The Control of the Synthesis of Isocitrate Lyase in a Thermophilic *Bacillus*

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From a strain of *Bacillus stearothermophilus*, devoid of active pyruvate carboxylase, a mutant (NG-15) was selected that grew on acetate in the presence of glucose. This mutant differed from its parent organism in possessing high activities of isocitrate lyase when grown on all carbon sources tested except nutrient broth, in possessing unusually low activities of NADP⁺-dependent isocitrate dehydrogenase and in containing increased amounts of isocitrate. Revertants of mutant NG-15 which regained the ability to synthesize active pyruvate carboxylase also synthesized isocitrate lyase and isocitrate dehydrogenase to the same extent as the wild-type strain. These results suggest that the regulatory mechanism for the synthesis of isocitrate lyase in the thermophile may be different from that in mesophilic bacilli.

INTRODUCTION

Isocitrate lyase is a key enzyme of the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957). The synthesis of this enzyme in *Escherichia coli* appears to be under the control of a metabolite repressor, thought to be phosphoenolpyruvate or pyruvate (Kornberg, 1965, 1966), but this may not be so in all organisms (Herman & Bell, 1970; Higgins & Sariaslani, 1973). Like many other micro-organisms, a thermophilic strain of *Bacillus* isolated by Epstein & Grossowicz (1969) is induced to synthesize isocitrate lyase during growth on acetate (Sundaram & Kornberg, 1969; Griffiths & Sundaram, 1973). The growth on acetate of mutants of this thermophile that lack pyruvate carboxylase is inhibited by glucose. Further mutants can be isolated which, though still devoid of pyruvate carboxylase, are resistant to the growth inhibition brought about by glucose. Many such mutants are constitutive for isocitrate lyase (Sundaram, 1973). However, one such mutant, designated NG-15, has been found which may not be truly constitutive for the enzyme. The properties of this mutant are described here and the results are discussed in relation to a possible regulatory system for isocitrate lyase synthesis in this thermophilic strain of *Bacillus*.

METHODS

Isolation of mutant. A mutant designated PC-2, devoid of active pyruvate carboxylase, was isolated from wild-type cells of a thermophilic strain of *Bacillus* (Sundaram, 1973). This organism was used as a source of further mutants which were not inhibited by glucose when they were grown on acetate as carbon source. Cultures of strain PC-2 were grown in nutrient broth and were inoculated into flasks containing salts (Sundaram *et al.*, 1969), supplemented with biotin (0.1 µg ml⁻¹) and sodium acetate (50 mM). The cultures were harvested in the mid-exponential phase of growth by centrifuging at 10000 *g* for 10 min at 18 °C. The cells were resuspended to about 0.1 mg dry mass ml⁻¹ in the salts solution and samples (0.05 to 0.2 ml) of the suspensions were spread on to plates containing a mixture of sodium acetate (50 mM), glucose (10 mM), biotin (0.1 µg ml⁻¹) and nutrient broth (160 µg ml⁻¹), solidified with 1.5 % (w/v) agar. A crystal of 5'-methyl-5'-nitrosoguanidine was placed in the middle of each plate and the plates were incubated at 55 °C for

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36 h. A clone, designated NG-15, was obtained which was able to grow on the acetate/glucose plates. This was purified by repeated restreaking on acetate/glucose plates.

Growth of the organism and preparation of cell-free extracts. The organism was grown and extracts were prepared by lysozyme treatment as described previously (Griffiths & Sundaram, 1973).

Measurement of enzyme activities. Isocitrate lyase (EC 4.1.3.1) was assayed by the method of Kornberg (1965) as described by Griffiths & Sundaram (1973). An alternative method was adopted for the assay of isocitrate lyase in inhibition studies with pyruvate and phosphoenolpyruvate, both of which react with phenylhydrazine to form hydrazones. In this case, the assay method of John & Syrett (1968) was employed.

Pyruvate carboxylase (EC 6.4.1.1) was assayed at 45 °C by measuring the ^{14}C fixed from $\text{KH}^{14}\text{CO}_3$ or $\text{Na}_2^{14}\text{CO}_3$ with pyruvate as substrate (Sundaram *et al.*, 1969).

The activity of NADP⁺-dependent isocitrate dehydrogenase (EC 1.1.1.42) was determined by the method of Cleland *et al.* (1969).

The method of Dixon & Kornberg (1959) was used to assay the activity of malate synthase (EC 4.1.3.2).

Aconitase (aconitate hydratase; EC 4.2.1.3) activity was assayed by the method of Racker (1950).

Except where stated, all the assays were carried out at 30 °C. Protein concentrations were determined by the method of Lowry *et al.* (1951).

Preparation of cultures for the measurement of metabolite levels. Cultures were grown for six or more generations and samples for analysis were taken when the cultures had reached a density of approximately 0.1 to 0.2 mg dry mass ml⁻¹. Dry mass was measured as absorbance at 680 nm, an A_{680} of 1.0 corresponding to 0.33 mg dry mass ml⁻¹ (Harris & Kornberg, 1972). The cells were harvested by rapid filtration of 30 to 50 ml culture on to a Millipore filter (47 mm diam., 0.45 µm pore size) with suction. The filters were then treated with HClO_4 as described by Lowry *et al.* (1971).

Measurement of isocitrate concentration in extracts. Isocitrate present in extracts obtained by the above procedure was determined by mixing portions of them with 100 µmol imidazole/acetate buffer (pH 7.0), 0.1 µmol MnCl_2 , 0.1 µmol NADP, 2.5 µg isocitrate dehydrogenase (from pig heart, Sigma) and water to 1 ml. The initial A_{340} was measured spectrophotometrically and again 5 min after addition of the enzyme. The concentration of isocitrate was calculated from the amount of NADPH formed, a ΔA_{340} of 0.622 being taken as equivalent to 0.1 µmol isocitrate.

Measurement of glyoxylate concentration in extracts. The method of Klotzsch & Bergmeyer (1965) was used. The glyoxylate present in bacterial extracts was determined by mixing portions of them with 33 µmol phosphate buffer (pH 6.4), 0.24 µmol NADH, 20 µg glyoxylate reductase (EC 1.1.1.26; Boehringer) and water to 1 ml. The decrease in A_{340} was measured spectrophotometrically after addition of the enzyme. The concentration of glyoxylate was calculated from the amount of NAD formed, ΔA_{340} of 0.622 being taken as equivalent to 0.1 µmol glyoxylate.

Chemicals. Most biochemicals were from Sigma; coenzyme A and NADH were from Boehringer; Folin-Ciocalteu reagent and lysozyme were from BDH; 5'-methyl-5'-nitrosoguanidine was from Koch-Light; radiochemicals were from The Radiochemical Centre, Amersham. Acetyl-coenzyme A was prepared from coenzyme A and acetic anhydride (Cazzulo *et al.*, 1970).

RESULTS

Growth of thermophile mutants on various carbon sources

Mutant NG-15 was selected for its ability to grow on acetate in the presence of glucose. The parent organism, PC-2, was unable to grow on this medium as it lacked pyruvate carboxylase activity (Sundaram, 1973). Addition of glucose to wild-type cultures or cultures of strain NG-15 growing at 55 °C on acetate as sole carbon source resulted in an immediate acceleration in their growth rates. The mean generation time for the wild-type decreased from 120 min for growth on acetate to 73 min for growth on acetate plus glucose. Similarly, for strain NG-15 the mean generation time decreased from 124 min for growth on acetate to 98 min for growth on acetate in the presence of glucose. In contrast, addition of glucose to a culture of strain PC-2 growing on acetate caused growth to stop after 1 h. This was probably due to inhibition of isocitrate lyase activity by the C_3 compounds formed from glucose (Kornberg & Smith, 1969) and, in agreement with this view, could be reversed by the further addition of 10 mM-glyoxylate. Growth of strain PC-2 on acetate was also inhibited by adding pyruvate or lactate, but this was not so for strain NG-15.

Because of its lack of pyruvate carboxylase activity, mutant PC-2 was unable to grow on glucose as sole carbon source. Its mutant NG-15 was also devoid of pyruvate carboxylase

Table 1. *Enzyme activities in extracts of thermophiles grown on acetate as the sole carbon source*

Extracts were made from cells grown to late-exponential phase and assayed at 30 °C as described in Methods. Specific activities are expressed as $\mu\text{mol product min}^{-1} (\text{mg protein})^{-1}$.

Enzyme	Specific activity in extracts of:			
	Wild-type	Strain PC-2	Strain NG-15	Strain R-9
Aconitase	0.59	0.53	0.82	—
Isocitrate lyase	0.20	0.21	0.22	0.09
Malate synthase	0.68	0.88	0.74	—
NADP ⁺ -dependent isocitrate dehydrogenase	0.078	0.082	0.003	0.082
Pyruvate carboxylase	0.109	0.001	0.001	0.051

—, Not tested.

Fig. 1. Effect of glucose addition to acetate-grown cells on the rate of isocitrate lyase synthesis in strains of the thermophile. Samples were removed at various times throughout the growth cycle from cultures of wild-type (a) and strain NG-15 (b) growing on acetate (●, ■) or on acetate in the presence of glucose (○, □). Organisms were harvested and cell-free extracts were prepared and assayed for isocitrate lyase activity as described in Methods. Total isocitrate lyase activity is expressed as the product of the specific activity of the enzyme in the extract [$\mu\text{mol glyoxylate produced min}^{-1} (\text{mg protein})^{-1}$] and cell density [$\text{mg dry mass (ml culture)}^{-1}$].

(Table 1), but was able to grow on glucose, albeit slowly, after preincubation on acetate medium. Presumably the glyoxylate cycle could function as the anaplerotic route under these conditions in mutant NG-15, although it could not do so in the parent organism.

Both PC-2 and NG-15 mutants grew on intermediates of the tricarboxylic acid cycle, such as succinate and fumarate, at rates similar to those of the wild-type organism on the same substrates.

Synthesis of isocitrate lyase during growth on various substrates

Unlike the wild-type organism, strain NG-15 continued to synthesize isocitrate lyase when growing on acetate in the presence of glucose (Fig. 1). By plotting the total activities of isocitrate lyase (defined as the product of the cell density of the culture and the specific activity of the enzyme in extracts of cells harvested at those densities) in extracts of the wild-type organism and mutant NG-15 against the cell densities at which the samples were taken, the rates of induced synthesis of the enzyme in these organisms could be calculated (Monod, 1956).

The activities of isocitrate lyase in the wild-type and mutant strains of the thermophile

Table 2. *Isocitrate lyase activity in extracts of thermophiles grown on various carbon sources*

Cells were grown to late-exponential phase on the appropriate carbon source and harvested by centrifugation. The isocitrate lyase activity present in the extracts was determined at 30 °C as described in Methods. Values are $\mu\text{mol glyoxylate formed min}^{-1} (\text{mg protein})^{-1}$.

Carbon source	Isocitrate lyase activity in extracts of:				
	Wild-type	Strain PC-2	Strain NG-15	Strain R-9	Strain NG-35
Acetate	0.196	0.213	0.217	0.092	0.531
Acetate + glucose	0.001	NG	0.205	0.013	—
Glucose	0.002	NG	—	—	0.161
Citrate	0.166	NG	—	—	—
Succinate	0.057	0.067	0.115	—	0.196
Fumarate	0.007	0.037	0.372	0.002	—
Malate	0.001	—	0.196	0.008	—
Nutrient broth	0.003	0.018	0.016	—	0.137

NG, No growth; —, not tested.

Fig. 2. Synthesis of isocitrate lyase by wild-type cells of the thermophile and *Bacillus licheniformis* during growth on citrate. Cells of the wild-type strain of the thermophile (a) and the mesophile *B. licheniformis* A5 (b) were grown on salts/acetate (●, ■) or salts/citrate (○, □) media. Samples of the cultures were removed at intervals and the cells were harvested. Cell-free extracts were prepared and assayed for isocitrate lyase activity. The total isocitrate lyase activity was plotted against the cell density of the culture at the time the cells were removed, as described in Fig. 1.

grown on various carbon sources are shown in Table 2. Mutant NG-15 produced high activities of the enzyme during growth on all the media examined except nutrient broth. In contrast, the parent organism PC-2 contained isocitrate lyase at the same activity as found in wild-type organisms grown on the same substrate. On all media examined, mutant NG-35 also produced isocitrate lyase to a high specific activity. This mutant synthesized isocitrate lyase to a much greater activity than the other strains when grown on acetate.

The activity of isocitrate lyase was low in extracts of wild-type organisms grown on all the media except acetate, citrate and, to a lesser extent, succinate. However, the wild-type strain grew poorly on citrate and the continued synthesis of the enzyme on this medium (Fig. 2a) may indicate a partial relief of catabolite repression consequent upon this poor growth. However, a mesophilic strain of *Bacillus* (*B. licheniformis*) also grew slowly on citrate, but without the concomitant synthesis of isocitrate lyase (Fig. 2b).

The isocitrate lyase activities of mutant NG-15 and the wild-type strain were similar. Both were inhibited by pyruvate and by phosphoenolpyruvate with approximate inhibition constants (K_i) of 0.8 mM for phosphoenolpyruvate and 0.5 mM for pyruvate.

Table 3. *NADP⁺-dependent isocitrate dehydrogenase activity in extracts of thermophiles grown on various carbon sources*

Cells were grown to late-exponential phase on the appropriate substrate. The NADP^+ -dependent isocitrate dehydrogenase activity present in cell-free extracts was assayed by following the reduction of NADP^+ at 30 °C. Values are $\mu\text{mol NADP reduced min}^{-1} (\text{mg protein})^{-1}$.

Carbon source	Isocitrate dehydrogenase activity in extracts of:			
	Wild-type	Strain PC-2	Strain NG-15	Strain R-9
Acetate	0.078	0.082	0.003	0.082
Acetate + glucose	0.075	—	0.001	—
Succinate	0.087	0.101	0.009	0.109
Fumarate	0.475	1.129	0.121	0.397
Malate	0.387	—	0.095	0.477
Nutrient broth	0.222	0.146	0	—

—, Not tested.

Enzyme activities in mutants of the thermophile

The activities of certain enzymes in extracts of acetate-grown cultures of wild-type cells and of mutants PC-2 and NG-15 are shown in Table 1. Isocitrate lyase and malate synthase activities were similar in all three organisms. Only extracts derived from the wild-type strain contained pyruvate carboxylase activity.

Extracts of mutant NG-15 contained considerably less NADP^+ -dependent isocitrate dehydrogenase activity than those of the wild-type strain or of its mutant PC-2. No NAD^+ -dependent isocitrate dehydrogenase was detected in any of the organisms.

Assays with mixed extracts of strains PC-2 and NG-15 showed that the low activities of the NADP^+ -dependent isocitrate dehydrogenase in extracts of the mutant NG-15 did not result from the presence of other inhibitors in the extract. The lack of isocitrate dehydrogenase activity in the mutant was, therefore, due to a genetic lesion.

Aconitase activity in extracts of strain NG-15 was higher than those of wild-type and strain PC-2 extracts. This agreed with the findings of Rutberg & Hoch (1970), who showed that mutants lacking isocitrate dehydrogenase had enhanced aconitase activities.

The specific activity of isocitrate dehydrogenase in extracts of NG-15 that had been grown on carbon sources other than acetate was consistently lower than that found in similar extracts of wild-type and PC-2 cultures (Table 3). However, appreciable amounts of isocitrate dehydrogenase were synthesized by NG-15 when grown on malate or fumarate.

Isocitrate concentrations in the mutant of the thermophile

The isocitrate concentrations of extracts of the wild-type thermophile after growth on acetate, and on acetate in the presence of glucose, were 16.2 and 6.6 $\mu\text{mol (g dry mass cells)}^{-1}$, respectively. Similarly, the values for the mutant NG-15 were 24.6 and 15.7 $\mu\text{mol (g dry mass cells)}^{-1}$. (These results are the average for four individual extracts from two different cultures.) The concentrations of isocitrate in extracts of strain NG-15 were considerably higher than in similar extracts of wild-type cells. The increase appeared to be a consequence of the reduced ability of strain NG-15 to channel isocitrate through the NADP^+ -dependent isocitrate dehydrogenase.

Glyoxylate concentrations in the mutant of the thermophile

The glyoxylate concentrations in extracts of the wild-type organism grown on acetate averaged 1.3 $\mu\text{mol (g dry mass cells)}^{-1}$. The glyoxylate concentration in strain NG-15 extracts grown on the same carbon source was 0.8 $\mu\text{mol (g dry mass cells)}^{-1}$.

Revertants from NG-15 capable of good growth on glucose

A culture of mutant NG-15 grown on acetate was plated on to salts/glucose plates, and the larger colonies which appeared after incubation at 55 °C for between 24 and 36 h were removed and streaked on to fresh glucose plates. Single colony isolates were obtained and extracts of these mutants were assayed for pyruvate carboxylase activity. Fourteen of the colonies tested in this way had regained pyruvate carboxylase activity. Further examination of nine of these revertants showed that, whereas they synthesized isocitrate lyase normally during growth on acetate as sole carbon source, they had lost the ability to synthesize the enzyme when grown on acetate in the presence of glucose. These mutants had also regained the ability to synthesize wild-type levels of NADP⁺-dependent isocitrate dehydrogenase activity. The properties of one such revertant, designated R-9, are shown in the Tables; it is evident that the revertant had regained all the characteristics of the wild-type organism. It thus appears that the unusually high activities of isocitrate lyase observed when mutant NG-15 was grown on substrates other than acetate are directly related to the decreased activity of isocitrate dehydrogenase and the concomitantly increased concentrations of isocitrate.

DISCUSSION

Organisms growing on glucose as the sole carbon source fix CO₂ with a C₃-acid as an anaplerotic reaction. In the wild-type strain of a thermophilic *Bacillus*, CO₂ was fixed into oxaloacetate via pyruvate carboxylase (Cazzulo *et al.*, 1970). Mutant PC-2 which was devoid of pyruvate carboxylase was, therefore, unable to grow on glucose as the sole carbon source (Sundaram, 1973). However, mutant PC-2 grew normally on acetate with the 'induction' of isocitrate lyase being unimpaired. Addition of glucose to acetate-grown cells of mutant PC-2 inhibited growth because C₃ compounds repressed and inhibited isocitrate lyase (Kornberg & Smith, 1969). Mutant NG-15 was unusual in that it grew on acetate in the presence of glucose, although it was devoid of pyruvate carboxylase activity. Indeed, it grew on glucose alone, albeit poorly (Griffiths, 1974). This suggested that the regulation of isocitrate lyase synthesis, or control of its activity, must be different in this strain. Mutant NG-15 is similar to mutant NG-35 studied by Chell & Sundaram (1975) in that both can grow on acetate in the presence of glucose, despite their lack of pyruvate carboxylase activity. There are, however, some notable differences between the two mutants. Mutant NG-35 is constitutive for isocitrate lyase as the enzyme is synthesized in cells grown on all media tested (Sundaram, 1973; Chell & Sundaram, 1975). Although strain NG-15 also synthesizes isocitrate lyase on all media tested (Table 2), the activity of the enzyme in nutrient broth cultures is the same as that of the parent organism PC-2 which is not constitutive for the enzyme. The activity of isocitrate lyase in extracts of mutant NG-35 grown on nutrient broth is about 10 times greater than the activity in extracts of mutant NG-15 grown on the same medium (Table 2). Also, the isocitrate lyase activity in extracts of NG-35 grown on acetate appears to be about 2.5 times that of similar extracts of wild-type, PC-2 and NG-15. Thus strain NG-15 may not be truly constitutive for isocitrate lyase synthesis. This would indicate that there was an altered induction system for isocitrate lyase in mutant NG-15 or, alternatively, that there was a lack of inhibition of the isocitrate lyase from the mutant NG-15 by C₃ compounds. The latter did not appear to be the case as the enzymes from the wild-type strain and mutant NG-15 were both inhibited by phosphoenolpyruvate and/or pyruvate.

Mutant NG-15 was also characterized by very low activities of NADP⁺-dependent isocitrate dehydrogenase during growth on all media examined. Extracts of NG-35 also have lower activities of NADP⁺-dependent isocitrate dehydrogenase than the wild-type organism, although not as low as mutant NG-15 (Chell & Sundaram, 1975). The activity of this enzyme is still appreciable in strain NG-35 grown in nutrient broth. In contrast, there is no

detectable isocitrate dehydrogenase activity in extracts of strain NG-15 grown in nutrient broth. Synthesis of the NADP⁺-dependent isocitrate dehydrogenase in the wild-type organism was repressed during growth on acetate and this agrees with the findings of Bennett & Holms (1975).

Another difference between mutants NG-15 and NG-35 is that NG-35 grows much more slowly than the wild-type organism on acetate (relative growth rate of 0.26) (Chell & Sundaram, 1975) whereas on the same carbon source NG-15 grows at the same rate as the wild-type organism. This slow growth of mutant NG-35 may be due to a deficiency of glutamate (Chell & Sundaram, 1975) being caused by glyoxylate and oxaloacetate inhibiting and repressing isocitrate dehydrogenase. The build up of glyoxylate or a metabolite resembling it in mutant NG-35 has been reported by these authors. However no such build up of glyoxylate was observed in mutant NG-15 during growth on acetate, although the concentrations of isocitrate in mutant NG-15 were much higher than those in the wild-type organism grown under the same conditions. This suggested that the residual isocitrate dehydrogenase activity served to supply the glutamate needed for growth of mutant NG-15, but was not adequate for the supply of energy and anaplerotic intermediates. Hence, isocitrate lyase was needed to play this role and clearly this would not be possible unless the regulatory system for isocitrate lyase synthesis had been altered. It was not clear whether the lack of isocitrate dehydrogenase was necessarily associated with this altered isocitrate lyase regulation. One possibility was that the accumulation of isocitrate (or possibly citrate) might have induced the synthesis of isocitrate lyase in mutant NG-15. This hypothesis was supported by the finding that the wild-type strain of the thermophile synthesized isocitrate lyase to a considerable extent during poor growth on citrate. Ashworth & Kornberg (1964) demonstrated that *E. coli* W could produce up to 30% of the activity of isocitrate lyase found in acetate-grown cells during growth on substrates that allowed only slow growth; in contrast, a mesophilic strain of *Bacillus*, *B. licheniformis*, also grew slowly on citrate, but without the synthesis of isocitrate lyase. Moreover, other organisms which grow on citrate, such as *Salmonella* sp., do not synthesize the enzyme on this medium (Kornberg, 1966). Isocitrate has been proposed as an inducer of isocitrate lyase in *Neurospora crassa* (Flavell & Woodward, 1971) and it may be that isocitrate can act in this manner in cells of the thermophilic strain of *Bacillus*.

The possibility exists that the lesion in mutant NG-15 occurs in the gene specifying the synthesis of isocitrate dehydrogenase, whereas the mutation of strain NG-35 of Chell & Sundaram (1975) is in the system regulating the synthesis of the glyoxylate cycle enzymes. However, the nature of these lesions remains unclear.

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