Factors Preventing the Metabolism of Carbohydrates by *Bacillus sphaericus* 2362

by

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(Abstract)

Bacillus sphaericus 2362 is a mosquito pathogenic bacterium. Its greatest industrial potential may be in developing countries where mosquitos are often vectors for diseases. This strain is typical of the species in that it is unable to grow using carbohydrates as a sole source of carbon. The goal of this research was to determine the metabolic deficiency(s) responsible for the inability of this organsim to grow on carbohydrates. Compounds that supported light growth of this organism on an agar-solidified, defined medium included acetate, glycerol, and gluconate. Growth in a defined liquid medium with acetate as the source of carbon was much slower than growth in a complex, protein-based broth. *B. sphaericus* grew poorly in a defined, liquid medium with glycerol or gluconate as the carbon source. Activity of enzymes responsible for the initiation of metabolism of some substrates was not detected in cell extracts. These

enzymes were: glucokinase, hexokinase, beta-galactosidase, and amylase. Growth of this bacterium on glycerol as a sole source of carbon implies the presence of the enzymes from the lower half of the Embden-Meyerhof-Parnas (EMP) pathway. Two enzymes of the upper half of the EMP pathway, phosphofructokinase and fructose diphosphate aldolase, were undetected in cell extracts. In addition, glucose dehydrogenase activity was not detected. The inability to form glucose-6-phosphate from glucose prevents the catabolism of this and related substrates via the Entner-Doudoroff (ED), hexose monophosphate (HMP), and EMP pathways. Oxygen uptake studies indicated that B. sphaericus oxidized gluconate slightly but only when the cells were grown in a complex, protein-based medium supplemented with gluconate. Although gluconokinase activity was detected in cell extracts, no activity was detected for the key enzymes of the ED (phosphogluconate dehydratase/KDPG aldolase), or HMP (6-phosphogluconate dehydrogenase) pathway. It is unclear how B. sphaericus grows on a defined medium with gluconate as the sole source of carbon. In addition to enzymatic deficiencies, whole cells were unable to accumulate [^{14}C]glucose or [^{14}C]sucrose.

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INTRODUCTION

The lack of mosquito control is a major factor preventing any improvement in the standard of living in some developing countries. In these countries, mosquitos are vectors for diseases like malaria, yellow fever, dengue, filariasis, and encephalitis. The use of insecticides is one practical approach to the control of these insects and the diseases carried by them. However, the purchase and importation of insecticides is difficult in countries having few foreign currency reserves. The World Health Organization and the United Nations Industrial Development Organization encourage developing countries to produce their own insecticides instead of buying them elsewhere. This approach is financially feasible and creates employment opportunities for the citizens. However, the lack of advanced technology may limit the ability of some countries to produce chemical insecticides. A practical alternative to buying or manufacturing complex chemical insecticides is the production of microbially-derived insecticides from organisms like Bacillus sphaericus and B. thuringiensis. The biological control of pests is a desirable alternative to chemical control not only because the biological materials may be less expensive and easier to make but also because they have minimal impact on the environment. The environmental advantage is due to their narrow host range,

their limited toxicity and persistence in the environment, and the apparent failure of insects to develop resistance to microbial pesticides.

The most effective and widely used microbial insecticides have been composed of bacteria from the genus Bacillus. B. popilliae causes milky spore disease in some species of scarabaeid beetles (such as Japaneese beetles) and has been used for their control (9). It has been marketed under the trade name DOOM. The greatest commercial successes have come from the manufacture and use of strains of B. thuringiensis. B. thuringiensis serovar kurstaki has been effective in the control of the larval stage of many agriculturally important insects belonging to the order Lepidoptera. These pests include the cabbage looper, tobacco budworm, cotton bollworm, and gypsy moth. Several B. thuringiensis preparations have been marketed under the names Biotrol, Dipel, and Thuricide (18). More recently, B. thuringiensis serovar H14 (israelensis) was isolated (18). This strain is not pathogenic to lepidopterans but will kill mosquito and blackfly larvae belonging to the order Diptera. B. thuringiensis produces a toxin as a parasporal crystal during sporulation, and this toxin is lethal upon ingestion by the susceptible insects (1).

Pathogenicity in Bacillus sphaericus was first reported by Kellen et al. (30). This strain, Kellen K, was of little industrial interest because it killed mosquito larvae slowly

and lost activity when stored at room temperature. Interest has been revived since the isolation of strains 1593 and 2362 which are significantly more toxic. The B. sphaericus toxin is similar to the toxin of B. thuringiensis in that in strains having very high toxicity it resides in a parasporal inclusion. Strain 2362 has higher toxicity than 1593 and can also produce toxin at higher growth temperatures. This favors the production of strain 2362 in tropical environments where fermentor cooling water may be in limited supply (56).

The protein toxin of strain 2362 is deposited in the parasporal body immediately following completion of exponential growth of the bacterium. One toxic protein, having a molecular mass of about 125 kilodaltons (kDa), is converted to a protein of 110 kDa during sporulation. A second toxic peptide is of 43 kDa and upon ingestion by mosquito larvae is further degraded by larval gut proteases to a 40 kDa protein. This latter proteolytic conversion increases toxicity of the protein for cells in culture and presumably for the target larval gut epithelial cells as well (8).

One important factor which may limit the production of B. sphaericus in developing countries is its inability to grow using carbohydrates as a carbon source. Carbohydrates are readily available in the forms of molasses (sucrose), cheese whey (lactose), starch (glucose), or cellulose

hydrolysates (glucose). Complex protein sources such as fish meal or soy meal support good growth of B. sphaericus but they are more expensive and often less readily available than carbohydrates. In addition, in developing countries, they may be used for animal or human food. The reason B. sphaericus cannot use carbohydrates as a carbon source is unknown.

The goal of this research was to discover the metabolic lesion(s) responsible for the failure of B. sphaericus to use carbohydrates. Three possibilities were explored: i) the bacteria are unable to transport carbohydrates into the cell; ii) carbohydrates are transported into the cell unphosphorylated and a kinase is not present to initiate metabolism; iii) there is a deficiency in one or more of the enzymes of carbohydrate metabolism. In the case of a single enzymatic deficiency, genetic engineering could possibly be applied to supply a missing enzyme and thus allow good growth on carbohydrates.

LITERATURE REVIEW

The Species

Bacillus sphaericus has been described as a species whose boundaries are "diffuse and arbitrary" (26). It consists of strains which are both pathogenic and nonpathogenic to mosquito larvae. Krych et al. (33) studied sixty-two strains of pathogens and nonpathogens and found there was little difference between them in the mol% guanine plus cytosine content (34-37%). However, DNA homologies showed little similarity among strains. Only three of 62 strains had greater than 70% homology to the type strain, ATCC 14577. Strains with at least 70% homology are commonly considered to be one species. Despite this lack of homology, there are no distinguishing phenotypic characteristics on which to base a reclassification.

A Survey of Carbon Sources that Support Growth of *B. sphaericus*

Several researchers have studied the nutritional requirements of *B. sphaericus* and all have found that the bacteria are unable to grow using carbohydrates as a sole carbon source (3, 12, 29, 31, 48, 53). Singer et al. (48) studied a pathogenic strain, Kellen K, which was unable to grow in a defined medium with 0.3% dextrin, fructose, glucose, glycerol, glycogen, inulin, or starch as the sole

source of carbon. This strain did grow with 0.3% acetate, fumarate, alpha-ketoglutarate, malate, pyruvate, or succinate as a sole carbon source. Supplementation of the medium with casein hydrolysate enhanced growth, probably because it supplied arginine, glutamate, and proline as carbon sources. In addition to an appropriate carbon source, the vitamins biotin and thiamine were essential for the growth of Kellen K (48). This is true for many pathogenic strains (55).

de Barjac et al. (12) studied 35 strains of B. sphaericus including pathogens and nonpathogens, in an attempt to define the species by phenotypic characteristics. They tested 160 substrates for their ability to support growth as a sole source of carbon. The substrates included 32 carbohydrates, 20 amino acids and their derivatives, 16 alcohols and glycols, 9 dicarboxylic acids, and 10 organic acids. Of the 160 substrates, only 30 were utilized by any strain. More than 90% of the strains utilized aspartate, glutamate, ornithine, and histidine. Through numerical analysis of 8 phenotypic characteristics and growth on the substrates, de Barjac divided the 35 strains into three groups. All nine pathogenic strains tested were in a single group which also included two nonpathogenic strains.

White and Lotay (53) studied the nutritional requirements of 26 nonpathogenic strains of B. sphaericus.

Three strains grew and sporulated in a defined medium with sodium acetate as the carbon source but lacking vitamin supplementation. An additional 13 strains grew in the same medium supplemented with the vitamins biotin and thiamine. The remaining 10 strains required one or more amino acids instead of acetate as a carbon source and, in some cases, the addition of adenine and/or guanine was also required. A nonpathogen, strain NCTC 9602 was tested more extensively in the study. No growth was observed on ethanol, glycolate, glycine, L-serine, N-methylurea, DL-lactate, pyruvate, alpha-ketoglutarate, glucose, glycerol, succinate, fumarate, malate or propionate. Jelley (29) grew this same strain with glutamate, histidine, proline, succinate, or malate as a sole source of carbon on an agar-solidified medium supplemented with vitamins. Strain 9602 also grew on pyruvate as the sole source of carbon. This is probably because, unlike White and Lotay, Jelley added biotin and thiamine to the medium.

Jelley (29) tested a total of 45 strains of pathogens and nonpathogens for growth on a defined, agar-solidified medium which included biotin, thiamine, and 0.5% of the carbon source. After a 96-hour incubation, most pathogenic strains grew on glycine, acetate, succinate, pyruvate, malate, and those amino acids that enter the TCA cycle at alpha-ketoglutarate i.e. arginine, glutamate, histidine, and proline. Some pathogens exibited slight growth on

asparagine, glutamine, serine, glycerol, alanine, leucine, and threonine. The nonpathogens grew on fewer substrates. The substrates that supported heaviest growth of the nonpathogens were glutamate, histidine, proline, acetate, succinate, pyruvate, and malate. Jelley (29) also tested the ability of exponential phase cells of pathogenic strains 2297, 1593, and 2362 to oxidize a variety of substrates. All strains used oxygen in the presence of glutamate, acetate, pyruvate, malate, succinate, arginine, glycerol, glutamine, histidine, and proline, though there was some variability in the degree. No oxygen uptake was observed by the same strains in the presence of glucose or citrate.

The utilization of substrates by ten pathogenic strains of B. sphaericus was tested by Baumann, et al. (3). Strain 2362 was not included in this study. The cells were grown on an agar-solidified mineral medium containing 0.05% yeast extract, 0.3% of the substrate to be tested, and a colorless electron acceptor, 2,3,5-triphenyltetrazolium chloride (TTC). Electrons generated by the catabolism of a utilizable substrate reduced the TTC to a brightly colored formazan. Those compounds that tested positive included acetate, succinate, malate, citrate, alpha-ketoglutarate, pyruvate, glycerol, ribose, and gluconate. This indicates that at least one dehydrogenase enzyme responsible for the oxidation of these compounds is functioning in these

organisms. However, the study did not show that the enzymes were present in concentrations sufficient to support growth of the organism using the substrate as the sole carbon source in the absence of yeast extract. It is notable that these data are the only evidence suggesting B. sphaericus has an enzyme for the oxidation of ribose. If the enzyme(s) for ribose oxidation is (are) actually present, it is probably in too low concentration to allow for growth of the bacterium.

Transport of Carbohydrates into Bacterial Cells

Carbohydrate molecules enter bacterial cells by one of three types of carrier-mediated transport systems: facilitated diffusion, active transport, or group translocation. Facilitated diffusion requires no energy from the cell and therefore, does not move substrates against a concentration gradient. Glycerol is the only carbohydrate known to be transported by facilitated diffusion in bacteria (15). This type of transport has been identified in B. subtilis (46). Active transport and group translocation both require energy from the cell and can move substrates against a concentration gradient. The type of active transport is defined by the form of energy that drives the transport. An example of this type of transport is lactose-proton symport, driven by energy derived from the movement of protons across the cell

membrane. Group translocation is a mechanism by which a substrate is not only transported into a cell but is changed chemically as it is released to the interior of the cell. Carbohydrates are group translocated by phosphoenolpyruvate-carbohydrate phosphotransferase systems (PTS) (41, 47). Phosphoenolpyruvate-carbohydrate phosphotransferase systems consist of three or four separate proteins depending on the particular organism and substrate. Phosphoenolpyruvate is the source of energy and phosphate driving the transport and phosphorylation of the substrate. Glucose PTS's are found more frequently in anaerobes and facultative anaerobes than in strict aerobes. Achromobacter parvulus and B. subtilis, both strict aerobes, do possess a glucose PTS. However, both organisms belong to genera that consist predominantly of facultative anaerobes (43).

Each type of transport is dependent upon facilitator or carrier proteins (15). Absence or disfunction of these proteins in a bacterium would prevent its growth on the corresponding substrate even if all the enzymes for metabolism were active. Cells with this characteristic are referred to as cryptic.

Pathways of Carbohydrate Metabolism

The first step in the catabolism of a substrate is the phosphorylation of that substrate by either a PTS or a phosphorylating enzyme. Glucokinase and hexokinase both

phosphorylate glucose in the C-6 position producing glucose-6-phosphate. Glucose-6-phosphate can be further catabolized by three major pathways: the Embden-Meyerhof-Parnas (EMP), the hexose monophosphate (HMP), or the Entner-Doudoroff (ED) pathway (see Figs. 1, 2, and 3). A single microorganism may use one or a combination of these pathways for its metabolism. The key enzymes of each pathway are those which are unique to that pathway.

In the EMP pathway, three enzymes convert glucose-6-phosphate into glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. First, glucose-6-phosphate is isomerized by phosphoglucose isomerase to fructose-6-phosphate. This is then phosphorylated by phosphofructokinase, a key enzyme of this pathway, forming fructose diphosphate. Another key enzyme, fructose diphosphate aldolase, splits fructose diphosphate into the two three-carbon compounds glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. The enzyme triose phosphate isomerase maintains an equilibrium between glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. Glyceraldehyde-3-phosphate is ultimately converted to pyruvate through five enzymatic steps in the lower half of the EMP pathway. Glycerol is also metabolized in the lower half of the EMP pathway after conversion to dihydroxyacetone phosphate.

In the ED and HMP pathways, glucose-6-phosphate is

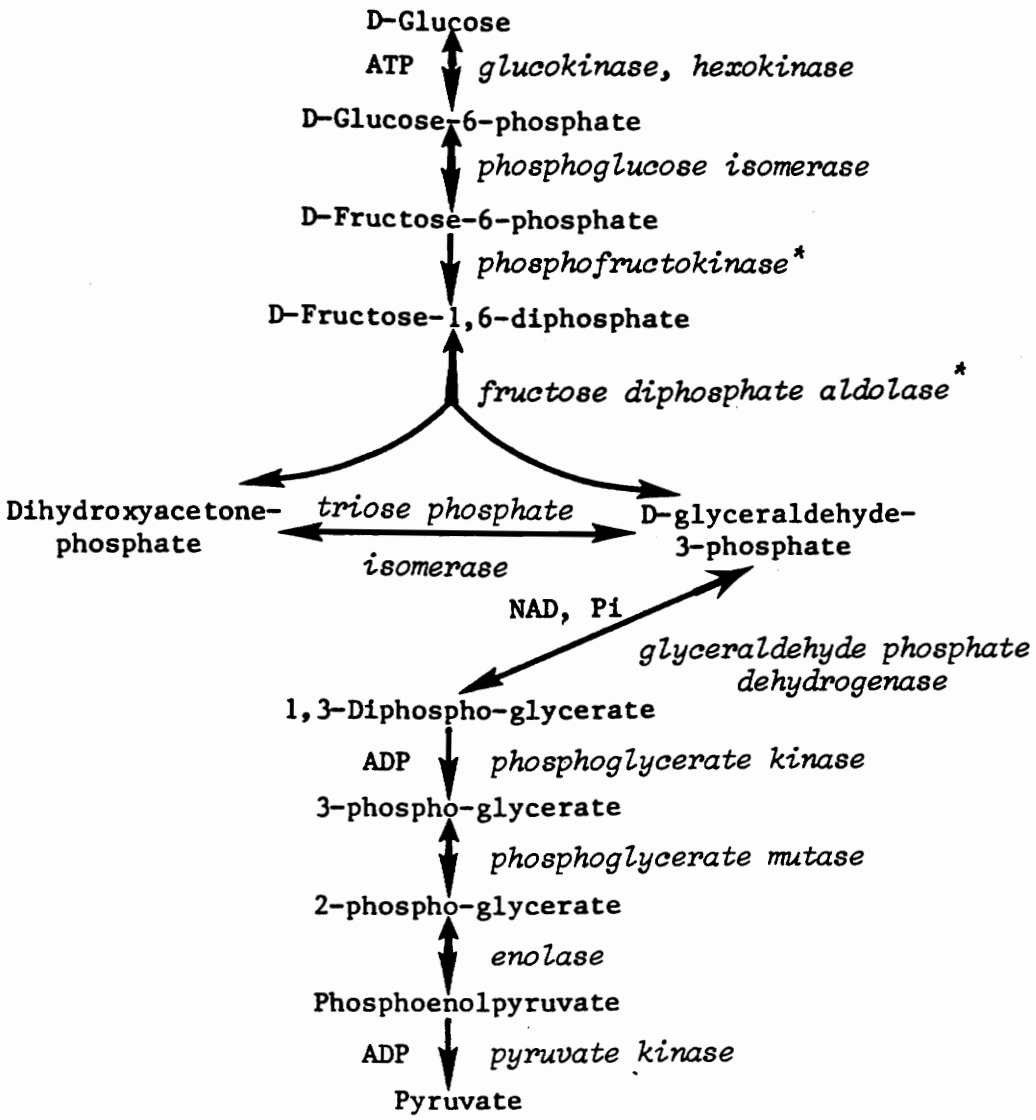


Figure 1. The Embden-Meyerhof-Parnas pathway.
A * denotes a key enzyme.

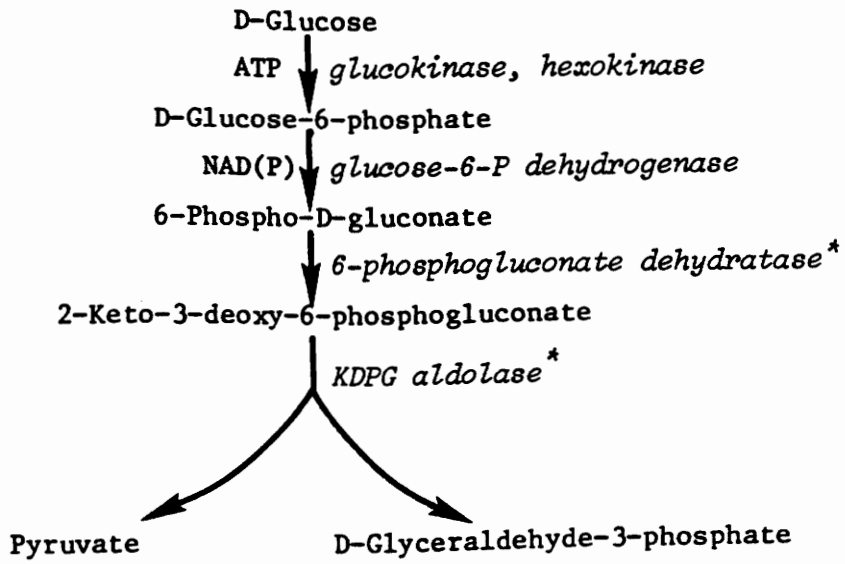


Figure 2. The Entner-Doudoroff pathway.
A * denotes a key enzyme.

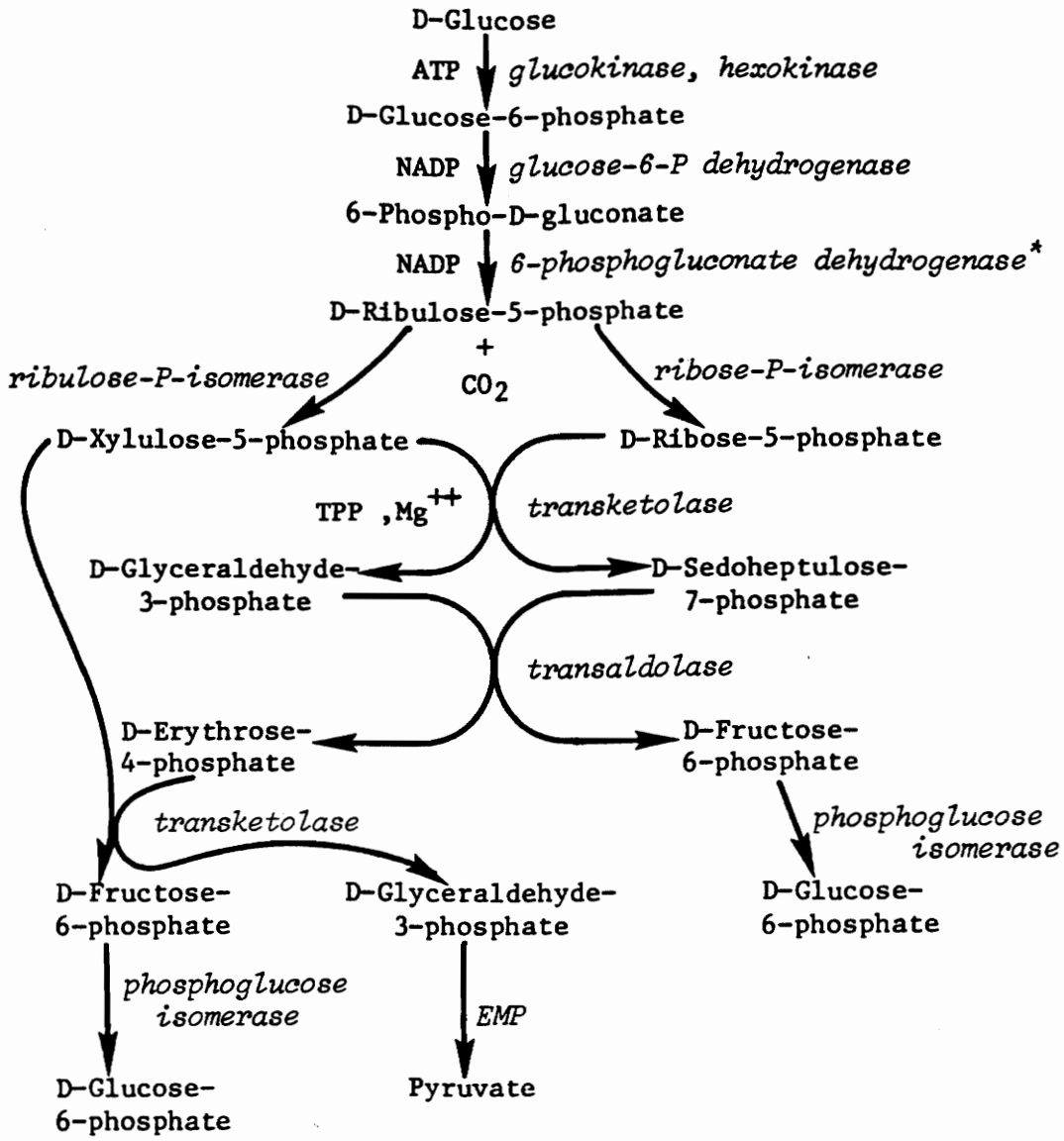


Figure 3. The hexose monophosphate pathway.
A * denotes a key enzyme.

first oxidized forming glucono-delta-lactone-6-phosphate and instantly hydrolyzed to 6-phosphogluconate by glucose-6-phosphate dehydrogenase and gluconolactonase, respectively. At this point the ED and the HMP pathways diverge.

6-Phosphogluconate enters the HMP pathway through oxidative decarboxylation to ribulose-5-phosphate and CO_2. This is catalyzed by the key enzyme of the HMP pathway, 6-phosphogluconate dehydrogenase. Ribose-5-phosphate and xylulose-5-phosphate are formed from ribulose-5-phosphate by ribulose phosphate-3-epimerase and ribulose phosphate isomerase, respectively. Three transketolase/transaldolase steps result in the formation of fructose-6-phosphate and glyceraldehyde-3-phosphate. Fructose-6-phosphate can be converted to glucose-6-phosphate and cycled back through the pathway forming more pentose phosphates. This pathway is particularly important for the production of pentose phosphates and other biosynthetic intermediates.

The first of two key enzymes of the ED pathway, 6-phosphogluconate dehydratase, dehydrates 6-phosphogluconate forming 2-keto-3-deoxy-6-phosphogluconate (KDPG). KDPG is split into two three-carbon compounds, glyceraldehyde-3-phosphate and pyruvate, by a second key enzyme, KDPG aldolase. The glyceraldehyde-3-phosphate can be used to produce pyruvate through the EMP pathway. Unlike the other two pathways discussed, the ED pathway is unique to

procaryotes.

Gluconate enters the ED or HMP pathway at 6-phosphogluconate through phosphorylation by the enzyme gluconokinase. Gluconate is synthesized from glucose by the enzyme glucose dehydrogenase. This enzyme has been found in some species of Bacillus at the onset of sporulation (45, 51). The role of this enzyme in the sporulation process is unknown.

There are two additional pathways of carbohydrate metabolism. They are the pentose phosphoketolase pathway (the heterofermentative pathway) and the hexose phosphoketolase pathway (the bifidum pathway) (16, 25). Both of these pathways are in part dependent upon the HMP pathway.

The pentose phosphoketolase pathway (PPK) has been found in some heterofermentative lactobacilli. In this pathway, the metabolism of glucose is dependent upon the HMP pathway for the formation of pentose phosphates. Since these bacteria lack the enzyme transketolase further catabolism of the pentose phosphates through the HMP pathway is impossible. Catabolism of the pentose phosphates by the PPK pathway results in the formation of acetate and glyceraldehyde-3-phosphate. In some organisms posessing the appropriate phosphorylating enzymes; ribose, xylose, and arabinose are catabolized directly by the PPK pathway.

The hexose phosphoketolase pathway is found in the genus Bifidobacterium. These organisms lack the enzymes fructose diphosphate aldolase and glucose-6-phosphate dehydrogenase. Glucose is catabolized in this pathway by enzymatic conversion to fructose-6-phosphate. Fructose-6-phosphate is cleaved by a phosphoketolase enzyme producing erythrose-4-phosphate and acetyl phosphate. Pentose phosphates can be formed by the reversal of the HMP pathway through the enzymes transaldolase and transketolase.

Each pathway offers advantages to the cell. The EMP pathway produces two moles of ATP per mole of glucose by substrate level phosphorylation, twice as much as the others. Although, organisms that use solely the EMP pathway for glucose catabolism will have specific growth requirements for precursors for the synthesis of ribose-5-phosphate and erythrose-4-phosphate, which themselves are precursors for nucleotide and amino acid biosynthesis. An organism using the HMP pathway for hexose catabolism must have that part of the EMP pathway from the formation of glyceraldehyde-3-phosphate through pyruvate. No microorganism is known to use only the HMP pathway (with the possible exception of Gluconobacter). The ED pathway can function independently of the EMP and HMP pathways. This is due to its ability to directly form pyruvate from glucose. The ratio of pathways in a particular microorganism is dependent upon the enzymatic capabilities

of the cell and the environmental conditions (16).

The Tricarboxylic Acid Cycle and the Glyoxylate Pathway

Pyruvate formed in the EMP, HMP, and ED pathways can be converted to acetyl CoA by the pyruvate dehydrogenase enzyme complex. Acetyl CoA can then enter the tricarboxylic acid (TCA) cycle (see Fig. 4). The TCA cycle is present in most aerobic and many facultative organisms. The condensation of acetyl CoA with a catalytic molecule of oxalacetate by citrate synthetase results in the formation of citrate. One molecule of citrate is metabolized through the TCA cycle (as described in Fig. 4) resulting in the the generation of two molecules of CO_2, eight reducing equivalents (NADH and FADH), and one molecule of oxalacetate. The TCA cycle is essential for the formation of several intermediates for cell biosynthesis and for generation of reducing equivalents which are essential for the synthesis of energy yeilding ATP. Acetyl CoA may be metabolized by an alternate route known as the glyoxylate pathway. In this pathway, isocitrate formed in the TCA cycle is enzymatically cleaved by isocitrate lyase producing succinate and glyoxylate. The enzyme malate sythetase catalyzes the condensation of acetyl CoA and glyoxylate forming the TCA cycle intermediate malate. Acetate is often catabolized through the glyoxylate pathway. This allows a part of the TCA cycle to continue

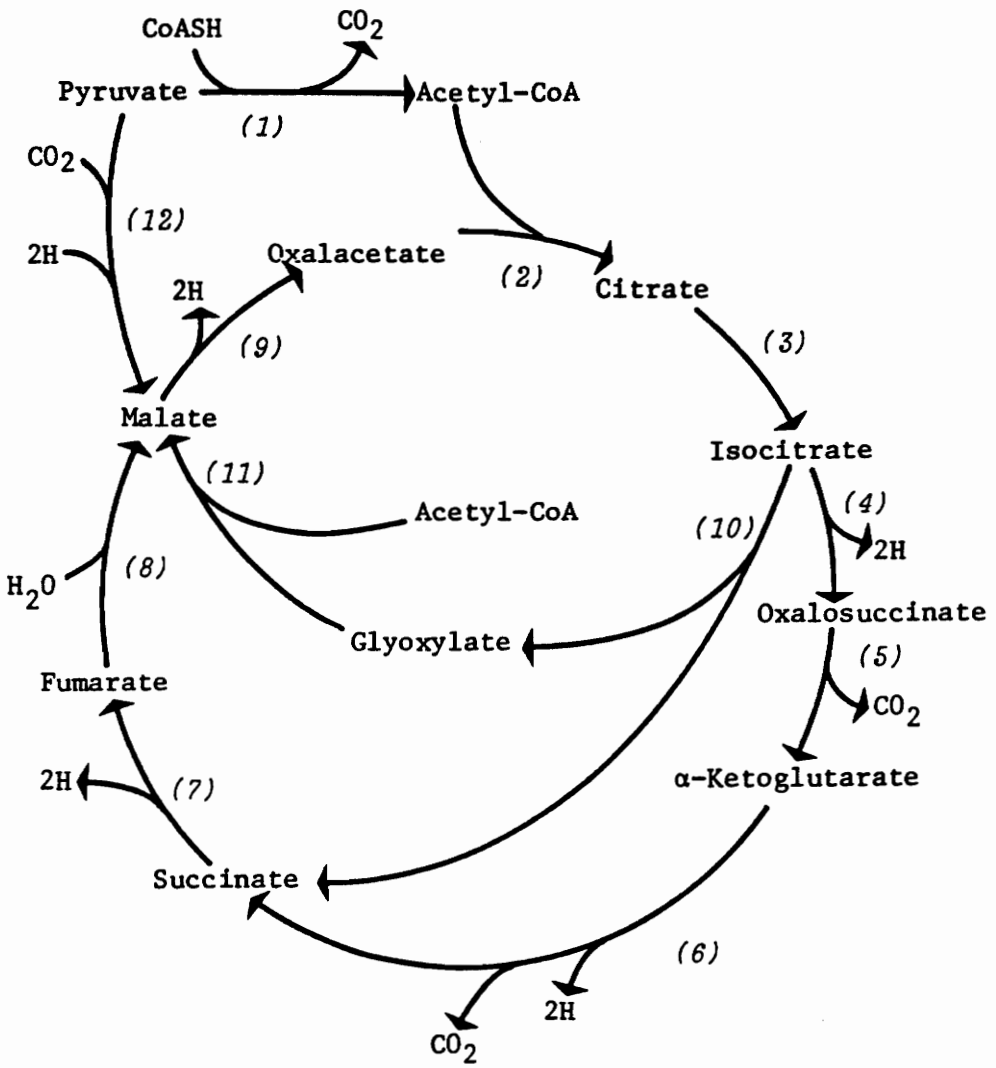


Figure 4. The tricarboxylic acid cycle and the glyoxylate pathway. The enzymes are as follows: (1) pyruvate dehydrogenase, (2) citrate synthetase, (3) aconitase, (4) isocitrate dehydrogense, (5) isocitrate dehydrogenase (6) alpha-ketoglutarate dehydrogenase, (7) succinate dehydrogenase, (8) fumarase, (9) malate dehydrogenase, (10) isocitrate lyase, and (12) malic enzyme

functioning in the absence of the catalytic molecule of oxalacetate or while alpha-ketoglutarate and succinate are used as precursors for cell biosynthesis.

Metabolic Intermediates Required for Cell Biosynthesis

Cell biosynthesis can only occur in the presence of precursor metabolites for the synthesis of cell structural and catalytic materials. In bacteria, these needs can be met by twelve compounds commonly found as intermediates in carbohydrate metabolism: glucose-6-phosphate, fructose-6-phosphate, glyceraldehyde-3-phosphate, 3-phosphoglycerate, phosphoenolpyruvate, pyruvate, ribose-5-phosphate, erythrose-4-phosphate, acetyl CoA, alpha-ketoglutarate, succinyl CoA, and oxalacetate (28). Glucose-6-phosphate is a precursor for the synthesis of lipopolysaccharide and is, therefore, a requirement only for gram-negative cells.

There are several ways by which cells acquire these metabolites. Catabolism of glucose and other carbohydrates through the EMP, HMP, or ED pathways results in the formation of many of these compounds. Acetyl CoA produced from pyruvate is shuttled into the TCA cycle where the remaining compounds, alpha-ketoglutarate, succinyl CoA, and oxalacetate are intermediates.

Cells grown on compounds like glycerol (whose catabolism is dependent upon the enzymes of the lower half of the EMP pathway) or acetate (whose catabolism begins at

the formation of acetyl CoA) face a problem obtaining intermediates synthesized early in the EMP pathway. This is overcome by a reversal of the EMP pathway called gluconeogenesis. This pathway produces glyceraldehyde-3-phosphate and dihydroxyacetone phosphate from phosphoenolpyruvate. The condensation of glyceraldehyde-3-phosphate and dihydroxyacetone phosphate forms fructose diphosphate. This is then dephosphorylated to fructose-6-phosphate by an enzyme not present in the EMP pathway, fructose diphosphate phosphatase.

Finally, when exogenously supplied with these intermediates, some bacteria will simply incorporate them into cellular components. This may occur when the cell does not have the metabolic capabilities required for their biosynthesis.

Metabolism in Bacillus sphaericus

Some research has been done to determine the pathways of glucose catabolism in the genus Bacillus. Enzyme assays, isotope, and inhibitor studies have demonstrated that the major pathway of glucose catabolism in resting cells of B. popilliae and B. lentimorbus in an air atmosphere is the EMP pathway, with the HMP pathway responsible for about 2% of the glucose metabolized (38). Through radiorespirometric studies on B. subtilis marburg C4 and B. cereus terminalis, Goldman and Blumenthal (23, 24) established the primary metabolic pathway of glucose

catabolism as the EMP pathway with the HMP pathway responsible for 2%-30% of the glucose utilized. This combination of pathways supplies the cells with a relatively high amount of ATP and important precursors for biosynthesis.

The TCA cycle is believed to be partially or fully functional in B. sphaericus since many of the compounds that support growth of this species are either TCA cycle intermediates or compounds metabolized through a TCA cycle intermediate. White and Lotay (53) found activity for the TCA cycle enzymes isocitrate dehydrogenase and alpha-ketoglutarate dehydrogenase in extracts of the nonpathogen NCTC 9602. Jelley (29) found citrate synthetase, aconitase, and isocitrate dehydrogenase activity in extracts of the pathogenic strain 1593 when it was grown in a complex medium.

White and Lotay (53) proposed that strain NCTC 9602 grew on acetate as a sole carbon source by use of the glyoxylate cycle. This was supported by their finding of isocitrate lyase and malate synthetase in cell extracts. Since many strains of B. sphaericus can grow with acetate as a sole carbon source, the glyoxylate cycle is probably widely distributed in the species. White and Lotay (53) also reported the absence of pyruvate dehydrogenase activity in strain NCTC 9602. However, Jelley found the enzyme to be present at a very low level in strains NCTC

9602 and 1593.

Gluconate is oxidized by some strains of B. sphaericus (3). This indicates the presence of those enzymes required for phosphorylation and dehydrogenation of this compound. Glycerol is also catabolized by some strains of B. sphaericus including strain 2362 (29). Strain 1593 grown in a complex medium supplemented with glycerol accumulated acetate during exponential phase. The acetate was then used by the cells during stationary phase. Acetate did not accumulate in the complex medium lacking glycerol (29). Metabolism of glycerol and acetate implies the presence of the EMP pathway enzymes from glyceraldehyde-3-phosphate through pyruvate. Supplementation of a complex medium with acetate resulted in no accumulation of organic acids in strain 1593. The acetate is believed to be completely oxidized to CO_2 (29). It is not known whether strain 1593 posesses a glyoxylate cycle for acetate utilization (29) but the use of acetate as sole carbon source suggests the presence of this pathway.

Bacteria Incapable of using Carbohydrates as a Carbon Source

Bacillus sphaericus is not unique in its inability to grow on carbohydrates. A variety of bacteria, both wild-type and mutant strains, can be found having enzymatic and/or transport deficiencies that reduce or prevent their

growth on certain substrates.

In Bergey's Manual of Systematic Bacteriology (32) most members of the aerobic/microaerophilic, motile, helical/vibrioid, gram-negative bacteria are described as incapable of catabolizing carbohydrates. This catagory includes the genera Campylobacter and Aquaspirillum. C. fetus can neither phosphorylate nor transport glucose. It derives its energy from amino acids and TCA cycle intermediates (49). The two species in the genus Azospirillum, A. lipoferum and A. brasilense, both grow on fructose but only A. lipoferum will grow on glucose. Both species possess a complete EMP pathway, including glucokinase, but A. brasilense is cryptic to glucose (21).

Pseudomonas acidovorans and Veillonella sp. do not use carbohydrates due solely to enzymatic deficiencies. All pseudomonads lack 6-phosphofructokinase and thus are unable to use the EMP pathway (10). Their catabolism of glucose and other carbohydrates is dependent upon the ED pathway. P. acidovorans lacks glucokinase and glucose dehydrogenase. This blocks the ED pathway and glucose catabolism. It can instead use gluconate as a carbon source (52).

Members of the genus Veillonella lack glucokinase and hexokinase. They derive their energy primarily from the fermentation of lactate. Veillonella sp. grown on lactate produce hexose phosphates and other biosynthetic intermediates through gluconeogenesis. Exogenously

supplied ribose and fructose are not fermented but can be incorporated into nucleic acid, lipopolysaccharide, and cell wall material (13).

Bacterial mutants have been isolated with a wide variety of metabolic deficiencies. These mutants have provided a significant amount of data that have helped in the elucidation of metabolic pathways and in understanding how these pathways are regulated.

The effects of some mutations are obvious. A phosphoglucose isomerase mutant of E. coli grows more slowly than the wild-type on sugars whose catabolism is via glucose-6-phosphate. These compounds are instead shuttled through the HMP pathway. The mutants lacking both phosphoglucose isomerase and glucose-6-phosphate dehydrogenase fail to grow on glucose (20). A fructose diphosphate phosphatase mutant of E. coli is unable to grow on succinate, and acetate because its growth is dependent upon gluconeogenesis for the formation of biosynthetic intermediates. This mutant still grows on sugars when growth is not dependent upon gluconeogenesis (19).

Other mutants show effects that are more difficult to explain. A triose phosphate isomerase mutant of E. coli is unable to grow on carbohydrates. One may expect this deletion to simply slow carbohydrate catabolism. Some evidence suggests that in this mutant methylglyoxyl is formed enzymatically from dihydroxyacetone phosphate and

that this compound is toxic to the cell (11). A fructose diphosphate aldolase mutant of E. coli is unable to grow on hexoses and their derivatives but does grow on glycerol (6). The aldolase should be necessary for the formation of biosynthetic intermediates through gluconeogenesis during growth on glycerol. It was proposed that the enzyme was present at a low level sufficient only for the minor use of the enzyme in gluconeogenesis but not for the major use of growth on hexoses.

MATERIALS AND METHODS

Strain

Bacillus sphaericus strain 2362 was isolated by J. Weiser from an insect collected in Nigeria. The strain was numbered 290-8 in his collection.

Source of Chemicals

The following reagent grade compounds were obtained from Sigma Chemical Company, St. Louis, MO: biotin, thiamine, 3-[N-morpholino]propanesulfonic acid (MOPS), fructose, galactose, sodium gluconate, glucosamine, sodium glutamate, glycerol, mannose, sorbose, trehalose, xylose, o-nitrophenyl galactoside (ONPG), sodium carbonate, NAD, NADP, NADH, NADPH, bovine serum albumen, Tris[hydroxymethyl]aminomethane(Tris), beta-mercaptoethanol, ATP, glucose-6-phosphate, fructose-6-phosphate, fructose diphosphate, 6-phosphogluconate, glycylglycine, potassium acetate, phosphoglucoisomerase (cat.# P-9010), 6-phosphogluconate dehydrogenase (cat. # P-0632), glucose-6-phosphate dehydrogenase (cat.# G-7878), fructose diphosphate aldolase (cat. # A-7145), alpha-glycerophosphate dehydrogenase (cat. # G-6880), and alpha-glycerophosphate dehydrogenase/triose phosphate isomerase (cat. # G-6755).

The following reagent grade compounds were obtained from the Fisher Scientific Company, Pittsburgh, PA:

ammonium sulfate, ferrous sulfate, magnesium chloride, sodium acetate, sucrose, and sodium sulfate.

The following reagent grade compounds were obtained from the J. T. Baker Chemical Company, Phillipsburg, PA: sodium phosphate, potassium phosphate, glucose, and lactose.

Agar, nutrient broth, and yeast extract were obtained from Difco Laboratories, Detroit, MI.

Reagent grade calcium chloride and manganese sulfate were obtained from Allied Chemical Company, Morristown, NJ.

[U-^{14}C]sucrose and [U-^{14}C]glucose were obtained from DuPont Chemical, Boston, MA.

Culture Media

BACT medium (biotin, ammonium sulfate, carbon source, thiamine) was prepared as follows. The base consisted of (per liter): $Na_2HPO_4 \cdot 2H_2O$, 5.57 g; KH_2PO_4, 2.40 g; $(NH_4)_2SO_4$, 2.00 g; and agar, 20.0 g (in the agar-solidified medium only). The remaining ingredients were (per liter) $MnCl_2 \cdot 4H_2O$, 4 mg; $FeSO_4 \cdot 7H_2O$, 2.8 mg; $CaCl_2 \cdot 2H_2O$, 1.5 mg; thiamine, 200 mg; biotin, 20 ug; and either 1 g or 40 mM carbon source. The biotin, thiamine, and carbon source were prepared as filter-sterilized stock solutions. The Mg^{++}, Mn^{++}, Ca^{++}, and Fe^{++} salts were prepared as an acidified (0.03% H_2SO_4 [vol/vol]) filter-sterilized stock solution. The biotin, thiamine, carbon source, and salts

were added to the autoclaved base after it cooled to at least 50° C.

NY broth consisted of nutrient broth (8 g/liter) and yeast extract (0.5 g/liter). NYSM broth was supplemented with salts at a final concentration of 5 x 10^{-5}M $MnCl_2$, 7 x 10^{-4}M $CaCl_2$, and 10^{-3}M $MgCl_2$. These salts were prepared as a concentrated stock solution. NY and NYSM broth were supplemented with 2% agar to make agar-solidified media.

GYS broth was used to grow B. cereus T, the control in the glucose dehydrogenase assay. The base contained (per liter): glucose, 1.0 g; $(NH_4)_2SO_4$, 2.0 g; yeast extract, 2.0 g. Filter-sterilized salts with the same composition and concentration as those added to NYSM, were added to the autoclaved base.

Dry Weight Determinations

The dry weight of cells used in some experiments was determined by adding 2 ml of cell suspension to dried, preweighed, aluminum weighing pans. The filled pans were dried at 110° C for at least 48 hours. When the cells were suspended in buffer instead of water the dry weight of the buffer was subtracted from the dry weight of cells plus buffer. The average dry weight of cells was calculated from three pans.

Growth Conditions

All cultures were incubated at 30° C unless otherwise

stated. Flasks were shaken at 180 rpm on the New Brunswick model G25 shaker incubator (New Brunswick Scientific Co., New Brunswick, NJ) or the New Brunswick gyratory water bath shaker incubator. Growth was measured on a Klett-Summerson photoelectric colorimeter (Klett Mfg. Co., NY). Growth in NY and NYSM media was read using a red filter (660 nm) and growth in BACT medium was read using a blue filter (420 nm). Klett units were converted to absorbance units for growth curves.

Preparation of Cells for Sole Carbon Source Studies

Five ml of NY broth was inoculated from a slant of B. sphaericus. The bacteria grew overnight in the New Brunswick model G25 shaker incubator. The cells were centrifuged at 12,100 xg for 10 minutes in sterile, cotton-stoppered Corex centrifuge tubes using a Beckman model J-21C centrifuge at 4^{o} C for 10 minutes. The cells were washed once by centrifugation in sterile 0.05 M MOPS buffer, pH 7.2, and then resuspended in 5 ml sterile MOPS.

Sole Carbon Source Study on an Agar-Solidified Medium

BACT plates were streaked with the above cell preparation and incubated for a total of 5 days. The carbon sources tested at a concentration of 40 mM were sodium acetate, arabinose, fructose, galactose, sodium gluconate, glucosamine, glucose, sodium glutamate, glycerol, lactose, mannose, sucrose, sorbose, trehalose, and xylose. NYSM agar

was streaked to verify that the cells survived the washing process and were viable.

Sole Carbon Source Study in a Liquid Medium

This study was designed to quantitate the growth observed on the BACT agar-solidified media. Fifty ml of BACT in a 500 ml side-arm flask was inoculated with 1 ml of the washed cell preparation. The cells were incubated in the Beckman model G25 shaker incubator. Growth was measured on the Klett over a 50 hour period. The carbon sources tested were 40 mM sodium acetate, sodium gluconate, and glycerol. Growth was also followed in NYSM broth to verify that the cells used as the inoculum were viable.

Attempts were made to increase the cell density of the BACT plus gluconate broth cultures. One attempt employed the Roux bottle technique in which cells were incubated in a biphasic medium (NYSM) both stationary and while shaking at 30° C. In another attempt, an aliquot of cells incubated for 48 hours in BACT plus gluconate broth was transferred to a second flask of BACT plus gluconate. At 48 hour intervals aliquots of these cells were transferred to a third and fourth flask.

Oxygen Uptake

Strain 2362 was tested for its ability to take up oxygen in the presence of different substrates. Five ml of NY broth was inoculated with B. sphaericus and incubated

overnight on a roller. This 5 ml culture was added to 100 ml NYSM or NYSM + 0.1% carbon source in a 500 ml side-arm flask. These cells were incubated on the New Brunswick model G25 shaker incubator until mid exponential phase (A_{660}=0.2). The cells were harvested by centrifugation at 12,100 x g for 10 minutes in the Beckman J-21C centrifuge at 4° C. The cells were washed two times in sterile MOPS buffer (0.05 M, pH 7.2) and resuspended in 50 ml MOPS.

Oxygen uptake was measured with a Clark oxygen electrode, maintained at 30° C, and a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH). The Clark electrode was calibrated according to the method of Robinson and Cooper (42).

Endogenous oxygen uptake by the cell suspension was monitored for 2 minutes. All substrates were prepared as 1.7 M stock solutions except lactose which was 1 M. One hundred ul of substrate was added to the cell suspension in the Clark cell resulting in a final substrate concentration of 0.12 M (0.07 M for lactose). These substrate concentrations allowed for maximum oxygen uptake. Substrate induced oxygen uptake was measured for at least 2 minutes. The QO_2 (microliters of oxygen consumed/hour/mg dry cell weight) was calculated by 1) multiplying the change in divisions per minute on the recorder paper by the microliters of oxygen each division represented (determined by electrode calibration) for both endogenous oxygen uptake

and oxygen uptake in response to the substrate, 2) subtracting endogenous oxygen uptake from oxygen uptake in response to the substrate, 3) dividing this value by the dry weight of the cells (mg/ml) and, 4) multiplying by 60 minutes per hour.

Preparation of Cell Extracts for Enzyme Assays

The enzymes assayed were (i) those from the EMP pathway whose presence was not implied by the ability of B. sphaericus to convert glycerol to acetate (29), (ii) the key enzymes of the HMP and ED pathways, and (iii) some enzymes related to these pathways.

Cells were prepared for all enzyme assays (except amylase and beta-galactosidase) in the following manner. Five ml NY was inoculated with B. sphaericus or the control organism and placed on the New Brunswick model G25 shaker incubator for about 10 hours. One-hundred ml of NY in a 500 ml flask was inoculated with 1 ml of this culture and incubated in the New Brunswick water bath shaker incubator for 4 hours. Twenty ml of these cells were added to each of two 2 L flasks containing 230 ml NYSM supplemented with 0.1% carbon source. The cultures were incubated on the New Brunswick model G25 shaker to mid exponential phase (A_{660}=0.2). The cells were harvested by centrifugation at 9,828 x g for 10 minutes in the Beckman J-21C centrifuge at 4° C. The pellets from the two flasks were combined and

washed twice in the buffer to be used in the enzyme assay. Cells were resuspended in 15 ml of the same buffer and were kept on ice prior to breaking.

The cells for the glucose dehydrogenase assay were incubated for 9 hours instead of to mid-log stage. At this time most of the cells had begun to sporulate but the majority were not yet refractile spores. Otherwise, they were treated as described above.

Cells were broken with a Fisher sonic dismembrator model 300 (Fisher Scientific, Co., Pittsburg, PA) using the large probe at the maximum setting for two 30 second intervals. This broke more than 80% of the cells as judged by phase contrast microscope observation. The cells and the probe were kept cold with ice during disruption. The preparation was centrifuged at 48,400 x g for 15 minutes on the Beckman J-21C centrifuge at 4° C. The supernatant was ultracentrifuged at 120,000 x g for 1 hour at 4° C using a Beckman model L-550 preparative ultracentrifuge and a model 50-Ti fixed-angle titanium rotor. The resulting cell-free extracts were kept on ice. All extracts were assayed within a few hours of preparation.

Spectrophotometric enzyme assays

Enzyme activity was measured for the following enzymes: (i) glucokinase (E.C. 2.7.1.2), (ii) hexokinase (E.C. 2.7.1.1), (iii) gluconokinase (E.C. 2.7.1.12), (iv)

phosphoglucose isomerase (E.C. 5.3.1.9), (v) 6-phosphofructokinase (E.C. 2.7.1.11), (vi) fructose diphosphate aldolase (E.C. 4.1.2.13), (vii) glucose dehydrogenase (E.C. 1.1.1.47), (viii) glucose-6-phosphate dehydrogenase (E.C. 1.1.1.49), (ix) 6-phosphogluconate dehydrogenase (E.C. 1.1.1.44), and (x) 6-phosphogluconate dehydratase (E.C. 4.2.1.12) /KDPG aldolase (E.C. 4.1.2.14).

The substrate KDPG and the enzymes 6-phosphogluconate dehydratase and KDPG aldolase were not commercially available. As a result, neither KDPG aldolase nor 6-phosphogluconate dehydratase could be assayed conveniently. As an alternative, the two enzymes were assayed in a single reaction mixture where the oxidation of NADH was dependent upon the presence of both enzymes.

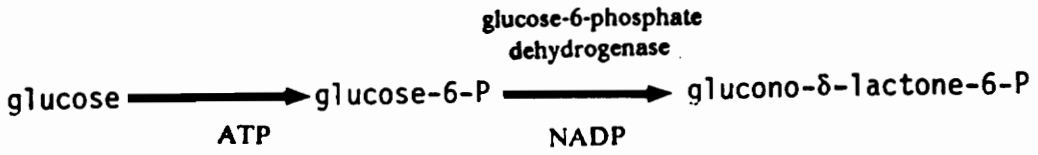
All reaction mixtures were prepared in a quartz cuvette with a 1 cm light path. During the assay, cell extracts and commercial enzymes were kept on ice. The remaining reactants were maintained at room temperature. The commercial enzymes were tested for activity with the appropriate substrates and in the absence of cell extract for each assay.

Enzyme activity was monitored by an increase or decrease in absorbance at 340 nm on a Bausch and Lomb, Spectronic 2000 (Bausch and Lomb Inc., Rochester, NY). The change in absorbance was due to the appearance or disappearance of NAD(P)H. Specific activities were

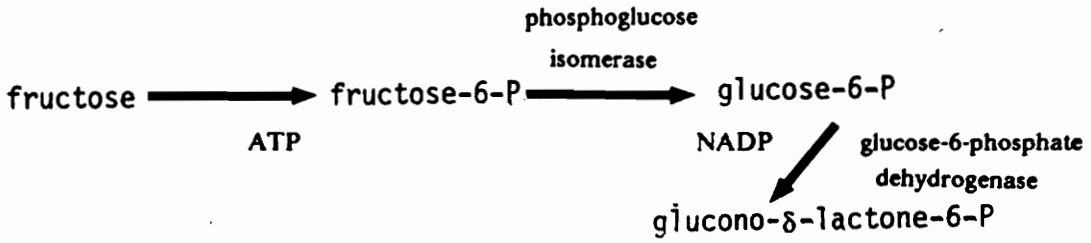
expressed as μmoles of substrate converted per minute (International units) per mg protein. Protein determinations were done according to the dye-binding method of Bradford (7) using the Bio-Rad reagent and bovine serum albumen as the standard.

Details of each enzyme assay are described in the Appendix. Schematic representation of each assay method is presented below.

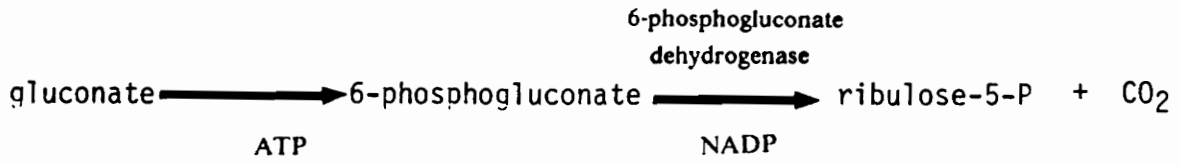
(i) Glucokinase



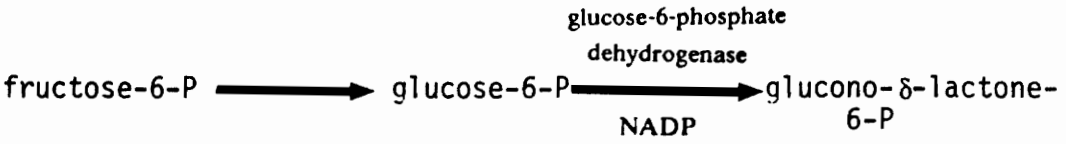
(ii) Hexokinase



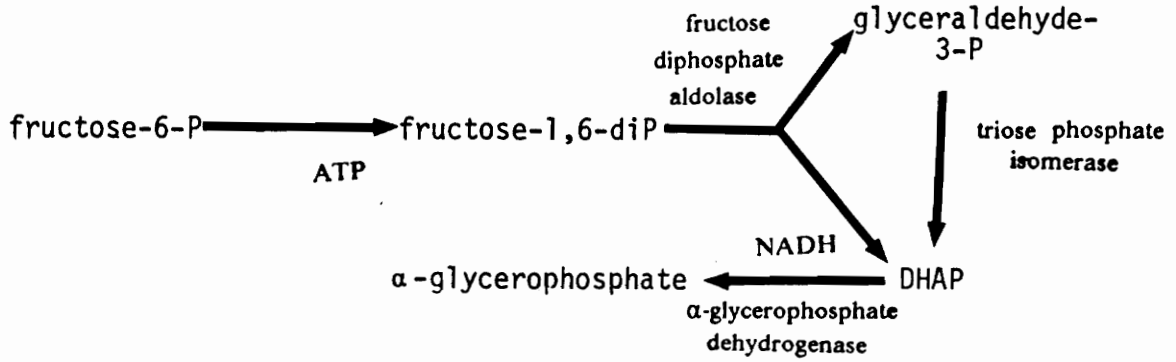
(iii) Gluconokinase



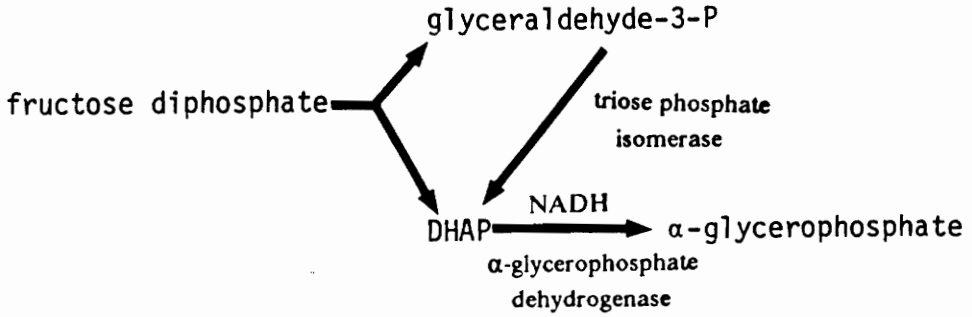
(iv) Phosphoglucoisomerase



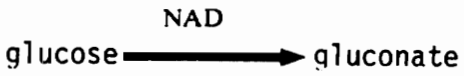
(v) 6-Phosphofructokinase



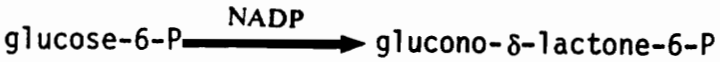
(vi) Fructose diphosphate aldolase



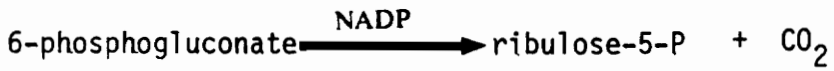
(vii) Glucose dehydrogenase



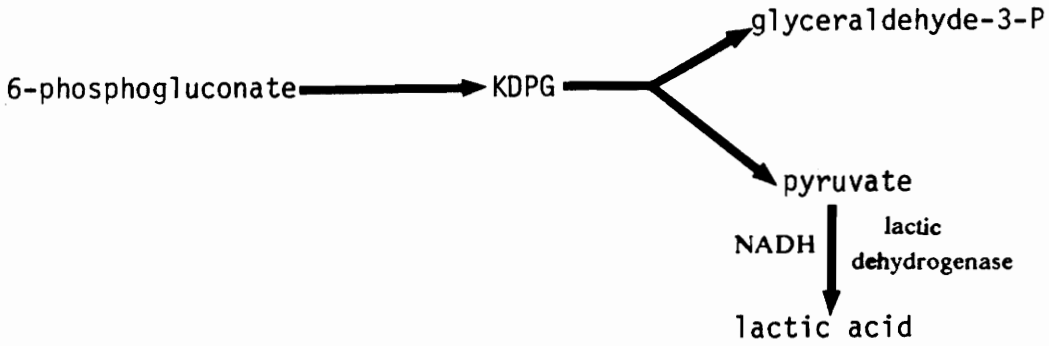
(viii) Glucose-6-phosphate dehydrogenase



(ix) 6-Phosphogluconate dehydrogenase



(x) 6-Phosphogluconate dehydratase/ KDPG aldolase



Additional attempts to find activity for phosphogluconate dehydrogenase were made using the assay as described with the following modifications: i) incubation of the cells in NYSM + 0.5% gluconate instead of 0.1% gluconate, ii) breaking the cells using a French Press instead of by sonication, or iii) adding beta-mercaptoethanol to the buffer. In another experiment, cells grown on BACT + gluconate agar for three days were washed off the plates with buffer. About 80% of the cells harvested from these plates were in some stage of sporulation while about 20% were nonsporulated, motile cells. The cells were assayed as described.

Additional attempts to detect fructose diphosphate aldolase activity in extracts of B. sphaericus were done using the assay presented with the following modifications: i) the cells were broken and assayed with and without the addition of dithiothreitol to the buffer, ii) the cells were grown in BACT + acetate instead of the complex medium, or iii) the cells were broken with a French Press instead of by sonication.

Amylase Assay

A sterile wire was touched to growth on a bacterial slant of B. sphaericus and stabbed into NYSM agar containing 2% Argo brand corn starch. The starch caused the agar to appear cloudy. The culture was incubated for

48 hours. Break-down of the starch by amylase was observed as a clear zone in the agar surrounding the bacterial growth.

Beta-Galactosidase Assay

Five ml of NY was inoculated with B. sphaericus or the control organism E. coli K-12 and incubated stationary over night. One hundred ml of NYSM + 0.1% lactose in a 500 ml side-arm flask was inoculated with the 5 ml culture. These cells were incubated to mid exponential phase (about 0.2 abs.) and harvested by centrifugation at 9,820 x g for 10 minutes on the Beckman J-21C centrifuge at 22° C. The cells were washed one time in 50 mM MOPS, pH 7.2 and resuspended to 25 ml in the same buffer.

One-half ml of the cell suspension was transferred aseptically to a tube on ice containing 0.5 ml MOPS buffer and one drop of toluene. The tube was transferred from the ice to a 37° C water bath to evaporate most of the toluene. After toluene evaporation, 0.2 ml o-nitrophenyl galactoside (ONPG), a non-metabolizable analogue of lactose, was added to the tube. The tube was incubated stationary at room temperature for 30 minutes at which time 3 ml of 0.5 M sodium carbonate was added to stop the reaction. The absorbance of the reaction mixture was measured on the Spectronic 2000 at 420 nm. The negative control consisted of a B. sphaericus cell suspension boiled for two minutes

in a water bath. The boiled cells were then assayed as described. This control accounted for the amount of absorbance increase due to the turbidity of the cell suspension. This absorbance was subtracted from the absorbance of the B. sphaericus cells which had not been heat inactivated.

Beta-Galactosidase present in the reaction mixture cleaved the ONPG molecule and the free o-nitrophenol (ONP) exibited a yellow color. A standard curve of ONP was used to determine to amount of ONPG hydrolyzed (5). Enzyme activity was reported as umoles ONP produced /mg dry cell weight/minute.

Uptake of Radioactive Substrates by Whole Cells

Five ml of NY were inoculated with B. sphaericus or the control organism and incubated for about 10 hours while shaking. One-hundred ml of NY in a 500 ml side-arm flask was inoculated with 1 ml of the culture and incubated in the New Brunswick water bath shaker incubator for 4 hours. Twenty ml of this culture was transferred to a 2 L flask containing 230 ml NYSM + 0.1% glucose or sucrose. These cells were incubated in the New Brunswick model G25 shaker incubator to mid exponential phase. The cells were harvested by centrifugation at 7,520 x g for 10 minutes on a Beckman J-21C centrifuge at 22° C. They were washed one time

with potassium phosphate buffer (50 mM, pH 7.0) at room temperature. The cells were resuspended with buffer to 50 ml in a 250 ml flask.

The reaction mixture consisted of 2.5 ml of cells and 25 µl of radioactive glucose (2.5 µCi of [U-^{14}C]glucose in 1 ml of 0.5% nonradioactive glucose) or radioactive sucrose (2.5 µCi of [U-^{14}C]sucrose in 1 ml of 0.5% nonradioactive sucrose) in a sterile tube. The reaction mixture was incubated in the New Brunswick model G25 shaker incubator at 30^{o} C. At various time intervals each tube was vortexed and a 200 µl aliquot was removed. Each aliquot was filtered through a prewetted millipore filter (Cat. # HABG 25 00, type HA, 0.45 µm pore size) using a Millipore, model 1225 sampling manifold (Millipore Corporation, Bedford, MA). The filters were washed with 5 ml of the phosphate buffer and air dried. Radioactivity was determined by placing each filter in a plastic scintillation baggie with 3 ml of Ecoscint scintillation fluid (National Diagnostics, Manville, NJ). The baggies were heat sealed and placed in glass scintillation vials. Radioactivity was counted on a Beta Trac, model 6895 liquid scintillation system (Tm Analytic Inc., Elk Grove Village, IL) with a window setting of 350-620. All samples were taken in duplicate and the counts averaged. Cells starved for one hour in buffer prior to the addition of substrate were tested for uptake

of substrate in the same manner as described.

Background radioactivity was measured using two controls. In one control, a 200-µl aliquot of cell suspension, in the absence of substrate was filtered and washed. In the other control, 25 µl of the radioactive substrate was added to 2.5 ml of buffer instead of cell suspension. A 200-µl aliquot of this diluted substrate was filtered and washed. The filters were treated as described. The control with the higher number of counts per minute (cpm) was considered the background radioactivity value.

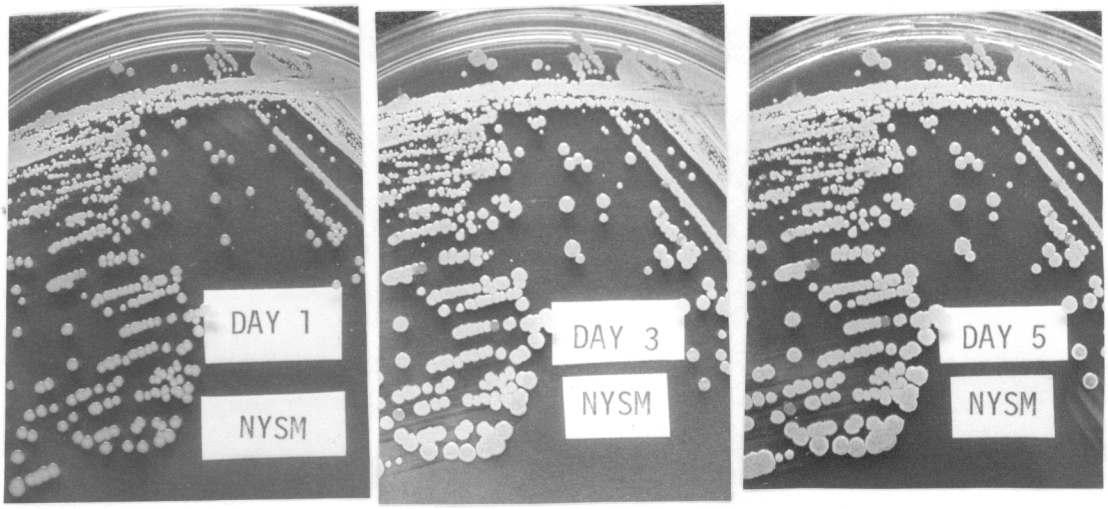
Calculation of nmoles of substrate taken up per mg dry weight of cells was done by: 1) subtracting the background cpm from the total cpm, 2) multiplying cpm by the efficiency of the scintillation counter (determined from standards) resulting in disintegrations per minute (dpm), 3) converting dpm into µCi (2.2×10^6 dpm = 1 uCi), 4) converting µCi into nmoles of substrate using the final specific activity (mCi/mmole) of the substrate, and 5) dividing this number by the dry weight of cells (mg/ml).

RESULTS

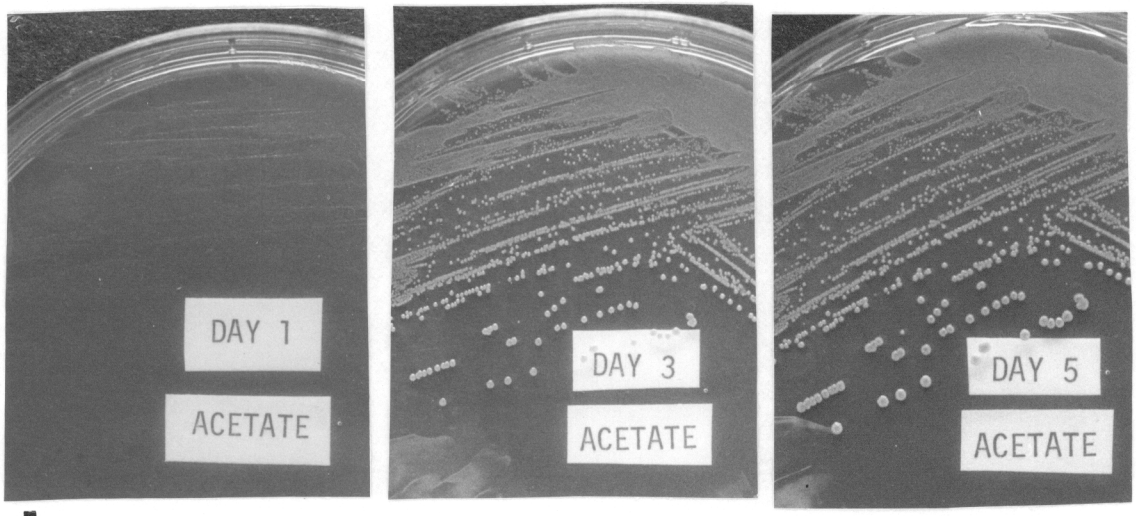
Sole Carbon Source Study on an Agar-Solidified Medium

Bacillus sphaericus 2362 was tested for its ability to grow on a defined, agar-solidified medium, BACT, with various compounds as the sole source of carbon. After a five day incubation, no growth was observed on media containing the following carbon sources: arabinose, fructose, galactose, glucosamine, glucose, lactose, mannose, sorbose, sucrose, trehalose, or xylose. Growth was observed on media containing acetate, glutamate, gluconate, or glycerol.

Photographs of colony growth on BACT containing acetate, glycerol, or gluconate are presented in Figs. 5b, c, and d. Growth on NYSM is presented in Fig. 5a for a comparison of growth on a complex, protein-based medium. After one day of incubation, no growth was present on the glycerol or gluconate plates. Slight growth was present on the acetate plate although distinct colonies were not yet apparent. The NYSM plate demonstrated heavy growth with the largest colonies having a diameter of about 2 mm. By day three, pin-point colonies were present on the glycerol and gluconate plates. The largest colonies on the acetate plates were about 1 mm in diameter and colonies on the NYSM plate were about 2.5 mm in diameter, their maximum size over

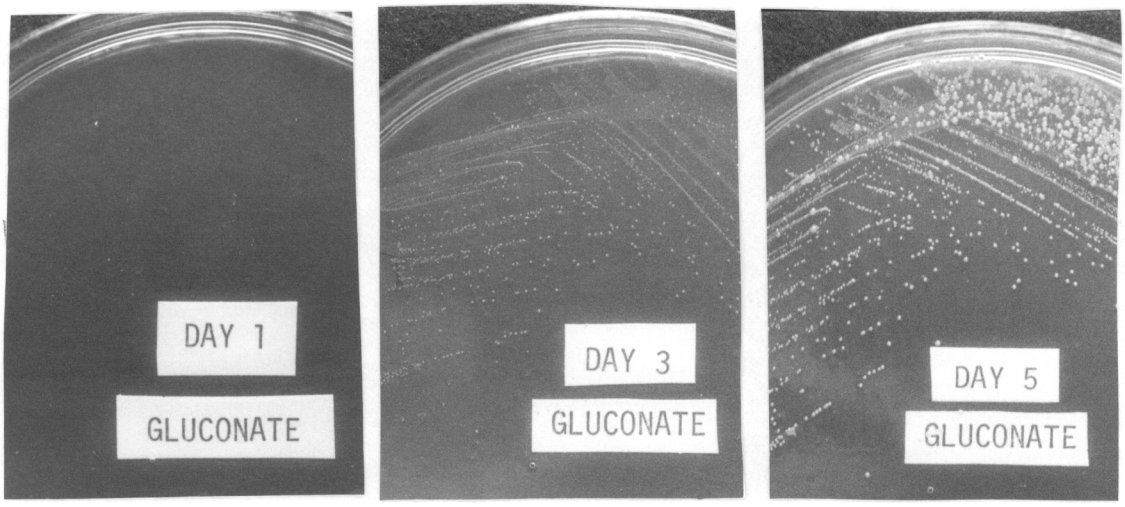


a

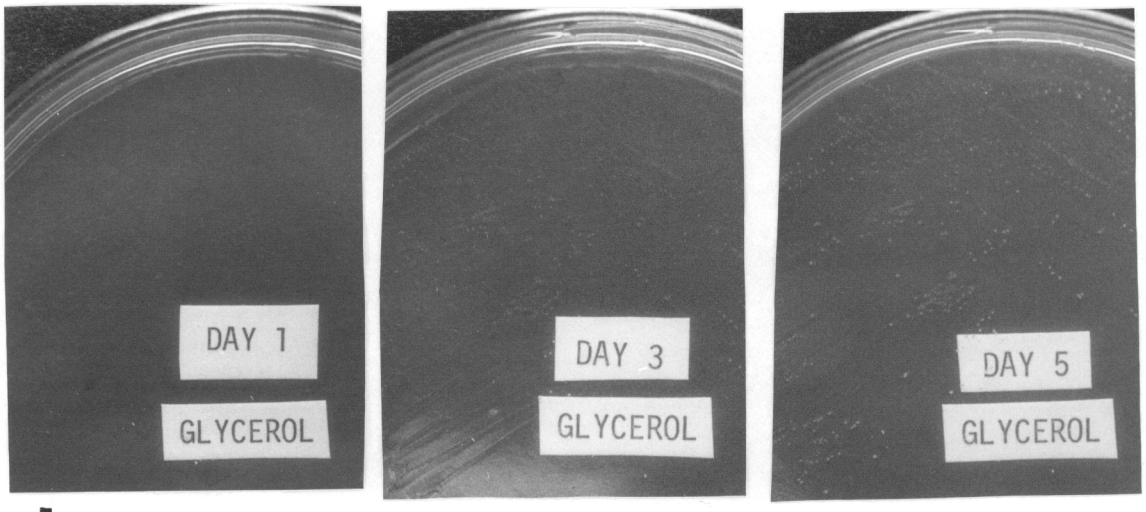


b

Figure 5a, b. Growth of B. sphaericus 2362 on defined agar-solidified media. All cultures were incubated at 30° C. (a) Growth on NYSM. (b) Growth on BACT + 40 mM sodium acetate. The figures presented are actual size.



c



d

Figure 5c, d. Growth of B. sphaericus 2362 on (c) BACT + 40 mM sodium gluconate and (d) BACT + 40 mM glycerol. The figures presented are actual size.

this five-day period. By day five, growth on glyerol was still poor and growth on gluconate was slightly better than that on glycerol. In both cases, maximum colony diameter remained less than 1 mm. At five days the colonies grown on acetate had diameters up to 1.5 mm. Growth of B. sphaericus on NYSM after one day appeared heavier than the growth on acetate, glycerol, or gluconate even after five days of incubation.

Sole Carbon Source Study in a Defined-Liquid Medium

Growth of B. sphaericus was quantitated using growth curves of the organism in BACT broth with acetate, glycerol, or gluconate as the sole source of carbon (see Fig. 6). Growth in NYSM broth was followed for comparison. The relative amount of growth in liquid media was similar to that observed on agar-solidified media.

Growth on glycerol and gluconate progressed very slowly. An exponential phase was not apparent in the growth curve and the cell density, and presumably the cell number, did not even double in fifty hours. Somewhat better growth was observed in the broth containing acetate. An exponential phase was apparent with these cells although growth progressed significantly slower than cells grown on NYSM. Mid-exponential phase occurred at about 4 hours on NYSM but at about 20 hours

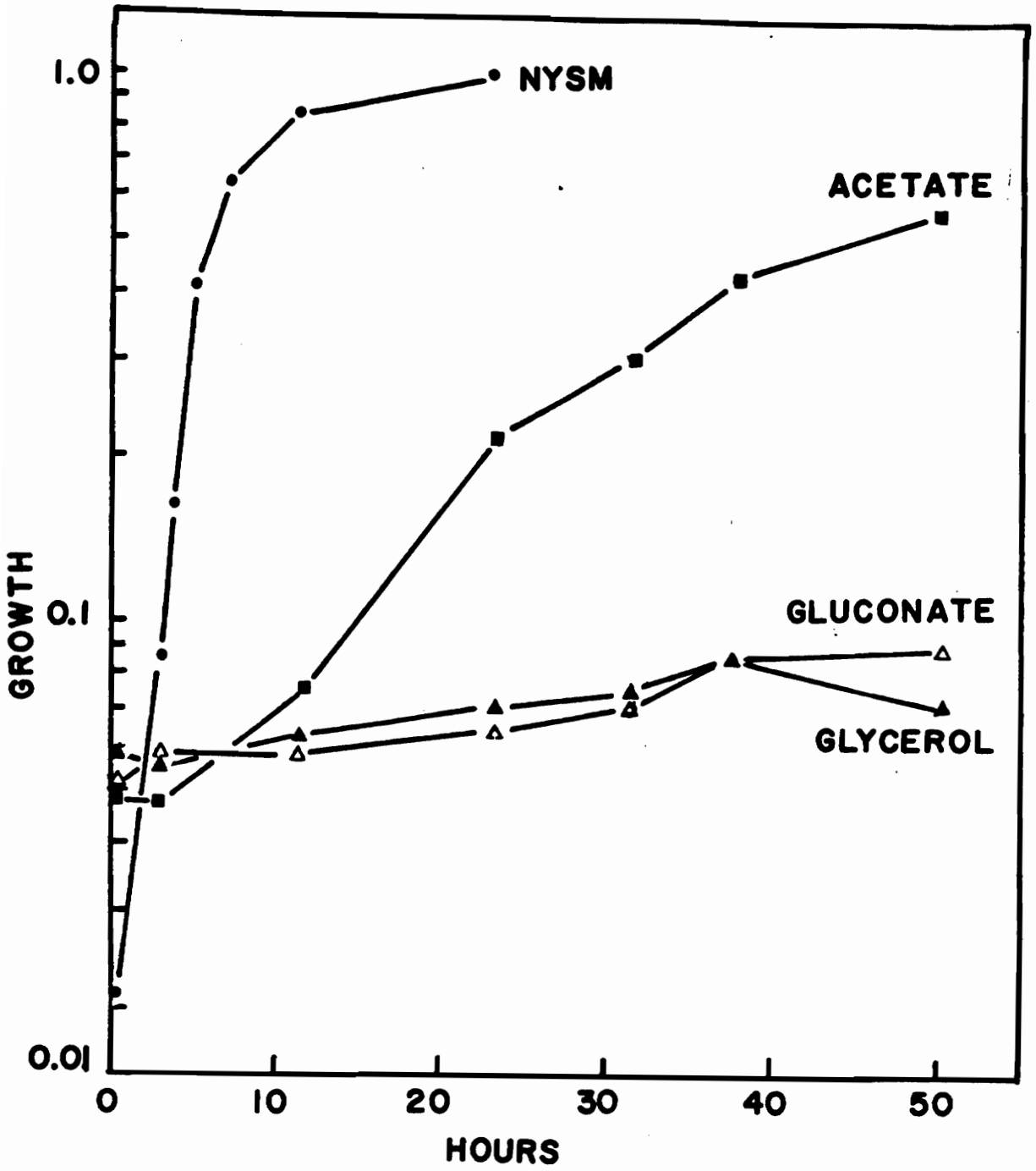


Figure 6. Growth of B. sphaericus 2362 in defined liquid media. All cultures were incubated at 30° C on a shaker. The acetate, gluconate, and glycerol grown cells were incubated in BACT + 40 mM of the carbon source. Turbidity was measured at 420 nm for BACT cultures and at 660 nm for NYSM cultures. Growth in NYSM is presented for comparison.

on the defined medium plus acetate.

Improved growth on gluconate was not observed when cells were transferred from one flask to another at 48 hour intervals or with the Roux bottle technique. Some increase in cell density was observed in the BACT + gluconate + yeast extract culture but this increase was similar to that of a BACT + yeast extract (no other carbon source) control culture. Therefore, any increase in turbidity was probably due to the use of the yeast extract by the organism as the source of carbon.

Oxygen Uptake by Whole Cells

B. sphaericus 2362 was tested for its ability to oxidize a variety of substrates. Cells grown in NYSM demonstrated little or no oxidation of glucose, sucrose, lactose, gluconate, or ribose. Cells grown in NYSM supplemented with a carbon source showed oxidative ability similar to that of cells grown in NYSM alone in all cases but one. Unlike cells grown NYSM only, cells grown in NYSM plus 0.1% gluconate were able to oxidize gluconate (see Table 1). The activity of one or more enzymes essential for gluconate metabolism may be induced by the presence of gluconate in the growth medium. The cells prepared in each medium were tested for their ability to oxidize glutamate to assure that they posessed normal oxidative abilities. The QO_2 for each cell

Table 1. Oxygen Uptake by Bacillus sphaericus 2362.

Growth medium	Substrate	QO_2 ± S.D.[c]
NYSM[b]	glutamate	41 ± 1
NYSM	glucose	3 ± 3
NYSM + 0.1% glucose	glucose	8 ± 3
NYSM	sucrose	0
NYSM + 0.1% sucrose	sucrose	1 ± 2
NYSM	lactose	0
NYSM + 0.1% lactose	lactose	1 ± 1
NYSM	glycerol	25 ± 4
NYSM + 0.1% glycerol	glycerol	18 ± 7
NYSM	gluconate	0 ± 1
NYSM + 0.1% gluconate	gluconate	11 ± 7
NYSM	ribose	3 ± 3
NYSM + 0.1% ribose	ribose	3 ± 3

[a] QO_2 represents the ul O_2 consumed/mg dry wt./hour. All means and standard deviations represent each assay run in duplicate on two separate batches of cells.

[b] NYSM is nutrient broth plus 0.05% yeast extract and salts ($CaCl_2$, 0.7 mM; $MgCl_2$, 1 mM; $MnCl_2$, 0.05 mM).

[c] S.D. = standard deviation

preparation was similar to that presented in Table 1 for the oxidation of glutamate by cells grown in NYSM.

Spectrophotometric Enzyme Assays

The activity of certain enzymes of carbohydrate catabolism was assayed in cell extracts of B. sphaericus 2362 (see Table 2). No activity was detected for any of the enzymes commonly found in the upper half of the EMP pathway i.e. glucokinase, hexokinase, phosphoglucose isomerase, phosphofructokinase, and fructose diphosphate aldolase. The key enzymes of the HMP and ED pathways phosphogluconate dehydrogenase and phosphogluconate dehydratase/KDPG aldolase were also undetected in cell extracts. In addition, no activity was detected in cell extracts for the enzymes glucose-6-phosphate dehydrogenase and glucose dehydrogenase. Activity was detected for each of these enzymes in a control organism thus demonstrating the proper functioning of the assay system itself. Gluconokinase activity was detected in B. sphaericus. The specific activity of this enzyme was approximately 10-fold lower than that of the gluconokinase in E. coli K-12. The average amount of protein in the B. sphaericus extracts was 1 mg/ml which was similar to that in the control extracts.

NADH oxidase activity was monitored in cell extracts for the enzyme assays having NADH in the reaction

Table 2. Specific Activities of Enzymes of Carbohydrate Catabolism in Bacillus sphaericus 2362[a]

Enzyme	Specific activity of: B. sphaericus	Control[b]	
Glucokinase	ND[d]	0.021 ± 0.001	(1)
Hexokinase	ND	0.023 ± 0.002	(1)
Phosphoglucose isomerase	ND	2.67 ± 0.040	(1)
Phosphofructokinase	ND	0.079 ± 0.003	(1)
Fructose diphosphate aldolase	ND	0.087 ± 0.008	(1)
Glucose-6-phosphate dehydrogenase	ND	0.181 ± 0.008	(2)
Glucose dehydrogenase	ND	0.060 ± 0.001	(3)
Gluconokinase	0.012 ± 0.00	0.127 ± 0.003	(1)
Phosphogluconate dehydrogenase	ND	0.139 ± 0.002	(1)
Phosphogluconate dehydratase/ KDPG aldolase[c]	ND	0.017 ± 0.00	(4)

[a] Specific activity = umoles of NAD(H) or NADP(H) produced/minute/mg protein.

[b] The number in parenthesis indicates the control bacterium:
(1) Escherichia coli K-12
(2) Bacillus subtilis 168
(3) Bacillus cereus T
(4) Pseudomonas fluorescens ATCC 13525

[c] The substrate for KDPG aldolase was not available; consequently this enzyme was assayed using 6-phosphogluconate as substrate and depending upon the presence of phosphogluconate dehydratase in the cell. Thus, the lack of activity in B. sphaericus could be due to the absence of either enzyme.

[d] ND = not detected = <0.001

mixture. NADH oxidase activities in B. sphaericus, E. coli, and P. fluorescens extracts were about 0.002, 0.003, and 0.001 µmole NADH oxidized/mg protein/hour respectively.

All enzyme activities reported in Table 2 were linear with respect to time and enzyme concentration. Therefore, these low levels of NADH oxidase activity probably did not interfere with the measurement of activity of the enzymes being assayed unless those enzymes were present at exceedingly low levels.

The activity of three dehydrogenase enzymes, glucose-6-phosphate dehydrogenase, glucose dehydrogenase, and 6-phosphogluconate dehydrogenase was monitored in the cell extracts of B. sphaericus. Each enzyme was tested with NADP and NAD as the coenzyme. In each case, no activity was detected in the extracts of B. sphaericus using either coenzyme.

Amylase

Amylase is a hydrolytic enzyme which breaks starch molecules. Amylase production is easily visualized by a region of starch hydrolysis surrounding the bacterial growth on medium containing starch. Extracellular amylase activity was not detected in B. sphaericus (see Fig. 7), yet it was apparent in the control organisms.

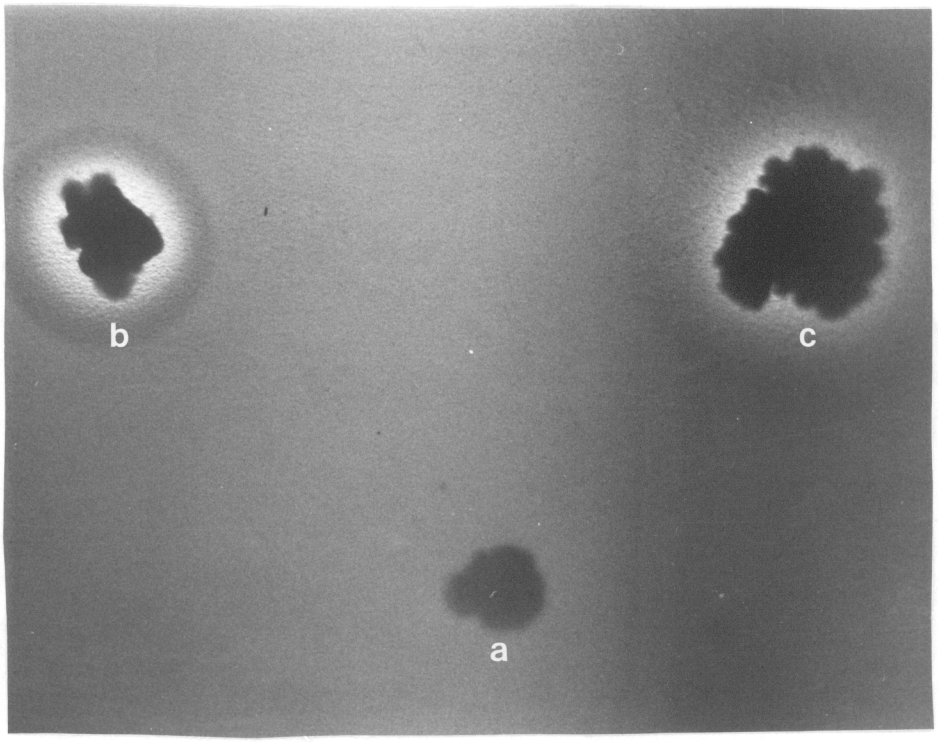


Figure 7. Amylase is seen on this NYSM + starch plate as a zone of starch hydrolysis surrounding the bacterial growth. This plate was incubated for 48 hours at 30° C. The organisms shown are (a) B. sphaericus 2362, (b) B. subtilis 168, (c) B. thuringiensis israelensis.

Beta-Galactosidase

Beta-Galactosidase breaks the disaccharide lactose into its subunits glucose and galactose. Beta-galactosidase activity was not detected in B. sphaericus when grown in the presence of lactose. E. coli K-12 had an average activity of 1.08 µmoles of ONP produced/mg dry cell weight/minute.

Uptake of [^{14}C]Glucose and [^{14}C]Sucrose by Whole Cells

Whole cells of B. sphaericus 2362 were tested for their ability to take up radioactively labeled glucose or sucrose. Uptake was monitored for at least 45 minutes for both substrates. Exponential stage cells were unable to transport either substrate (see Figs. 8 and 9). The starvation of the cells for one hour prior to adding the substrate had no effect on the results. The slight amount of radioactivity detected with the B. sphaericus cells was probably due to substrate adsorbing to the cell surface.

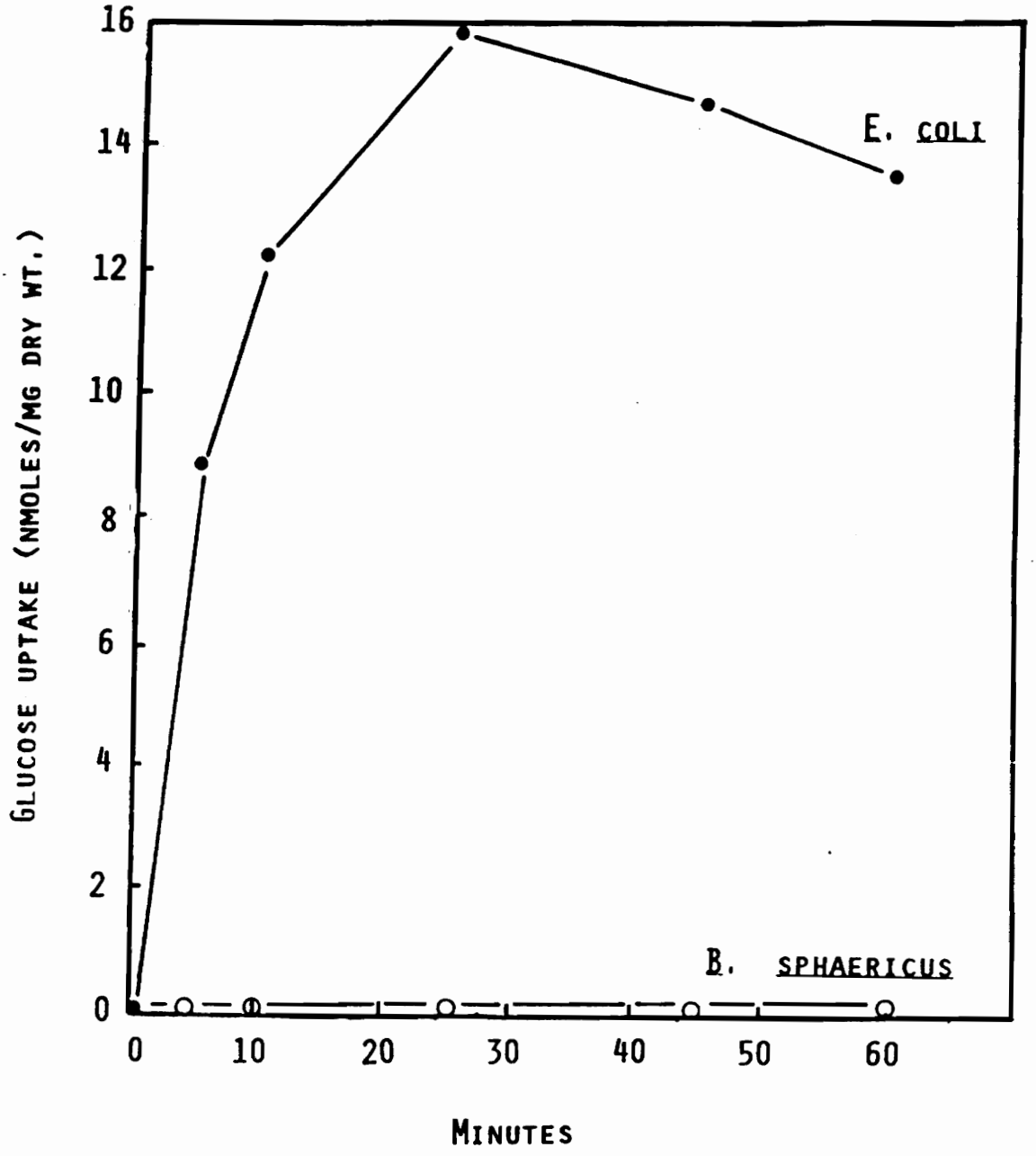


Figure 8. Uptake of [^{14}C]glucose by whole cells. The glucose had a specific activity of 0.71 mCi/mmole. These results are representative of uptake tested on two separate batches of cells. They are similar to the results of uptake by cells starved for one hour in buffer.

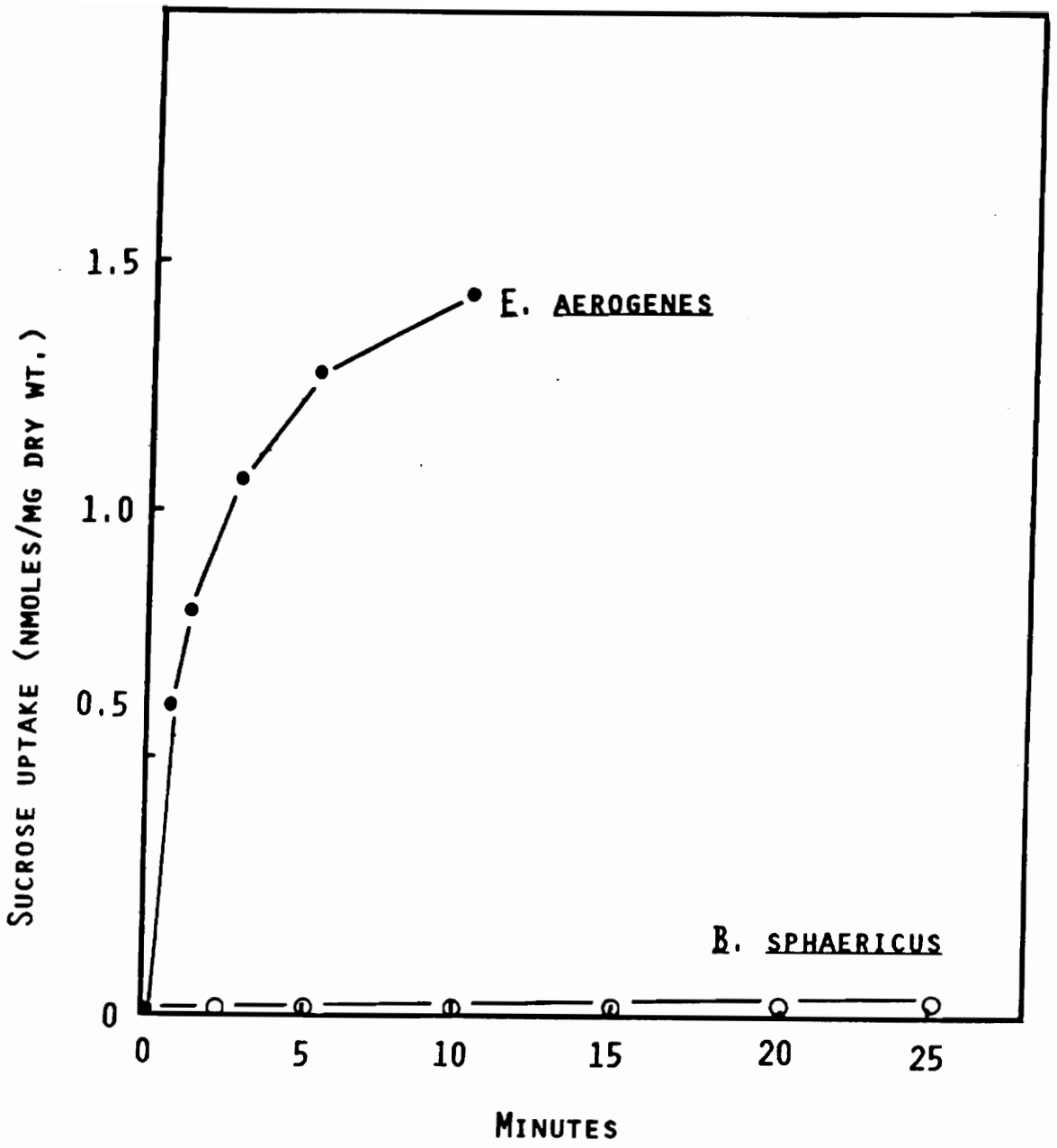


Figure 9. Uptake of [^{14}C]sucrose by whole cells. The sucrose had a specific activity of 1.67 mCi/mmole. These results are representative of uptake tested on two separate batches of cells. They are similar to the results of uptake by cells starved for one hour in buffer.

DISCUSSION

The goal of this research was to determine why B. sphaericus 2362 is unable to grow using carbohydrates as a carbon source. There appear to be several reasons. Activity of the enzymes responsible for the initiation of metabolism of some carbohydrates was undetected in cell extracts of B. sphaericus. These enzymes were glucokinase, hexokinase, beta-galactosidase, and amylase. The lack of beta-galactosidase and amylase is enough to prevent the catabolism of the substrates lactose and starch respectively. This is because carbohydrates must be in monosaccharide form to be further catabolized in a cell. Glucokinase or hexokinase would not be essential for glucose catabolism if the cells posessed a glucose PTS. The PTS would allow for the transport and phosphorylation of glucose. In this organism, glucose-6-phosphate would probably not be catabolized through the EMP pathway due to the low or lack of activity of the enzymes fructose diphosphate aldolase and phosphofructokinase. In addition, the lack of glucose-6-phosphate dehydrogenase activity is one factor preventing the catabolism of glucose through the ED or HMP pathway. The catabolism of other substrates including sucrose and fructose would be prevented for similar reasons. Finally, the experiments on the uptake of labeled glucose and sucrose by whole cells demonstrated the inability of

this organism to accumulate these substrates. Therefore, even if all the enzymes were active, the cells would be cryptic toward glucose and sucrose.

It is not known whether the activity of several of the enzymes of carbohydrate metabolism was not detected because the genes coding for the proteins are absent, because they are present but expressed at very low levels, or because they are not expressed at all. Absence of the genes could be due to a very large deletion from the chromosome if the genes had been linked in a single region. Unfortunately, no work has been done on mapping of the B. sphaericus chromosome due to the lack of information about mechanisms of genetic exchange in this species and to the previous obscurity of the bacteria. It is possible to make some limited and qualified predictions based upon the chromosome map of B. subtilis if it is assumed that members of the same genus would have similar genomes. However, it must be recognized that the species within this genus have been shown to be rather divergent (50) and the chromosome map of B. subtilis may or may not be predictive of B. sphaericus. Unfortunately, only a few of the genes for the enzymes in question have been located on the B. subtilis chromosome. Genes essential for sucrose and glucose transport in B. subtilis are located at 335 and

13 degrees respectively. Those for phosphofructokinase and hexokinase are located at 250 and 51 degrees respectively (39). The dispersed location of these genes and the assumption that they may be similar in B. sphaericus supplies no support for the hypothesis of a possible large deletion as the cause of multiple missing enzymes. Several of the enzymes involved in carbohydrate metabolism have been mapped in E. coli. Among these enzymes are fructose diphosphate aldolase, glucokinase, phosphoglucoisomerase, and phosphofructokinase. These genes are also dispersed throughout the chromosome (2). There is no way to know at this time how much, if any, resemblance there is between the B. sphaericus and E. coli genomes. If these genes are dispersed throughout the chromosome in B. sphaericus it unlikely that each gene would have been independently deleted from the chromosome. It is more likely that a factor involved in the regulation of these genes is dysfunctional.

It is now understandable why this bacterium cannot use carbohydrates as a source of carbon. Some interesting questions remain concerning the metabolism of B. sphaericus.

This research extended the observation by Baumann (that B. sphaericus could use gluconate as a source of electrons to reduce tetrazolium) by demonstrating actual

(though weak) growth on gluconate as the sole source of carbon. Gluconate is catabolized through enzymatic conversion to 6-phosphogluconate by gluconokinase. This enzyme has been detected in cell extracts at a relatively low level. At this time it is unclear how 6-phosphogluconate is catabolized by B. sphaericus. In other microorganisms, 6-phosphogluconate is catabolized through the ED or HMP pathway. However, no activity for phosphogluconate dehydratase/KDPG aldolase and phosphogluconate dehydrogenase was detected in cell extracts of this organism. These enzymes may be present at a level below the sensitivity of the assay and this would account for the weak growth response. The presence of 6-phosphogluconate dehydrogenase is somewhat more likely since no evidence has been presented in the literature showning the ED pathway functioning in the genus Bacillus. The missing enzyme might be produced at a higher (detectable) level by the cells grown in a defined medium with gluconate as the sole source of carbon. At the present time the growth of B. sphaericus in this type of liquid medium is too poor to provide enough cells for enzyme assay. The development of a defined medium that supports better growth of B. sphaericus using gluconate as the carbon source would be useful. This medium would possibly contain the

biosynthetic intermediates that are produced in growth limiting concentrations when the cells are supplied with gluconate as the sole source of carbon. The addition of these compounds to the medium should promote growth provided they can be transported into the bacterial cell. It would also be of interest to determine the rate of gluconate transport into the cells as this may limit the rate of growth.

From the results of the oxygen uptake experiments it appears that enzymes related to gluconate metabolism may be induced by the presence of gluconate in the culture medium. However, growth of B. sphaericus on gluconate would be of little industrial significance since gluconate is not available naturally in large quantities. Gluconate is produced industrially by the fermentative oxidation of the aldehyde group of glucose by some bacteria. In addition, although growth of B. sphaericus is apparent after three days on an agar-solidifed, defined medium, growth in a defined medium containing gluconate is negligible in broth cultures. The inability of the bacterium to grow in the broth culture would prevent its mass production in a fermentor.

Although Baumann (3) reported tetrazolium reduction by B. sphaericus in response to ribose in yeast extract-supplemented medium, B. sphaericus 2362 failed to oxidize

this sugar. This might be related to the differences in strains.

Another question remaining concerns the catabolism of glycerol and acetate. The reversal of the reactions of the upper half of the EMP pathway is necessary for the formation of fructose-6-phosphate and ribose-5-phosphate, important precursors for the synthesis of peptidoglycan and nucleic acids respectively. Fructose-6-phosphate production would be possible if the bacteria posessed fructose diphosphate aldolase and fructose diphosphate phosphatase activity. The phosphatase was not assayed and no activity for the aldolase was detected in cell extracts. Bock and Neidhardt (6) observed an E. coli mutant lacking fructose diphosphate aldolase which was unable to grow on hexoses but which did grow on glycerol as a sole source of carbon. They believed the enzyme was present at a level capable of promoting gluconeogenisis but not hexose catabolism. If so, this may also be the case in B. sphaericus.

SUMMARY AND CONCLUSIONS

1. This research confirmed that Bacillus sphaericus 2362 is typical of the species in that it is unable to grow on carbohydrates as a sole source of carbon. This strain grows slowly on an agar-solidified, defined medium containing glycerol or gluconate as the sole source of carbon. It grows somewhat better on the defined medium with acetate as the sole source of carbon. In a defined liquid medium it grows slowly when supplied with acetate as the sole source of carbon and growth is very slow when supplied with glycerol or gluconate as the sole carbon source.

2. Several enzymatic deficiencies contribute to the inability of B. sphaericus 2362 to utilize carbohydrates as a source of carbon. It is unknown if the enzymes are completely absent from the bacterium of if they are expressed in levels below the sensitivity of the assay. These undetected enzymes include; i) some enzymes responsible for the initiation of metabolism: glucokinase, hexokinase, beta-galactosidase, and amylase, ii) some enzymes of the upper half of the EMP pathway; phosphofructokinase and fructose diphosphate aldolase, and iii) glucose-6-phosphate dehydrogenase. This last enzyme is essential for the catabolism of carbohydrates

via the ED or HMP pathway. It is unlikely that genetic engineering could be employed to relieve these extensive enzymatic deficiencies so that readily available sugars could be used for the production of large quantities of these larvicidal bacteria.

3. Whole cells of B. sphaericus were unable to transport [^{14}C]glucose or [^{14}C]sucrose. This is sufficient to prevent growth of the organism on these substrates even if all the enzymes were active.

4. B. sphaericus 2362 exibited little or no oxidation of glucose, sucrose, lactose, or ribose as measured by a Clark oxygen electrode. Some oxidation of glycerol was observed. Cells grown in NYSM, a complex protein-based medium, exibited no oxidation of gluconate. However, cells grown in the same medium supplemented with gluconate did oxidize gluconate. This suggests that the activity or synthesis of one or more enzymes essential for gluconate catabolism may be induced by the presence of gluconate in the culture medium.

5. Gluconokinase activity was detected in B. sphaericus., but the key enzymes of the ED or HMP pathways were not detected. These results provide no explanation as to how this bacterium uses gluconate as a carbon source. It is possible that when the cells were

grown in a complex medium supplemented with gluconate, the enzyme(s) in question was produced a a level below the sensitivity of the assay. Unsuccessful attempts were made to increase the cell density of B. sphaericus grown in a defined medium containing gluconate as the sole source of carbon. The development of a defined medium with gluconate as the source of carbon may be useful for the elucidation of the pathway of gluconate metabolism in B. sphaericus.

6. It is unclear how this bacterium grows on glycerol or acetate as the sole carbon source since no fructose diphosphate aldolase activity was detected. Fructose diphosphate aldolase is essential for gluconeogenesis which is responsible for the formation of fructose-6-phosphate. This compound is a precursor of peptidoglycan synthesis. It is possible that the aldolase is produced at a level below the sensitivity of this assay.

LITERATURE CITED

1. Aronson, A. I., W. Beckman, P. Dunn. 1986. Bacillus thuringiensis and related insect pathogens. Microbiol. Rev. 50:1-24.

2. Bachmann, B. J. 1983. Linkage map of Escherchia coli K-12, edition 7. Microbiol. Rev. 47:180-230.

3. Baumann, L., K. Okamoto, B. M. Unterman, M. J. Lynch, and P. Baumann. 1984. Phenotypic characterization of Bacillus thuringiensis (Berliner) and B. cereus (Franklaund and Franklaund). J. Invertebr. Pathol. 44:329-341.

4. Baldwin, S. A., R. N. Pernam. 1978. Novel kinetic and structural properties of the class-I D-fructose 1,6-diphosphate aldolase from Escherichia coli (Crookes strain). Biochem. J. 169:643-652.

5. Bhowmik, T., M. C. Johnson, and B. Ray. 1987. Factors influencing synthesis and activity of beta-galactosidase in Lactobacillus acidophilus: J. Indust. Microbiol. 2:1-7.

6. Bock, A. and F. C. Neidhardt. 1966. Isolation of a mutant of Escherichea coli with a temperature-sensitive fructose 1,6-diphosphate aldolase activity. J. Bacteriol. 92:464-469.

7. Bradford, M. M. 1976. A rapid and sensitive method for the quanitation of microgram quantities of protein utilizing the pringiples of protein-dye binding. Anal. Biochem. 72:248-254.

8. Broadwell, A. H., P. Baumann. 1987. Proteolysis in the gut of mosquito larvae results in further activation of the Bacillus sphaericus toxin. Appl. Environ. Microbiol. 53:1333-1337.

9. Bulla, L. A., R. N. Costilow, E. S. Sharpe. 1978. Biology of Bacillus popilliae. Adv. Appl. Microbiol. 23:1-5.

10. Clarke, P. H. and L. N. Ornston. 1975. Metabolic pathways and regulation (I and II). In Clarke and Richmond (Eds.), Genetics and Biochemistry of Pseudomonas, J. Wiley and Sons, London.

11. Cooper, R. A. 1984. Metabolism of methylglyoxyl in microorganisms. Annu. Rev. Microbiol. 38:49-68.

12. deBarjac, H., M. Vernon, V. Cosmao-Dumanoir. 1980. Caracterisation biochemique et serologique de souches de Bacillus sphaericus pathogenes ou non pour les moustiques. Annual Microbiology. (Paris). 131B:191.

13. Delwiche, E. A. 1985. The Veillonellas: gram-negative cocci with a unique physiology. Annu. Rev. Microbiol. 39:175-193.

14. DeMoss, R. D. 1955. Glucose-6-phosphate and 6-phosphogluconate dehydrogenases as from Leuconostoc mesenteroides, pp. 328-334 In S. P. Colowick and N. O. Kaplan (Eds.), "Methods in Enzymology", vol. 1. Academic Press, Inc., New York.

15. Dills, S. S., A. Apperson, M. R. Schmidt, M. H. Saier Jr. 1980. Carbohydrate transport in bacteria. Microbiol. Rev. 34:385-418.

16. Doelle, H. W. 1969. "Bacterial Metabolism". Academic Press, New York.

17. Eisenberg, R. C., W. J. Dobrogosz. 1967. Gluconate metabolism in Escherichia coli. J. Bacteriol. 93:941-949.

18. Faust, R. M. 1974. Bacterial diseases, pp. 87-138. In G. C. Cantell (Ed.), "Insect Diseases". Marcel Dekker, Inc., New York.

19. Fraenkel, D. G., B. L. Horecker. 1965. Fructose-1,6-diphosphatase and acid hexose phosphatase of E. coli. J. Bacteriol. 90:837-842.

20. Fraenkel, D. G., Levinsohn. 1967. Glucose and gluconate metabolism in an Escherichia coli mutant lacking phosphoglucose isomerase. J. Bacteriol. 93:1571-1578.

21. Goebel, E. M., N. R. Krieg. 1984. Fructose catabolism in Azospirillum brasilense and Azospirillum lipoferum. J. Bacteriol. 159:86-92.

22. Goldberg, L. J. and J. Margalit. 1977. A bacterial spore demonstrating rapid larvicidal activity against Anopheles sergentii, Uranotaenia unguiculata, Culex univitattus, Aedes aegyptii and Culex pipiens. Mosq. News. 37:355-358.

23. Goldman, M. and J. J. Blumenthal. 1963. Pathways of glucose catabolism in Bacillus subtilis J. Bacteriol. 86:303.

24. Goldman, M. and H. J. Blumenthal. 1964. Pathways of glucose catabolism in Bacillus cereus. J. Bacteriol. 87:377.

25. Gottschalk, G. 1986. "Bacterial Metabolism", second edition. Springer-Verlag, Inc., New York.

26. Gibson, T., R. E. Gordon. 1974. Endospore-forming rods and cocci. Family I. Bacillaceae. Genus I. Bacillus. In Buchanan, R. E., Gibbons N. E. (Eds.), "Bergey's Manual of Determinative Bacteriology", 8th edition, Williams and Wilkins Co., Baltimore.

27. Hylemon, P. B., P. V. Phibbs, Jr. 1972. Independent regulation of hexose catabolizing enzymes and glucose transport activity in Pseudomonas aeruginosa. Biochem. Biophys. Res. Comm. 48:1041.

28. Ingraham, J. L., O. Maaloe, and F. C. Neidhardt. 1983. "Growth of a Bacterial Cell". Sinauer Associates, Inc., Sunderland, Massachusetts.

29. Jelley, S. 1985. Effects of carbon source on growth, sporulation, and development of mosquito toxicity by Bacillus sphaericus. M.S. Thesis. Virginia Polytechnic Institute and State University, Blacksburg.

30. Kellen, W., T. Clark, J. Lindegren, B. Ho, M. Rogoff, S. Singer. 1965. Bacillus sphaericus Neide as a pathogen of mosquitos. J. Invertebr. Pathol. 7:442.

31. Knight, B. C. J., H. Proom. 1950. A comparative survey of the nutrition and physiology of mesophilic species in the genus Bacillus. J. Gen. Microbiol. 4:508-538.

32. Krieg, N. R. 1984. Aerobic/microaerophilic, motile, helical/vibrioid, Gram-negative bacteria, p. 71 .In J. R. Holt and N. R. Krieg, (Eds.), Bergey's Manual of Systematic Bacteriology. vol. 1. Williams and Wilkins, Baltimore, MD.

33. Krych, V. R., J. L. Johnson, and A. A. Yousten. 1980. Deoxyribonucleic acid homologies among strains of Bacillus sphaericus. Int. J. Syst. Bacteriol. 30:476-484.

34. Laughon, B. E., and N. R. Krieg. 1974. Sugar catabolism in Aquaspirillum gracile. J. Bacteriol. 119:691-697.

35. Lessie, T. G. 1984. Alternative pathways of carbohydrate utilization in Pseudomonads. Annu. Rev. Microbiol. 38:359-387.

36. Ling, J. V., V. Paetkav, F. Marcus, and H. A. Lardy. 1966. Phosphofructokinase. I. Skeletal muscle, pp. 425-429. In W. A. Wood (Ed.), "Methods in Enzymology", vol. 9. Academic Press, Inc., New York.

37. Noltman, E. A. 1964. Isolation of crystalline phosphoglucose isomerase from rabbit muscle. J. Biol. Chem. 239:1545-1550.

38. Pepper, R. E., R. N. Costilow. 1964. Glucose catabolism by B. popilliae and B. lentimorbus. J. Bacteriol. 87:303-310.

39. Piggot, P. F., J. A. Hoch. 1985. Revised genetic linkage map of Bacillus subtilis. Microbiol. Rev. 49:158-179.

40. Pontremoli, S., and E. Grazi. 1966. 6-Phosphogluconate dehydrogenase-crystalline, pp. 137-141. In W. A. Wood (Ed.), "Methods in Enzymology", vol. 9. Academic Press, Inc., New York.

41. Postma, P. W., and J. W. Lengeler. 1985. Phosphoenolpyruvate: carbohydrate phosphotransferase system of bacteria. Microbiol. Rev. 49:232-269.

42. Robinson, J., J. M. Cooper. 1969. Method of determining O_2 concentration in biological media, suitable for calibration of the oxygen electrode. Anal. Biochem. 33:390-399.

43. Romano, A. H., J. D. Trifone, M. Brustolon. 1970. Distribution of the phosphoenolpyruvate: glucose phosphotransferase systems in bacteria. J. Bacteriol. 139:93-97.

44. Rutter, W. J., and J. R. Hunsley. 1966. Fructose diphosphate aldolase. I. Yeast, pp. 480-486. In W. A. Wood (Ed.), "Methods in Enzymology", vol. 9. Academic Press, Inc., New York.

45. Sadoff, J. L. 1966. Glucose dehydrogenase-soluble. I. Bacillus cereus, pp. 103-104. In W. A. Wood (Ed.), "Methods in Enzymology", vol. 9, Academic Press Inc., New York.

46. Saheb, S. A. 1972. Permeation du glycerol de sporulation chez Bacillus subtilis. Can. J. Microbiol. 18:1307-1313.

47. Saier, M. H., Jr. 1977. Bacterial phosphoenolpyruvate: sugar phosphotransferase systems: structural, functional and evolutionary interrelationships. Bacteriol. Rev. 41:856-871.

48. Singer, S., N. S. Goodman, and M. H. Rogoff. 1966. Defined media for the study of bacilli pathogenic to insects. Ann. N. Y. Acad. Sci. 139:16-23.

49. Smibert, R. M. 1978. The genus Campylobacter. Annu. Rev. Microbiol. 32:673-709.

50. Stackenbrandt, E., W. Ludwig, M. Weizenegger, F. Dorn. T. Fox, C. R. Woese, W. Schubert, and K. H. Scleifer. 1987. Comparative 16S rRNA oligonucleotide analysis and murein types of round-spore-forming bacilli. J. Gen. Micro. 133:2523-2529.

51. Warren, S. C. 1968. Sporulation in Bacillus subtilis. Biochemical changes. Biochem. J. 109:811-818.

52. Wettermark, M. H., J. R. Taylor, M. L. Rogers, H. E. Heath. 1979. Metabolism of carbohydrate derivatives by Pseudomonas acidovorans. J. Bacteriol. 138:418-424.

53. White, P. J., H. K. Lotay. 1980. Minimal nutritional requirements of B. sphaericus NCTC 9602 and 26 other strains of this species: the majority grow and sporulate with acetate as a sole major source of carbon. J. Gen. Microbiol. 118:13-19.

54. Wood, W. A. 1971. Assay of enzymes representative of metabolic pathways, pp. 411-424. In J. R. Norris and D. W. Ribbons (Eds.), "Methods in Microbiology", vol. 6A. Academic Press, Inc., New York.

55. Yousten, A. A. 1984. Bacillus sphaericus: Microbiological factors related to its potential as a mosquito larvicide. Adv. Biotech. Proc. 3:315-345.

56. Yousten, A. A., Madhekar, D. A. Wallis. 1984. Fermentation conditions affecting growth, sporulation, and mosquito larval toxin formation by B. sphaericus. Dev. Indust. Microbiol. 25:757-762.

APPENDIX-ENZYME ASSAYS

Preparation and Storage of Reagents

All substrates were prepared in distilled water and stored at -8° C. NAD and NADP were prepared in distilled water and stored at -8° C for no more than one month. NADH and NADPH solutions were prepared in distilled water, wrapped in foil to protect them from light, and stored at -8° C for no more than one month. Commercial enzymes were prepared in the buffer used in the assay. The enzymes were also stored at -8° C.

Assay Procedures

(i) Glucokinase. Method of Hylemon and Phibbs (27).

(a)	Tris-HCl buffer, 0.1 M with 10 mM beta-mercaptoethanol, pH	500 µl
(b)	ATP, 0.01 M	50 µl
(c)	$MgCl_2$	30 µl
(d)	NADP, 5 mM	60 µl
(e)	glucose-6-phosphate dehydrogenase, 0.2 units	10 µl
(f)	glucose, 0.5 M	50 µl
(g)	cell extract and buffer	300 µl
		1 ml

The cells were grown in NYSM + 0.1% glucose. The reaction was initiated by the addition of glucose. The reduction of NADP was monitored as an increase in absorbance at 340 nm.

(ii) Hexokinase. Method of Hylemon and Phibbs (27).

(a)	Tris-Hcl buffer, 0.1 M	506 µl
(b)	ATP, 0.1 M	50 µl
(c)	$MgCl_2$, 0.3 M	30 µl
(d)	NADP, 5 mM	60 µl
(e)	fructose, 0.5 M	50 µl
(f)	glucose-6-phosphate dehydrogenase, 0.2 units	2 µl
(g)	phosphoglucosisomerase, 0.2 units	2 µl
(h)	cell extract and buffer	300 µl
		1 ml

The cells were grown in NYSM + 0.1% fructose. The reaction was initiated by the addition of fructose. The reduction of NADP was monitored as an increase in absorbance at 340 nm.

(iii) Gluconokinase. Method of Hylemon and Phibbs (27) modified by Laughon and Krieg (34).

(a) Tris-HCl buffer, 0.1 M with 10 mM beta-mercaptoethanol, pH 8.0.....568 µl
(b) ATP, 0.1 M.....50 µl
(c) gluconate, 0.5 M.....50 µl
(d) $MgCl_2$, 0.1 M.....30 µl
(e) NADP, 10 mM.....50 µl
(f) 6-phosphogluconate dehydrogenase, 0.1 unit..2 µl
(g) cell extract and buffer.....250 µl
1 ml

Cells were grown in NYSM + 0.1% gluconate. The reaction was initiated by the addition of gluconate. Enzyme activity was monitored by an increase in absorbance at 340 nm.

(iv) Phosphoglucoisomerase. Method of Noltman (37).

(a) Tris-HCl buffer, 0.1 M with 10 mM beta-mercaptoethanol, pH 8.0.....615 µl
(b) fructose-6-phosphate, 0.2 M.....25 µl
(c) NADP, 5 mM.....50 µl
(d) glucose-6-P dehydrogenase, 0.2 units.....10 µl

(e) cell extract and buffer.....300 µl

1ml

The cells were grown in NYSM + 0.1% glucose. Fructose-6-phosphate was added to initiate the reaction. Enzyme activity was monitored by the increase in absorbance at 340 nm.

(v) 6-Phosphofructokinase. Method of Ling et al. (36).

(a) Tris-HCl buffer, 0.1 M with
10 mM beta-mercaptoethanol, pH 8.0.....514 µl

(b) ATP, 0.1 M.....50 µl

(c) $MgCl_2$, 0.3 M.....30 µl

(d) NADH, 2 mM.....50 µl

(e) fructose diphosphate aldolase, 0.2 units....2 µl

(f) triose phosphate isomerase, 0.2 units.....2 µl

(g) alpha-glycerophosphate dehydrogenase,
0.2 units.....2 µl

(h) fructose-6-phosphate, 0.2 M.....50 µl

(i) cell extract and buffer.....300 µl

1ml

The cells were grown in NYSM + 0.1% glucose. The blank was prepared without the NADH and fructose-6-phosphate. NADH was added to the blank and NADH oxidase activity was followed by a decrease in absorbance at 340 nm. The addition of fructose-6-phosphate initiated the reaction. Change in absorbance due to NADH oxidase was subtracted from the substrate induced change. Enzyme activity was calculated from the net decrease in absorbance at 340 nm.

(vi) Fructose diphosphate aldolase. Method of Rutter and Hunsley (44).

(a) buffer-K-SH solution: glycylglycine, pH 7.5, 0.1 M; potassium acetate, 0.2M; beta-mercaptoethanol, 50 mM.....500 µl
(b) NADH, 2 mM.....100 µl
(c) alpha-glycerophosphate/triose phosphate isomerase, 2-20 units.....10 µl
(d) fructose diphosphate, 20 mM.....100 µl
(e) cell extract and buffer.....290 µl

1 ml

Cells were grown in NYSM + 0.1% glucose. The blank was prepared without NADH and fructose diphosphate. NADH was added and NADH oxidase activity was monitored by a decrease in absorbance at 340 nm. Fructose diphosphate was added to initiate the raction. Change in absorbance due to NADH oxidase was subtracted from substrate induced change. Enzyme activity was calculated from the net decrease in absorbance at 340 nm. It was assumed that two moles of NAD were produced by the cleavage of one mole of fructose diphosphate.

(vii) Glucose dehydrogenase. Method of Sadoff (45).

(a) Tris-HCl buffer, 1 M, pH 8.0.....600 µl
(b) glucose, 1 M.....100 µl
(c) NAD, 20 mM.....100 µl
(d) $MnSO_4$, 0.1 mM.....100 µl
(e) cell extract and buffer.....100 µl

1 ml

The cells were grown in NYSM + 0.1% glucose. The reaction was initiated by the addition of glucose. Activity was monitored by an increase in absorbance at 340 nm.

(viii) Glucose-6-phosphate dehydrogenase. Method of DeMoss (14).

(a) Tris-HCl buffer, 0.1 M with
10 mM beta-mercaptoethanol, pH 8.0.....1.5 ml
(b) NADP, 10 mM.....0.2 ml
(c) $MgCl_2$, 0.1 M.....0.1 ml
(d) glucose-6-phosphate, 0.2 M.....0.1 ml
(e) cell extract and buffer.....1 ml
3 ml

Cells were grown in NYSM + 0.1% glucose. The reaction was initiated by the addition of glucose-6-phosphate. The reduction of NADP was followed by an increase in absorbance at 340 nm.

(ix) 6-Phosphogluconate dehydrogenase. Method of Pontremoli and Grazi (40).

(a) glycylglycine, 0.05 M, pH 7.6.....560 µl
(b) NADP, 10 mM.....30 µl
(c) $MgCl_2$, 0.1 M.....200 µl
(d) 6-phosphogluconate, 0.15 M.....10 µl
(e) cell extract and buffer.....200 µl
1 ml

The cells were grown in NYSM + 0.1% gluconate. The reaction was initiated by the addition of 6-phosphogluconate. The reduction of NADP was followed by an increase in absorbance at 340 nm.

(x) 6-Phosphogluconate dehydratase/KDPG aldolase.
Method of Wood (54).

(a) Tris-HCl buffer, 0.1 M, pH 8.0.....808 µl
(b) NADH, 10 mM.....20 µl
(c) lactic dehydrogenase, 1.6 units.....2 µl
(d) $MnCl_2$, 3 mM.....10 µl
(e) 6-phosphogluconate, 0.15 M.....10 µl
(f) cell extract and buffer.....150 µl

1 ml

Cells were grown in NYSM + 0.1% gluconate. The blank was prepared with out NADH and 6-phosphogluconate. NADH was added and NADH oxidase activity was monitored by a decrease in absorbance at 340 nm. 6-Phosphogluconate was added to initiate the raction. Change in absorbance due to NODH oxidase was subtracted form substrate induced change. Enzyme activity was calculated form the net decrease in absorbance at 340 nm.

VITA

Brenda Lurline Russell was born in Bryn Mawr, Pennsylvania on February 26, 1961. She spent much of her childhood in Downingtown, Pennsylvania. She graduated from Downingtown High School in June of 1979. She entered Bloomsburg State College (now Bloomsburg University of Pennsylvania) in the fall of 1979 and graduated with a B.A. in Biology in August of 1983. She was employed as a research assistant at the Pennsylvania Department of Agriculture from May of 1983 to November of 1983. At this time she conducted research on the effects of simulated rain pH on the deposition of Colletotricum coccodes on tomato fruit surface. In November of 1983 she was transferred to the United States Department of Agriculture, Avian Influenza Task Force in Lancaster, Pennsylvania where she worked until December of 1984. While employed by U.S.D.A., Ms. Russell did lab and field work and organized data for epidemiological studies. In January of 1985 she received a U.S.D.A. Outstanding Employee award. Ms. Russell spent the summer of 1985 as a laboratory technician at Walter Reed Army Institute of Research (W.R.A.I.R.), Division of Immunology and Desease Control in Whashington, D. C. While working at W.R.A.I.R. she cloned the majority of the gene library of

Plasmodium falciparum. She began work on her M. S. degree at Virginia Polytechnic Institute and State University (V.P.I. & S.U.) in September of 1985. While at V.P.I. &. S.U. she held both teaching and research assistantships. Her research on carbohydrate metabolism in *Bacillus sphaericus* was sponsored by the World Health Organization.