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Malate dehydrogenase of *Saccharopolyspora erythraea* CA340: Purification and effect of carbon source on its synthesis.

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ABSTRACT. Malate dehydrogenase (MDH) was purified to homogeneity from cell-free extracts of *Saccharopolyspora erythraea,* an erythromycin producer, and found to be a dimer with an apparent monomer mass of 40 kDa. The enzyme catalyzed preferentially the reduction of oxaloacetic acid with NADH; K_m values for oxaloacetate and NADH were 0.015 mM and 0.056 mM, respectively. *S. erythraea* grown in minimal medium with either glucose, galactose or lactose as carbon sources showed similar levels of MDH specific activity, which were not affected by the growth phase. On the other hand, growth on fructose as carbon source resulted in elevated levels of MDH specific activity that were two-fold higher than in the other carbon sources.

Key words: Malate dehydrogenase, actinomycetes, citric acid cycle, *Saccharopolyspora erythraea*.

RESUMEN. La malato deshidrogenasa (MDH) de un productor de eritromicina, *Saccharopolyspora erythraea*, fue purificada a homogeneidad y mostró ser un dímero con una masa aparente de 40 kDa para el monómero. La enzima catalizó preferencialmente la reducción de oxaloacetato; los valores de K_m para este compuesto y NADH fueron 0.015 mM y 0.056 mM, respectivamente. Los perfiles de las actividades específicas de MDH de *S. erythraea* obtenidos en medio mínimo con glucosa, galactosa o lactosa como fuentes de carbono no mostraron cambios ni fueron afectados por la fase de crecimiento. Por otro lado, la adición de fructosa como fuente de carbono resultó en niveles de MDH que fueron dos veces más altos que en las otras fuentes de carbono.

Palabras clave: Malato deshidrogenasa, actinomicetos, ciclo del ácido cítrico, *Saccharopolyspora erythraea*.

INTRODUCTION

Polyketides are a large and diverse group of natural products that have a wide variety of biological activities and, include many compunds with medically important such antibiotic, antifungal and anticancer properties. *Saccharopolyspora erythraea* produces the polyketide antibiotic erythromycin by condensation of one propionyl-CoA starter unit with six methylmalonyl-CoA extender units (Pfeifer *et al*., 2001). Methylmalonyl-CoA is synthesized from succinyl-CoA, an intermediate in the tricarboxylic acid (TCA) cycle; therefore carbon flux through the TCA cycle could influence the antibiotic yield. In this sense, we are interested in the regulation of the TCA cycle enzymes.

Malate dehydrogenase (MDH; L-malate-nicotine adenine dinucleotide [NAD] oxidoreductase; EC 1.1.1.37) is a key enzyme that plays an important metabolic role in aerobic energy producing pathways and in the malate shuttle. It catalyzes the pyridine nucleotide-dependent interconversion of malate and oxaloacetate (Labrou and Clonis 1997). MDH has been isolated from bacteria, archaea, fungi, plants, mammals, and from subcellular organelles such as mitochondria, chloroplasts, glyoxysomes, and peroxisomes (Goward and Nichols 1994). In most organisms it is a homodimeric protein, including all eukaryotes examined so far. In most organisms, including all eukaryotes examined to date, it is a homodimeric protein. On the other hand, members of the *Bacillus* genus and the phototrophic green bacteria *Chlorobium vibrioforme*, *Chlorobium tepidum*, and *Chloroflexus aurantiacus* have a homotetrameric MDH (Wynne *et al*., 1996).

In *Escherichia coli* it has been shown that MDH levels are reduced by anaerobiosis, and by the presence of glucose, and induced by acetate and malate. In addition, MDH activity is elevated in *arcA* mutants that are defective in the ArcA regulatory protein, which controls the levels of many aerobically expressed enzymes in the cell. Also, MDH expression showed dependence on the availability of oxygen and heme groups, the carbon source used for cell growth, and the cell growth rate (Park *et al*., 1995). To date, there are no reports about the regulation of MDH synthesis in the actinomycetes, in spite of the importance of this enzyme.

This work reports the effect of carbon source and growth phase on MDH synthesis by *Saccharopolyspora erythraea* CA340 and the biochemical characterization of the enzyme.

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MATERIALS AND METHODS

Strain and culture conditions

Saccharopolyspora erythraea CA340 was obtained from Abbott Laboratories (North Chicago, IL), and grown on minimal medium under conditions described previously (Bermúdez *et al.*, 1998).

Preparation of cell-free extracts

The mycelium was recovered at different times of growth by centrifugation and washed with 0.01 M Tris-HCl, pH 7.0 containing 35 mg PMSF ml^{-1}, 0.3 mg EDTA ml^{-1} and 1 mM DTT. The pellet was resuspended in a minimal volume of the same buffer and disrupted by sonication with a Vibra-Cell Model 9130 sonicator for a total of 1 min in 15s intervals while standing on ice. Cell debris was removed by centrifugation at 22,000 X *g* for 30 min at 4 °C and the supernatant used for enzyme activity determination.

MDH assay

Malate dehydrogenase activity was assayed as reported for *Streptomyces aureofaciens* by following the oxidation of NADH at 25°C measuring the absorbance at 340 nm (Mikulásová *et al.*, 1998). Protein concentration was determined by the Bradford method (Bradford, 1976). Specific activity is reported as µmoles of NADH reduced per milligram of protein per minute.

Zymograms

For detection of enzyme activity in gels, non-denaturing electrophoresis was performed in 12.5 % w/v polyacrylamide gels (1.5 mm thick); 70 ml of electrophoresis buffer with 15 mg of NADH were used for running each gel. After electrophoresis, gels were washed twice with Tris-HCl 0.01 M, pH 7.0 for 10 min and incubated at room temperature in 15 ml of the same buffer containing 10 mg of oxaloacetate and 5 mg of NADH. The activity bands were visualized under ultraviolet illumination (Manchenko 1994).

Table 1. Induction of MDH in *S. erythraea* by fructose.

	Growth* (mg protein ml^{-1})	Specific Activity* (µmoles mg^{-1} min^{-1})
Fructose	0.50 ± 0.1	12.18 ± 1.2
Fructose + Clm	0.13 ± 0.015	8.2 ± 0.7
Glucose	0.54 ± 0.008	8.46 ± 0.81
Glucose + Clm	0.14 ± 0.01	9.15 ± 0.5

Purification of MDH

The band corresponding to MDH activity was cut out from the gel and the protein eluted in Tris-HCl buffer (0.01 M, pH 7.0). Enzyme purity was checked by native and denaturing PAGE. The protein band was visualized using Coomassie brilliant blue R-250 or by silver staining.

Molecular mass determination

Molecular mass was determined by SDS-PAGE using protein standards as markers.

Data presentation

All experiments were repeated at least three times and the mean values are shown in the tables and figures.

RESULTS

Growth and MDH activity profile

When *S. erythraea* was grown on glucose as the sole carbon source, growth was linear until 48 h and then

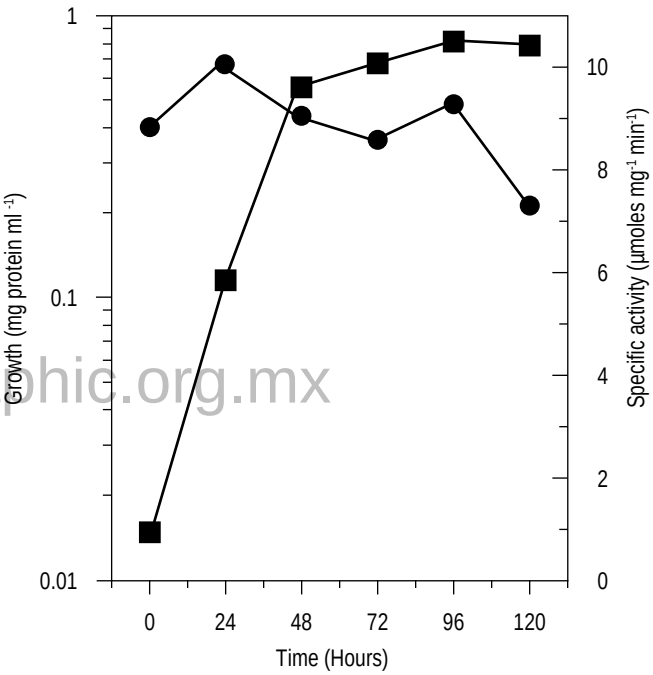

Figure 1. *Growth (■) and MDH activity (●) of S. erythraea CA340 grown in minimal medium with 1% (w/v) glucose and 20 mM glycine as carbon and nitrogen sources, respectively, under conditions described in materials and methods.*

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ceased; under these conditions, the MDHspecific activity was independent of the growth phase and remained relatively constant (Figure 1). When other carbon sources were used, such as galactose or lactose, a similar activity profile and similar MDH levels were obtained, even though less growth was supported by these carbon sources. On the other hand, fructose as a sole carbon source led to a very good growth, and under these conditions MDH specific activity showed a constant increase until 96 h and then diminished slightly (Figure 2); in this case, activity levels were up to two-fold higher than those observed with the other carbon sources.

Effect of fructose on synthesis of MDH

In order to find out more about this effect of fructose, *S. erythraea* was grown on different concentrations of this sugar. As can be seen in Figure 3, growth rates were identical in fructose concentrations ranging from 0.5% to 4% (w/v); the total biomass obtained was higher in cultures grown in 1%, 2% and 4% fructose. MDH activity increased linearly with maximal activity obtained at 96 h, and the activity values decreased as the sugar concentration increased. In addition, cultures grown in 2 and 4% fructose showed a 24 h delay in the onset of MDH activity increase, suggesting that high concentrations of this sugar inhibit the synthesis of the enzyme.

Apparent molecular mass of MDH and biochemical characteristics

MDH activity from cell-free extracts of *S. erythraea* could be detected in non-denaturing polyacrylamide gels. The band showing activity was cut out from the gel and the protein was eluted to purify the enzyme, which had an apparent molecular mass of 40 kDa, as determined by SDS-PAGE (Figure 4). The specific activity of the purified MDH was 192.6 µmoles of NADH oxidized mg^{-1} min^{-1}. The apparent K_m values obtained for oxaloacetate and NADH were 0.015 and 0.056 mM, respectively, with a V_{max} of 235 µmoles mg^{-1} min^{-1}. Concentrations of oxaloacetate higher than 0.3 mM inhibited MDH activity; no activity was detected with NADPH (data not shown).

DISCUSSION

S. erythraea is the main producer of the antibiotic erythromycin, whose precursors methylmalonyl-CoA and propionyl-CoA are provided by primary metabolism. In spite of its importance, there is little information about primary metabolism of this species and of actinomycetes in general.

In this work, we showed that *S. erythraea* possesses a highly active cytoplasmic malate dehydrogenase, which biochemically resembles the enzyme from *Streptomyces aureofaciens* and other bacteria (Mikulásová *et al.*, 1998). The enzyme is a homodimer with a molecular mass of 40 kDa

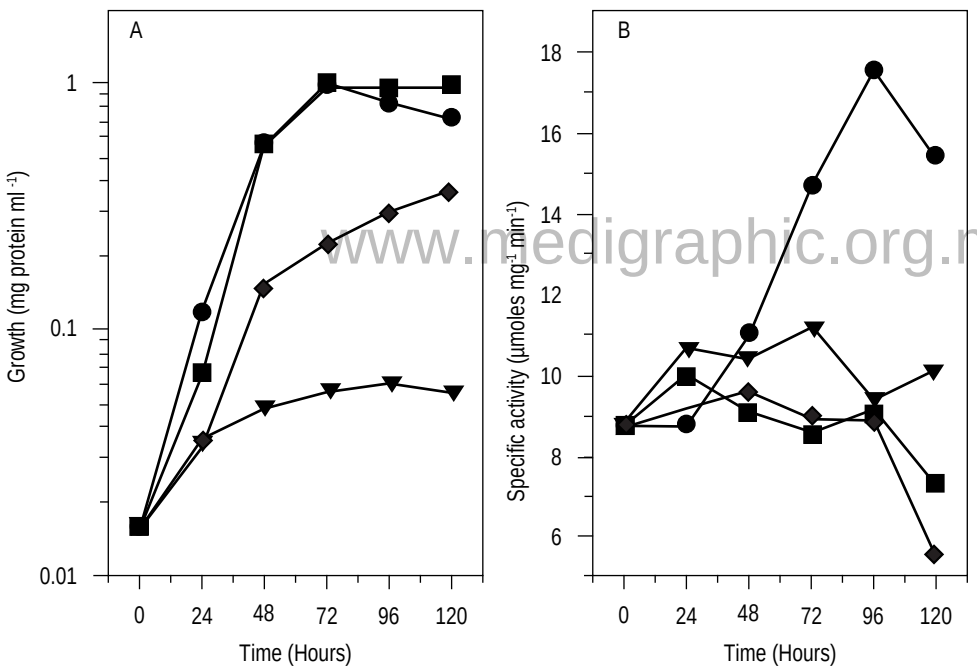

Figure 2. *Growth (Panel A) and MDH activity (Panel B) of S. erythraea CA340 grown in minimal medium with glucose (■), fructose (●), galactose (◆) and lactose (▼) as carbon sources. All sugars were added at a concentration of 1% (w/v).*

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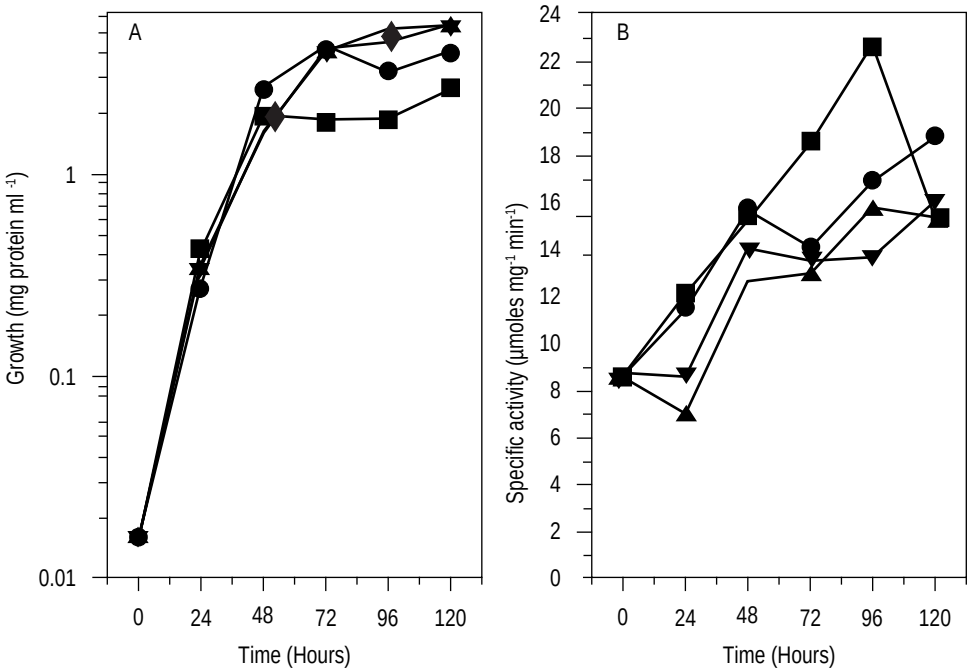

Figure 3. *Growth (Panel A) and MDH activity (Panel B) of S. erythraea CA340 grown in minimal medium with 0.5% (■), 1% (●), 2% (▲) and 4% (▼) fructose as carbon source, under conditions described in materials and methods.*

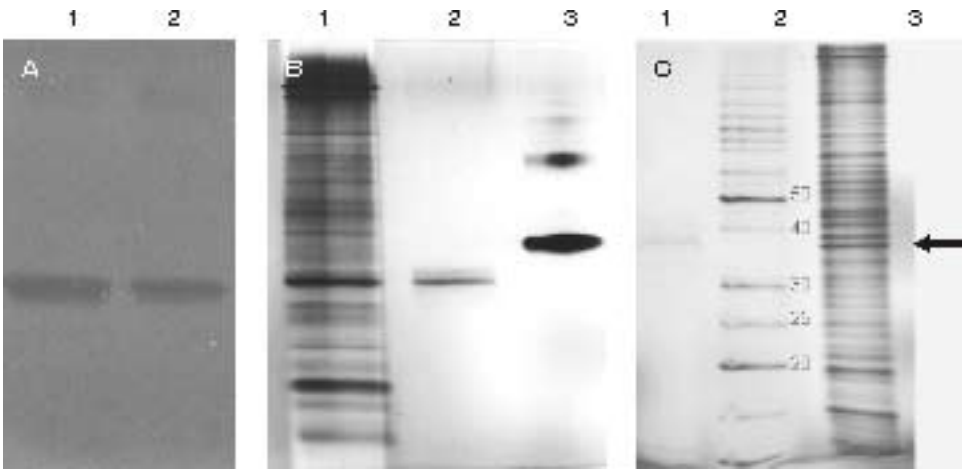

Figure 4. *Purification of MDH from S. erythraea CA340. (A) Zymogram of MDH from a cell-free extract (Lane 1) and purified MDH (Lane 2); (B) non denaturing gel stained with silver nitrate lane 1, cell-free extract; lane 2, purified MDH; lane 3, bovine serum albumin; (C) denaturing gel stained with silver nitrate lane 1, purified MDH; lane 2, molecular weight markers; lane 3, cell-free extract. The position of the MDH monomer is indicated by an arrow.*

for the monomer, and catalyzes preferentially the reduction of oxaloacetate with NADH. The K_m values obtained for *S. erythraea* MDH are within the range reported for other microorganisms (Langelandsvik *et al.*, 1997; Pitson *et al.*, 1999; Thompson *et al.*, 1998).

The MDH activity profile revealed a constant level of expression when *S. erythraea* was grown on glucose, galactose or lactose, suggesting that the synthesis of the enzyme is constitutive during all growth phases. Constitutive synthesis of MDH has also been found in the thermophilic, filamentous fungus *Talaromyces emersonii* (Maloney *et al.*, 2004). In contrast, *S. aureofaciens* showed a peak of MDH activity at 20 h, when the bacteria reached their highest growth rate (Mikulásová *et al.*, 1998). Our results also show that glucose does not repress synthesis of MDH in *S. erythraea*, as occurs in *Corynebacterium glutamicum*, *Bacillus subtilis* and many other bacteria (Hederstedt 1993; Molenaar *et al.*, 2000; Vogel *et al.*, 1987).

On the other hand, the activity profile when fructose was used as carbon source showed growth phase dependent *de novo* synthesis of MDH, which reached levels higher than with the other carbon sources. Induction of MDH synthesis

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by fructose has not been reported for any other microorganism. We have previously reported high levels of isocitrate dehydrogenase in *S. erythraea* when either glucose or fructose were used as carbon sources and showed that glucose induces its synthesis; therefore it is likely that fructose also induced ICDH synthesis (Alvarado and Flores 2003). Fructose is transported by the fructose-PTS system in *Streptomyces coelicolor* and then metabolized by a fructose 1-phosphate kinase (FruA) which is subsequently converted to fructose 1,6-bisphosphate by FruK (Nothaft *et al.*, 2003). On the other hand, Loo *et al.*, (2003) have suggested that the regulation of fructose transport and metabolism in *Streptococcus gordonii* is intricately tied to carbon catabolite control and the ability to form biofilms. Thus, fructose transport and metabolism could be regulating the flow of carbon via metabolic pathways in *S. erythraea* and therefore methylmalonyl and erythromycin synthesis.

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