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NUTRITIONAL REQUIREMENTS OF *BACILLUS POPILLIAE*[1]

CHARLES J. SYLVESTER[2] AND RALPH N. COSTILOW

Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan

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ABSTRACT

SYLVESTER, CHARLES J. (Michigan State University, East Lansing), AND RALPH N. COSTILOW. Nutritional requirements of *Bacillus popilliae*. J. Bacteriol. **87**:114-119. 1964.—*Bacillus popilliae* grew consistently well in a semisynthetic medium containing a "vitamin-free," salt-free, acid hydrolysate of casein supplemented with dextrose, DL-tryptophan, and thiamine. However, when the casein hydrolysate was replaced with a complete array of amino acids, no growth occurred. The addition of barbituric acid to this synthetic medium resulted in consistent growth. A number of purines, pyrimidines, and other nitrogen-containing compounds were tested, but none was found that would replace barbituric acid. In synthetic media, *B. popilliae* was found to require biotin, thiamine, and 11 amino acids for growth. Three additional amino acids were stimulatory. Preliminary studies indicated that the nutritional requirements of *B. lentimorbus*, another species known to be pathogenic for Japanese beetle (*Popillia japonica*) larvae, were similar to those for *B. popilliae*.

Bacillus popilliae and *B. lentimorbus*, the causative agents of "milky disease" in Japanese beetle (*Popillia japonica*) larvae, are members of the fastidious group of bacilli. Dutky (1940) and Steinkraus (1957) devised laboratory media which supported the growth of *B. popilliae*, and Haynes et al. (1961) succeeded in growing both species in vitro and in preserving them by lyophilization of vegetative cells. These organisms are known to require simple carbohydrates as an energy source (Dutky, 1947; Steinkraus, 1957), and Dutky (1947) reported that thiamine was an essential growth factor.

At best, these organisms grow poorly, have very limited viability, and sporulate poorly or not at all in laboratory cultures (Steinkraus and Tashiro, 1955; Steinkraus and Provvidenti, 1958; Rhodes et al., 1963). Conversely, spore populations attained in the larvae of the beetle average about 2×10^9 per larvae (Beard, 1945), which would equal a population of about 4×10^{10} spores per ml of body fluid. It appears possible that the failure of these organisms to sporulate in vitro may be a result of the failure of laboratory cultures to attain similar population levels. This nutritional study represents one of the fundamental approaches being made to this problem. Detailed investigations of the vitamin and amino acid requirements of *B. popilliae* were made, and simplified semisynthetic and synthetic media developed. The adequacy of these media to support growth of several strains of *B. lentimorbus* was determined.

MATERIALS AND METHODS

Cleaning of glassware. Pipettes were cleaned by soaking them in hot detergent solution consisting of T Kal plus B-R 5512-S (Du Bois Chemical, Inc., E. Rutherford, N.J.) for 20 to 30 min and thoroughly rinsing them in tap and distilled water. They were dried in a gas-heated convection oven for 12 hr at 180 C or higher. Other glassware was scrubbed with synthetic detergent, rinsed thoroughly in tap and distilled water, and air dried.

Cultures. The cultures used in this study were obtained through the courtesy of W. C. Haynes, Northern Regional Research and Development Division, Agricultural Research Service, Peoria, Ill. *B. popilliae* NRRL B-2309 was used in most studies, but additional NRRL cultures (B-2043, B-2519, B-2524) were tested for growth in a synthetic medium. *B. lentimorbus* NRRL B-2522 was used for nutritional studies, and NRRL B-2521 and B-2530 were tested for growth in various media. The original source of these cultures was described by Haynes et al. (1961). Separate culture lines used in some tests resulted from the maintenance of stock cultures initiated from different lyophil tubes of the same strain.

[1] Journal Article No. 3222, Michigan Agricultural Experiment Station.

[2] Present address: Henry Ford Hospital, Detroit, Mich.

Stock cultures were carried on slants of a medium, designated B-4, comprised of 1.5% yeast extract, 0.2% glucose, 0.6% K_2HPO_4, and 1.5% agar (H. H. Hall, *personal communication*). Subcultures on this medium were made two or three times weekly.

Inocula. Inocula were prepared in two ways. In earlier experiments, 100 ml of B-4 broth in a 500-ml Erlenmeyer flask were inoculated with a loopful of cells from the slant of a 48-hr stock culture. The flasks were incubated on a rotary shaker oscillating at 200 rev/min. For some of these earlier tests, 100 ml of a semisynthetic medium, designated SS-2, were used instead of B-4 broth. The composition of SS-2 is described in Results. After 24 hr of incubation at 30 ± 1 C, the cells were centrifuged and resuspended in 0.85% saline.

For later tests, inocula were prepared by adding 5 ml of B-4 broth to a 48-hr slant culture, suspending the cells by agitation, and then using the entire suspension to inoculate 95 ml of B-4 broth. After incubating the broth culture for 24 hr, the cells were harvested by centrifugation, washed once in 0.85% saline, and resuspended in saline. Viable-cell counts of *B. popilliae* NRRL B-2309 and *B. lentimorbus* NRRL B-2522 stored in 0.85% saline indicated that no drastic reduction in viable cells occurred during the harvesting and inoculating procedures.

Regardless of the method of preparation, a 1% inoculum containing approximately 3×10^8 viable cells per ml was used.

Media. Preliminary investigation demonstrated that a semisynthetic medium (SS-1) containing 1.5% acid hydrolysate of casein (salt-free, "vitamin-free"; Nutritional Biochemicals Corp., Cleveland, Ohio), 1.0% glucose, 0.2% K_2HPO_4, and 0.01% DL-tryptophan supplemented with the nine vitamins listed in Table 1 (concentrations shown in table; pH 7.4) would support growth of *B. popilliae* comparable with that in B-4 broth. Attempts to substitute inorganic nitrogen sources, Vitamin Free Casamino Acids (Difco), or enzymatic hydrolysate of casein (General Biochemicals, Inc., Chagrin Falls, Ohio) for the acid hydrolysate were completely unsuccessful. Also, attempts to increase the amount of growth by increasing the K_2HPO_4 to 0.6% and by adding salts and trace elements were not successful. No growth occurred in this medium when DL-tryptophan was omitted, and very little growth was evident when the glucose level was reduced to 0.2%. Vitamin requirements were determined in this medium by deleting vitamins one at a time. Stimulatory effects were determined by adding

TABLE 1. *Composition of synthetic medium no. 1 (S-1)*

Ingredient	Concn	Ingredient	Concn
	g/liter		*mg/liter*
Glucose	10	DL-Norvaline	200
K_2HPO_4	6	DL-Phenylalanine	200
		L-Proline	200
	mg/liter	DL-Serine	100
DL-Alanine	400	DL-Threonine	100
L-Arginine	400	DL-Tryptophan	100
L-Asparagine	800	L-Tyrosine	100
DL-Aspartic acid	200	DL-Valine	200
L-Cysteine·HCl	200		
L-Cystine	200		*μg/liter*
L-Glutamic acid	800	*p*-Aminobenzoic acid	200
L-Glutamine	40	Biotin	2
Glycine	200	Calcium pantothenate	400
L-Histidine HCl	100	Folic acid	2
Hydroxy-L-proline	100	Myoinositol	2,000
L-Isoleucine	50	Niacin	400
L-Leucine	100	Pyridoxine·HCl	400
L-Lysine HCl	200	Riboflavine	200
DL-Methionine	200	Thiamine·HCl	400
DL-Norleucine	100		

TABLE 2. *Influence of various vitamins on the growth of Bacillus popilliae in semisynthetic media**

Vitamin	OD† when deleted from SS-1	OD† when added to SS-2
Controls, complete media	0.82	0.50
Biotin	0.46	—
Folic acid	0.81	—
Biotin and folic acid	—	0.61
p-Aminobenzoic acid	0.76	0.50
Myoinositol	0.72	0.58
Pyridoxine·HCl	0.78	0.54
Niacin	0.55	0.49
Calcium pantothenate	—	0.48
Riboflavine	—	0.33
Pantothenate and riboflavine	0.85	—
Thiamine·HCl	*0.04*	—

* The basal semisynthetic medium contained 1.5% salt-free, "vitamin-free," casein hydrolysate, 0.2% K_2HPO_4, 1.0% glucose, and 0.01% DL-tryptophan. SS-1 contained all of the vitamins in the concentrations listed in Table 1, and SS-2 contained only thiamine·HCl. Individual and pairs of vitamins were deleted or added as indicated.

† Growth was estimated by the optical density at 620 mμ after 72 to 78 hr of incubation on a rotary shaker. Values are averages of duplicate determinations. The value which is less than one-half the control is in italics.

the individual vitamins to a semisynthetic medium (SS-2) containing only the essential vitamin.

The composition of the initial synthetic medium devised (S-1) is given in Table 1. The amino acid composition is similar to that used in a synthetic medium devised by Sauberlich and Baumann (1948). Although a number of other synthetic media were developed, the concentrations of the individual compounds were not changed. Vitamin and amino acid requirements were determined by deleting them singly and in groups from synthetic media.

The water used in preparation of media was deionized and double-distilled in a glass unit for early experiments; in the final work with synthetic media, two additional distillations were performed, and potassium permanganate was present during the third distillation.

Media were prepared in 100-ml volumes and dispensed in 500-ml Erlenmeyer flasks plugged with gauze-wrapped cotton rolls. They were sterilized by autoclaving at 121 C for 15 min. B-4 medium was autoclaved with all ingredients combined. The synthetic media were autoclaved without glucose, and a 20% glucose solution was autoclaved separately and added aseptically to the cooled medium. The basal semisynthetic media were sterilized by filtration through an asbestos Seitz filter pad.

Growth measurements. All test cultures were incubated at 30 ± 1 C on a rotary shaker set at 200 rev/min. Growth was recorded as optical density (OD) with a Bausch and Lomb Spectronic-20 colorimeter at a wavelength of 620 mμ. The instrument was set at zero OD with a sample of sterile medium or a sample of inoculated medium clarified by centrifugation.

RESULTS

Vitamin requirements of B. popilliae in a semisynthetic medium. Thiamine was the only vitamin found absolutely essential for growth when vitamins were omitted singly from a semisynthetic medium with a rich vitamin supplement (SS-1). A simpler medium (SS-2) containing only thiamine supported good growth (Table 2). However, growth in the vitamin-rich medium was significantly greater than that in SS-2, and the data indicated some stimulatory effect of biotin, myoinositol, and niacin. The addition of riboflavine to SS-2 proved inhibitory.

Development of a synthetic medium. Initial experiments indicated that synthetic medium S-1 (Table 1) was adequate to support consistent growth of *B. popilliae*. However, on initiating studies of vitamin and amino acid requirements with this medium, it was found that the complete medium failed to yield consistent results and, in many cases, failed to support any significant growth. Various purines, pyrimidines, and other nitrogen-containing compounds were added individually to this medium in a search for some missing factor. Only urea and barbituric acid were found to stimulate growth, and the results with urea were not consistent (Table 3). Therefore, the synthetic medium was altered by incorporation of 0.1% barbituric acid, which was as effective as 0.5%. This medium is referred to as S-2. Neither barbituric acid nor urea influenced growth in the vitamin-rich semisynthetic medium (SS-1).

Vitamin requirements of B. popilliae in synthetic media. Thiamine and biotin proved to be the only vitamins essential for growth in S-2 medium when

the vitamins were deleted individually (Table 4), and a synthetic medium containing only these two vitamins (S-3) proved to be as effective as the control (S-2). Four other strains of *B. popilliae* including two culture lines of three of the strains were tested for growth in synthetic medium S-3. At least one culture of each strain grew well, but one culture line of two strains did not grow.

Amino acid requirements of B. popilliae. Of the amino acids included in synthetic medium S-3, 11 proved essential or stimulatory when deleted individually (Table 5). However, when the other 11 amino acids plus asparagine and glutamine were deleted, the medium would not support growth of *B. popilliae*. By deleting in groups, it was found that 6 of the 11 remaining amino acids could be deleted without impairing growth, and this synthetic medium was designated as S-4.

TABLE 3. *Effect of urea and barbituric acid on the growth of Bacillus popilliae in synthetic medium (S-1)*

Additive	OD*			
	Trial 1	Trial 2	Trial 3	Trial 4
None	0.00	0.00	0.00	0.00
Urea†	0.19	0.09	0.00	0.00
Barbituric acid†	0.16	0.10	0.31	0.13
Barbituric acid + urea	0.12	0.09	0.21	—

* Optical density readings after 70 to 72 hr of incubation.

† The concentration was 0.5% for trial 1 and 0.1% on subsequent trials.

TABLE 4. *Growth response of Bacillus popilliae in a synthetic medium (S-2) from which various vitamins were omitted*

Vitamin omitted	OD*
None	0.21
p-Aminobenzoic acid	0.25
Biotin	*0.03*
Calcium pantothenate	0.22
Folic acid	0.21
Myoinositol	0.20
Niacin	0.24
Pyridoxine·HCl	0.25
Riboflavine	0.20
Thiamine·HCl	*0.00*
All except biotin and thiamine	0.22

* Optical density after 72 hr of incubation. All values less than one-half the control are italicized.

TABLE 5. *Effect of deletion of individual amino acids from synthetic media on the growth of Bacillus popilliae*

Amino acid omitted	OD*	
	Medium S-3†	Medium S-4‡
None	0.17	0.18
DL-Alanine	0.16	—
L-Arginine	*0.01*	*0.00*
L-Asparagine	0.19	*0.00*
DL-Aspartic acid	0.26	0.24
L-Cysteine·HCl	0.16	*0.02*
L-Cystine	0.15	0.20
L-Glutamic acid	0.14	0.27
L-Glutamine	0.20	0.20
Glycine	*0.01*	*0.02*
L-Histidine	*0.01*	*0.00*
Hydroxy-L-proline	0.16	—
L-Isoleucine	*0.03*	*0.05*
L-Leucine	*0.01*	*0.00*
L-Lysine·HCl	0.18	—
DL-Methionine	0.15	*0.01*
DL-Norleucine	0.14	—
DL-Norvaline	0.15	—
DL-Phenylalanine	*0.08*	*0.08*
L-Proline	*0.01*	*0.00*
DL-Serine	*0.01*	*0.00*
DL-Threonine	0.13	—
DL-Tryptophan	*0.00*	*0.00*
L-Tyrosine	*0.05*	*0.10*
DL-Valine	*0.01*	*0.00*

* OD after 70 to 72 hr of incubation on a rotary shaker. All values less than one-half the control are italicized.

† S-3 medium varied from the composition of S-1 (Table 1) by the absence of all vitamins except biotin and thiamine, and by the addition of 0.1% barbituric acid. Values given are averages of duplicate determinations.

‡ S-4 medium differed from S-3 by the absence of the six amino acids indicated by the dash.

In deleting the amino compounds singly from this medium, ten amino acids and asparagine were found to be absolutely essential for growth, and three acids were definitely stimulatory (Table 5).

A simplified synthetic medium (S-5) was then devised with the following composition (per liter): glucose, 10 g; K_2HPO_4, 2 g; barbituric acid, 1 g; biotin 2 μg; thiamine·HCl, 400 μg; L-arginine, 400 mg; L-asparagine, 800 mg; L-cysteine·HCl, 200 mg; glycine, 200 mg; L-histidine·HCl, 100 mg; L-isoleucine, 50 mg; L-leucine, 100 mg; DL-

methionine, 200 mg; DL-phenylalanine, 200 mg; L-proline, 200 mg; DL-serine, 100 mg; DL-tryptophan, 100 mg; L-tyrosine, 100 mg; DL-valine, 200 mg; pH 7.4.

This medium supported growth of *B. popilliae* B-2309 on both initial inoculations and on subculture.

Studies with B. lentimorbus. Limited studies with *B. lentimorbus* indicated that the nutritional pattern was similar to that of *B. popilliae*. Thiamine was required for growth in semisynthetic and synthetic media, and biotin and niacin were stimulatory. Folic acid appeared to be interchangeable with biotin. Barbituric acid or urea was required for growth in a synthetic medium (S-1). Again, considerable strain and culture variation was evident.

DISCUSSION

The nutritional patterns of *B. popilliae* and *B. lentimorbus* are quite similar to those of a number of species of this genus; i.e., they require mixtures of amino acids, thiamine, biotin, and occasionally niacin for growth (Knight and Proom, 1950; Proom and Knight, 1955). There is no reference to a requirement for barbituric acid by other bacilli; but, in a number of instances, the nutrient requirements have not been studied in synthetic media and a requirement for this factor would not have been observed.

The barbituric acid requirement of *B. popilliae* and *B. lentimorbus* for growth in synthetic media leads to considerable speculation. It is tempting to suggest that it is utilized as a precursor for pyrimidine synthesis, but it is difficult to explain why uracil and other pyrimidines would not substitute for it. Hayaishi and Kornberg (1951) and Hayaishi (1952) demonstrated the oxidation of uracil to barbituric acid, and the cleavage and oxidation of this to malonic acid and urea by *Mycobacterium* and *Corynebacterium* species. Wang and Lampen (1952) isolated a *Bacterium* from soil that utilized barbituric acid as well as uracil, thymine, or cytosine as the sole source of C, N, and energy. However, it is not likely that barbituric acid is being utilized as an energy source by *B. popilliae* and *B. lentimorbus*, because the synthetic media contained glucose which these organisms oxidize readily (Pepper and Costilow, J. Bacteriol. *in press*). It is possible that the reaction is going in the reverse direction, with barbituric acid being reduced to uracil. However, one would expect uracil to be more effective than the acid if this were true, unless there is a selective permeability problem or unless the function as an electron acceptor is of prime importance. This area is worthy of more experimentation.

The need of *B. popilliae* for a nitrogen source other than amino acids is not unique in this genus. Proom and Knight (1955) found urea essential for six strains of *B. lentus* when grown in a synthetic medium containing seven amino acids, biotin, and thiamine. However, when nine additional amino acids were added to the medium, three strains grew without urea and the remaining three strains required either urea or ammonium chloride. Katznelson and Lochhead (1948) found a number of strains of *B. larvae*, an insect pathogen, to require a purine for growth in synthetic medium, with xanthine and guanine being interchangeable. However, *B. larvae* failed to grow on subculture in a fluid synthetic medium containing vitamins, a purine, and 18 amino acids; serial transfer was accomplished only on the addition of washed agar to give either a semisolid or solid medium and inoculation with a heavy suspension of cells. The amino acid requirements appeared different in the medium with washed agar. It would be of interest to determine the response of *B. lentus* and *B. larvae* to barbituric acid in synthetic media.

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