Minimal Nutritional Requirements of *Bacillus sphaericus* NCTC 9602 and 26 other Strains of this Species: the Majority Grow and Sporulate with Acetate as Sole Major Source of Carbon

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Bacillus sphaericus NCTC 9602 grew and sporulated in a simple chemically defined minimal medium containing only $\text{KH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$ buffer, pH 7.2, 15 mM-ammonium sulphate, inorganic salts and sodium acetate (as sole source of carbon); no vitamins were needed. The organisms contained the characteristic enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase) but lacked pyruvate dehydrogenase. Isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase were both present.

The minimal nutritional requirements of 26 other strains of *B. sphaericus* were investigated. Three of these (including the type culture) grew and sporulated in the above minimal medium, and a further 13 strains grew and sporulated when only biotin and thiamin were added. Two more strains grew in the minimal medium plus these vitamins and glutamic acid. Four of the remaining strains needed several amino acids, but not acetate, and four other strains required a purine as well as amino acids.

INTRODUCTION

Bacillus sphaericus is a rod-shaped, spore-forming, strictly aerobic bacterium; its spores are spherical and cause obvious terminal swelling of the sporangium. Closely related species are *Bacillus pasteurii*, *B. fusiformis*, *B. loehnisii* and *B. rotans*; the last three species are now usually regarded as variants of *B. sphaericus* (Gordon *et al.*, 1973). These five species are among the most numerous representatives of the genus *Bacillus* found in soils (Buchanan & Gibbons, 1974).

Powell & Strange (1957) examined three strains of *B. sphaericus* (including NCTC 9602) and found that the vegetative walls of all contained lysine (and aspartate) rather than diaminopimelate, though the peptidoglycan of the spore did contain diaminopimelate. In recent years, the biochemistry of the wall and of spore formation in *B. sphaericus* 9602 has been extensively studied (e.g. Hungerer & Tipper, 1969; Tipper & Linnett, 1976), but the organisms have always been grown in an undefined complex medium. The present work began with an attempt to grow this strain in a chemically defined medium, as a preliminary to a study of the diaminopimelate in its spores.

Relatively little work has been done on the nutrition of *B. sphaericus*. Knight & Proom (1950) found that none of 19 strains of *B. sphaericus* grew in a simple chemically defined medium with glucose, ammonium, phosphate and sulphate ions as principal nutrients. All grew when this medium was supplemented with acid-hydrolysed casein, biotin and thiamin. The acid-hydrolysed casein could be replaced by a mixture of 14 amino acids for growth of all but one of the strains, or by a mixture of only 7 amino acids for growth of most of

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the strains. Bornside & Kallio (1956) confirmed that amino acids plus thiamin or plus thiamin and biotin were needed by six strains of *B. sphaericus*, whereas four strains of *B. loehnisii* required only amino acids. Iyer & Kallio (1958) described one strain of *B. sphaericus* that grew with *N*-methylurea as sole major source of carbon, though yeast extract was also present in the medium. Chan *et al.* (1973) described a minimal defined medium (glucose, glutamate, salts and EDTA) which supported abundant growth of the one strain of *B. sphaericus* that they examined. Hastie & Brinton (1979) grew *B. sphaericus* 9602 in a defined medium (with amino acids and vitamins) similar to that of Knight & Proom (1950).

The minimal medium that has now been devised for *B. sphaericus* 9602 contains no vitamins and only acetate as carbon source. The type culture (*B. sphaericus* NCIB 9370) also grows in this simple medium. The study has been extended to numerous other strains of *B. sphaericus* to establish whether all are less exacting nutritionally than had previously been supposed.

METHODS

Organisms. The strains of *Bacillus sphaericus* used in this study are listed in Table 1. Strain 9602 was from the NCTC and strains 8216, 8217, 8218 and 9370 from the NCIB. All other strains were provided by Dr Ruth Gordon (Rutgers University, N.J., U.S.A.). This collection of strains is the same (apart from the addition of NCTC 9602) as that used in a previous general survey of the properties of the species (Gordon *et al.*, 1973). All strains were maintained on slopes of nutrient agar which were incubated at 30 °C and then stored at 2 °C.

Media. The defined medium A (White, 1972) was modified by using sodium acetate. 3H₂O (5 g l⁻¹) as principal carbon source in place of glycerol or glucose; trisodium citrate. 2H₂O (20 mg l⁻¹) was also added unless otherwise stated. This medium was called A3. All components of the medium (apart from salts of Mg²⁺, Fe²⁺ and Mn²⁺) were combined before autoclaving, except where stated otherwise. Sodium acetate and glutamic acid were always added as solutions, adjusted to pH 7. Solid medium contained 2% (w/v) Difco agar and was dried at 37 °C for 24 h to prevent spreading of surface growth. Various supplements of L-amino acids were used, generally at the following concentrations (mg l⁻¹): Difco 'vitamin-free' Casamino acids (acid-hydrolysed casein), 5000; alanine, 40; arginine.HCl, 160; aspartate, 50; asparagine, 40; cystine.HCl, 250; glutamate, 500; glutamine, 40; glycine, 80; histidine.HCl, 160; isoleucine, 40; leucine, 100; lysine.HCl, 50; methionine, 80; phenylalanine, 100; proline, 30; serine, 40; threonine, 30; tryptophan, 100; tyrosine, 140; valine, 160. Vitamin supplements were (mg l⁻¹): biotin, 0.002; thiamin, 10; pyridoxine, 10; pyridoxal, 10; pyridoxamine. 2HCl, 10; nicotinic acid, 10; sodium pantothenate, 10; riboflavin, 10; calcium 5-formyltetrahydropteroylglutamate, 0.01. Adenine and guanine were each added to give 20 mg l⁻¹.

Growth. In liquid cultures, growth was measured turbidimetrically in an EEL colorimeter, as described by White (1972). A reading of 1.0 was equivalent to 0.4 mg dry wt ml⁻¹ (1 × 10⁸ organisms ml⁻¹).

Tests for nutritional requirements. Strains were first screened on solid medium A3 plus biotin and thiamin, at both 25 and 30 °C. Strains that grew well in this first test were next examined on medium A3 without vitamins, and sometimes with either vitamin added singly. Strains that failed to grow in the first test after 48 h were examined on medium A3 containing Casamino acids, complete vitamin mixture, adenine and guanine, a medium on which all the more exacting strains grew. Progressive omissions were then made to find the minimal requirements. These tests were done in liquid medium (50 ml) in shaken 250 ml flasks incubated at 30 °C. With every strain, growth at 30 °C was faster (on complex or defined media) than at 25 °C, while growth at 37 °C was always much poorer.

Sporulation. Strains that grew vegetatively on medium A3 plus only biotin and thiamin were tested for sporulation on the same solid medium supplemented with 1 mM-CaCl₂ (added as a sterile solution to the autoclaved medium). Cultures were incubated at 30 °C and examined microscopically at intervals. Sporulating organisms were suspended in water, heated at 60 °C for 30 min, and then plated on nutrient agar and incubated at 30 °C overnight. The appearance of colonies of *B. sphaericus* confirmed that heat-stable spores had been formed in the simple defined medium.

Enzymic assays. *Bacillus sphaericus* 9602 was grown at 30 °C on a mechanical shaker in liquid medium A3 (500 ml per 2 l flask). Organisms were harvested late in the exponential phase of growth, when few (if any) spores were visible, and were washed and resuspended in the buffer that was appropriate for the enzymic assay in which the extract was subsequently to be used. Extracts were made at 2 °C by passing the suspended organisms through the pressure cell of Milner *et al.* (1950), operated at 140 MPa, and then removing the debris by centrifuging at 20000g for 20 min at 2 °C. Protein in the extracts was assayed by the Lowry method, with bovine serum albumin as standard.

Enzymic activities in the extracts were measured at 30 °C. Pyruvate dehydrogenase complex (overall reaction) was assayed by recording the anaerobic (see White, 1979) pyruvate-dependent reduction of NAD⁺ in a reaction mixture similar to that of Guest & Creaghan (1973); 2-oxoglutarate dehydrogenase complex (overall reaction) was assayed similarly. Pyruvate dehydrogenase E1 (EC 1.2.4.1) was assayed spectrophotometrically at 420 nm and pH 6.3 or 7.0 with ferricyanide as electron acceptor (Hager & Kornberg, 1961), and 2-oxoglutarate dehydrogenase E1 (EC 1.2.4.2) was assayed similarly. Isocitrate lyase (EC 4.1.3.1) was assayed as described by Kornberg (1965), with the dinitrophenylhydrazones of glyoxylic acid identified as described by Salem *et al.* (1973). Malate synthase (EC 4.1.3.2) was assayed by the method of Cox & Quayle (1976). Isocitrate dehydrogenase (EC 1.1.1.41) was assayed by the method of Plaut (1962). Glutamate dehydrogenase (EC 1.4.1.4) and glutamate synthase (EC 1.4.1.13) were assayed anaerobically at pH 7.6 as described by Meers *et al.* (1970) and White (1979) with NADPH as cofactor.

Peptidoglycans of walls and spores of B. sphaericus 9602. Organisms were grown at 30 °C in medium A3 as described above, and walls were isolated from exponential phase bacteria by the procedure of Day & White (1977). A suspension of walls (10 mg ml⁻¹) in 0.1 M-ammonium acetate, pH 6.5 was incubated with lysozyme (100 µg ml⁻¹) at 37 °C for 1 h. Insoluble material was removed by centrifuging; the supernatant liquid was mixed with an equal volume of 11.6 M-HCl and heated at 105 °C for 18 h or for 48 h in sealed tubes. The hydrolysates were dried in a desiccator over NaOH pellets, and acid was removed by repeated addition and evaporation of water.

Spores were obtained (after incubation at 30 °C for 3 d) from cultures in medium A3 plus 1 mM-CaCl₂, grown as above. The mixture of spores and organisms was harvested by centrifuging and suspended in 0.1 M-Na₂HPO₄/NaH₂PO₄ buffer, pH 6.5. The suspension was passed through a pressure cell (Milner *et al.*, 1950) at 100 MPa (to break vegetative organisms) and then centrifuged at 20000 g for 15 min at 2 °C. The pellet (spores and debris) was resuspended in 0.1 M-Na₂HPO₄/NaH₂PO₄ buffer, pH 6.5, autoclaved at 121 °C for 30 min (to inactivate lytic enzymes) and then incubated with lysozyme (100 µg ml⁻¹) for 1 h at 37 °C. The solid residue (mostly spores) was collected by centrifuging and the spores were isolated by solvent partition (Sacks & Alderton, 1961).

Spores were broken by shaking with glass beads and then incubated at 37 °C with ribonuclease followed by trypsin, both in 0.05 M-Tris/HCl buffer, pH 7.6. The resulting spore integuments were resuspended in 0.1 M-ammonium acetate, pH 6.5 and incubated at 37 °C overnight with lysozyme (300 µg ml⁻¹) in the presence of toluene. The material digested by lysozyme (i.e. cortical peptidoglycan) was hydrolysed at 105 °C for 18 h with 6 M-HCl and then dried repeatedly, as described above.

Amino acids in each hydrolysate were separated by paper chromatography with the solvent methanol/pyridine/11.6 M-HCl/water (80:10:2.5:17.5, by vol.; Rhuland *et al.*, 1955) and spots were detected with ninhydrin. Quantitative measurements of amino acids were made with an autoanalyser, and by the appropriate methods of Work (1957).

RESULTS

Nutritional requirements of 27 strains of B. sphaericus

Spores of *B. sphaericus* 9602 were obtained from complex medium, as described by Hungerer & Tipper (1969), and were used as inoculum throughout the initial tests on solid media. Medium A salts plus only sodium acetate.3H₂O (5 g l⁻¹) and L-glutamate or L-aspartate (each 1 g l⁻¹) proved adequate to support good vegetative growth in liquid culture from heat-shocked spores (80 °C for 30 min). When vegetative organisms were used as inoculum the glutamate could be omitted, although growth was then slower. Increasing the concentration of acetate did not improve growth. Neither spores nor vegetative organisms grew in minimal salts plus L-glutamate (1 to 5 g l⁻¹) when acetate was omitted. Growth with butyrate or DL-β-hydroxybutyrate (Na⁺ salts; 3 g l⁻¹) as sole carbon source was slower but the final yield of organisms was higher than with acetate. Omission of citrate from medium A3 had no apparent effect on growth.

The unexpectedly simple requirements of strain 9602 prompted a survey of other strains. The minimal nutritional requirements of 27 separate strains of *B. sphaericus* are shown in Table 1. There are 31 strains listed, but some (ATCC 4978 and NRS 633; ATCC 7054, ATCC 12300 and NRS 866; ATCC 12123 and NRS T111) are supposedly identical strains, obtained from different sources (Gordon *et al.*, 1973).

Table 1. *Strains of B. sphaericus and their minimal nutritional requirements*

Procedures are described in Methods.

Designation of strain as obtained*	Other names	Minimal requirements†	Sporulation on minimal medium + Ca ²⁺	Growth in medium A2 + biotin + thiamin
NCIB 8216	NRS 250	E	ND	ND
NCIB 8217	NRS 344	E	ND	ND
NCIB 8218	ATCC 4525; NRS 348; NCTC 2609	E	ND	ND
NCIB 9370	ATCC 14577; NCTC 10338 (Type culture)	A (C better)	+	ND
NCTC 9602		A (C better)	+	—
ATCC 245	<i>Bacillus lactimorbi</i>	B, 1+2	+	—
ATCC 4978 ^a	NRS 633; <i>Bacillus rotans</i>	E	ND	—
ATCC 7054 ^b	NRS 339, 866; ATCC 12300; NCTC 2608; <i>Bacillus fusiformis</i>	B, 1	+	—
ATCC 7055	NRS 350	B, 1+2	+	—
ATCC 7063	NRS 668 <i>Bacillus serosittitis</i>	E	ND	ND
ATCC 10208	NRS 966	A	+	—
ATCC 12123 ^c	NRS T111	B, 1	+	—
ATCC 12300 ^b	ATCC 7054 etc.	B, 1+2	+	—
ATCC 12844	NCIB 8867; <i>Bacillus rotans</i>	B, 1+2	+	—
NRS T111 ^c	ATCC 12123	D (B, 1+2 poorer)	+	—
NRS 400		B	+	ND
NRS 592		E	ND	ND
NRS 633 ^a	ATCC 4978 etc.	E	ND	ND
NRS 672	<i>Bacillus loehnisii</i>	E	ND	ND
NRS 717		B, 2	+	—
NRS 718		B, 1+2	+	—
NRS 719	<i>Bacillus pseudotetani</i>	B, 1+2	+	—
NRS 752		B, 1+2	+	—
NRS 800		D (B, 1+2 poorer)	+	—
NRS 866 ^b	ATCC 7054 etc.	B	+	ND
NRS 967		A	+	ND
NRS 1089		B	+	ND
NRS 1184		B	+	ND
NRS 1189		D	ND	ND
NRS 1223		E	ND	ND
NRS 1307		B	+	ND

ND, Not determined.

* Superior letters indicate supposedly identical strains that have different numbers.

† Minimal requirements in medium A salts (+citrate) were: A, sodium acetate.3H₂O (5 g l⁻¹); B, acetate + biotin (2 µg l⁻¹) + thiamin (20 mg l⁻¹) [for those strains where a 1 and/or 2 is shown, the requirements for each vitamin (biotin 1, thiamin 2) were determined separately]; C, acetate + L-glutamate (1 g l⁻¹); D, acetate + glutamate + biotin + thiamin; E, other than above (see Table 2).

Most strains grew with acetate as sole major source of carbon (though biotin and thiamin were usually needed too), and all of these strains sporulated in their minimal medium plus Ca²⁺. All of these strains could, of necessity, use NH₄⁺ (30 mM in medium A3) as sole major source of fixed nitrogen. Among the strains that grew with acetate as sole major source of carbon, none (of those tested) could grow in medium A2 (glucose as major carbon source) supplemented with vitamins (Table 1). Addition of glutamate to medium A3 (with biotin and thiamin) allowed growth of two more strains. Eight strains had more exacting requirements (Table 2). None of these showed an absolute requirement for acetate, but four needed adenine and/or guanine for growth. These are the first instances of purine-requiring wild-type strains in the genus *Bacillus*. The minimal requirements for individual amino acids were found for only three strains. Omission of any one of the listed amino acids caused much poorer growth, but addition of one or more other amino acids would restore growth, i.e. the requirements were not sharply defined (cf. White, 1972).

Table 2. *Minimal nutritional requirements of the more exacting strains of B. sphaericus*

Procedures for determining the minimal requirements are described in Methods. Numbers in parentheses are the concentrations (in mg l⁻¹) used in the minimal media, and were the same throughout for a given substance. All amino acids were L-isomers. Addition of the substances shown in square brackets slightly stimulated growth.

Strain	Minimal requirements in medium A salts (+ citrate)
NCIB 8216	Alanine (350); asparagine (40); glutamate (1000); methionine (100); guanine (20); thiamin (10)
NCIB 8217	Alanine; glutamate; methionine; phenylalanine (100); adenine (20); guanine
NCIB 8218	Alanine; asparagine; glutamate; serine (200); guanine; thiamin; pyridoxine.HCl (20)
ATCC 4978*	Casamino acids (5000); guanine
NRS 633*	
ATCC 7063	Casamino acids [sodium acetate.3H ₂ O (5000)]
NRS 592	Casamino acids; biotin (0.002); thiamin
NRS 672	Casamino acids [acetate]
NRS 1223	Casamino acids; biotin; thiamin

* Supposedly identical strains that have different numbers.

Other properties of B. sphaericus 9602

In medium A3 the doubling time during exponential growth was approximately 2 h. Growth stopped at a dry weight equivalent of about 1.0 mg ml⁻¹; the final pH value of the medium was 8.0 to 8.5. Neither the vegetative organisms nor the spores could be distinguished (by phase contrast microscopy) from those grown in complex media – the organisms (rods, about 5 µm long and very motile) occurred singly, and produced spherical, terminal spores.

Various other carbon sources [ethanol, glycollate, glycine, L-serine, N-methylurea, DL-lactate, pyruvate, 2-oxoglutarate, glucose, glycerol, succinate, fumarate, L-malate, propionate (Na⁺ salts, where appropriate)] were tested (at concentrations between 1 and 10 g l⁻¹) as replacements for acetate in minimal salts medium, but none supported growth. When added with a sub-optimal concentration of sodium acetate.3H₂O (1 g l⁻¹) none of the above compounds (added singly) caused quicker or heavier growth. Addition of DL-lipoic acid (2 to 10 µg l⁻¹) together with pyruvate (which was added to the autoclaved medium as a sterile-filtered solution) did not promote growth without acetate.

Extracts were made (see Methods) and various enzymes were assayed (Table 3). The characteristic enzymes of the glyoxylate cycle (isocitrate lyase and malate synthase) were found. Because isocitrate dehydrogenase was also present, it was necessary to show (as was done) that the product formed in the assay for isocitrate lyase was indeed glyoxylate (by identification of its dinitrophenylhydrazones) rather than 2-oxoglutarate. The relatively high activity of glutamate dehydrogenase and absence of glutamate synthase suggest that the former enzyme may be the means of assimilation of ammonia. Since the enzymes needed for synthesis of glutamate are present with good activity in the vegetative organisms grown on acetate alone, it is surprising that addition of glutamate should stimulate growth, especially as this amino acid (15 mM) did not support growth when it was sole source of nitrogen, in medium A3 from which (NH₄)₂SO₄ was omitted and replaced by Na₂SO₄. 10H₂O (4 g l⁻¹).

Vegetative walls were isolated and hydrolysed as described in Methods. No diaminopimelate was found in the hydrolysate (48 h, 6 M-HCl), which contained as principal components [µmol (mg wall)⁻¹]: alanine, 0.75; glutamate, 0.55; aspartate, 0.62; lysine, 0.51. The hydrolysate of peptidoglycan from the spore cortex contained diaminopimelate as well as lysine, though in relatively small quantities (molar proportions: alanine, 1.36; glutamate, 1.00; aspartate, 1.22; diaminopimelate, 0.34; lysine, 0.45).

Table 3. *Enzymic activities in B. sphaericus 9602*

Organisms were grown and extracts were made as described in Methods. Procedures for the various assays are described in Methods. The pH values, substrate concentrations etc. used in the assays are not necessarily optimal for the enzymes from *B. sphaericus*. All assays that showed low activities were repeated at least once with another fresh extract.

Enzyme	Activity [nmol min ⁻¹ (mg protein) ⁻¹]	
Isocitrate lyase	350	
Malate synthase	97	
Isocitrate dehydrogenase	55	
2-Oxoglutarate dehydrogenase (overall reaction)	4	44*
2-Oxoglutarate dehydrogenase E1	34 (pH 6.3)	192* (pH 6.3)
	0 (pH 7.0)	
Pyruvate dehydrogenase (overall reaction)	0	41*
Pyruvate dehydrogenase E1	0 (pH 6.3)	30* (pH 6.3)
	0 (pH 7.0)	15* (pH 7.0)
Glutamate dehydrogenase	400	
Glutamate synthase	0	

* Activity in an extract of *Escherichia coli* ATCC 9637 which was used to check that the assay system was functioning properly. The organisms were grown in medium A3 (at 37 °C) and extracts were prepared as for *B. sphaericus*.

DISCUSSION

Bacillus sphaericus 9602 grew readily and sporulated in a simple chemically defined medium with acetate as sole source of carbon. The organisms were indistinguishable morphologically and in the composition of their peptidoglycans (vegetative and spore) from bacteria grown in complex media. Anaplerotic synthesis presumably occurs by means of the glyoxylate cycle. If this is so, then the normal tricarboxylic acid cycle should also be functional, since both isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase were present; other necessary enzymes are common to both cycles.

The absence of pyruvate dehydrogenase explains the nutritional requirement for acetate. Substrates that give rise to acetate by only this metabolic route (e.g. glucose, succinate) are unable to provide all the necessary intermediates for biosynthesis. However, it is surprising that such substrates do not increase the rate or extent of growth when a limited supply of acetate is available. A compound such as succinate would be expected to furnish reducing power, ATP and biosynthetic intermediates, and so spare the requirement for acetate. Of the compounds tested, glutamate or aspartate alone improved growth with acetate. Possibly the organisms are selectively permeable to these amino acids. Whether *B. sphaericus* 9602 is really a strict acetotroph has not been established, but no other single carbon source among the small number tested was found to support growth, other than butyrate and β -hydroxybutyrate from which acetate can be generated without pyruvate dehydrogenase.

Numerous other strains of *B. sphaericus* grew with acetate as sole major carbon source, though most also required thiamin and/or biotin. However, several strains were considerably more exacting, yet no distinctions can be made between these strains and those with simpler requirements on the basis of their other characteristics, as reported by Gordon *et al.* (1973). The most exacting strains used amino acids as sources of carbon. Whether the amino acids are also principal nitrogen sources has not been shown.

It must be emphasized that the defined media used in this study are minimal media. With nearly every strain, growth was faster and heavier on nutrient agar than on the solid defined media. Considerably more work would be needed to develop optimal defined media and growth conditions for every strain.

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