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Metabolic Routes of Malonate in *Pseudomonas fluorescens* and *Acinetobacter calcoaceticus*

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Abstract: In malonate grown *Pseudomonas fluorescens*, malonate decarboxylase and acetyl-CoA synthetase were induced, whereas in *Acinetobacter calcoaceticus* malonate decarboxylase, acetate kinase, and phosphate acetyltransferase were induced. In both bacteria malonate decarboxylase was the first, key enzyme catalyzing the decarboxylation of malonate to acetate, and it was localized in the periplasmic space. Acetate thus formed was metabolized to acetyl-CoA directly by acetyl-CoA synthetase in *Pseudomonas*, and to acetyl-CoA *via* acetyl phosphate by acetate kinase and phosphate acetyltransferase in *Acinetobacter*.

Key words: *Acinetobacter calcoaceticus*, malonate decarboxylase, malonate metabolism, *Pseudomonas fluorescens*.

The use of malonate, a competitive inhibitor of succinate dehydrogenase, by microorganisms has been reported (Bentley, 1952). Hayaishi (1955) suggested a metabolic conversion of malonate to malonyl-CoA in the presence of CoA and ATP in malonate adapted *Pseudomonas*. In glucose grown *Bradyrhizobium*, malonyl-CoA synthetase was isolated and characterized, indicating a possible involvement of malonyl-CoA during malonate metabolism (Kim and Chae, 1991). However, the existence of malonate decarboxylase, which catalyzes direct decarboxylation of malonate to acetate and CO_2, was reported in a crude extract of *Malonomonas rubra* grown anaerobically on malonate (Hilbi *et al.*, 1992). Recently, a novel type of malonate decarboxylase was also purified from *A. calcoaceticus* grown on malonate (Kim and Byun, 1994). These results suggest that the first step of malonate assimilation in the experimental organisms is the decarboxylation of malonate to acetate instead of the formation of malonyl-CoA. However, little is known about further metabolic processes.

In this report evidence is presented that the metabolic route of malonate assimilation in *P. fluorescens* is partly different from that in *A. calcoaceticus*.

Materials and Methods

Materials

P. fluorescens ATCC 11250 was obtained from the Genetic Engineering Research Institute, KIST (Taeduck, Korea). *A. calcoaceticus var. Kim* is a bacterium isolated from soil and identified on the basis of malonate consumption (Kim and Kim, 1985). Sodium malonate, MOPS, p-nitroblue tetrazolium, and 5-bromo-3-indolyl phosphates were purchased from Sigma Chem. Co. (St. Louis, USA) [1,3-^{14}C]malonate (9.5 mCi/mmol) was obtained from Amersham (Amersham, UK) and [2-^{13}C] malonate (99 atom % ^{13}C) was purchased from Sigma Aldrich (Milwaukee, USA). Immobilon NC nitrocellulose transfer membrane was purchased from Millipore Co. (Milford, USA). All other reagents were of analytical grade.

Growth of bacteria

P. fluorescens and *A. calcoaceticus* were grown aerobically at 30℃ for 18 h and 6 h, respectively on malonate as a sole carbon and energy source. The growth medium contained 0.6% malonic acid, 0.3% KH_2PO_4, 0.3% NH_4Cl, 0.04% $MgSO_4 \cdot 6H_2O$, and 0.01 % $FeSO_4 \cdot 7H_2O$. The pH was adjusted to 6.8 using KOH for the *A. calcoaceticus* medium, and to the same pH using NaOH for the *P. fluorescens* medium. The yields were approximately 3 g and 6 g wet cell/l of medium for *P. fluorescens* and *A. calcoaceticus*, respectively.

Malonate decarboxylase assay

Enzyme assays were based on a determination of the amount of CO_2, and were performed in 10 ml tubes sealed with rubber stoppers containing 20 μmol MOPS buffer, pH 6.8, 1 μmol sodium malonate, 2 nmol (0.019 μCi) of [1,3-^{14}C]malonic acid, 2 nmol

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malonyl-CoA, and the enzyme solution in a 0.2 ml reaction mixture. Decarboxylation was started by adding malonate, followed by incubation at 30℃ for 10 min. The reaction was stopped by injection of 0.1 ml of 20% (w/v) trichloroacetic acid through the rubber caps. Evolved $^{14}CO_2$ was trapped and its radioactivity counted by the general method used for other decarboxylases (Bartos *et al.*, 1993).

Acetate kinase assay

Acetate kinase was assayed by measuring the rate of acetyl phosphate formation. The formation of acetyl phosphate was determined by measurement of the amount of the acetohydroxamate-Fe^{3+} complex at 540 nm, according to the method of Lipmann and Tuttle (1945).

Phosphate acetyltransferase assay

Enzyme activity was assayed by measuring the increase of absorbance at 232 nm for the formation of thioester bonds of acetyl-CoA by the method of Ko and Kim (1987).

Acetyl-CoA synthetase assay

Enzyme activity was assayed by measuring the amount of acetohydroxamate formed from acetyl-CoA and hydroxylamine. A reaction mixture containing (in micromoles) potassium phosphate buffer, pH 7.2, 50; sodium malonate, 20; $MgCl_2$, 10; coenzyme A, 0.25; NH_2OH, 100; and enzyme and water in a 0.5 ml final volume was incubated at 30℃ for 30 min. After 0.5 ml of ferric chloride (10%, w/v) was added, the color developed by the formation of the acetohydroxamate-Fe^{3+} complex was measured at 540 nm.

Decarboxylation of malonate by bacteria

The formation of acetate from malonate was monitored by two different methods. First, cells harvested at the late exponential growth stage were washed and resuspended in 20 mM MOPS, pH 6.8 (1.6×10^{10} cell/ml). The cell suspension (0.5×10^{10} cells) was incubated with 25 μmol of [2-^{13}C]malonate at 30℃ in a final volume of 0.5 ml containing 10 μmol MOPS, pH 7.2, and 15% (v/v) D_2O. ^{13}C-NMR spectra were obtained on a Varian Gemini-300 7.05 T Spectrometer operating in the Fourier transform mode at 300 MHz at 25℃ in a 5 mm tube. A total of 256 transients were accumulated using a pulse of 45° (the 90° pulse was 16 s) and an acquisition time of 3 s.

Second, a reaction mixture containing the cell suspension (0.5×10^{10} cells), 5 μmol malonate, Triton X-100 (0 to 0.1%, v/v), and 50 μmol MOPS, pH 6.8, in a final volume of 0.5 ml was incubated at 30℃ for 10 min. After incubation the mixture was centrifuged at 15,000×g for 5 min and the acetate content in the supernatant measured spectrophotometrically using acetate kinase.

Induction of the enzymes

P. fluorescens and *A. calcoaceticus* were grown aerobically at 30℃ on malonate, or the three other carboxylates succinate, tartarate, or acetate, as a sole carbon and energy source. LB and YM media (0.1% Yeast extract and 1% mannitol) were also used. Bacteria were harvested at the late log phase and stored at −70℃. Cells were suspended (10 ml of buffer per gram wet weight of cells) in 20 mM MOPS, pH 7.2, containing 5% glycerol, and exposed to ultrasonic waves to prepare crude extracts. The enzymatic activities of the crude extracts were assayed.

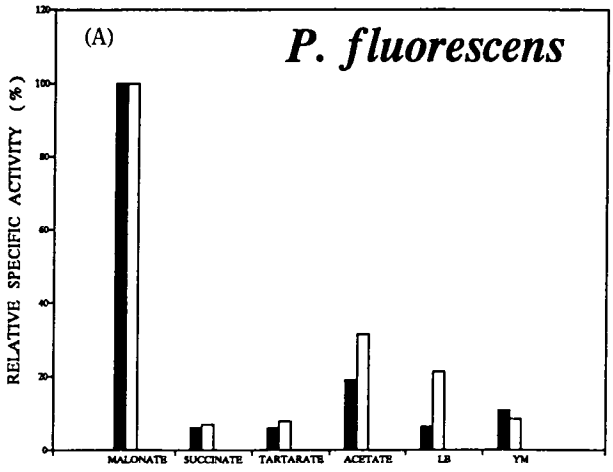
Immunoblot analysis

Antisera against the purified malonate decarboxylases from *P. fluorescens* and *A. calcoaceticus* were prepared in New Zealand White rabbits. IgG fractions were prepared by ammonium sulfate precipitation and DEAE-Sephacel chromatography. Crude extracts were subjected to SDS/PAGE, and the proteins were transferred electrophoretically to nitrocellulose filters according to the method of Sambrook, *et al.* (1989). Immunodetection was carried out with two antibodies prepared against malonate decarboxylases purified from two different bacteria as the first antibodies (1 : 5000 dilution), and alkaline phosphate-conjugated goat anti-rabbit antibody as the second antibody. p-Nitroblue tetrazolium and 5-bromo-3-indolyl phosphates were used as substrates, according to the Protoblot immunoscreening system protocol.

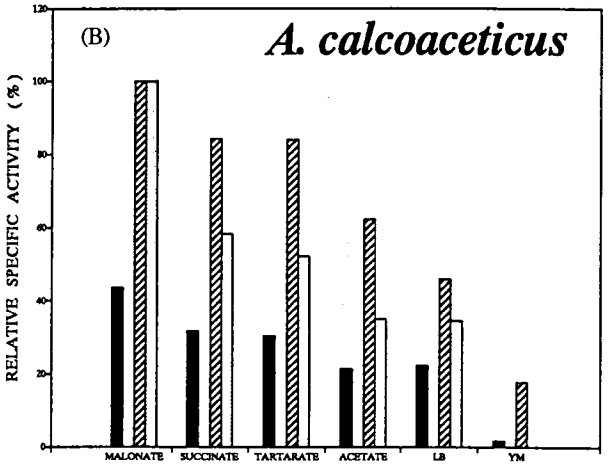
Results and Discussion

Induction of enzymes

Malonate decarboxylase and acetyl-CoA synthetase were induced in *P. fluorescens* grown on malonate as a sole carbon and energy source, whereas the production of these enzymes was repressed in media containing succinate or tartarate, as well as in LB or YM media (Fig. 1A). In malonate grown *A. calcoaceticus*, not only malonate decarboxylase, but also acetate kinase and phosphate acetyltransferase were induced instead of acetyl-CoA synthetase (Fig. 1B). Relatively high activities for these enzymes were also detectable in *Acinetobacter* cells grown on succinate, tartarate, acetate or LB, indicating that induction may be controlled differently from induction in *Pseudomonas*. However, induction levels of malonate decarboxylase and acetyl-



MEDIA / ENZYME	MALONATE	SUCCINATE	TARTARATE	ACETATE	L B	Y M
MDC (■)	1.280*	0.076	0.074	0.244	0.080	0.139
ACS (□)	0.203*	0.014	0.016	0.064	0.043	0.017
AK (▨)	0	0	0	0	0	0
PAT (▦)	0	0	0	0	0	0



MEDIA / ENZYME	MALONATE	SUCCINATE	TARTARATE	ACETATE	L B	Y M
MDC (■)	0.557	0.407	0.389	0.274	0.286	0.019
ACS (□)	0	0	0	0	0	0
AK (▨)	0.604*	0.508	0.502	0.377	0.277	0.106
PAT (▦)	0.120*	0.070	0.063	0.042	0.041	0

Fig. 1. Induction of enzymes for malonate assimilation in *P. fluorescens* (A) and *A. calcoaceticus* (B) grown on various media. The numbers in the table attached to figure represent the specific activities (U/mg) of enzymes in crude extracts (●: This specific activity was used as a denominator for the calculation of percentage).

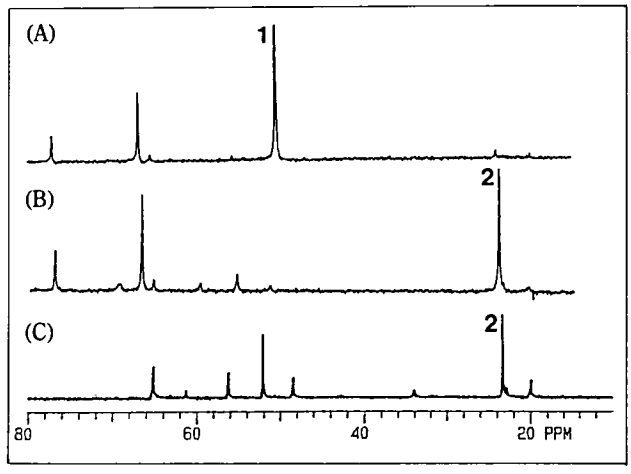


Fig. 2. Decarboxylation of [2-^{13}C]malonate by bacterial cells. Cells (0.5×10^{10}), *Pseudomonas* or *Acinetobacter*, were incubated with 25 μmol of [2-^{13}C]malonate in a final volume of 0.5 ml at 30℃ (A, [2-^{13}C]malonate; B, [2-^{13}C]malonate+*A. calcoaceticus*; C, [2-^{13}C]malonate+*P. fluorescens*). The appearance of [2-^{13}C]acetate was monitored by NMR, immediately after the addition of [2-^{13}C]malonate to the cell suspensions. 1, [2-^{13}C]malonate; 2, [2-^{13}C]acetate.

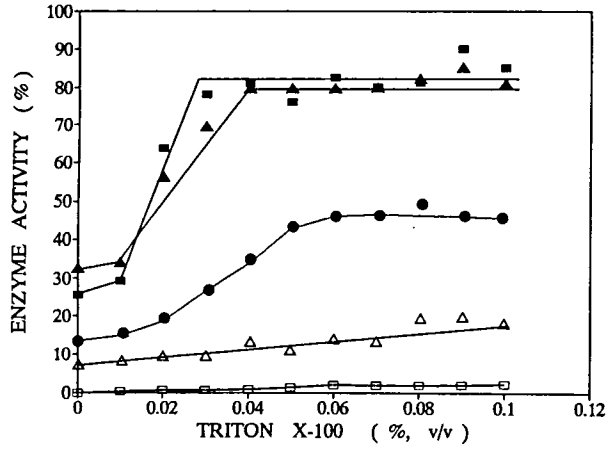


Fig. 3. Formation of acetate from malonate by bacteria in the presence of a detergent. Reaction mixture containing 50 μmol MOPS, pH 6.8, 5 μmol malonate, and cell suspension (0.5×10^{10} cells) of *P. fluorescens* or *A. calcoaceticus* in a total volume of 0.5 ml were incubated at 30℃ for 10 min with 0 to 0.1% (v/v) of Triton X-100. After the centrifugation, acetate content in the supernatants of *P. fluorescens* (▲) or *A. calcoaceticus* (■) were determined spectrophotometrically using acetate kinase. For the periplasmic marker enzyme, cAMP-phosphodiesterase (●) was used for both bacteria, whereas acetyl-CoA synthetase (△) or acetate kinase (□) were assayed as a cytoplasmic marker enzyme for *P. fluorescens* or *A. calcoaceticus*, respectively.

CoA synthetase in *P. fluorescens*, and those of malonate decarboxylase, acetate kinase, and phosphate acetyltransferase in *A. calcoaceticus*, were similar in various media. This result indicates that expression of the two enzymes in *P. fluorescens* and the three enzymes in *A. calcoaceticus* may be closely coordinated.

Decarboxylation of malonate

The first step of malonate assimilation in both of the experimental microorganisms might be the decarboxylation of malonate to acetate. Evidence for this first enzyme step was obtained by monitoring the formation of acetate from malonate with intact cells in

an NMR tube. As shown in Fig. 2, the formation of ^{13}C-acetate from ^{13}C-malonate was directly monitored with cells grown on malonate and resuspended in a medium containing ^{13}C-malonate. However, the further metabolic processes of acetate in both bacterial cell lines could not be monitored immediately in the NMR tube, suggesting a different location for malonate decarboxylase than the metabolic enzymes involved in the further, unmonitored processes. The cellular locations of malonate decarboxylase in the two bacteria were determined by measuring the enzyme activity of cells treated with detergent. The activities of acetyl-CoA synthetase in *Pseudomonas* and acetate kinase in *Acinetobacter*, however, were not detected in detergent treated cells (Fig. 3). Periplasmic enzymes were demonstrated by this method in *Rhizobium* by Streeter (1989).

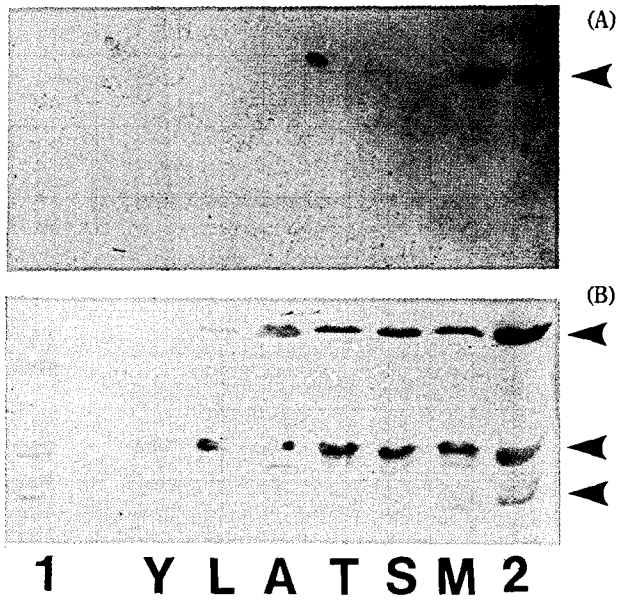


Fig. 4. Immunoblot analysis of malonate decarboxylase by using two different anti-malonate decarboxylase antibodies. Crude extracts were prepared from *P. fluorescens* (A) or *A. calcoaceticus* (B) grown on malonate (lane M), succinate (lnae S), tartarate (lane T), acetate (lane A), LB (lane L), or YM (lane Y), and the same amount of the extracts (50 μg) were loaded on SDS/PAGE. On the lane 2 in A and lane 1 in B, or lane 1 in A and lane 2 in B, the purified malonate decarboxylase from *P. fluorescens* or from *A. calcoaceticus* were applied, respectively. After electrotransfer of proteins from gel to nitrocellulose filter, the antibodies prepared against *Pseudomonas* and *Acinetobacter* enzymes were used for plate A and B, respectively, as the first antibodies for immunoblot.

In order to determine whether the increase in malonate decarboxylase activity resulted from enzyme production or activation of the enzyme, immunoblot analysis was performed. As shown in Fig. 4, malonate decarboxylase proteins were induced in a malonate medium. At present the real inducer of the enzyme is not known, but an inducer might regulate the plasmid encoded malonate decarboxylase gene (Kim and Kim, 1994). Antibody prepared against the malonate decarboxylase from *Acinetobacter* did not show any cross reactivity to the malonate decarboxylase from *Pseudomonas*, and *vice versa*, suggesting that they are immunologically different.

The proposed metabolic pathways

Based on previous results (Kim and Byun, 1994; Kim and Park, 1988; Ko and Kim, 1987; Chae and Kim, 1987; Park *et al.*, 1986; Jang and Kim, 1982) and the results of this study, it is proposed that malonate assimilation in *Pseudomonas* and *Acinetobacter* is as follows (Fig. 5). Conversion of malonate to other metabolites has been also reported in glucose grown *Rhizobia* taxa, such as *Bradyrhizobium japonicum*, *Rhizobium trifolii*, and *Rhizobium meliroti*. In these bacteria malonate is converted to malonyl-CoA by malonyl-CoA synthetase (Kim and Kwon, 1993), and to malonamate by malonamidase (Kim and Kang, 1993). The presence of these enzymes suggests that there are

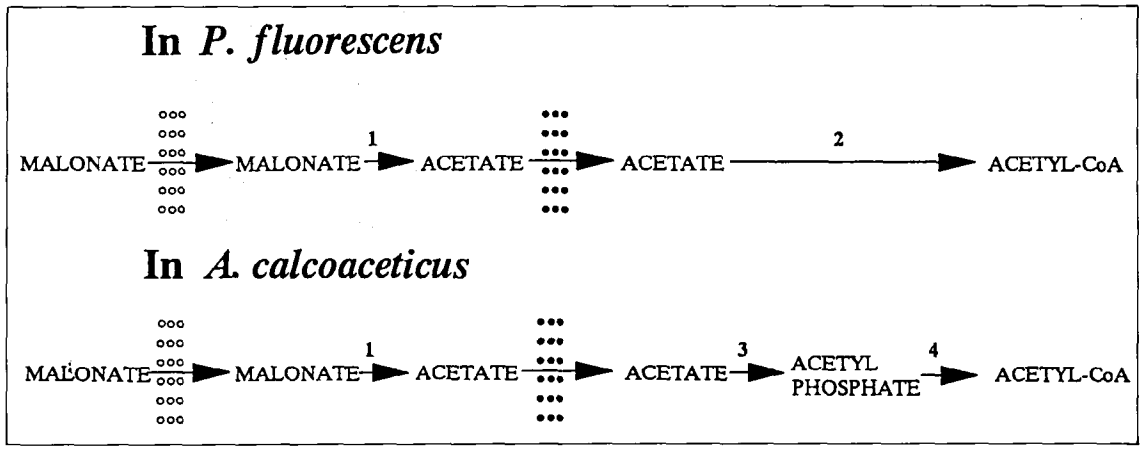


Fig. 5. The proposed metabolic routes for malonate assimilation in *P. fluorescens* and *A. calcoaceticus*. 1, Malonate decarboxylase; 2, Acetyl-CoA synthetase; 3, Acetate kinase; 4, Phosphate acetyltransferase. ○○○, outer membrane; ●●●, inner membrane.

at least three alternative metabolic pathways for malonate use in biological systems. However, when malonate is used by microorganisms as a sole carbon source, the first step of the metabolic reaction is believed to be the decarboxylation of malonte to acetate. Acetate, thus formed from malonate, is further metabolized to acetyl-CoA directly in *Pseudomonas*, and *via* acetyl phosphate in *Acinetobacter*. Acetate is metabolized to acetyl-CoA directly by acetyl-CoA synthetase in animals, whereas it is metabolized indirectly to acetyl-CoA *via* acetyl phosphate by acetate kinase and phosphate acetyltransferase in bacteria (Walsh, 1977). However, the phylogenetically similar bacteria *P. fluorescens* and *A. calcoaceticus* (Ko, 1989) use different metabolic pathways for malonate assimilation. Acetyl-CoA, thus formed in both bacteria, is metabolized *via* the anaplerotic glyoxylate cycle (Jang and Kim, 1982; Park *et al.*, 1986).

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