The Nutrition of *Bacillus megaterium* and *Bacillus cereus*

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SUMMARY

Out of nine strains of *Bacillus megaterium* from culture collections, only five grew in a minimal medium that contained glucose and ammonium sulphate as principal nutrients. One strain required biotin, while three other strains required two or more amino acids for growth. A freshly isolated strain of *B. megaterium* required three amino acids for growth.

Three strains of *Bacillus cereus* from a culture collection each required two or more amino acids for growth. From two of these strains, substrains independent of amino acids were developed by serial transfers. Ability to grow in a minimal medium is therefore not so valuable as a distinction between *B. megaterium* and *B. cereus* as previously had been supposed.

INTRODUCTION

Strains of *Bacillus megaterium* examined previously grew in a simple chemically defined medium with glucose and ammonium salts as principal nutrients (Knight & Proom, 1950; Koser, 1968). This ability of *B. megaterium* has been used as an important (e.g. Wolf & Barker, 1968) or supplementary (e.g. Breed, Murray & Smith, 1957) means of identification of the species, and as a distinction from *B. cereus*, which resembles *B. megaterium* in several respects, but requires amino acids as growth factors (Knight & Proom, 1950; Koser, 1968).

White, Lejeune & Work (1969) grew *Bacillus megaterium* (strain 7581) as a source of the enzyme diaminopimelate epimerase. In chemically defined medium, growth in shaken flasks at 37°C from a large inoculum was heavy after about 40 h. However, from a smaller inoculum there was no visible growth, even after prolonged incubation. Biotin has now been found necessary for growth of this strain from a small inoculum.

Another strain (13632) of *Bacillus megaterium* grew very poorly in the defined medium until it was supplemented with amino acids.

These unexpected findings prompted an examination of the nutrition and bacteriological properties of other strains of *Bacillus megaterium* from culture collections and of one that was freshly isolated. Because some of these resembled *B. cereus* nutritionally as well as morphologically, strains of this latter species were also studied to establish the similarities and differences between them and *B. megaterium*. The minimal amino acid requirements of *B. cereus* have not before been determined. When two strains were found each to need only two amino acids, a successful attempt was made to develop amino acid independent substrains of *B. cereus*.

METHODS

Organisms. *Bacillus megaterium*: strains 4821, 7581, 8291, 8508, 9323 and 9376 were from the National Collection of Industrial Bacteria (NCIB); strain 13632 from the American Type Culture Collection (ATCC); strain KM from Dr D. Ellar, Sub-Department of Chemical Microbiology, Department of Biochemistry, Tennis Court Road, Cambridge; strain 216

from Dr G. Coleman, Department of Biochemistry, University of Sheffield. Strain 16 was newly isolated during a class experiment by plating soil pasteurized at 60°C on solid medium and incubating at 30°C.

The origins of strains from the NCIB are described in the catalogue (1964) of that collection. Strain 9376 is the proposed neotype of *Bacillus megaterium* (Smith, Gibson, Gordon & Sneath, 1964). Strain 13632 of the ATCC and strain KM (Northrop, 1951; Weibull, 1953) are supposedly identical, but in our hands the two strains were clearly different (see Results). Strain 216 is from the collection of Professor G. Ivanovics, The Medical University, Szeged, Hungary.

Bacillus cereus strains 6349, 7464 and 9373 were from the NCIB. Strain 9373 is the proposed neotype of *B. cereus* (Smith *et al.* 1964).

All organisms were maintained on slopes of nutrient agar that were incubated at 37°C (except for *Bacillus megaterium* 13632 which was incubated at 30°C) for 8 to 16 h and then kept at 2°C. Stock cultures were transferred monthly, and freeze-dried suspensions of organisms of every strain were kept in sealed ampoules.

Media. All media were made in glass-distilled water. Medium W was the defined medium of White *et al.* (1969) for *Bacillus megaterium* and contained glycerol (1%, w/v) as principal carbon source. Medium A was modified from medium W and the medium of Alföldi (1958) and contained: Na₂HPO₄·12H₂O, 11·2 g; KH₂PO₄, 2·4 g; (NH₄)₂SO₄, 2·0 g; MgSO₄·7H₂O, 50 mg; MnCl₂·4H₂O, 4 mg; FeSO₄·7H₂O, 2·8 mg; either glycerol, 10 g (medium A1) or glucose, 10 g (medium A2); water to 1 l; the pH value was 7·2. The basal medium (without salts of Mg²⁺, Mn²⁺ and Fe²⁺ and carbon source) was autoclaved for 10 min at 121°C. The salts (dissolved together at ×50 strength in 0·01 N-H₂SO₄) were autoclaved separately, as was the carbon source (as a 25%, w/v, aqueous solution); these solutions (20 ml and 40 ml respectively for 1 l of complete medium) and the basal medium were mixed after autoclaving and cooling. Solid media were made by adding Difco agar (1%, w/v).

In tests of nutritional requirements, supplements were added to medium A. All quantities are mg/l of medium. Vitamins were: pyridoxine HCl, 2; pyridoxal HCl, 2; pyridoxamine diHCl, 2; riboflavin, 1; thiamin, 1; Na pantothenate, 1; nicotinic acid, 1; biotin, 0·013; calcium 5-formyl tetrahydropteroylglutamate (leucovorin), 0·01. Purines were: adenine sulphate, 10; guanine HCl, 10. Trace salts were: CaCl₂, 1; CoSO₄·7H₂O, 1; ZnSO₄·7H₂O, 1; CuSO₄·5H₂O, 0·1; H₃BO₃, 0·1; Na₂MoO₄·2H₂O, 0·1; added after autoclaving (as a ×100 strength solution in 0·01 N-H₂SO₄). Amino acids were: Difco 'vitamin-free' acid-hydrolysed casein, 4000; L-cysteine HCl, 50; DL-tryptophan, 100. Difco yeast extract (0·001% final concentration) was also used as a separate supplement to medium A.

In determining requirements for individual amino acids, medium A was supplemented with (mg/l): DL-aspartic acid, 60; DL-glutamic acid, 140; DL-proline, 60; DL-methionine, 30; L-arginine HCl, 70; glycine, 30; DL-serine, 15; DL-alanine, 30; DL-phenylalanine, 30; DL-tryptophan, 10; DL-tyrosine, 60; DL-histidine, 40; L-isoleucine, 40; L-leucine, 40; DL-threonine, 60; DL-valine, 70; L-cysteine HCl, 50; L-lysine HCl, 50.

Glucose nutrient agar was nutrient agar containing glucose (1%, w/v, added after autoclaving).

Fermentations were studied in a basal medium that contained (g/l): Na₂HPO₄·12H₂O, 1·1; KH₂PO₄, 0·24; (NH₄)₂SO₄, 2·0; Difco 'vitamin-free' Casamino acids, 0·1; Difco yeast extract, 0·1. The basal medium (5 ml containing a Durham tube) with indicator (0·05 ml of 0·4% bromothymol blue/tube) was autoclaved (121°C for 10 min) and cooled before adding salts (0·1 ml of the solution used for medium A) and carbohydrate (0·25 ml of a 20%, w/v, solution).

Conditions for growth experiments. Medium W or medium A (500 ml after all additions) in 2 l conical flasks were incubated, usually at 37°C, on a mechanical shaker (model VS gyro-rotatory shaker; New Brunswick Scientific Co., New Brunswick, New Jersey, U.S.A.) at about 200 gyrations/min. Samples (5 ml) were removed at intervals to measure turbidity and for other purposes. The standard inoculum (1.0 ml/500 ml of medium) was a suspension in sterile water of organisms (about 0.4 mg dry wt/ml) from a fresh culture grown for 8 to 16 h at 37°C (30°C for *Bacillus megaterium* 13632) on a nutrient agar slope.

For tests of amino acid requirements in liquid medium A cultures (50 ml after all additions) were shaken as above in 250 ml conical flasks and 0.1 ml of standard inoculum was used. Growth in the minimal medium of Knight & Proom (1950) was similarly tested.

Assessment of growth. Turbidities of liquid cultures were measured in 6 mm diameter tubes in an EEL photoelectric colorimeter (Evans Electroselenium Co. Ltd, Halstead, Essex) with neutral-density filter (no. 1.0) and with water for the zero setting. The relation between colorimeter reading and dry wt of organisms was linear for *Bacillus megaterium* 7581 from 0 to 4.0 on the colorimeter scale, and a reading of 1.0 was equivalent to 0.4 mg dry wt of organisms/ml and to about 2×10^7 organisms/ml. The dry wt of organisms was determined as described by White *et al.* (1969).

Determination of nutritional requirements. A loopful of the standard inoculum (see above) was streaked on to each of a series of plates comprising minimal medium (A1 or A2) and minimal medium singly supplemented by each of the following additions: vitamins; Difco yeast extract; purine solution; trace salts solution; and amino acids solution. One series of plates was incubated at 37°C and another at 30°C.

Vitamin requirements of *Bacillus megaterium* 7581 were determined by streaking organisms (as before) on to minimal medium supplemented by simpler mixtures of the vitamins at the same final concentrations as in the preliminary screening.

Amino acid requirements were determined similarly on solid minimal medium supplemented by groups of amino acids. Requirements for individual amino acids within a group were determined in liquid medium (see above), since the requirements were then often more sharply discernible than on solid medium.

Development of substrains of Bacillus cereus independent of amino acids. Organisms of strains 6439 and 9373 from nutrient agar slopes were streaked on to plates of unsupplemented medium A2. The plates were incubated at 37°C until faint confluent growth appeared (5 days). Organisms from the plates were then suspended in sterile water to give slight turbidity and streaked on to the same medium. After eight transfers organisms of both strains were growing well in two days on unsupplemented medium A2. At no transfer were individual colonies seen that grew more rapidly. The organisms from solid unsupplemented medium grew only slowly and lightly (less than 10^7 organisms/ml in 48 h) when transferred to liquid medium A2 (50 ml) as inocula of about 2×10^6 organisms. However, after three transfers in liquid medium growth was heavy (about 10^8 organisms/ml in 40 h) from an inoculum of 2×10^5 organisms. Single colonies (from organisms out of the last liquid cultures) were isolated on solid medium A2 and transferred to slopes of the same medium.

Bacteriological tests. Fresh cultures grown overnight at 37°C (*Bacillus megaterium* 13632 at 30°C) on slopes of nutrient agar were used for the catalase test and Gram staining. Spores were examined in older cultures by phase contrast microscopy.

Fermentation tests were incubated at 30°C and 37°C for a week before reading results.

To examine the lecithinase reaction, organisms were streaked on to plates of nutrient agar containing 'Oxoid' egg yolk emulsion (5%, v/v). Marked cloudiness under and round the streak after incubation overnight at 37°C indicated a positive reaction.

To recognize hydrolysis of starch, organisms were streaked on to plates of nutrient agar containing 0.2% soluble starch. After overnight incubation at 37°C the plates were flooded with a dilute solution of iodine in KI. Colourless areas round the streak indicated hydrolysis of the starch.

Organisms from nutrient broth cultures were examined by phase contrast microscopy for motility. Migration of organisms from the point of inoculation (in nutrient broth containing 0.2% agar) into a Craigie tube (Collins, 1967) was also used to demonstrate motility.

The Voges-Proskauer (V-P) and the catalase tests were done as described in the textbook of Cruickshank (1965).

Total and viable counts. Organisms were counted in a chamber by phase-contrast microscopy. The organisms occurred singly and in short chains (after growth in rich media). Since a chain of organisms would presumably form only one colony on a plate, each chain was counted as one organism.

Samples (0.1 ml) of dilute suspensions of organisms (about 1000/ml) were plated on solid medium A2 (in 85 cm diameter Petri dishes) and incubated overnight at 37°C. Total and viable counts made before the stationary phase of growth agreed well.

RESULTS

Growth of Bacillus megaterium 7581 in defined media

White *et al.* (1969) grew *Bacillus megaterium* 7581 in medium W from an inoculum of about 2 mg dry wt of organisms/500 ml of medium. A good yield of organisms (about 2.5 mg dry wt/ml) was obtained after incubation for 40 h. However, on decreasing the inoculum tenfold there was little or no visible growth after 48 h. When the growth curve resulting from an adequate inoculum was examined more closely, growth on medium W proved to be diphasic (Fig. 1).

Medium W contained DL-glutamic acid and glycerol as possible sources of carbon and energy. Medium A was devised as a medium with only one source of carbon and energy (glycerol or glucose) that would support good growth of strain 7581 (Fig. 1).

Requirement for biotin. In medium A *Bacillus megaterium* 7581 grew with a single exponential phase, yet growth failed in liquid medium A (containing glycerol or glucose) as in medium W unless the inoculum was large. When organisms (from a nutrient agar slope) were streaked out on to solid medium A (containing glucose or glycerol) a curious type of growth appeared after overnight incubation at 37°C. The zones of heaviest inoculation showed areas of heavy confluent growth, but always contained patches where there was only faint growth of very minute colonies and a few larger colonies. Growth on the second, less heavily inoculated, zone of agar was usually evenly confluent or sometimes patchy, and the single colonies on the third and fourth zones were all of normal size; no minute isolated colonies were seen. Organisms incubated at 30°C did not show patchy growth on any zone. Repeated streakings from large single colonies on to fresh medium A2 did not abolish the patchiness of the first zone, and, conversely, repeated transfers from patchy areas did not lead to any increase in the extent of patchy growth on the fresh plates.

The patchy growth at 37°C was abolished when solid medium A2 was supplemented with yeast extract or vitamins; the other supplements were ineffective. Biotin alone promoted even growth in the area of heaviest inoculation, and 0.02 µg of biotin/l was enough (lower concentrations were not tested) to give this effect.

In liquid medium A2 plus biotin (20 µg/l) growth was heavy after 24 h at 37°C from an inoculum of only 4×10^2 organisms/ml. Without biotin there was only very light growth

Fig. 1. Growth of *Bacillus megaterium* 7581 shaken in defined media (containing glycerol) at 37°C. Medium W or A 1 (500 ml) in a 2 l flask was inoculated with 1.0 ml of a suspension of organisms (3.0 mg dry wt) from a fresh slope culture on nutrient agar. The dry wt of organisms in the growing culture was determined from the turbidity, which was measured at intervals. O, Medium W; Δ, medium A 1.

after 24 h from an inoculum of 4×10^4 organisms/ml, though growth had become heavy after 40 h; there was no visible growth at all after 40 h in biotin-deficient medium from inocula of 1×10^4 organisms/ml or less.

Nutritional requirements of other strains of *Bacillus megaterium*

Strains KM, 216, 8291, 8508 and 9323 gave heavy confluent growth at 37°C on solid unsupplemented medium A2 and also behaved as prototrophes on repeated subculture in liquid medium A2. Strains 4821, 9376 and 13632 grew poorly on this minimal medium and needed amino acids for good growth; vitamins, purines, yeast extract or trace salts did not much improve growth on minimal medium. For growth in liquid medium A the following conditions were necessary (quantities are mg/l).

(i) Strain 4821 required L-asparagine (400), L-glutamic acid (200) and L-proline (100). At 30°C the lag phase before turbidity appeared was shorter by about 10 h with glycerol (and the amino acids) than with glucose or than with glycerol or glucose at 37°C. Growth after 42 h was heavier at 30°C with glycerol or glucose (about 2.5 mg dry wt/ml) than at 37°C (0.75 mg dry wt/ml with glycerol; 1.5 mg dry wt/ml with glucose). Although growth was most rapid and heavy at 30°C in medium A1 plus asparagine, glutamic acid and proline, omission of any one of these compounds only slightly delayed growth at this temperature, and even when any of them were added singly the yield of organisms (2.0 mg dry wt/ml) was the same after 48 h. There was no growth when all amino acids were omitted.

(ii) Strain 9376 required L-threonine (60) and L-valine (60) whether the organisms were grown at 30°C or 37°C with glucose or glycerol. Growth was most rapid at 30°C with glycerol.

(iii) Strain 13632 required L-leucine (60) plus either L-aspartic acid (60) or L-glutamic acid (120) for growth at 30°C with glucose; glycerol gave much poorer growth. At 37°C with this minimal supplementation there was no growth whether glucose or glycerol was used, although with the complete amino acid supplement there was light growth with glucose at 37°C.

Four strains of *Bacillus megaterium* out of the nine so far examined failed to grow freely on unsupplemented medium A. The same four strains (4821, 7581, 9376 and 13632) failed to grow from small inocula in the minimal medium of Knight & Proom (1950). Possibly repeated cultivation on crude media might have caused selection of auxotrophes that grew more rapidly in the presence of appropriate growth factors than did the original prototrophic organisms. A fresh strain of *B. megaterium* was therefore isolated from pasteurized soil and characterized by size, ability to form spores, spore morphology, Gram reaction, failure to grow anaerobically and negative V-P reaction (i.e. key of Wolf & Barker, 1968).

This new strain (16) grew at 37°C on solid medium A2 only when it was supplemented with amino acids. For growth at 37°C in liquid medium A2 strain 16 required (quantities are mg/l): L-aspartic acid (100), L-glutamic acid (120) and L-arginine HCl (120). Growth of strain 16 in medium A with these amino acids at 37°C was less if glucose was replaced by glycerol, and growth at 30°C was poor with either glucose or glycerol.

Bacteriological properties of Bacillus megaterium

Some of the strains that failed to grow in minimal medium might have been wrongly classified as *Bacillus megaterium* although those from culture collections have all been independently identified by other workers. Bacteriological tests on all the strains were therefore made to see whether any strain showed marked departures from the expected properties of *B. megaterium*, and whether any of the auxotrophic strains had any other atypical features in common. The tests used were chiefly those which are useful in distinguishing *B. megaterium* from *B. cereus* (Knight & Proom, 1950; Breed *et al.* 1957; Wolf & Barker, 1968). Tests that are reported to give varying results with different strains of the same species were not used with all strains.

For fermentation tests a special lightly buffered basal medium was devised, with $(\text{NH}_4)_2\text{SO}_4$ as main source of nitrogen and with supplements of acid-hydrolysed casein and yeast extract (see Methods). With a peptone-containing basal medium alkali production was excessive, and without the supplements some strains gave no visible growth (or indicator change) with any sugars tested.

The description of *Bacillus megaterium* in *Bergey's Manual* (Breed *et al.* 1957) very closely fitted strains KM, 216, 8291 and 8508 (Table 1). Strains 7581, 9376 and 13632 also closely matched the description and deviated only in their nutritional requirements. Strains 16, 4821 and 9323 deviated in their sugar fermentations, and 16 and 4821 were also nutritionally exacting though 9323 was not. All the strains seem to be best described as *B. megaterium*.

Bacteriological properties and nutrition of Bacillus cereus

Three strains of *Bacillus cereus* had in general properties corresponding to the description of this species (Breed *et al.* 1957), although none of the strains grew heavily anaerobically, and strain 9373 was negative in the Voges-Proskauer test (Table 1). All grew on solid medium

Table 1. Bacteriological properties of strains of *Bacillus megaterium* and *B. cereus*

Property	Literature*		Strain													
	<i>B. megaterium</i>	<i>B. cereus</i>														
Gram reaction	+ [± or —]	+	7464	+	5.3	+	O	NS	—	+	±	±	+	+	+	+
Length in μm (Gram stained)	2 to 4	3 to 5	6349 AI	±	6.0	1.0	+	C	NS	.	+	±	±	+	+	+
Width in μm (Gram stained)	≥ 0.9†	≥ 0.9	6349	+	4.8	1.0	+	O	NS	—	.	±	±	+	+	+
Catalase	+	+	9373 AI	±	5.5	1.1	+	O	NS	.	+	±	±	—	+	+
Spores (under aerobic conditions)	Oval (O) or cylindrical (C), sporangia not swollen (NS)	Oval NS	9373	+	6.0	1.0	+	O	NS	+	+	±	±	+	+	—
Motility	+ [—]	+ [—]	16	+	3.5	1.3	+	C	NS	—	+	—	—	—	—	—
Vacuolation on glucose agar	+	+	9323	±	4.0	1.5	+	.	.	+	+	—	—	—	—	—
Anaerobic growth:			KM	±	4.0	1.0	+	.	.	—	+	—	—	—	—	—
glucose agar	—†	+	216	±	5.0	1.5	+	O	NS	—	+	—	—	—	—	—
glucose broth	—	+	829 I	+	5.0	1.5	+	O	NS	+	+	—	—	—	—	—
Voges—Proskauer	—†	+	8508	±	3.8	1.8	+	O	NS	—	+	—	—	—	—	—
Starch hydrolysis	+	+	482 I	±	4.0	1.2	+	O	NS	—	+	—	—	—	—	—
Lecithinase	—†	+	13632	±	3.8	1.0	+	O	NS	+	+	—	—	—	—	—
Growth with glucose + NH ₄ ⁺	+	—	758 I	±	4.5	1.0	+	C	NS	—	+	—	—	—	—	—
Fermentations			9376	±	4.5	1.5	+	O	NS	+	+	—	—	—	—	—
Glucose	Acid (A), no gas†	Acid, no gas										A	A	A	A	A
Arabinose	Acid, no gas†	No acid or gas										A	A	A	A	A
Xylose	Acid, no gas	No acid or gas										A	A	A	A	A
Mannitol	Acid, no gas	No acid or gas										A	A	A	A	A

* Knight & Proom (1950); Breed *et al.* (1957); Wolf & Barker (1968). Properties in square brackets are common variations.† Features of *B. megaterium* valuable in distinguishing *B. megaterium* from *B. cereus* (or from other species of *Bacillus*).

A2 at 37°C only when amino acids were added. For growth in liquid medium A2 at 37°C the following additions were required (quantities are mg/l):

- (i) strains 6349 and 9373 both required glycine (50) and L-methionine (50);
- (ii) strain 7464 grew with DL-aspartic acid (100), DL-glutamic acid (240), DL-histidine (60), glycine (50) and DL-alanine (100). The minimal requirements were difficult to define precisely since, for instance, aspartic and glutamic acids could be omitted if DL-serine (25) and DL-proline (100) were present (with histidine, glycine and alanine).

Amino acid independent substrains of Bacillus cereus

By repeated transfer of strains 6349 and 9373 in medium A2 (see Methods) two substrains were developed, called 6349 AI (amino acid independent) and 9373 AI, that grew at 37°C in unsupplemented medium A2. Apart from being less nutritionally exacting, the substrains resembled their parent strains in every respect that has been examined (Table 1), and do not seem to be contaminants.

DISCUSSION

In the present study five among ten strains of *Bacillus megaterium* failed to grow at 30°C or 37°C from a small inoculum in a liquid minimal medium whether the carbon source was glucose or glycerol. By contrast, Knight & Proom (1950) found that all the strains (eleven) of *B. megaterium* that they examined grew (with glucose) in a similar minimal medium. This discrepancy may have several possible explanations: (i) some of the strains used in the present work or by Knight & Proom (1950) were incorrectly designated as *B. megaterium*; (ii) some of the strains used in the present work may have become auxotrophic after repeated subcultivation on rich media; (iii) some nutritional requirements of the strains they examined were missed by Knight & Proom (1950); (iv) nutritionally exacting strains of *B. megaterium* do occur (five from a total of twenty-one) but by chance none was included among those examined (eleven) by Knight & Proom (1950) while five were among those studied (ten) in the present work.

The first explanation is very unlikely. Knight & Proom (1950) identified their strains as *Bacillus megaterium* by numerous bacteriological tests, as was done (with similar tests) in the present work. Furthermore, nine of the strains identified as *B. megaterium* in the present study were also characterized as *B. megaterium* by other workers (although strain 4821 was originally described as '*B. carotarum*'). The only species of the genus *Bacillus* that Knight & Proom (1950) and Proom & Knight (1955) found to grow in their unsupplemented medium were *B. megaterium*, *B. subtilis* and *B. licheniformis*. *Bacillus polymixa* and *B. pumilus* grew in medium supplemented only with biotin. These four latter species can all be readily distinguished from *B. megaterium*. *B. brevis* and *B. cereus* were the only species that required supplements of amino acids and no vitamins. *Bacillus brevis* is unlikely to be confused with *B. megaterium* or *B. cereus*.

The second explanation may be more possible but seems rather unlikely. Eight of the eleven strains examined by Knight & Proom (1950) were freshly isolated. In the present study only one strain of *Bacillus megaterium* was freshly isolated, but this strain did require amino acids. The organisms were not transferred on rich medium any more often than was essential to provide inocula, and were kept freeze-dried when not in use, although the strains which were not new isolates may have earlier been transferred frequently by other workers before deposition in culture collections. Knight & Proom (1950) remarked, however, that nutritional requirements of *Bacillus* species did not seem to become more exacting after subcultivation on rich media. Strain KM, which is a much-used strain, did not require growth factors, and

strain 7581 has not altered its growth factor requirement (for biotin alone) during 2 years in which it has been subcultured on nutrient agar at monthly intervals.

The third explanation is also possible but unlikely. Knight & Proom (1950) in their tests transferred organisms from the simplest minimal medium that supported growth into the same medium to avoid carrying nutrients from a rich medium with the inoculum. All the strains which in the present work required amino acids failed to grow when transferred directly from nutrient agar into minimal medium. A similar marked requirement by any of the strains tested by Knight & Proom (1950) would almost certainly have been recognized. However, the requirement of strain 7581 for biotin was less obvious because the organisms could be propagated (from a large inoculum) for several transfers at 37°C without addition of the vitamin in liquid culture. A nutritional requirement similar to this might perhaps have been missed by Knight & Proom (1950). In their tests, the inoculum was 0.05 ml of a slightly turbid suspension in 5 ml of liquid medium. This may be roughly equivalent to about 1 ml of a distinctly turbid suspension in 500 ml of medium, which was just sufficient to allow growth of strain 7581 without added biotin. Also, the requirement for biotin was seen at 37°C and not at 30°C, though those strains that needed amino acids did so at both of these temperatures.

The fourth explanation may be the most probable. If the frequency of occurrence of nutritionally exacting strains of *Bacillus megaterium* is really about 1 in 4, then it is rather surprising but not very remarkable that Knight & Proom (1950) might have examined 11 strains all of which were non-exacting – the odds against doing this would be about 20 to 1.

Knight & Proom (1950) suggested that the differences they found in the nutritional requirements of *Bacillus megaterium* and *B. cereus* (no growth factors and amino acids respectively) might be useful in distinguishing these two species, which have several points of similarity, especially morphology. Since exacting strains of *B. megaterium* have now been found, and non-exacting substrains of *B. cereus* developed, nutritional requirements do not seem to afford such a clear distinction between the two species.

Strain 9376 has been proposed (Smith *et al.* 1964) as the neotype of *Bacillus megaterium*, though its nutrition has not previously been examined. It is in fact an exacting strain; threonine and valine are needed as growth factors.

Strain 9373 has been proposed (Smith *et al.* 1964) as the neotype of *Bacillus cereus*. This strain did, as expected from the findings of Knight & Proom (1950), require amino acids (glycine and methionine) as growth factors. However, this requirement could be lost without other change in the properties of the strain.

Nothing is known of the biochemical and genetical means by which two strains of *Bacillus cereus* developed the ability to grow without added amino acids. The independence appeared gradually during repeated subcultivation on solid and liquid medium. Probably, therefore, several mutational steps were involved. Mutations would not be expected to lead to the development of new regions of the genome able to specify the formation of the new enzymes needed to synthesize the amino acids. Presumably, these regions existed in the genome of the parent (exacting) strain but did not give active enzymes until some controlling mechanisms were altered by mutations.

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