MINIMAL MEDIA FOR QUANTITATIVE STUDIES WITH *BACILLUS SUBTILIS*

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Received for publication November 7, 1957

The tremendous advances in our knowledge of bacterial genetics made during the past 15 years have been the result of experiments on a small number of species, most of which are fairly closely related. At present, several laboratories are attempting to extend such phenomena as genetic recombination, transformation, and transduction to other genera. The limiting factor in such programs will probably be one of technique involving, for example, the formulation of chemically defined minimal agar media which will allow each cell plated to form a visible colony. Using *Bacillus subtilis*, we have found that previously described "minimal" media do not satisfy this requirement. The present paper describes the development of truly minimal media which allow maximal colony formation by spores and vegetative cells of this organism, as determined on nutrient agar.

MATERIALS AND METHODS

Culture. The organism used was the Marburg strain of *B. subtilis* (ATCC6051). Washed spore suspensions were prepared by inoculating nutrient broth (Difco) tubes with some growth from a nutrient agar slant. The broth tubes were incubated at 37 C on a rotary shaker (220 rpm) for 24 hr. Blake bottles containing nutrient agar were inoculated with 0.75 ml of this broth and were incubated for 4 days. The growth from each Blake bottle was harvested with glass-distilled water using glass beads. The suspension was pasteurized for 20 min at 80 C, filtered through glass wool and washed twice with glass-distilled water. The washed spores were stored at 4 C as a suspension in glass-distilled water. They were diluted to approximately 200 spores per ml for plating experiments.

Preparation of vegetative cell suspensions. A 20 by 175 mm colorimeter tube containing 10 ml of nutrient broth was inoculated with 10^7 to 10^8 washed spores and was incubated on the shaker until an optical density of 0.5–0.6 (Lumetron colorimeter, model 402E, 660 mμ filter) was reached. The cells were then washed twice in the minimal salts solution described below and plated immediately at 10^{-4}, 10^{-5}, and 10^{-6} dilutions.

Mineral salts mixture. The concentrated salts mixture contained the following amounts per L: K_2HPO_4, 30 g; KH_2PO_4, 10 g; NH_4Cl, 5 g; NH_4NO_3, 1 g; Na_2SO_4, 1 g; $MgSO_4 \cdot 7H_2O$, 100 mg; $MnSO_4 \cdot 4H_2O$, 10 mg; $FeSO_4 \cdot 7H_2O$, 10 mg; $CaCl_2$, 5 mg; pH adjusted to 6.8–7.0. The water used in the mixture and in all the chemically defined media was redistilled in an all-glass apparatus. The salts mixture was used at $\frac{1}{10}$ the above concentration for washing the vegetative forms and for the newly devised chemically defined media.

Preparation of washed agar. All chemically defined media were prepared with washed agar. One pound of agar (Difco) was subjected to 15 successive washes, each composed of 10 L distilled water, followed by 3 washes each containing 3 L reagent grade acetone. The agar was collected on a large Buchner funnel using Arthur H. Thomas qualitative filter paper and air dried for 24 hr at 37 C.

Plate counts. The Marburg strain has the ability to spread rapidly at agar-air and agar-glass interfaces. Thus, it was impossible to use the spread plate technique and difficult to use pour plates. Instead, the three layer "sandwich" plate method was employed. The top and bottom protective layers contained only medium while the middle layer also contained the inoculum. Plates were prepared in duplicate and counted daily for 3 days. The counts listed in the tables are the maximum counts observed.

All glassware was washed with acid-dichromate solution and rinsed thoroughly with distilled water before use.

RESULTS

A. Colony formation by washed spores. Several workers have previously described "minimal"

media for spores of the Marburg strain of *B. subtilis*. The first was a glucose-mineral salts liquid medium devised by Burkholder and Giles (1947). Adding washed agar, we found that such a medium supported colony formation from only a small percentage of the spores present. Thus, counts on a complex medium such as nutrient agar were about 100 times as great. Since Burkholder and Giles listed asparagine at 1.5 mg per ml as an optional component, we tried this modification. A higher but still unsatisfactory count was obtained. Morowitz (1953) modified the Burkholder and Giles medium (with asparagine) by adding a small amount of DL-threonine whereas Teas (1950) added low concentrations of DL-glutamic acid and L-arginine to the medium lacking asparagine and used sucrose instead of glucose. In our hands, these supported no greater colony formation than the Burkholder and Giles medium containing asparagine. The best available medium was that used by Guthrie and Saperstein (1949) which contained glutamic acid and asparagine. However, this medium also failed to give counts equal to those obtained on the complex medium.

(1) Effect of supplements:—Addition of a mixture of vitamins, purines and pyrimidines failed to increase the counts obtained on a basal medium of 1 per cent glucose plus mineral salts. However, 0.5 per cent enzyme- or acid-hydrolyzed casein (vitamin-free, Nutritional Biochemicals Corp.) produced counts equivalent to those given by nutrient agar. When a synthetic mixture of amino acids was found to replace the casein hydrolyzates, the problem became one of obtaining the simplest amino acid mixture capable of giving high counts. A mixture of L-alanine, L-glutamic acid, and L-asparagine gave excellent results when each amino acid was added at 10^{-2} M. Lower counts were obtained at 10^{-3} and 10^{-4} M. All 3 amino acids were required for maximum counts. The amino acids were most effective when they were autoclaved in the absence of the salts mixture and agar and added aseptically. They were therefore stored as a neutral sterile mixture with each at 10^{-1} M. The mixture was prepared monthly. The requirement for the amino acids was not dependent upon the cleanliness of the spore suspensions. Both unwashed spores and spores which had been repeatedly washed in water or in versene solution gave maximal colony formation only when the medium contained the 3 amino acids.

TABLE 1

Comparison of spore minimal medium with previously described chemically defined media and nutrient agar

Medium or Reference	Counts per ml
Spore minimal medium	217
Burkholder and Giles (1947) without asparagine	1
Burkholder and Giles (1947) with asparagine	38
Teas (1950)	32
Morowitz (1953)	36
Guthrie and Saperstein (1949)	131
Nutrient agar	184

(2) The spore minimal medium:—From the above experiments a new spore minimal medium was devised. It contained the following components per L: glucose, 10 g; L-alanine, 890 mg; L-glutamic acid, 1470 mg; L-asparagine, 1320 mg; mineral salts mixture, 100 ml; agar, 25 g. The glucose and the amino acid mixtures were autoclaved separately and added aseptically. The pH of the medium before autoclaving should be 6.8 to 7.0. This required no adjustment.

(3) Effectiveness for different spore batches:—The new medium was tested with two other spore batches in addition to the crop used to develop the medium. Full colony formation occurred with all 3 suspensions.

(4) Comparison with previously described media:—The spore minimal medium was compared to those previously used by other workers for growth of the Marburg strain. The results are shown in table 1. As expected, the new medium allowed greater colony formation than any previously described.

(5) Role of alanine, asparagine, and glutamic acid:—Some insight into the roles of the 3 amino acids was obtained by preparing liquid chemically defined media containing various combinations of these compounds each at 10^{-2} M. Duplicate colorimeter tubes containing 10 ml medium were inoculated with about 10^9 washed spores and the changes were observed turbidimetrically. Germination was followed by the initial drop in

[1] The term "germination" is used here to represent the initial stage in the conversion of spore to vegetative cell. The term "growth" refers to the remaining stage up to and including the increase in mass of the vegetative population i. e., outgrowth and vegetative growth.

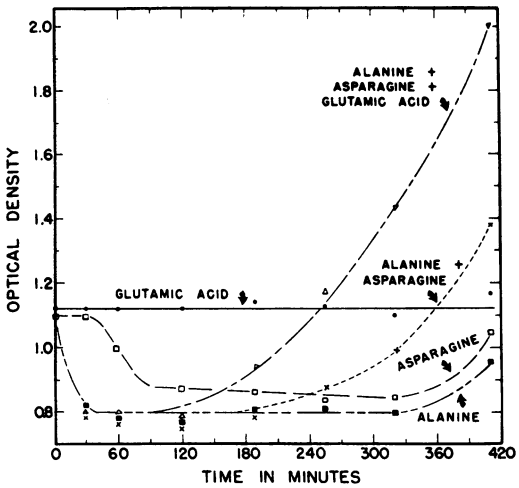

Figure 1. Germination and growth of spores in glucose-mineral salts liquid medium containing various combinations of alanine, asparagine, and glutamic acid.

optical density while growth was observed as the subsequent OD increase.[1] The curves of figure 1 reveal the following facts: (a) L-alanine allowed rapid and extensive germination; (b) L-asparagine had less germinating ability; (c) L-glutamic acid allowed no germination during the time interval studied; (d) addition of L-asparagine or L-glutamic acid to L-alanine did not affect the rate or extent of germination; (e) the long lag in growth of germinated spores observed with L-alanine or L-asparagine was decreased when both were present; (f) the value of glutamic acid lies in its stimulation of growth of germinated spores. Further experimentation showed that the stimulatory effect of the combination of alanine and asparagine was not due to the increased total amino acid concentration i. e., 2×10^{-2} M alanine or 2×10^{-2} M asparagine did not show the shortened lag produced by 10^{-2} M alanine plus 10^{-2} M asparagine.

These data correlate fairly well with those of Hachisuka *et al.* (1955) with *B. subtilis* strain PC1219. These workers found that in liquid cultures, L-glutamic acid stimulated only vegetative growth whereas DL-alanine and L-asparagine increased both germination and growth. L-alanine, however, was ineffective.

(6) Other bacilli:—The new medium was compared to nutrient agar and glucose-salts agar with respect to colony formation by washed spores of *Bacillus cereus* (ATCC6464), *Bacillus brevis* (ATCC18), *Bacillus macerans* (ATCC7069) and *Bacillus natto*. Table 2 shows that all except *B. cereus* gave counts on the chemically defined minimal medium which were greater than the nutrient agar counts. None of the cultures were able to form colonies on glucose-salts agar.

B. Colony formation by washed vegetative forms. (1) Diluents:—Winslow and Brooke (1927) reported that *B. cereus* and *Bacillus megaterium* died rapidly in water or saline but not in nutrient broth. In the present investigation, we found the same phenomenon to occur with *B. subtilis*. The data in table 3 show that rapid death ensued in tap distilled water even when unwashed nutrient broth-grown cells were used. Glass-distilled water was equally poor as a diluent. Saline and tap water offered no protection. However, the mineral salts mixture described above was found to be capable of protecting cells for at least 75 min. Its most active component in this respect was K_2HPO_4 which, at a concentration of 0.02 M, stabilized cells for periods up to 30 min. The phosphate solution was therefore used as a diluent for plating vegetative cells. Longer proce-

TABLE 2

Colony formation by washed spores of other bacilli

Culture	Counts per ml		
	Spore minimal medium	Nutrient agar	Glucose-salts agar
Bacillus cereus	5	126	0
Bacillus brevis	360	157	2
Bacillus macerans	278	121	0
Bacillus natto	174	152	0

TABLE 3

*Survival of unwashed vegetative cells in various diluents**

Diluent	Initial Count per ml	Survival	
		20 min	40 min
		%	%
Tap distilled water	450	2	2
0.85% NaCl	470	4	4
Tap water	500	36	12
Salts	630	103	94

* The cells had been prepared in nutrient broth. The counts were made on nutrient agar after storage at room temperature for the times indicated.

dures, such as washing and centrifuging cells, were done in the salts mixture. Spores were stable in distilled water and required no special diluents.

(2) Plating experiments:—Washed vegetative cells showed extremely poor colony formation on glucose-salts agar, and the chemically defined media previously used by other workers did not support colonial growth. Furthermore, the spore minimal medium was ineffective for vegetative cells.

(3) Effect of supplements:—Using glucose-salts agar as a basal medium, it was found that vitamin and purine-pyrimidine mixtures did not increase colony formation but that amino acid supplement was highly stimulatory. Auxanographic plates were then prepared using the spore minimal medium containing a heavy suspension of washed vegetative cells. Crystals of the individual amino acids were then spotted on the plates. After 16 hr of incubation, only the cysteine spot showed heavy growth.

When L-cysteine·HCl was incorporated into the spore minimal medium, at least as many colonies were formed as on nutrient agar. Cysteine alone was found to be ineffective; the medium also required the presence of either glutamic acid or asparagine but not alanine. Glutamic acid was chosen since it allowed more rapid colony formation than did asparagine.

A vegetative minimal medium was thus prepared containing glucose, 1 per cent; L-glutamic acid, 0.147 per cent; L-cysteine·HCl, 0.1 per cent; mineral salts, 10 per cent v/v, and washed agar, 2.5 per cent. This was compared to the "minimal" media of other workers and was found to be superior (table 4).

(4) Attempts to replace cysteine:—The role of cysteine in stimulating colony formation by vegetative cells was studied by replacing it with other compounds in the above medium. Reducing agents were found to be ineffective. Glutathione was inactive at 0.1 and 1 μg per ml and toxic at higher levels. Ascorbic acid was toxic at the lowest concentration tested i. e., 0.1 μg per ml. Sodium thioglycolate showed a slight replacement effect between 100 and 1000 μg per ml but was toxic at 2000 μg. DL-Homocysteine also showed partial replacement at 100 μg per ml but was toxic at higher concentrations. British anti-Lewisite (1,2-dithioglycerol) was ineffective at 10 μg per ml and inhibited colony formation at 100 μg. It thus appeared that cysteine was not

TABLE 4

Comparison of vegetative minimal medium containing cysteine with previously described chemically defined media and nutrient agar

Medium or Reference	Counts per ml
Vegetative minimal medium containing cysteine	1.9×10^7
Burkholder and Giles (1947) without asparagine	$<10^4$
Burkholder and Giles (1947) with asparagine	4.2×10^5
Teas (1950)	1.2×10^5
Morowitz (1953)	1.7×10^5
Guthrie and Saperstein (1949)	2.2×10^6
Nutrient agar	1.7×10^7

acting as a reducing agent. Since this amino acid also can act as a chelating agent (Michaelis and Guzman Barron, 1929; Schade, 1949; Zentmeyer and Rich, 1956), an attempt was made to replace it with disodium ethylenediaminetetraacetate. This was successful, ethylenediaminetetraacetate being 20 times as active as cysteine on a molar basis. The lowest concentration of ethylenediaminetetraacetate (dihydrate) showing maximal colony formation was 10^{-4} M (40 μg per ml) while 2×10^{-3} M L-cysteine·HCl (300 μg per ml) was required.

Several experiments were conducted to determine why there was a need for a chelating agent. The first possibility considered was that the plating medium contained a toxic level of some mineral. Cysteine has been reported to chelate iron, copper, cobalt, and nickel. Of these, only iron is included in the medium. Thus, platings were conducted in the presence of the normal iron concentration, $\frac{1}{10}$ the normal concentration and without added iron. In all 3 media, ethylenediaminetetraacetate stimulated colony formation indicating that iron toxicity on the plates did not cause the effect. In a further experiment the entire salts mixture was used at normal concentration and at $\frac{1}{10}$ normal but ethylenediaminetetraacetate was effective in both media. Since the effect could not have been due to the salts in the plating medium, the next possibility was that the cells picked up a toxic mineral during washing in the salts mixture. However, this was shown not to be the case as unwashed cells and phosphate-washed cells also showed increased colony formation when plated with ethylenediaminetetraacetate. It is possible that toxicity

TABLE 5

Colony formation by washed vegetative cells of other bacilli

Culture	Counts per ml		
	Vegetative minimal medium	Nutrient agar	Glucose-salts agar
Bacillus cereus	2.4×10^6	3.8×10^7	$<10^4$
Bacillus brevis	1.5×10^8	1.7×10^8	$<10^4$
Bacillus macerans	2.9×10^7	4.0×10^7	$<10^4$
Bacillus natto	5.4×10^6	6.3×10^6	$<10^4$

is picked up during growth in the nutrient broth, but the point was not pursued further.

One more point of interest should be mentioned. In most experiments the minimal medium containing ethylenediaminetetraacetate gave higher counts than nutrient agar. However, the nutrient agar counts could also be increased by addition of ethylenediaminetetraacetate.

(5) The vegetative minimal medium:—A description of the new vegetative medium is as follows (per L): glucose, 10 g; L-glutamic acid, 1470 mg; disodium ethylenediaminetetraacetate·$2H_2O$,[2] 40 mg; mineral salts mixture, 100 ml; agar, 25 g. The glucose was autoclaved separately and added aseptically. The medium was brought to pH 6.8 to 7.0 before autoclaving.

(6) Other bacilli:—The 4 *Bacillus* species tested earlier on the spore minimal medium were plated on the new vegetative medium. Vegetative cells were developed in a manner similar to that used for *B. subtilis*. The results are shown in table 5. All the cultures showed a response to the glutamic acid and ethylenediaminetetraacetate. The poorest response was shown by *B. cereus* which had also grown poorly from spores. The other 3 cultures, however, gave counts on the minimal medium almost equal to that obtained on nutrient agar. Thus, with slight modification, the vegetative minimal medium devised for *B. subtilis* could probably be used for these 3 species.

[2] The ethylenediaminetetraacetate used for the development of the medium was a sample (grade: analytical reagent) obtained from the former Bersworth Chemical Co., Framingham, Mass. At present, reagent grade ethylenediaminetetraacetate can be purchased from Fisher Scientific Co., Fairlawn, N. J.

DISCUSSION

The data presented serve to emphasize the fact that whether or not a nutrient medium can be considered to be adequate depends on the manner in which growth is defined. If one considers the production of turbidity in liquid culture as the criterion of growth, then glucose-mineral salts is a satisfactory minimal medium even though only one cell out of a hundred develops. Thus, Knight and Proom (1950) found that the vast majority of strains of *B. subtilis* could grow with ammonia as the sole nitrogen source and in the absence of added growth factors. However, if the more sensitive criterion of colony formation is used, glucose-salts agar and the other previously described media are nutritionally inadequate.

Since most genetic and mutagenic experiments depend on quantitative conversion of cells to colonies, the newly devised media should be useful for such studies with *B. subtilis* and several other bacilli. Of particular importance will be the utility of these media in isolating nutritional mutants by the "delayed enrichment" technique of Lederberg and Tatum (1945). It is obvious that if a poor medium was used in such a method, only that small portion of the surviving population capable of forming colonies would be marked as prototrophs. Upon addition of the enriched layer, not only would the mutants form colonies but also that large number of prototrophs which would not develop on the insufficient "minimal" medium. This, of course, would lead to very low mutant:isolate ratios and would decrease the efficiency of the procedure.

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. David Hendlin for his continued interest and frequent suggestions. Thanks are also offered to Dr. Robert Guthrie of the Roswell Park Memorial Institute for his stimulating discussions and for details of the composition of his medium.

SUMMARY

The need for a suitable minimal medium for colony formation by *Bacillus subtilis* (Marburg strain) was demonstrated. With this goal in mind, the nutrition of colonial growth was studied.

Washed spore suspensions required L-alanine, L-asparagine, and L-glutamic acid. The main

role of alanine and asparagine was that of stimulating germination while glutamic acid was needed for outgrowth and vegetative growth. The newly devised spore minimal medium gave complete colony formation with *B. subtilis* and also supported colonial growth of *Bacillus brevis*, *Bacillus macerans*, and *Bacillus natto* but it was deficient for *Bacillus cereus*.

For colony formation by washed vegetative cells, glutamic acid and either ethylenediaminetetraacetate or cysteine were required. A new vegetative minimal medium, containing such a supplement, gave maximal plate counts of *B. subtilis*. The medium was slightly less effective for *B. brevis*, *B. macerans*, and *B. natto* whereas *B. cereus* developed poorly on it.

These studies also emphasized the need for mineral salts during washing and diluting vegetative cells. Tap water, distilled water, and physiological saline killed the vegetative cells at an extremely rapid rate.

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