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Full Length Research Paper

Studies on carbohydrate metabolism in *Bacillus sphaericus* 1593

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***Bacillus sphaericus* 1593 was found to grow poorly on glucose when provided as sole carbon source. However, growth was significantly much higher when acetate was provided as the carbon source, as compared to glucose. The activities of aconitase, isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and isocitrate lyase were detectable which suggests the operation of TCA cycle, hexose monophosphate pathway and glyoxylate by pass. Only one form of isocitrate dehydrogenase and two forms of glucose-6-phosphate dehydrogenase were observed by activity staining. Further studies were substantiated by low respiration rate observed, when variety of intermediates of biochemical pathways were checked. Some metabolites, like 2-oxoglutarate, pyruvate, oxaloacetate and malate showed inhibitory effect on isocitrate lyase activity.**

Key words: *Bacillus sphaericus*, carbohydrate metabolism, glycolytic enzymes.

INTRODUCTION

Bacillus sphaericus has been reported to lack glucose metabolism due to absence of some of the early enzymes of glycolysis, TCA cycle, ED pathway and hexose monophosphate pathway (Russell et al., 1989). Lotay and White (1980) studied the minimal nutritional requirements of 26 strains of *B. sphaericus* and found that none of the strain was able to grow in medium containing glucose, while the strains of *B. sphaericus* could grow with acetate as main carbon source. So the minimal medium that has now been devised for *B. sphaericus* contains acetate as the sole carbon source (Massie et al., 1985). The specific requirement for acetate suggests that *B. sphaericus* might have developed the ability to grow particularly well on acetate, so that the bacteria can rapidly use a nutrient which may become available in soil close to decaying plant materials. So when a medium containing only acetate as the major carbon source was inoculated with pasteurized soil. *B. sphaericus* would grow most rapidly, being an efficient user of acetate. They would out grow competing microorganism which metabolise this substrate slowly.

Chemically defined media containing only acetate or acetate and glutamate as the major carbon source, with or without biotin and thiamine, were inoculated with pasteurized soil and the bacteria that grew most rapidly were indeed *B. sphaericus* (Massie et al., 1985). Russell et al. (1989) showed the inability of *B. sphaericus* 2362 to transport glucose or sucrose into the cell, and the lack of glucokinase and hexokinase activities. In addition, it lacked phosphoglucose isomerase, phosphofructokinase and glucose-6-phosphate dehydrogenase. Critical enzymes of the Entner-Doudoroff pathway were also shown to be absent. Although growth occurs with gluconate as the carbon source, but this material is not a potential substrate for large scale production of the bacteria for use as a mosquito larvicide. It is not known whether the lack of enzyme activity of various metabolic pathways is caused by the absence of appropriate genes or the production of non-functional proteins or due to some regulatory defect (Russell et al., 1989). This inability to utilize carbohydrates is characteristic of all the members of the *B. sphaericus* even though they have been shown by DNA homology studies to be very diverse genetically.

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This metabolic defect seems to be a unifying feature in a species (Carboulce and Priest, 1989; Alexander and Priest, 1990).

MATERIALS AND METHODS

Bacterial strains

The bacterial strain used in the present study, *B. sphaericus* 1593 was obtained from Pasteur Institute of Paris, France.

Media

The media used for the growth of *B. sphaericus* 1593 were (a) TAP (Tryptone Acetate Phosphate; Massie et al., 1985); (b) TGP (Tryptone Glucose Phosphate); (c) Defined A medium (Russell et al., 1989); (d) Defined G medium; (e) Synthetic A medium (modified form of defined A medium); (f) Synthetic G medium; and (g) Luria Bertanii (LB) medium. All these media were sterilized by autoclaving at 10 psi for 20 min. Filter sterilized streptomycin (100 µg/l) was added to all these sterilized media. Filter sterilized biotin and thiamine hydrochloride at final concentration of 0.003 and 0.03 g/l, respectively, were added in Defined A and Defined G media.

Growth conditions

The bacteria were grown in 250 ml Erlenmeyer flasks containing 100 ml of medium and inoculated with 1.0 ml of culture having O.D. of 1.0 at 600 nm. The flasks were incubated on rotary shaker (180 rpm) at 30 ± 2^0 C for 24 h or as stated in the experiments. Control was also kept without inoculating the culture and after inactivating the culture.

Growth of *B. sphaericus* 1593 in tryptone phosphate medium as well as minimal medium containing different carbon sources was checked by measuring O.D. at 600 nm after 24 and 48 h.

Preparation of cell free extract

For enzyme assays, cell free extract were prepared in 0.1 M Tris-HCl buffer (pH 7.2) by sonicating the cells for 30 s 6 times, at an interval of 30 sec at 0-5°C. The cell free extract was then centrifuged at 10 k for 20 min at 0-5°C. The supernatant was used as the source of enzyme.

Biochemical assays

Protein estimation of the cell free extracts was done by the method of Lowry et al. (1951) using bovine serum albumin as standard. Aconitase assay was done as described by Anfinsen (1955). Isocitrate dehydrogenase (NAD) was assayed according to the method described by Ochoa (1955). Isocitate lyase was assayed according to the method of Dixon and Kornberg (1957). Glucose-6-phosphate dehydrogenase was assayed as described by Kornberg and Horecker (1955).

Native polyacrylamide gel electrophoresis

Proteins from cell free extracts were separated on polyacrylamide gels according to the procedure of Davis (1964) using Tris-glycine as electrode buffer. Proteins were loaded on the gel at a concentration of 100 µg/well. The current was adjusted to 3 mA/well. The gels were subjected to the following activity staining:

- Isocitrate dehydrogenase activity on gel was visualized by the tetrazolium method by incubating gel in a solution containing isocitrate, 30 mM; NAD, 3 mM; nitroblue tetrazolium, 0.5 mM; phenazinemethosulphate, 3 mM; Tris-HCl buffer (pH 7.2), 100 mM and $MgCl_2$, 10 mM (Hammond JBW, 1985).
- For glucose-6-phosphate dehydrogenase activity on gel, the reaction system used was as described above, except that isocitrate was replaced with glucose-6-phosphate and NAD was replaced with NADP at the same concentration.

Whole cell respiration

Cells of *B. sphaericus* were grown at 30 ±2°C on a rotary shaker in TAP medium. Mid - exponential phase cells (12 h) were recovered by centrifugation, washed once with 20 mM potassium phosphate buffer (pH 7.0) and resuspended in 10 ml of the same buffer and kept on a rotary shaker (120 rpm) at 30 ± 2°C for 2 h. Different substrates were added to a system containing 0.76 ml of 20 mM phosphate buffer (pH 7.0) and 0.2 ml of cells after the completion of endogenous respiration. The respiratory rates were monitored using an Oxygraph. Different substrates used were glucose, glucose-6-phosphate, fructose-1,6-diphosphate, glycerol, pyruvate, citrate, isocitrate, 2-oxoglutarate, malate and acetate. The unit of respiration rate is defined as the ppm of oxygen taken up per min at 30°C per mg protein.

RESULTS AND DISCUSSION

B. sphaericus 1593 has gained importance because of its mosquitolarvicidal activity. It is active against Culex and Anopheles mosquitolarvae. Advantages of *B. sphaericus* over other larvicidal bacterial strains like *Bacillus thuringiensis* var *israelensis* is that it can grow even in polluted water (Baumann et al., 1991). The nutritional requirement of *B. spherical* are very simple but carbohydrate metabolism is not yet well studied. Russell et al. (1989) studied the carbohydrate metabolism in mosquito pathogenic strain *B. sphaericus* 2362. This bacterium was found to be unable to transport glucose or sucrose into the cell and it lacked glucokinase and hexokinase activities. In addition, it lacked phosphoglucoisomerase, phosphofructokinase and glucose-6-phosphate dehydrogenase, which are enzymes of Embden-Myerhof-Parnas and hexose monophosphate shunt pathways. The enzymes of the Entner-Doudoroff pathway were also shown to be absent (Table 1). Lotay and White (1980) investigated the minimal requirements of 26 strains of *B. sphaericus*. None of the 26 strains of *B. sphaericus* grew when glucose was used as a sole carbon source. Three of these strains grew and sporulated in the minimal medium containing only phosphate buffer (pH 7.2), 15 mM ammonium sulphate, inorganic salts and sodium acetate as a sole source of carbon, and 13 strains grew and sporulated when the above medium was supplemented with biotin and thiamine. Some strains grew in the minimal medium supplemented with biotin, thiamine, glutamic acid, while other strains needed seve-

Table I. Enzymes of carbohydrate catabolism in *Bacillus sphaericus* 2362 (Russell et al 1989).

Enzyme	Specific activity [a] (± SD)	
	B. sphaericus	**Control [b]**
Glucokinase (EC 2.7.1.2)	ND [c]	0.021±0.001(A)
Hexokinase (EC 2.7.1.1)	ND	0.023±0.002(A)
Phosphoglucoisomerase (EC 5.3.1.9)	ND	2.670±0.040(A)
6-Phosphofructokinase (EC 4.1.2.13)	ND	0.079±0.003(A)
Fructose-bisphosphate aldolase (EC 4.1.2.13)	0.030±0.002	0.087±0.008(A)
Glucose 6-phosphate dehydrogenase (EC 1.1.1.49)	ND	0.181±0.010(B)
Glucose dehydrogenase (EC 1.1.1.47)	ND	0.060±0.000(C)
Gluconokinase (EC 2.7.1.12)	0.012±0.0	0.127±0.003(A)
6-Phosphogluconic dehydrogenase (EC 2.7.1.12)	0.011±0.002	0.222±0.002(A)
Phosphogluconate dehydratase/ phospho-2-keto-3-deoxygluconate aldolase (EC 4.1.2.12/4.1.2.14)	ND	0.017±0.000(D)

[a] Micromolecules per milligram of protein
[b] The letter indicates the control bacterium : A - *E. coli*; B - *B. subtilis*, C-*B. cereus* T; D - *Pseudomonas fluorescens* ATCC 13525
[c] ND - Not detected.

Table 2. Effect of different carbon source in tryptone phosphate medium on the growth of *Bacillus sphaericus* 1593.

Carbon Source	Growth (24 hrs) (OD at 600nm)
Control (Tryptone only)	0.739 ± 0.069
Acetate	0.830 ± 0.224
Glucose	0.782 ± 0.224
Glutamate	0.807 ± 0.130
Glycerol	0.799 ± 0.169
Sucrose	0.754 ± 0.043

SD ± 3 determinations.

Table 3. Growth of *Bacillus sphaericus* 1593 in different minimal medium using glucose or acetate as carbon source.

Medium	Growth (48hrs) (OD at 600nm)
Defined A	0.096 ± 0.070
Defined G	0.060 ± 0.043
Synthetic A	0.050 ± 0.049
Synthetic G	0.029 ± 0.028

SD ± 3 determinations
A : Medium containing acetate
G : Medium containing glucose.

several amino acids, some required purine as well as amino acids.

Attempts were therefore made to see whether *B. sphaericus* 1593 obtained from Pasteur Institute of Paris could utilize glucose as carbon source in the complex medium such as tryptone phosphate medium. Initially the growth of *B. sphaericus* 1593 was checked in tryptone acetate phosphate (TAP) medium, but when acetate was replaced by different carbon sources such as glucose, glutamate, glycerol and sucrose having equimolar carbon concentration, considerable growth was detected. It was however observed that there was no significant difference in the growth without carbon source which suggests that tryptone may be playing important role (Table 2). Since, the TP medium contains tryptone in addition to the different carbon source, there is the possibility that the bacteria might be growing because of the presence of tryptone apart from other carbon source. Tryptone phosphate medium without any additional carbon source was kept as a control. So the ability of *B. sphaericus* 1593 to grow on defined as well as on synthetic minimal medium supplemented with glucose/acetate as a carbon source was checked (Table 3). Very poor growth was observed in all these media as compared to TGP or TAP media used earlier (Table 2). In contrast to this, Russell et al (1989) have reported that *B. sphaericus* 1593 is unable to utilize glucose due to absence of uptake system.

To show further the possibility of the presence of TCA cycle, glyoxylate by pass, hexose monophosphate pathway, the activities of isocitrate dehydrogenase, aconitase, isocitrate lyase and glucose-6-phosphate dehydrogenase were carried out from cell free extract of cultures grown in TAP and TGP medium. The activity of aconitase was found to be higher in TAP medium than in TGP medium. Similarly higher activity of isocitrate dehydrogenase was found in TGP medium than in TAP medium. TGP grown cells showed double the activity compared to TAP grown cells (Table 4). Isocitrate dehydrogenase showed a single activity band by polyacrylamide gel electrophoresis (Figure 1a). Activity of isocitrate lyase, an enzyme of glyoxylate by pass pathway was also checked. The activity of isocitrate lyase was found to be maximum with acetate grown culture than with glucose grown culture. To show the possibility of the presence of hexose mono-

Table 4. Various enzyme activities from *Bacillus sphaericus* 1593.

Medium	Aconitase	IDH	Isocitrate lyase	Glucose-6-phosphate dehydrogenase
TGP	6.8	4.21	11.85	4.98
TAP	10.7	2.63	28.76	8.79

Enzyme activity- units/mg protein

Table 5. Rate of respiration of *Bacillus sphaericus* 1593 with various substrate using oxygraph.

Metabolic Pathway	Substrate	Rate of Respiration (Oxygen Uptake) (ppm/min/mg protein)
Glycolysis	Glucose	8.21 ± 2.06
	Glucose-6 – phosphate	9.75 ± 0.12
	Fructose-1,6-diphosphate	22.79 ± 8.76
	Glycerol	12.66 ± 4.46
	Pyruvate	9.44 ± 1.75
TCA cycle	Citrate	13.57 ± 8.12
	Isocitrate	9.87 ± 0.11
	2-Oxoglutarate	15.76 ± 5.63
	Malate	8.66 ± 0.30
Glyoxylate bypass	Acetate	14.86 ± 3.07

SD ± 3 determinations.

Figure 1. Activity staining of a) Isocitrate dehydrogenase (IDH) and b) Glucose-6-phosphate dehydrogenase (G6PD).

phosphate pathway, the activity of glucose-6-phosphate dehydrogenase was carried out from the cell free extract of acetate grown culture. Glucose-6-phosphate dehydrogenase activity was found to be present (Russell et al., 1989). Zahner et al. (1994) reported the absence of this enzyme in *B. sphaericus* strains. Further confirmation was done by activity staining of glucose-6-phosphate dehydrogenase which showed two activity staining bands on polyacrylamide gel electrophoresis (Figure 1b).

In our studies we have shown that *B. sphaericus* 1593 was able to grow on glucose though very poorly and acetate as carbon sources. Attempts were further made to see the rate of respiration with variety of substrates like glucose, glucose-6-phosphate, fructose-1,6-diphosphate, glycerol, pyruvate, citrate, isocitrate, 2-oxoglutrate, malate and acetate. All these substrates were found to be oxidized. Low oxidation rate with glucose, glucose-6-phosphate, fructose-1,6-bisphosphate, glycerol and pyruvate, was obtained which suggests the possibility of an operation of glycolysis to some extent in this organism. Oxidation of citrate, isocitrate, 2-oxoglutarate and acetate suggest the presence of TCA cycle and glyoxylate bypass (Table 5). All these studies suggest that *B. sphaericus* 1593 can take up glucose and metabolise it through glycolysis and TCA cycle. However, metabolism of acetate is more efficient.

Attempts were made to check the effect of the various TCA cycle intermediates and other metabolites on isocitrate lyase activity from *B. sphaericus* 1593. The cell free extract was dialyzed and preincubated with the different metabolites. Isocitrate lyase activity could be detected and was found to be inhibited by some TCA cycle intermediates and other metabolites. Oxalate did not show inhibitory effect on isocitrate lyase from *B.*

Table 6. Effect of various compounds on isocitrate lyase activity from *Bacillus sphaericus* 1593.

Compounds	Concentration (µmoles)	Isocitrate lyase activity (units/mg protein)	% Activity
Control	-	28.78	-
Oxalate	10	31.18	108.34
Malate	10	22.45	78.00
2-oxoglutarate	10	3.20	11.12
Pyruvate	10	7.86	27.31
Oxaloacetate	10	4.89	16.99

sphaericus 1593 as shown in Table 6. Competitive inhibition by oxalate in *Pseudomonas indigofera* was reported by (Rao and McFadden, 1965). Malate was found to inhibit isocitrate lyase activity in *B. sphaericus* 1593. Competitive inhibition of malate on isocitrate lyase activity from *Brevibacterium flavum* was reported by Ozaki and Shiio (1968). Pyruvate also acts as an inhibitor of isocitrate lyase from *B. sphaericus* 1593. Similar observations were also made (Kornberg, 1966) in case of *Escherichia coli* and *Arthrobacter crystallopoetes* (Cioni et al., 1981).

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