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Metabolic response of *Bacillus stearothermophilus* chemostat cultures to a secondary oxygen limitation

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With variously limited chemostat cultures of *Bacillus stearothermophilus*, the glucose consumption rate increased markedly as the concentration of dissolved oxygen (d.o.t.; dissolved oxygen tension) was lowered from 50% to 1% air saturation. Concomitantly, the specific rate of acetate production increased and lactate, which was not present in the fully aerobic cultures, accumulated in large amounts. Moreover, whereas at a high d.o.t. only an ammonia-limited culture excreted 2-oxoglutarate, all glucose-sufficient cultures excreted this metabolite at a d.o.t. of 1% air saturation, even more being produced by a K^+-limited culture than by the ammonia-limited one. The activities of those enzymes of glycolysis that were measured increased in parallel with the glucose consumption rate, as did the activities of enzymes of the Entner–Doudéroff pathway. Similarly, the activities of lactate dehydrogenase and acetate kinase (which were synthesized constitutively also) reflected the corresponding metabolite production rates. Tricarboxylic acid (TCA) cycle activity markedly diminished with a lowering of the available oxygen supply and again (with the exception of aconitase and 2-oxoglutarate dehydrogenase) this was mirrored in decreases in the activities of TCA cycle enzymes. Assessments of energy flux in terms of ATP equivalents suggested that it was energetically more expensive to synthesize biomass at a low d.o.t. than at a high one. However, the presence of enzymes of the methylglyoxal bypass (methylglyoxal synthase and glyoxylase) at high activities in cells grown at a low d.o.t. render assessments of ATP flux rates unreliable.

Introduction

Although some strains of *Bacillus stearothermophilus* are known to be facultatively anaerobic, fermenting glucose to lactate, acetate and ethanol (Gibson & Gordon, 1974), *B. stearothermophilus* var. *non-diastaticus* is reportedly an obligate aerobe (Epstein & Grossowicz, 1969). Thus, whereas aerobically this organism will grow readily and rapidly in a glucose simple salts medium (Epstein & Grossowitz, 1969; Pennock & Tempest, 1988), it will not grow anaerobically either in this medium or in a complex medium, or in the presence of added electron acceptors such as nitrate or fumarate. Nevertheless, during studies of the growth of this organism in glucose-sufficient chemostat culture it was noticed that whenever the concentration of dissolved oxygen (d.o.t., dissolved oxygen tension) was caused to fall below 10% air saturation, a marked stimulation of catabolic rate occurred accompanied by excretion of much acid. Because this was a response more typical of a facultative anaerobe than an obligate aerobe, we decided to study the influence of dissolved oxygen concentration on the physiology of this organism in more detail, using the controlled conditions of chemostat culture. The results obtained are contained herein.

Abbreviations: d.o.t., dissolved oxygen tension (concentration); KDPG, 2-keto-3-deoxy-6-phosphogluconate; TCA cycle, tricarboxylic acid cycle.

Methods

Organism and culture conditions. The organism used in this study was a near-prototrophic strain of *Bacillus stearothermophilus* that, apart from the carbon and energy source, required only biotin as an organic supplement. It was obtained from the Laboratory of Microbiology, University of Amsterdam, and named *B. stearothermophilus* Amsterdam. In all its characteristics it appeared to be identical with the strain *B. stearothermophilus* var. *non-diastaticus* described by Epstein & Grossowicz (1969).

Organisms were routinely grown in chemostats (LH Fermentation 500 series, 1 litre growth vessel with a 700 ml working volume) in defined simple salts media (Evans *et al.*, 1970) at 55 °C and pH 7·0. Glucose was provided as the carbon and energy source and, in general, all non-limiting nutrients were present in at least a 3-fold excess. The basal medium (excluding glucose) was prepared in 20 l batches and sterilized by autoclaving at 121 °C for 30 min. The required amount of glucose was made up as a 50% (w/v) solution (slightly acidified with HCl), autoclaved at 121 °C for 30 min and added aseptically to the bulk medium after cooling. The temperature and pH values of the cultures were controlled automatically and foaming was suppressed by the

addition of a silicone-based antifoaming agent, on demand, as sensed by a foam probe. Dissolved oxygen was monitored by means of a galvanic oxygen electrode (Uniprobe Instruments) and its concentration adjusted and maintained at the desired degree of saturation by varying the stirrer speed.

Procedure. Organisms were grown at a specific rate of 0·2 h^{-1} (i.e. 0·15 μ_{max}) under fixed steady-state conditions (55 °C, pH 7·0) in five different glucose-sufficient media (growth limiting with respect to ammonia, sulphate, phosphate, magnesium and potassium), as well as in a glucose-limiting medium. The d.o.t. was first set, and controlled, at about 50% saturation, then subsequently lowered to 8–10% saturation, and finally to 1–2% saturation. After equilibration at each d.o.t. for 1–2 d, samples of culture (10–20 ml) were withdrawn from the fermenter and analysed for glucose and products. From the results of these assays, the rates of glucose consumption and products formation were determined, and carbon balances constructed.

Analyses. Oxygen consumed and carbon dioxide produced by the cultures were determined by passing the effluent gas through an oxygen analyser (Taylor Servomex type OA 272) and a carbon dioxide analyser (Servomex IR gas analyser PA 404). The rates of respiration were then calculated as specified by Pennock & Tempest (1988), bacterial dry weights being assessed by the procedure of Herbert *et al.* (1971). Production of overflow metabolites was detected, and quantitatively determined, by an HPLC apparatus (LKB) fitted with an Aminex HPX 87H organic acid analysis column (Biorad), a 2142 refractive index detector (LKB) and an SP4270 integrator (Spectra Physics). The column temperature was 55 °C and the eluent was 5 mM-H_2SO_4. Glucose was determined enzymically, following the procedure of Bergmeyer & Bernt (1974) as well as by HPLC. Acetate was determined enzymically also, using a commercial test kit supplied by Boehringer.

Enzyme activities. In the preparation of cell-free extracts, suspensions of organisms from steady state chemostat cultures were centrifuged (5000 ***g*** for 10 min), washed with 50 mM-potassium phosphate buffer (pH 7) and sonified six times for 30 s at 75 W in a B-12 sonifier (Branson) with intermittent 30 s periods of cooling in an ice/water mixture. The extracts were then centrifuged at 20 000 ***g*** for 10 min and the supernatant fraction used for the determination of enzyme activities. Activities were determined according to the following procedures: phosphoglucose isomerase (Noltmann, 1966), phosphofructokinase (Racker, 1946), glucose-6-phosphate dehydrogenase (Bergmeyer *et al.*, 1974), 2-keto-3-deoxy-6-phosphogluconate (KDPG) aldolase (Pennock, 1988), pyruvate kinase (Bücher & Pfleiderer, 1955), acetate kinase (Nishimura & Griffith, 1981), lactate dehydrogenase (Hillier & Jags, 1982), methylglyoxal synthase and glyoxylase I and II (Cooper & Anderson 1970), citrate synthase, aconitase and isocitrate dehydrogenase (Reeves *et al.*, 1971), 2-oxoglutarate dehydrogenase (Amarasingham & Davis, 1965), succinate dehydrogenase (Arrigoni & Singer, 1962), fumarase (Hill & Bradshaw, 1969), fumarate reductase (Jones & Lascelles, 1967) and malate dehydrogenase (Reeves *et al.*, 1971). Protein was determined using the method of Lowry, as modified by Peterson (1977). Except for succinate dehydrogenase and fumarate reductase, all enzyme activities are expressed in units of μmol min^{-1} (mg protein)$^{-1}$.

Results and Discussion

Influence of dissolved oxygen concentration on metabolic rate and metabolite over-production

With a glucose-limited culture growing under standardized conditions (55 °C, pH 7·0, $D = 0{\cdot}2\ h^{-1}$), progress-

Table 1. *Influence of d.o.t. on the rates of metabolite flux in variously limited chemostat cultures of B. stearothermophilus*

Cultures were grown on glucose at 55 °C and pH 7·0 and at a dilution rate of 0·2 h^{-1}. All rates are expressed as mmol h^{-1} (g dry wt cells)$^{-1}$.

		D.o.t. (% saturation)		
Limitation	Parameter	50	8	1
Glucose-	$Q_{Glucose}$	2·9	4·1	14·2
	Q_{Oxygen}	9·1	14·2	10·9
	$Q_{Carbon\ dioxide}$	9·5	14·8	11·4
	$Q_{Acetate}$	0·0	0·0	5·5
	$Q_{Lactate}$	0·0	0·0	18·0
	$Q_{2\text{-}Oxoglutarate}$	0·0	0·0	0·0
	Carbon recovery*	102·5	94·0	99·4
	Q_{ATP}†	39·4	61·5	74·4
Ammonia-	$Q_{Glucose}$	5·3	4·7	25·6
	Q_{Oxygen}	15·8	13·0	11·6
	$Q_{Carbon\ dioxide}$	14·8	14·0	11·1
	$Q_{Acetate}$	0·4	0·8	9·1
	$Q_{Lactate}$	0·1	0·2	31·8
	$Q_{2\text{-}Oxoglutarate}$	0·7	1·0	4·1
	Carbon recovery*	87·2	104·7	100·1
	Q_{ATP}†	69·4	58·2	98·6
Sulphate-	$Q_{Glucose}$	7·7	6·3	11·5
	Q_{Oxygen}	35·3	26·6	6·2
	$Q_{Carbon\ dioxide}$	34·4	26·3	8·6
	$Q_{Acetate}$	1·8	1·5	2·5
	$Q_{Lactate}$	0·0	0·0	13·5
	$Q_{2\text{-}Oxoglutarate}$	0·0	0·0	1·3
	Carbon recovery*	100·3	99·6	99·9
	Q_{ATP}†	155·9	117·7	45·0
Phosphate-	$Q_{Glucose}$	8·3	7·1	27·8
	Q_{Oxygen}	30·6	27·8	10·9
	$Q_{Carbon\ dioxide}$	30·9	26·9	10·9
	$Q_{Acetate}$	5·2	3·6	8·5
	$Q_{Lactate}$	0·0	0·0	36·3
	$Q_{2\text{-}Oxoglutarate}$	0·0	0·0	1·3
	Carbon recovery*	99·7	99·6	90·9
	Q_{ATP}†	141·2	126·4	98·1
Magnesium-	$Q_{Glucose}$	9·2	8·5	27·8
	Q_{Oxygen}	43·3	36·9	18·7
	$Q_{Carbon\ dioxide}$	43·7	35·4	19·2
	$Q_{Acetate}$	0·8	3·9	7·6
	$Q_{Lactate}$	0·0	0·3	43·0
	$Q_{2\text{-}Oxoglutarate}$	0·4	0·2	2·9
	Carbon recovery*	100·8	104·8	111·6
	Q_{ATP}†	189·0	166·7	137·6
Potassium-	$Q_{Glucose}$	8·5	7·4	28·4
	Q_{Oxygen}	41·4	25·6	11·9
	$Q_{Carbon\ dioxide}$	41·3	26·7	17·2
	$Q_{Acetate}$	0·1	5·6	15·4
	$Q_{Lactate}$	0·0	0·0	36·1
	$Q_{2\text{-}Oxoglutarate}$	0·1	0·0	4·5
	Carbon recovery*	98·7	104·1	109·8
	Q_{ATP}†	179·5	120·2	114·9

* Carbon recovery = 100 × product carbon/substrate carbon.

† The rate of ATP formation (Q_{ATP}) is based on the formula: $4{\cdot}33 \times Q_{Oxygen} + 1{\cdot}67 \times Q_{Acetate} + 0{\cdot}34 \times Q_{2\text{-}Oxoglutarate} + Q_{Lactate}$, and assumes the formation of 2 mol ATP per mol-atom of oxygen consumed.

ively lowering the d.o.t. from 50% saturation first to 8–10%, and then to 1–2% saturation caused the specific rate of glucose consumption to increase progressively (Table 1). The respiration rate also increased as the d.o.t. was lowered to 8% saturation, but then declined as growth became overtly oxygen-limited. Under these latter conditions some residual glucose was present in the extracellular fluids and there was a substantial excretion of lactate and acetate. These findings are similar to those reported by Harrison (1976) for both *Klebsiella pneumoniae* and *Escherichia coli* cultures when grown with a restricted oxygen supply.

With cultures of *B. stearothermophilus* that were limited in their growth rate by the availability of an anabolic substrate (i.e. were either N-, S-, P-, Mg^{2+}- or K^+-limited), with glucose present in excess of the growth requirement, a common, though slightly different, pattern of response to d.o.t. was manifest (Table 1). In contrast to the glucose-limited culture, the glucose consumption rate initially decreased slightly as the d.o.t. was lowered from 50% to 8% saturation, but then rose steeply as the d.o.t. was further decreased to 1% saturation. At the same time, the specific rate of oxygen consumption and of CO_2 production declined progressively, and that of acetate production generally, though not invariably, rose; however, it was invariably maximal at 1% oxygen saturation. The specific rate of lactate production was negligible at high levels of aeration and also, in general, at 8% oxygen saturation, but was invariably substantial at a d.o.t. of 1% saturation, accounting for up to 70% of the glucose carbon metabolized.

A further point of note with all glucose-sufficient cultures (Table 1) was an excretion of 2-oxoglutarate into the medium when organisms were growing at low oxygen tensions. This metabolite is associated generally with growth under N(ammonia)-limiting conditions (Neijssel & Tempest, 1979), but as evident in Table 1, when growing under conditions of secondary oxygen limitation, the rate of production of 2-oxoglutarate was even higher in the K^+-limited culture than in the one that was ammonia-limited. Excretion of this metabolite implies a restriction in the operation of the TCA cycle at, or beyond, the 2-oxoglutarate dehydrogenase reaction when growing at a low d.o.t.

Finally, it should be mentioned that with both glucose-limited and glucose-sufficient chemostat cultures of *B. stearothermophilus*, virtually all the glucose carbon consumed could be accounted for by cells, CO_2, acetate, lactate and 2-oxoglutarate, and that other intermediary metabolites such as pyruvate, succinate, fumarate and ethanol were present in the extracellular fluids in never more than trace amounts, as assessed by HPLC analysis.

Energetic considerations

Assessments of the influence of d.o.t. on the energy flux in variously limited chemostat cultures of *B. stearothermophilus* gave differing results (Table 1). With the glucose-limited culture the estimated rate of ATP generation virtually doubled from about 39 to 74 mmol ATP equivalent h^{-1} (g dry wt cells)$^{-1}$ as the d.o.t. was lowered from 50% to 1% saturation. In contrast, those of cultures that were either S-, P-, K^+ or Mg^{2+}-limited declined progressively. These assessments, however, assume no lowering in the efficiency of respiratory energy conservation under oxygen-limiting conditions, which could occur if alternative terminal oxidases with a higher affinity for oxygen were then present and functioning (see Jones *et al.*, 1977).

A further imponderable arises in assessing energy fluxes when lactate is a major overflow product. If this lactate is derived from the reduction of pyruvate, as redox considerations might suggest, then there would be a substantial increase in the rate of ATP generation via substrate-level phosphorylation reactions to augment the lowered rate of respiratory ADP phosphorylation in oxygen-limited environments. This was assumed to be the case in deriving the values of ATP flux in Table 1. However, lactate can be formed from dihydroxyacetone phosphate via the methylglyoxal bypass (Cooper, 1984; Teixeira de Mattos *et al.*, 1984) and this reaction sequence circumvents both sites of substrate-level phosphorylation in glycolysis (i.e. phosphoglycerokinase and pyruvate kinase). It is known that *B. stearothermophilus* can synthesize enzymes of the methylglyoxal bypass under conditions where there is an overproduction of triose phosphates (Burke & Tempest, 1990), and if this bypass was functional it would cause a marked decrease in the ATP flux rate. Measurements of lactate dehydrogenase, methylglyoxal synthase and glyoxylase in cell-free extracts (Table 2) showed substantial activities of all three in cells that were oxygen-limited, activities that generally were higher than the rate of lactate formation *in situ*. Hence, on the basis of these data alone, it is not possible to assess the contribution of the methylglyoxal bypass to the overall rate of lactate synthesis, and thus the contribution of substrate-level phosphorylation reactions to the overall rate of ATP generation. In this connection, it was found that most, if not all, of the lactate formed was the D-isomer (as would be produced from methylglyoxal by glyoxylase): however, cells cultured micro-aerophilically possessed an active pyruvate reductase that also generated D-lactate.

Enzymes of glycolysis

In order to determine whether the observed changes in glucose flux rate, following a lowering of the d.o.t.,

Table 2. *Influence of d.o.t. on the rate of lactate production, from glucose, and on the activities of those enzymes in B. stearothermophilus that might be implicated in its synthesis*

Cultures were grown on glucose at 55 °C and pH 7·0 and at a dilution rate of 0·2 h^{-1}. The rate of lactate production ($Q_{Lactate}$) and enzyme specific activities are expressed as μmol min^{-1} (mg protein)$^{-1}$.

Limitation	D.o.t. (% sat.)	$Q_{Lactate}$	Enzyme specific activity*		
			Ldh	MG synthase	Glyoxylase I and II
Glucose-	50	0·00	0·15	0·81	0·78
	1	0·60	1·79	2·22	1·14
Ammonia-	50	0·00	0·29	0·39	0·76
	1	1·06	1·33	0·75	1·25
Sulphate-	50	0·00	0·16	0·78	0·86
	1	0·45	2·41	1·04	1·21
Phosphate-	50	0·00	0·26	1·07	0·71
	1	1·22	1·42	1·15	1·24
Magnesium-	50	0·00	0·20	0·74	0·61
	1	1·43	2·50	1·18	1·06
Potassium-	50	0·00	0·38	0·84	0·86
	1	1·20	3·90	0·82	1·34

* Ldh, lactate dehydrogenase; MG synthase, methylglyoxal synthase.

Table 3. *Influence of d.o.t. on the rate of glucose metabolism, and on the activities of selected enzymes of glycolysis in B. stearothermophilus*

Cultures were grown on glucose at 55 °C and pH 7·0 and at a dilution rate of 0·2 h^{-1}. The rate of glucose consumption ($Q_{Glucose}$) and enzyme specific activities are expressed as μmol min^{-1} (mg protein)$^{-1}$.

Limitation	d.o.t. (% sat.)	$Q_{Glucose}$	Enzyme specific activity*		
			PGI	PFK	PK
Glucose-	50	0·10	0·95	0·14	0·43
	1	0·49	2·73	1·29	1·00
Ammonia-	50	0·18	1·74	0·33	0·47
	1	0·74	3·90	1·52	0·56
Sulphate-	50	0·38	1·15	0·39	0·20
	1	0·72	1·76	0·71	0·46
Phosphate-	50	0·43	0·49	0·25	0·34
	1	0·93	1·54	0·52	0·58
Magnesium-	50	0·31	0·87	0·18	0·28
	1	0·92	2·10	1·60	1·15
Potassium-	50	0·28	1·10	0·26	0·42
	1	0·95	1·93	0·65	1·10

*Abbreviations: PGI, phosphoglucose isomerase; PFK, phosphofructokinase; PK, pyruvate kinase.

implicated changes in cellular enzyme content or activity, or both, measurements were made of the activities of selected enzymes of glycolysis in cell-free extracts (Table 3). In all cases there was a substantial increase in specific activity following a decrease in the d.o.t., though the increase was not uniformly proportional to the corresponding increase in glucose flux rate. In this connection, the nature of the primary glucose phosphorylating reaction remains to be elucidated unequivocally. Despite reports that glucose uptake by *B. stearothermophilus* involves a PEP-dependent glucose phosphotransferase system (Harris & Kornberg, 1972), no such activity could be detected in cells of the strain used in this study that had been decryptified and assayed following the procedure of Kornberg & Reeves (1972). Moreover, although the glucokinase activity of glucose-limited cells was more than sufficient to account for the rate of glucose metabolism *in situ*, it was insufficient to account for the rates of glucose consumption expressed by either K^+- or Mg^{2+}-limited cells (Pennock, 1988).

Besides the enzymes of the Embden–Meyerhof–Parnas pathway being present in substantial amounts, so too were enzymes of the Entner–Doudoroff pathway (Table 4). Again, activities were substantial and generally increased when cultures were exposed to a secondary oxygen limitation. However, there was little correlation between the activities of 6-phosphogluconate dehydratase and KDPG aldolase (measured simultaneously) and the rate of glucose consumption. It should be pointed out that the above result contrasts sharply with earlier study

Table 4. *Influence of d.o.t. on the activities of selected enzymes of the Entner–Douderoff pathway and on acetate kinase activity in B. stearothermophilus*

Cultures were grown on glucose at 55 °C and pH 7·0 and at a dilution rate of 0·2 h^{-1}. The rate of acetate production ($Q_{Acetate}$) and enzyme specific activities are expressed as μmol min^{-1} (mg protein)$^{-1}$.

Limitation	D.o.t. (% sat.)	Enzyme specific activity*			$Q_{Acetate}$
		G6P-dh	6-PGdh +KDPGA	Acetate kinase	
Glucose-	50	0·07	0·17	0·06	0·00
	1	0·08	0·41	0·34	0·18
Ammonia-	50	0·05	0·13	0·40	0·01
	1	0·21	0·12	1·62	0·62
Sulphate-	50	0·07	0·11	0·61	0·06
	1	0·14	0·15	1·20	0·12
Phosphate-	50	0·07	0·17	0·37	0·17
	1	0·20	0·48	1·43	0·28
Magnesium-	50	0·08	0·32	0·43	0·03
	1	0·13	0·82	1·52	0·25
Potassium-	50	0·07	0·18	0·70	0·33
	1	0·28	0·73	3·80	1·20

*Abbreviations: G6Pdh, glucose-6-phosphate dehydrogenase; 6-PGdh, 6-phosphogluconate dehydratase; KDPGA, 2-keto-3-deoxy-6-phosphogluconate aldolase.

of this organism (Pennock, 1988) where, using the same assay procedure, no activity was manifest.

All glucose-sufficient cultures excreted acetate, and all cultures exposed to a secondary oxygen limitation excreted lactate. Measurements of acetate kinase and lactate dehydrogenase (Tables 2 and 4) revealed that the activities of both enzymes increased with increases in the rates of product excretion.

Enzymes of the TCA cycle

Excretion of acetate implies that the activity of the TCA cycle was insufficient to oxidize acetyl-CoA at a rate equal to that at which it was being formed. A decrease in TCA cycle activity is, of course, inevitable when the oxygen supply becomes limiting because cells then are no longer able to rapidly oxidize the NAD(P)H arising from the isocitrate- and 2-oxoglutarate dehydrogenase reactions, and thus these reactions are hindered kinetically. It does not necessarily follow, however, that there should be a corresponding change in the cellular content of TCA cycle enzymes, even though this is known to occur when cultures of facultatively anaerobic organisms like *E. coli* are grown anaerobically (Spiro & Guest, 1990).

All the enzymes of the TCA cycle were assayed in cells from variously limited cultures of *B. stearothermophilus* that had been grown first with an excess of oxygen and then with oxygen 'shadowing' (i.e. at an oxygen concentration of 1% saturation). With notable exceptions, the cellular content of TCA cycle enzymes decreased substantially following growth in oxygen-depleted environments (Table 5), averaging a 40–50% reduction. The exceptions were aconitase and, most unexpectedly, 2-oxoglutarate dehydrogenase. The measured activity of the latter enzyme was low, and insufficient to match the assessed overall rate of TCA cycle activity. Nevertheless, with all the glucose-sufficient cultures there was a marked increase in the activity of this enzyme following a lowering of the d.o.t.

With facultatively anaerobic organisms, switching from an aerobic to an anaerobic environment activates the global regulator gene *fnr* (Spiro & Guest, 1990). The product of this gene regulates the synthesis of enzymes such as fumarate reductase and nitrate reductase that mediate the transfer of electrons from respiratory chain carriers to fumarate and nitrate, respectively, thereby allowing the respiratory chain to function anaerobically with either fumarate or nitrate as terminal electron acceptor. As mentioned previously, *B. stearothermophilus* var. *non-diastaticus* does not grow anaerobically either in the presence or absence of fumarate or nitrate. Nevertheless, it clearly does exhibit elements of the so-called FNR response in that, at low oxygen tensions, synthesis of succinate dehydrogenase was markedly diminished whilst fumarate reductase activity (which was absent from cells grown completely aerobically) was manifest (Table 6).

It is clear that the physiological response of *B.*

Table 5. *Influence of d.o.t. on the overall rate of TCA cycle activity, and on the activities of some of its component enzymes, in B. stearothermophilus*

Cultures were grown on glucose at 55 °C and pH 7·0 and at a dilution rate of 0·2 h^{-1}. The rate of TCA cycle activity (Q_{TCA}) and enzyme specific activities are expressed as μmol min^{-1} (mg protein)$^{-1}$.

Limitation	D.o.t. (% sat.)	Q_{TCA}†	Enzyme specific activity* Acon	ICdh	2-OGdh	Fum	Mdh
Glucose-	50	0·11	0·37	1·78	0·06	2·60	2·28
	1	0·07	0·45	0·85	0·02	1·87	1·12
Ammonia-	50	0·16	0·17	0·69	0·05	1·80	1·36
	1	0·02	0·85	0·44	0·09	0·47	0·64
Sulphate-	50	0·36	0·50	0·95	0·02	0·82	0·96
	1	0·07	0·87	0·89	0·04	0·45	0·82
Phosphate-	50	0·29	0·22	1·36	0·02	1·97	1·53
	1	0·03	0·75	0·71	0·08	0·81	0·85
Magnesium-	50	0·48	0·26	1·59	0·02	0·97	1·15
	1	0·13	0·30	0·72	0·03	0·86	1·04
Potassium-	50	0·46	0·21	2·03	0·03	1·49	2·13
	1	0·02	0·34	0·60	0·09	1·02	0·71

*Abbreviations: Acon, aconitase; ICdh, isocitrate dehydrogenase; 2-OGdh, 2-oxoglutarate dehydrogenase; Fum, fumarase; Mdh, malate dehydrogenase.

† Calculated rate of oxidation of C_2 units via the TCA cycle.

Table 6. *Influence of d.o.t. on the activities of succinate dehydrogenase and fumarate reductase in B. stearothermophilus*

Cultures were grown on glucose at 55 °C and pH 7·0 and at a dilution rate of 0·2 h^{-1}. Enzyme specific activities are expressed as μmol h^{-1} (mg protein)$^{-1}$. ND, Not determined; NA, no detectable activity.

Limitation	D.o.t. (% sat.)	Enzyme specific activity: Succinate Dehydrogenase	Fumarate Reductase
Glucose-	50	11·40	NA
	1	2.70	0·36
Ammonia-	50	ND	NA
	1	3·48	0·13
Sulphate-	50	7·20	NA
	1	3·90	0·50
Phosphate-	50	9·00	NA
	1	3·30	0·28
Magnesium-	50	19·20	NA
	1	2·10	0·77
Potassium-	50	8·40	NA
	1	1·80	1·74

stearothermophilus var. *non-diastaticus* to a lowering of the culture d.o.t. is precisely that of a bona fide facultative anaerobe. So why does it not grow in the total absence of molecular oxygen? The problem is unlikely to be one of energy generation, and the fact that this strain will not grow anaerobically in a complex medium militates against it being a problem of synthesis of some key anabolite such as nicotinic acid. In an attempt to resolve this paradox, we are now studying the response of this organism to d.o.t. when growing on alternative (both fermentative and non-fermentative) substrates.

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