inoculations. An aqueous spore suspension, containing >99% ungerminated spores (Fig. 1A), was prepared as described previously (10). A 0.05-ml amount containing 1.6×10^7 spores (determined by model B Coulter counter) was inoculated into 5 ml of medium, heat-shocked 10 min at 80 C, cooled to room temperature, and incubated anaerobically.

Germination and outgrowth were studied by microscopic examination of spores inoculated in test and complete media. Germination was determined by complete spore darkening under phase-contrast optics (Fig. 1C). Swelling occurred concurrently with spore darkening and was considered to be part of germination rather than outgrowth. Outgrowth stages (emergence, elongation, and cell division) were determined from morphological changes (Fig. 1D, E) following spore darkening. In this study, cell division during outgrowth was considered the first division of the elongated cell (arrow, Fig. 1E), and was not considered vegetative cell multiplication.

Evaluation of nutritional requirements. Vegetative growth requirements were determined by inoculating vegetative cells in test and complete media and measuring growth through a minimum of four serial transfers at 35 C. The role of each deleted component was determined from the following criteria: (i) *required*, if vegetative growth did not persist through two serial transfers in the test (deletion) medium; (ii) *stimulant*, if maximal vegetative growth was less than 85% of that observed in the complete medium, after four serial transfers in the deletion medium; and (iii) *not required*, if vegetative growth was greater than 85% of that observed in the complete medium, after four serial transfers in the deletion medium.

Germination and outgrowth requirements were evaluated by inoculating spores in test and in complete media. When visual turbidity occurred within 6 days at 35 C, the test medium was considered adequate for both germination and outgrowth. If no visual turbidity was observed after 6 days, the test medium was considered deficient for at least one growth stage, and the inoculum was recovered by centrifugation and was examined under phase-contrast optics. Five

TABLE 1. *Chemically defined medium[a] for determining the nutritional requirements for germination, outgrowth, and vegetative growth of putrefactive anaerobe 3679h*

Compound	Concn
	M
L-Arginine	57.0×10^{-3}
L-Valine	68.3×10^{-3}
L-Cysteine	15.8×10^{-4}
L-Tyrosine	27.6×10^{-5}
L-Alanine	98.8×10^{-3}
L-Tryptophan	97.9×10^{-5}
L-Isoleucine	76.2×10^{-4}
L-Phenylalanine	48.4×10^{-3}
L-Threonine	84.0×10^{-4}
Glucose	27.8×10^{-3}
Biotin	40.9×10^{-10}
p-Aminobenzoic acid	14.6×10^{-8}
Pyridoxamine	41.5×10^{-7}
Nicotinic acid	16.3×10^{-6}
K_2HPO_4	49.7×10^{-4}
NaCl	99.2×10^{-4}
$MgSO_4$	16.2×10^{-5}
$FeSO_4$	72.0×10^{-7}
$MnSO_4$	66.6×10^{-7}
Tris(hydroxymethyl)aminomethane buffer, to final *p*H 7.0	50.0×10^{-3}

[a] Sterilized by filtration.

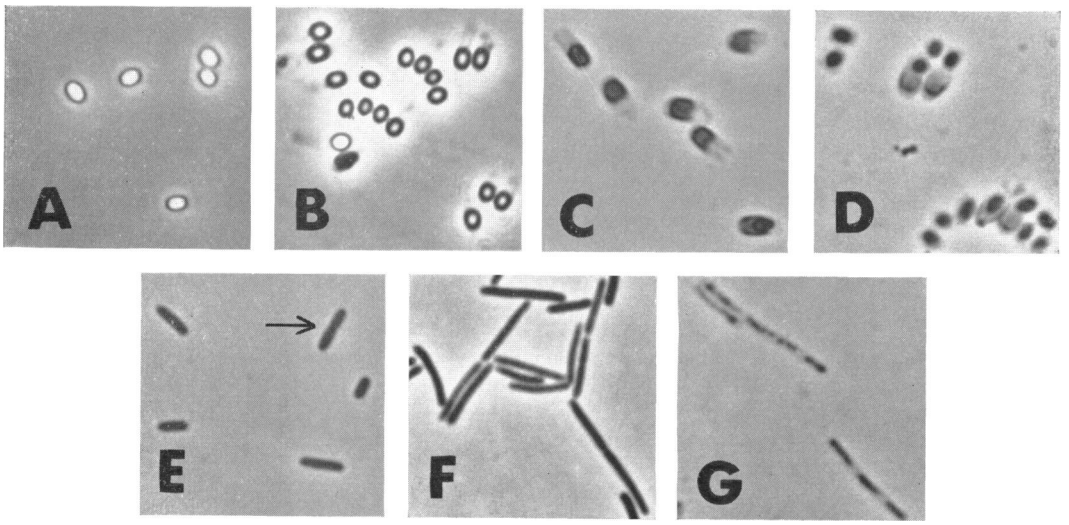

FIG. 1. *Phase-contrast photomicrographs of various growth stages of putrefactive anaerobe 3679h. (A) Ungerminated spores; (B) partially-germinated spores; (C) germinated spores (note prominent exosporia); (D) emerged spores (i.e., first phase of outgrowth); (E) elongated cells, including one (arrow) showing first cell division; (F) log-phase (28-hr) vegetative cells; (G) old (108-hr) vegetative cells, showing beaded appearance.* × *3,840.*

hundred cells were counted, and the per cent of each phase (Fig. 1A-E) was calculated. If more than 70% of the cells had not progressed beyond a given phase, the succeeding phase was considered to require the component deleted from the test medium. For example, if 90% of the cells were emerged only, the deleted component was required for elongation.

RESULTS

Vegetative growth from vegetative cell inocula. Growth from vegetative cell inocula occurred within 53 hr at 35 C in the complete chemically defined medium over an initial *p*H range of between 6.0 and 8.2. Maximal growth occurred between *p*H 7.0 and 7.5, but no growth was observed below *p*H 6.0 or above 8.2. At *p*H 7.0, growth occurred in the complete chemically defined medium within 3 days at incubation temperatures between 20 and 45 C, and was most rapid and abundant at 35 C. No vegetative growth was observed at 8, 47, or at 52 C, even after incubation for 3 weeks.

The growth curve (Fig. 2) of PA 3679h under optimal conditions (*p*H 7.0, 35 C) shows that growth commenced after a 15-hr lag period. Maximal growth (7×10^8 cells per milliliter) occurred after 36 hr, followed by a gradual decrease in absorbancy upon further incubation. After 48 hr, cells began to lose motility, and, by 84 hr, all cells had settled out of suspension and were nonmotile. Under phase-contrast optics, old cells were transluscent and beaded, but intact

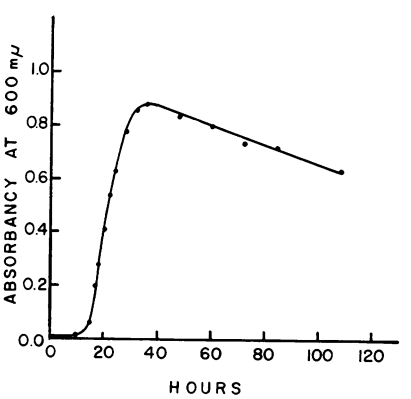

FIG. 2. *Growth curve of putrefactive anaerobe 3679h at 35 C in the chemically defined medium (pH 7.0).*

(Fig. 1G). The decline observed in absorbancy may have been due to leakage of cellular components from old cells, and not from autolysis, as observed for PA 3679h grown in complex media (7; Fujioka and Frank, *unpublished data*). In our complete chemically defined medium, autolysis of PA 3679h was probably inhibited by the high arginine concentration, as reported for *Clostridium botulinum* (1).

Nutritional requirements for vegetative growth at 35 C were studied in test (single-deletion) media (*p*H 7.0). Table 2 shows that arginine, cysteine, phenylalanine, threonine, valine, tryp-

tophan, K_2HPO_4, and NaCl were needed for vegetative growth. Isoleucine, tyrosine, nicotinic acid, and *p*-aminobenzoic acid were marked stimulants, whereas alanine, biotin, pyridoxamine, glucose, and salts C ($MgSO_4$, $FeSO_4$, and $MnSO_4$) were slight stimulants of vegetative growth.

No growth was observed in the complete chemically defined medium (*p*H 7.0) during aerobic incubation for 6 days at 35 C, showing that anaerobic conditions were required for vegetative growth.

Effect of initial pH on spore inocula. Vegetative growth occurred within 6 days at 35 C in spore-inoculated complete chemically defined medium over the *p*H range 6.0 to 8.2. No growth was observed when the initial *p*H of the medium was adjusted at 4.8 or at 8.5 and higher. Table 3 summarizes the phase-contrast appearance of spores inoculated in complete chemically defined medium adjusted to *p*H levels unfavorable for vegetative growth. At *p*H 4.8, essentially all the spores had germinated partially (Fig. 1B), indicating that low *p*H inhibited completion of germination. At *p*H 8.5, all the spores had germinated, but only 36% of these were emerged and 1% were elongated, indicating that outgrowth inhibition was responsible for the lack of growth. Previously, Uehara and Frank (9) reported that the optimal *p*H for germination of PA 3679h was 8.5.

TABLE 2. *Nutritional requirements for vegetative growth and outgrowth in putrefactive anaerobe 3679h*

Compound	Vegetative growth	Outgrowth (phase)
Cysteine	R[a]	R (emergence)
K_2HPO_4	R	R (emergence)
NaCl	R	R (elongation)
Arginine	R	R (cell division)
Valine	R	R (cell division)
Phenylalanine	R	N
Threonine	R	N
Tryptophan	R	N
Isoleucine	MS	N
Tyrosine	MS	N
Nicotinic acid	MS	N
p-Aminobenzoic acid	MS	N
Alanine	SS	N
Biotin	SS	N
Pyridoxamine	SS	N
Glucose	SS	N
Salts C[b]	SS	N

[a] Abbreviations: R = required; MS = marked stimulant; SS = slight stimulant; N = not required.

[b] $MgSO_4$, $FeSO_4$, and $MnSO_4$. See Table 1.

TABLE 3. *Effect of unfavorable pH on germination and outgrowth of putrefactive anaerobe 3679h spores*[a]

*p*H	Cells at various phases (per cent)					
	Germination			Outgrowth		
	Ungerminated	Partially germinated	Germinated	Emerged	Elongated	Divided
4.8	1	99	0	0	0	0
8.5	0	0	63	36	1	0
9.0	20	3	67	10	0	0
9.5	24	66	10	0	0	0
10.0	19	64	17	0	0	0

[a] After 6 days at 35 C.

At *p*H 9.0, 77% of the spores had germinated completely, including 10% that had also emerged, indicating that inhibition of germination was slight and that outgrowth was markedly inhibited at this *p*H. At *p*H 9.5 and 10.0, most of the spores were either partially germinated or ungerminated and very few were completely germinated, showing that germination was definitely inhibited at higher *p*H levels.

Effect of incubation temperature on spore inocula. Vegetative growth occurred within 6 days in spore-inoculated complete chemically defined medium (*p*H 7.0) at temperatures between 30 and 45 C, within 10 days at 20 C, but not at 8, 47, or 52 C, even after incubation for 3 weeks. Slow germination (32% after 10 days), but no outgrowth, was observed in spores incubated at 8 C. Only partially germinated spores (92 and 87%, respectively) were observed at 47 and 52 C, indicating that higher incubation temperatures inhibited complete germination.

Effect of aerobiosis on spore inocula. No vegetative growth was observed in spore-inoculated complete chemically defined medium (*p*H 7.0) after aerobic incubation for 6 days at 35 C. Phase-contrast examination showed that the inoculated spores had all germinated, and that 51% of these had emerged. Thus, germination and emergence occurred during lengthy aerobic incubation, and elongation was the first outgrowth phase requiring anaerobic conditions.

In all but one of the test (single-deletion) media, 100% germination occurred after 6 days of aerobic incubation. In the alanine-deficient medium, only 28% germination was observed under aerobic conditions, although 100% germination had occurred in this medium under anaerobic conditions. These results suggest that

germination via some inducer, other than alanine, in the complete chemically defined medium was inhibited by aerobic conditions. Therefore, alanine may be unique by inducing germination of PA 3679h spores under either aerobic or anaerobic conditions, as observed previously with a solution containing only L-alanine and sodium pyrophosphate (9).

Nutritional requirements for germination. Spore inocula germinated completely after incubation for 6 days at 35 C in all test (single-deletion) media. During shorter incubation (15 hr), 95% of the spores germinated in the complete chemically defined medium, 84% in arginine-deficient, 75% in mineral-salts (salts C plus NaCl)-deficient, only 23% in alanine-deficient medium, and over 90% in all other test media. Therefore, alanine was considered to be the primary germinant, and arginine and mineral salts, secondary germinants, in the chemically defined medium; all other components were regarded as minor or negligible germinants.

Spores were inoculated in a simplified germination medium (*p*H 7.0) composed of alanine, arginine, mineral salts, and phenylalanine (concentrations as shown in Table 1). After 67 hr at 35 C, 96% of the spores had germinated, including 1% that had emerged. Poor germination occurred when any one component was omitted from this simplified germination medium.

Nutritional requirements for phases of outgrowth. Different nutritional requirements were also observed for various phases of outgrowth. Table 2 shows that cysteine and K_2HPO_4 were required for emergence, NaCl was required for elongation, and that arginine and valine were required for division of the elongated cell.

A minimal outgrowth medium (*p*H 7.0), which supported germination and outgrowth, but not vegetative growth, was made by adding cysteine, valine, K_2HPO_4, and tryptophan (concentrations as shown in Table 1) to the simplified germination medium. In this medium, virtually all inoculated spores completed outgrowth (elongation and cell division) after incubation for 67 hr at 35 C.

Ionic requirements. Since NaCl and K_2HPO_4 were required for both outgrowth and vegetative growth, a study was made to determine the precise growth phase affected by individual ions.

Previously, in the single-deletion experiments, NaCl was necessary for vegetative growth and elongation (Table 2). Table 4 shows that adding Na^+ (as Na_2HPO_4) to the NaCl-deficient medium resulted in maximal vegetative growth from either spore or vegetative cell inocula. We concluded, therefore, that Na^+, but not Cl^-, was essential for both vegetative growth and for elongation during outgrowth.

In single-deletion experiments, K_2HPO_4 was required for vegetative growth and emergence (Table 2), but not for germination. Table 4 shows that adding phosphate only (as Na_2HPO_4) to the K_2HPO_4-deficient medium did not support vegetative growth from either inoculum; under phase-contrast examination, spore inocula germinated but did not grow out. Supplementation with K^+ (as KCl) alone to the K_2HPO_4-deficient medium resulted in *slight* vegetative growth from both inocula. Since it was assumed that outgrowth was completed if vegetative growth occurred from a spore inoculum, we concluded that K^+ alone was required for outgrowth and *limited* vegetative growth. Maximal vegetative growth occurred only when both K^+ and phosphate ions were present (Table 4). Hence, phosphate appeared to be required for *maximal* vegetative growth, but not for outgrowth.

Table 4 also shows that when pyrophosphate was substituted for phosphate, no growth resulted from either a spore or vegetative cell inoculum. Under phase-contrast examination, all spores incubated 6 days at 35 C in the presence of pyrophosphate had germinated, but showed no evidence of outgrowth. Thus, pyrophosphate appeared to inhibit both outgrowth and vegetative growth. Pyrophosphate also inhibited outgrowth and vegetative growth, but not germination, when added to the complete chemically defined medium.

TABLE 4. *Effect of inorganic components on growth from vegetative cell and spore inocula of putrefactive anaerobe 3679h*

Complete chemically defined medium[a] minus	Supplement			Absorbancy at 600 mμ	
	KCl (10 mM)	Na_2HPO_4 (5 mM)	$Na_4P_2O_7$, (10 mM)	Vegetative cells[b]	Spores[c]
Nothing...	−[d]	−	−	0.88	0.56
NaCl.....	−	−	−	0.01	0.02
	−	+	−	0.80	0.58
K_2HPO_4..	−	−	−	0.01	0.02
	−	+	−	0.01	0.02
	+	−	−	0.19	0.10
	+	+	−	0.78	0.58
	+	−	+	0.01	0.02

[a] See Table 1.
[b] Measured after incubation for 32 hr at 35 C.
[c] Measured after incubation for 6 days at 35 C.
[d] Symbols: − = not added; + = added.

ACKNOWLEDGMENT

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