JOURNAL OF BACTERIOLOGY, Oct. 1975, p. 279-284

Vol. 124, No. 1
Printed in U.S.A.

Development of Defined and Minimal Media for the Growth of *Bacillus stearothermophilus*

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Received for publication 22 July 1975

Defined media, both solid and liquid, that support good growth of *Bacillus stearothermophilus* 1503 have been developed. Data are presented which indicate that manganese is required at relatively high concentrations for growth in a defined liquid medium. Phosphate concentrations higher than 5×10^{-3} M have been shown to inhibit colony formation on solid media. Maximum viable counts of approximately 10^9 colony-forming units per ml were obtained in both the defined and minimal liquid media. Glucose, fructose, sucrose, glycerol, and starch support the growth of this obligate thermophile in the defined media, whereas citrate, α-ketoglutarate, succinate, fumarate, malate, acetate, and lactate do not. The described media have been utilized to isolate several amino acid-requiring mutants of *B. stearothermophilus*.

Although defined and minimal liquid media for the growth of *Bacillus stearothermophilus* 1503 have been reported (1, 3), we have found them to be inadequate for physiological studies. The growth observed by other investigators probably resulted from carry-over of nutrients with inocula that had been grown originally in a complex medium. The problem of defining growth in *Bacillus* sp. on the basis of changes in absorbance is complicated by the fact that cells often sporulate when transferred from a complex to a defined medium. Thus, the increase in absorbance observed is often the result of increased refractility during sporulation rather than the result of vegetative growth. Importantly, when we prepared the media previously reported (1, 3) they failed to support growth in the agar form. The absence of a defined or minimal agar medium for the growth of this sporeforming obligate thermophile has restricted progress in the study of the genetics of this organism.

This report describes defined and minimal media, both liquid and solid, that support good growth of *B. stearothermophilus* 1503. Moreover, the solid media have been shown to be useful for the isolation of amino acid-requiring mutants of this organism.

A preliminary report of this work was presented at the 75th Annual Meeting of the American Society for Microbiology, New York, N.Y. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1975, I11, p. 118), and was taken in part from a thesis submitted by J.J.R. to the University of Kansas, Kansas City, in partial fulfillment of the requirements for the Ph.D. degree (1975).

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MATERIALS AND METHODS

Organisms. *B. stearothermophilus* NCA 1503 (ATCC 7954) was obtained from R. Downey. *B. stearothermophilus* NCA 1518 (ATCC 7953) was purchased from the American Type Culture Collection. *B. coagulans* KU, a facultative thermophile, was a gift from J. Akagi.

Chemicals. Amino acids were obtained from either Sigma Chemical Co. or Calbiochem. All other chemicals used in this study were of reagent grade. Deionized, distilled water was used in all procedures.

Media. Antibiotic medium 3 and nutrient broth were prepared as described by Difco and supplemented with 0.2% glucose; in solid form, 2% (wt/vol) agar was included.

Thermophilic defined liquid and agar media (TDLM and TDAM, respectively) were prepared as described in Tables 1 and 4. The thermophilic minimal liquid and agar media (TMLM and TMAM, respectively) were made in the same way as the defined media, but included only the amino acids L-glutamate, L-histidine, L-isoleucine, L-methionine, and L-valine. The procedures described in Tables 1 and 4 involve making each stock solution separately. However, the amino acids may be combined as a single stock solution (10× with respect to final concentration) with the exception of tyrosine and tryptophan. Tyrosine requires the addition of 1 N HCl for solubilization, and tryptophan requires the addition of 1 N NaOH. The components must be added in the order listed to minimize precipitation.

Growth conditions. TDAM or TMAM plates were spread with 0.1 ml of a 0.9% saline suspension of lyophilized cells (previously grown in TDLM), and the plates were incubated for 8 to 10 h at 60 C in a humid atmosphere. After incubation, the plates were harvested in a small volume (approximately 5 ml) of the growth medium, and the liquid medium (100 ml contained in a 500-ml Erlenmeyer flask) was inoculated with enough of this cell suspension to give an initial absorbance reading of at least 0.05 at 600 nm. The cultures were incubated with vigorous aeration at 60 C in a water bath-shaker until they reached the midexponential growth phase (absorbance of approximately 0.45). A transfer (5%, vol/vol) to 100 ml of fresh medium was made, growth was followed by turbidity to midexponential phase, and a third transfer (5%, vol/vol) was made to 100 ml of fresh medium. Growth in this final culture was monitored by turbidity and viable cell count (colony-forming units per milliliter).

Growth on various carbon sources. The ability of *B. stearothermophilus* to utilize various substrates as the sole source of carbon was determined on TMAM (without glucose) by using Difco concentration disks (diameter of 0.25 inch [ca. 0.64 cm]) saturated with various stock solutions as follows: 15% (wt/vol) of glucose, potassium acetate, sodium citrate, α-ketoglutarate, sodium succinate, potassium fumarate, and sodium malate; 15% (vol/vol) of glycerol and sodium lactate; 10% (wt/vol) of fructose, sucrose, and starch. Citrate, α-ketoglutarate, succinate, fumarate, and malate were titrated to pH 7.0 with 1 N NaOH prior to use.

Isolation of amino acid-requiring mutants. *B. stearothermophilus* was treated with the mutagen *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine as described previously (2). The mutagenized cells were plated on TDAM, and the plates were incubated at 60 C in a humid atmosphere for 36 h. Individual colonies (1,000) were replica picked with sterile toothpicks to TMAM, and the plates were incubated for 48 h at 60 C in a humid atmosphere. Those cells unable to form colonies on TMAM were classified as presumptive auxotrophic mutants. These presumptive mutants were then tested for specific amino acid requirements by the disk method.

RESULTS

Initially, several attempts were made to obtain good growth of *B. stearothermophilus* 1503 in previously described defined liquid media. Only one of these (1) supported the growth of the organism after several 10% transfers; however, growth was unsatisfactory, reaching a maximum population of only 10^8 colony-forming units per ml. In no instance did any of the previously reported defined liquid media support growth when used in the solid form. For these reasons, we initiated studies to design a new medium (that could be used both as a liquid and solid) for studies of this organism. Because satisfactory growth (approximately 10^9 colony-forming units per ml) was achieved in a casitone mineral salts medium, the quantitative amino acid composition of this medium was obtained from Difco Co. A defined liquid medium containing all 20 amino acids at a concentration equivalent to that found in the casitone medium was made. However, as was our experience with the medium described by Campbell and Williams (1), the maximum population obtained was only 10^8 colony-forming units per ml. It is well known that certain metals such as manganese and zinc are required for the activity of some key enzymes, and we therefore incorporated these two elements into our medium (Table 1). The dramatic effect of Mn^{2+} on the

TABLE 1. *Thermophilic defined liquid medium (TDLM) for growth of B. stearothermophilus 1503*

Ingredients[a]	Amount (ml)
L-Alanine	0.84
L-Arginine·HCl	0.64
L-Asparagine·H_2O	0.50
L-Aspartate (monopotassium salt)	1.30
L-Cystine	0.50
L-Glutamate·HCl	4.00
L-Glutamine	0.50
Glycine	0.50
L-Histidine·HCl·H_2O	0.42
L-Isoleucine	1.00
L-Leucine	1.64
L-Lysine·HCl	1.40
L-Methionine	0.52
L-Phenylalanine	0.86
L-Proline	1.00
L-Serine	1.40
L-Threonine	0.84
L-Tryptophan	0.30
L-Tyrosine	0.56
L-Valine	1.26
Biotin (10 mg/100 ml)	1.00
Thiamine·HCl (10 mg/100 ml)	1.00
Nicotinic acid (10 mg/100 ml)	1.00
Anhydrous $CaCl_2$ (5%)	0.01
$FeCl_2·6H_2O$ (0.05%)	0.01
$ZnSO_4·7H_2O$ (5%)	0.01
$MnCl_2$, 10 mM	0.01
Glucose (20%)	1.00
Mineral salts solution (10 g of NH_4Cl, 10 g of NaCl, 4 g of $MgSO_4$ per liter)	10.00
Potassium phosphate buffer (125 g of K_2HPO_4 and 30 g of KH_2PO_4 per 500 ml)	5.00
Total volume	100.00

[a] All ingredients were added in the order indicated above without stirring to prevent precipitation. All stock amino acid solutions were 1% (wt/vol). The final volume was adjusted to 100 ml with deionized water, and the solution was filter sterilized; the pH was 7.3.

growth of *B. stearothermophilus* 1503 is illustrated in Fig. 1. Even after a 5% (vol/vol) transfer into medium lacking Mn^{2+}, growth nearly equivalent to that obtained in the original medium was still observed. However, after a second 5% (vol/vol) transfer into a fresh batch of medium lacking Mn^{2+}, growth leveled off at an absorbance of 0.26. When a final 5% (vol/vol) transfer was made from this culture into one containing Mn^{2+}, optimal growth was again obtained. Examination of the viable counts (see Fig. 3A) revealed that this increase in absorbance represents true growth. From these experiments it can be concluded that Mn^{2+} became limiting after the second 5% transfer into medium lacking Mn^{2+}, indicating that this metal becomes growth limiting at a concentration of approximately 2.5×10^{-9} M.

This medium was made as agar, and plates were streaked with a loopful of a culture growing in the liquid form of the medium. However, no discrete viable colonies were observed after 48 h of incubation at 60 C. It was concluded that either additional factors were required for the organism to grow on the agar medium, or that the concentration of one or more of the components of the medium was too low to support colony formation. The components of the medium were divided into four groups as follows: (i) mineral salts; (ii) amino acids; (iii) vitamins; and (iv) phosphate. Each of these was incorporated into the medium one at a time at concentrations ranging from two- to threefold higher than that found in the liquid medium while all other components were maintained at 1× concentration. As another approach, all of the components were increased to three times the concentration found in the liquid medium shown in Table 1. The plates were inoculated with a loopful of cells grown in TDLM and incubated at 60 C for 48 h. No discrete colonies were observed. These media were also modified by the addition of various other growth factors (vitamins, pyrimidines, and purines); however, in none of these cases were discrete colonies obtained. We reasoned that one of the components of TDLM might be inhibiting colony formation on the agar medium. To test this possibility, various dilutions of the liquid medium were made, agar was added, and ability of this medium to support growth was tested by inoculation of plates with a loopful of a culture of *B. stearothermophilus* grown in TDLM. The results of this experiment are seen in Table 2. At a dilution of 1:20, discrete colonies that could be readily transferred by picking were observed after 24 h of incubation at 60 C. Further dilutions did not result in an increase in colony size. Four different media were made, each of which contained all of the ingredients at a 1× concentration, with the exception of one component which was diluted 1:20. These plates were streaked with a loopful of culture growing in TDLM, and the plates were incubated for 24 h at 60 C in a humid atmosphere. The results (Table 3) strongly suggested that phosphate inhibited growth when the medium was made in the solid form. However, to eliminate the possibility that potassium could be involved in the inhibition, we performed an analogous experiment employing sodium phosphate buffer, and obtained identical results. It can be concluded that under these conditons a phosphate concentration greater than 5×10^{-3} M inhibits the formation of discrete viable colonies on defined solid medium. Based on these data, a defined solid medium (TDAM) was devised containing the components listed in Table 4.

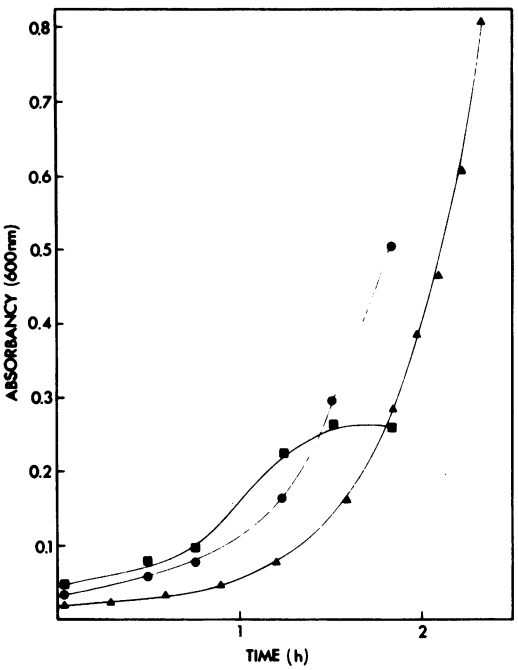

FIG. 1. *Effect of manganese on the growth of B. stearothermophilus in TDLM (see Table 1). All cultures (100 ml of TDLM contained in 500-ml Erlenmeyer flasks) were incubated at 60 C with shaking in a water bath-shaker.* ●, *Growth (by turbidity readings) in TDLM minus manganese; inoculated (5% vol/vol) with a culture grown to midexponential phase in TDLM plus manganese.* ■, *Growth in TDLM minus manganese; inoculated (5%, vol/vol) with the culture described for the first curve.* ▲, *Growth in TDLM plus manganese; inoculated (5%, vol/vol) with the culture described for the second curve.*

Further experimental procedures involved

TABLE 2. *Growth of B. stearothermophilus on dilutions of TDLM containing 2% agar*

Dilution	% Glucose	Colonies[a]
1:5	0.20	–
1:5	0.04	–
1:10	0.20	–
1:10	0.04	–
1:20	0.20	+
1:20	0.04	+
1:40	0.20	+
1:40	0.04	+
1:60	0.20	+
1:60	0.04	+

[a] +, Discrete colonies; –, complete absence of growth.

TABLE 3. *Growth of B. stearothermophilus on agar plates of TDLM with a 1:20 dilution of various components*

Dilution	Components	Colonies[a]
1:20	Mineral salts	–
1:20	Amino acids	–
1:20	Vitamins	–
1:20	Phosphate buffer	+

[a] +, Discrete colonies; –, complete absence of growth.

the systematic elimination of individual amino acids to determine those which were required for optimal growth of the thermophile. The organism was found to require glutamate, histidine, isoleucine, methionine, and valine for optimal growth. Although growth was observed in the absence of glutamate, longer incubation was required, and the colonies were much smaller when this amino acid was excluded. Figure 2 illustrates the growth of *B. stearothermophilus* 1503 on various solid media. The inocula for these plates were colonies that had been transferred several times on TDAM; the plates were incubated for 24 h at 60 C. As can be seen, the colonies formed on TDAM were larger than those on complex media incubated for the same period of time. However, incubation for 48 h was required before colonies of the size shown were obtained on TMAM. In contrast to the results cited above, cells grown in complex medium and plated directly on the four media indicated in Fig. 2 were able to form sizable colonies on complex media in 24 h; it required 48 h of incubation to obtain colonies comparable to the size shown in Fig. 2 on TDAM and TMAM.

In the previous reports (1, 3) of defined media for *B. stearothermophilus* 1503, growth was measured only in terms of turbidity changes, and the effect of serial transfer on growth was not reported. We have found that these earlier media were unsatisfactory in that they either yielded low maximum viable counts or could not support growth after one or two 10% transfers. This latter failure suggests that the growth observed resulted from carry-over of nutrients from the more complex initial growth medium. To evaluate such possibilities in our system and to determine whether equivalent plate counts could be obtained on both complex and defined

TABLE 4. *Thermophilic defined agar medium (TDAM) for growth of B. stearothermophilus*

Ingredients[a]	Amount (ml)
L-Alanine	0.84
L-Arginine·HCl	0.64
L-Asparagine·H_2O	0.50
L-Aspartate (monopotassium salt)	1.30
L-Cystine	0.50
L-Glutamate·HCl	4.00
L-Glutamine	0.50
Glycine	0.50
L-Histidine·HCl·H_2O	0.42
L-Isoleucine	1.00
L-Leucine	1.64
L-Lysine·HCl	1.40
L-Methionine	0.52
L-Phenylalanine	0.86
L-Proline	1.00
L-Serine	1.40
L-Threonine	0.84
L-Tryptophan	0.30
L-Tyrosine	0.56
L-Valine	1.26
Biotin (10 mg/100 ml)	1.00
Thiamine·HCl (10 mg/100 ml)	1.00
Nicotinic acid (10 mg/100 ml)	1.00
Anhydrous $CaCl_2$ (5%)	0.01
$FeCl_3 \cdot 6H_2O$ (0.5%)	0.01
$ZnSO_4 \cdot 7H_2O$ (5%)	0.01
$MnCl_2$, 10 mM	0.01
Glucose (20%)	1.00
Mineral salts solution (10 g of NH_4Cl, 10 g of NaCl, 4 g of $MgSO_4$ per liter)	10.00
KH_2PO_4, 1 M	0.10
Tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.8), 1 M	10.00
Agar (Difco)	2.00 g
Total volume	100.00

[a] All ingredients except the agar were added in the order indicated above without stirring to prevent precipitation. All stock amino acid solutions were 1% (wt/vol). The final volume was adjusted to 50 ml with deionized water, and the solution was filter sterilized. A 50-ml volume of 4% sterile agar (at 55 C) was mixed with 50 ml of the above mixture. The final pH was 7.8.

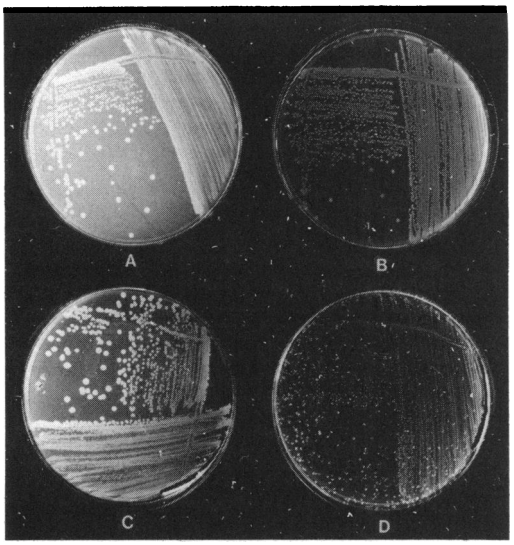

FIG. 2. *Colonies of B. stearothermophilus on: (A) TMAM, (B) antibiotic agar medium 3, (C) TDAM, and (D) nutrient agar medium.*

media, a viable growth curve was obtained for cells grown in TDLM and TMLM. The source of inocula for both systems consisted of 8- to 10-h plate growth either on TDAM or TMAM; cells harvested from plates older than 12 h exhibited decreased viability. Growth obtained in TDLM (Fig. 3A) showed a viable increase of 100-fold above the initial inoculum and reached a maximum viable count of 10^9 colony-forming units per ml. The maximum optical density obtained was 0.75 (Fig. 3B). Moreover, similar plate counts were obtained on both nutrient agar and TDAM. Experiments performed with cells grown in TMLM yielded comparable results. Again a 100-fold increase in viable cell numbers was observed. Appropriate dilutions of the culture sampled at various stages of growth were plated on TDAM and TMAM; comparable viable counts were obtained on both media (Fig. 4A). A turbidimetric measurement (Fig. 4B) indicated growth equivalent to that seen in TDLM (Fig. 3B). Slides made at various stages of growth in both TDLM and TMLM demonstrated no evidence of spore formation.

To determine the kinds of carbon sources that would support growth, a preliminary test was performed utilizing various compounds as sole sources of carbon (Table 5). The organism grew well on all carbon sources that are utilized via glycolysis. No growth was observed on any of the tricarboxylic acid cycle intermediates nor on acetate or lactate. These latter results may reflect the inability of this organism to transport these carbon sources across the cytoplasmic membrane, since a previous report (2) has demonstrated the presence of all of the enzymes of this cycle in *B. stearothermophilus* 1503.

Amino acid-requiring mutants for arginine, threonine, and tryptophan were isolated and purified by employing the solid media described in this communication. In addition, a serine-sensitive mutant was also isolated.

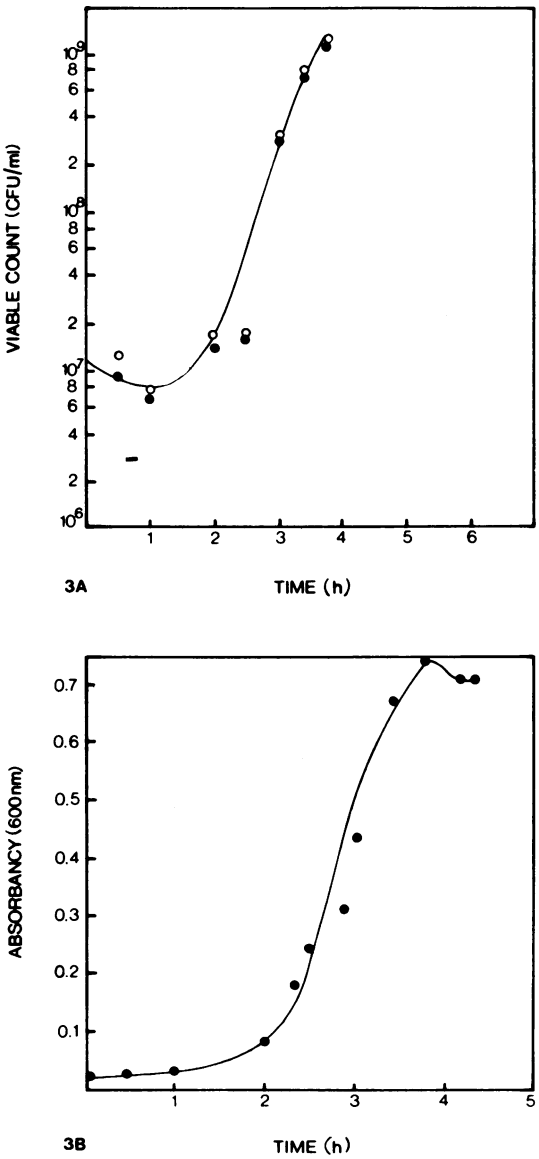

FIG. 3. *(A) Viable growth curve of B. stearothermophilus in TDLM. Plate counts on nutrient agar (●) and TDAM (○). (B) Growth of B. stearothermophilus by turbidity (600 nm) in TDLM.*

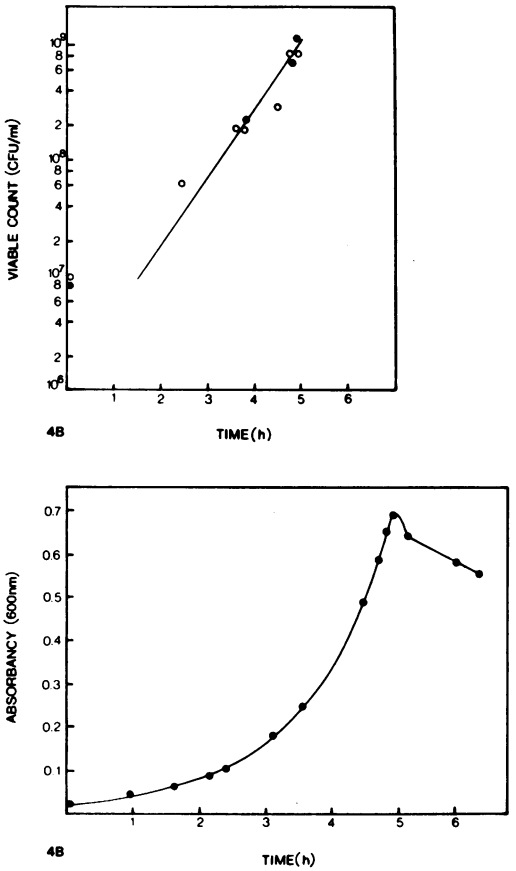

FIG. 4. *(A) Viable growth curve of B. stearothermophilus in TMLM. Plate counts on TDAM (●) and TMAM (○). (B) Growth of B. stearothermophilus (by turbidity, 600 nm) in TMLM.*

DISCUSSION

The results of this investigation suggest that the inability of the liquid media described previously to support good growth of the spore-forming obligate thermophile *B. stearothermophilus* 1503 was due to a unique requirement for manganese and an appropriate concentration of amino acids. Both the thermophilic defined and minimal liquid media (TDLM and TMLM, respectively) reported in this communication are able to support good growth of this organism as measured by turbidity and increase in viable cell numbers. Although manganese is probably a necessary element for the growth of other *Bacillus* sp., it is required in such trace amounts that it need not be added to the defined media.

The inhibition of colony formation by phosphate on both the thermophilic defined and minimal agar media (TDAM and TMAM, respectively) revealed in this study is particularly unusual for the genus *Bacillus*. A concentration of phosphate greater than 5×10^{-3} M inhibits the formation of colonies. In all previously reported liquid media, the phosphate concentrations used were much higher than that reported in this paper. Thus, the high level of phosphate used by other workers probably explains the inability of their media to support growth of the organism when used in solid form. Such inhibition is not observed in the liquid media. It is possible that, although the concentration of phosphate is theoretically the same in both liquid and solid form, the actual water of hydration within the agar medium is much lower, thus effectively increasing actual phosphate concentration in terms of the growth of the organism.

The lack of a defined solid medium has greatly restricted genetic and physiological studies of *B. stearothermophilus*. The amino acid auxotrophs that have been isolated in the study should prove useful in genetic studies of this organism. Furthermore, TDAM has also been shown to support good growth of *B. coagulans* KU (a facultative thermophile) and *B. stearothermophilus* 1518.

TABLE 5. *Growth of B. stearothermophilus on different carbon sources*

Carbon source	Growth[a]
Glucose	++
Fructose	++
Sucrose	++
Glycerol	++
Starch	+
Lactate	−
Acetate	−
Citrate	−
α-Ketoglutarate	−
Succinate	−
Fumarate	−
Malate	−

[a] ++, Good growth; +, growth; −, no growth.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant 5 TO1 AI00137 from the National Institute of Allergy and Infectious Diseases and by a grant from the American Cancer Society (Kansas Division).

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