Malonate Decarboxylase in Bacteria and Its Application for Determination of Intracellular Acyl-CoA Thioesters

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Pseudomonas putida is able to grow on malonate as a sole source of carbon and energy. Malonate decarboxylase is a key enzyme catalyzing malonate to acetate and CO2. The enzyme consists of the five different subunits, α (60 kDa), β (33 kDa), γ (28 kDa), δ (13 kDa), and ε (30 kDa). The smallest subunit δ is an acyl-carrier protein (ACP) possessing 2'-(5"-phosphoribosyl)-3'-dephospho-CoA as a prosthetic group. Acylation of ACP is the initial step triggering the decarboxylation of malonate in a cyclic manner, i.e., the δ subunit is activated to form acyl-δ-ACP by the ε subunit in the presence of acetyl-CoA or malonyl-CoA as an active acyl donor. The acetyl residue on the δ subunit is replaced with malonate by the α subunit, and the malonyl residue subsequently undergoes decarboxylation by the subunits β and γ, thereby regenerating acetyl-δ-ACP. This unique cyclic reaction mechanism that amplifies acetate as its product in proportion to the amount of a given acetyl-CoA and/or malonyl-CoA is allows one to detect pmol levels of the CoA derivatives. Moreover, one can separately measure the three CoA molecular species, acetyl-CoA, malonyl-CoA, and nonesterified CoA (CoASH), by a combination of the elimination of acetyl-CoA with citrate synthetase (EC 4.1.3.7) or acetylation of CoASH with phosphate acetyltransferase (EC 2.3.1.8). The micromethod, named the acyl-CoA cycling method, is useful to define the rapid changes in vivo in the size and composition of the CoA pool. By this micromethod, it has been demonstrated that there is a remarkable difference in the size and composition of intracellular pools of CoASH and CoA thioesters between aerobic bacteria and facultatively anaerobic bacteria. It has also been revealed that the composition of CoA pools in facultatively anaerobic bacteria drastically changes within minutes in response to the quality and quantity of the carbon source in the medium, growth phase, pH, incubation temperature, osmotic stress, or antibiotics to inhibit energy yielding systems and fatty acid biosynthesis.

Key words: malonate decarboxylase, acetyl-CoA, malonyl-CoA, CoASH, Pseudomonas putida

Introduction

Malonic acid, a homologue of succinic acid, is well known as a competitive inhibitor of succinate dehydrogenase in the tricarboxyl acid (TCA) cycle. Many plants contain a small amount of this three-carbon dicarboxylic acid. In leaf and root tissue of young soybean, malonate is present in high concentrations and is the predominant acid, but the metabolite does not accumulate as the dead-end product of a metabolic pathway. Also, malonate is observed in rat brain and participates in development. In both cases, it is likely that malonate is derived from malonyl-CoA produced by acetyl-CoA carboxylase. The inhibition of succinate dehydrogenase by malonate, therefore, occurs naturally in biological systems of plants and animals, suggesting that it plays a role as an important mediator in intracellular metabolism. On the other hand, little information...
on malonate accumulation is available in bacteria, although a few bacteria produce it via specially oxidative pathways of pyrimidine degradation\(^{39}\) and polyol (1,3-propanediol)\(^{21}\). However, there are quite a few bacteria that utilize malonic acid as a carbon source for growth. In these bacteria, malonate decarboxylase is the essential enzyme that catalyzes the conversion of malonate to acetate and \(CO_2\). In 1955, Hayaishi\(^{260}\) first discovered this enzyme in *Pseudomonas fluorescens* adapted to malonate. Using partially purified enzyme preparations, he found that malonate was activated to form malonyl-CoA in the presence of nonesterified CoA (CoASH) and ATP by malonate thiokinase, and then the malonyl-CoA was decarboxylated to acetyl-CoA and \(CO_2\) by malonyl-CoA decarboxylase. Although he detected the enzymatic activity of acetyl-CoA:malonate CoA transferase in the preparation, the significance of the enzyme was separate from the enzyme reaction. In 1981, Takamura and Kitayama\(^{43}\) reported that malonate decarboxylase purified from *P. putida* (originally *P. ovalis*) was a bifunctional enzyme with acetyl-CoA:malonate CoA transferase and malonyl-CoA decarboxylase. The decarboxylation of malonic acid progressed cyclically in the presence of a catalytic amount of acetyl-CoA by the functions of three different subunits. In 1992, Dimroth and his coworkers discovered an alternate malonate decarboxylase with an acyl-carrier protein (ACP) as a subunit in a microaerotolerant anaerobic bacterium, *Malonomonas rubra*\(^{29}\), and a novel reaction mechanism was proposed, that decarboxylation of malonate progressed cyclically with the participation of ACP instead of CoA\(^{59}\). Based on gene analyses as well as biochemical studies on subunits, it was concluded that acyl-S-ACP is an indispensable intermediate in the cyclic reaction of malonate decarboxylase\(^{6–8,13,14,20–29,31–33,49,50,78}\).

Another interesting feature of malonate decarboxylase is its unique cyclic reaction mechanism. We applied the characteristic of the *Pseudomonas* enzyme to a micromethod for the determination of acetyl-CoA, malonyl-CoA, and CoASH\(^{16,84}\). Numerous papers have been published on the pool sizes of cellular nucleotides\(^{41}\) such as ATP\(^{31}\), cAMP\(^{39}\), NAD, and NADP\(^{50}\). Consequently, adenylate energy charge\(^{3,9,10}\) and reduction charge\(^{1}\) have been proposed as parameters indicating in vivo metabolic state. In contrast, little is known about the changes in the size and composition of intracellular pools of CoA molecular species, even though they are also essential intermediates in numerous biosynthetic and energy-yielding metabolic pathways as well as regulators of several key metabolic reactions. The micromethod, named the acyl-CoA cycling method, is sufficiently sensitive to detect an intracellular CoA pool and allows one to monitor changes in the size and composition of intracellular pools.

In this paper, we will focus on the unique enzyme characteristics of malonate decarboxylases in bacteria as well as analyses of the gene (*mdc*) clusters. Furthermore, we deal with the in vivo behavior of acetyl-CoA, malonyl-CoA, and CoASH in aerobic and facultatively anaerobic bacteria determined by the acyl-CoA cycling method using this enzyme.

**Malate decarboxylase in bacteria**

**Distribution of malonate-assimilating bacteria**

Malonate is not a common substrate for organisms, however, some bacteria are known to utilize malonic acid as a carbon source for growth. A malonate-salts medium has been long on the market (Eiken Chemical Co. Ltd., Tokyo) for the biochemical examination of the taxonomy of bacteria to distinguish between the *Klebsiella-Enterobacter* group (positive) and genera *Escherichia* and *Serratia* (negative)\(^{77}\). It was also reported that *Alcaligenes faecalis* (positive) can be discriminated from *Acinetobacter* (negative) using this medium. However, *Alcaligenes faecalis* IB-14 isolated from soil by us utilizes malonate as an inducer of maleate cis-trans isomerase and a substrate of fatty acid synthesis without decarboxylation\(^{85}\). There are various bacteria including aerobes, facultative anaerobes, and anaerobes with diverse pathways for the metabolism of malonate that have not been elucidated yet. In recent years, malonate decarboxylases of *Pseudomonas putida*, *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus*, and *Malonomonas rubra* have been studied in detail as described below.

**Reaction mechanism of malonate decarboxylase**

We first reported that *Pseudomonas putida* malonate decarboxylase was an oligomeric enzyme with a molecular mass of 170 kDa composed of three subunits, \(\alpha\), \(\beta\), and \(\gamma\). The enzyme catalyzed decarboxylation of the acid in a cyclic manner, i.e., malonate was activated by acetyl-CoA:malonate CoA transferase to form malonyl-CoA which subsequently underwent decarboxylation by malonyl-CoA decarboxylase and the acetyl-CoA generated was used to activate free malonate in the second cycle as a CoA donor\(^{46}\). However, alternate malonate decarboxylases with ACP as a component of the enzyme were discovered in *Malonomonas rubra*\(^{5–7,20,29–31,76}\), *Klebsiella pneumoniae*\(^{20,32–35,78}\), *Acinetobacter calcoaceticus*\(^{24,43,49,50}\), and *P. fluorescens*\(^{24,44}\) and molecular biology-based analyses concerning the enzyme reaction mechanism have
been developed. In 1998, we reanalyzed the subunit composition of the *Pseudomonas* enzyme, and discovered subunits δ and ε in addition to subunits α, β, and γ[13]. Thus, it was found that malonate decarboxylase from *P. putida* (165 kDa) consists of the five different subunits α (60 kDa), β (33 kDa), γ (28 kDa), δ (13 kDa), and ε (30 kDa) with the composition 1:1:1:1:1. Since the ε subunit is loosely associated with the other four subunits, it is easily dissociated by purification such as a hydrophobic chromatography. The intact enzyme is catalytically active, while the enzyme lacking the ε subunit does not show any activity at all[14].

When the intact enzyme was incubated with [2-14C]malonyl-CoA, the δ subunit was labeled with radioactivity which in turn readily disappeared on incubation with cold malonate, indicating that the malonyl residue of [2-14C]malonyl-S-ACP was exchanged with cold malonate. The enzyme lacking the ε subunit could catalyze the transfer of the acyl residue of neither malonyl-CoA nor acetyl-CoA to the δ subunit at all. Thus, the subunits δ and ε were demonstrated to be ACP and malonyl-CoA:ACP transacylase, respectively.

Catalytic functions of the other three subunits were analyzed using the recombinant protein separately expressed in *Escherichia coli*, and the α subunit has been identified as an acetyl-S-ACP:malonate ACP transferase[11]. The complex of subunits β and γ functions as a malonyl-S-ACP decarboxylase, although the β subunit alone does not. The function of the γ subunit is still unclear, since the recombinant protein could not be obtained in soluble form despite many attempts.

From the results described above, we propose the decarboxylation mechanism of malonate as follows (Fig. 1A). First, the malonate is transferred to ACP (δ subunit) by exchanging the acetyl residue on the acetyl-S-ACP to form malonyl-S-ACP with the function of the α subunit, acetyl-S-ACP:malonate ACP transferase. Second, the malonyl-S-ACP is decarboxylated to regenerate acetyl-S-ACP by β and γ subunits, malonyl-S-ACP decarboxylase. The activation of malonate decarboxylase, however, is required in the initial step of malonate decarboxylation, i.e., the acylation of free ACP by the ε subunit, malonyl-CoA:ACP transacylase. The resulting acyl-S-ACP functions as the carrier of acyl residues in malonate decarboxylation in a cyclic manner.

There are two categories of malonate decarboxylases reported in bacteria. One is a biotin-dependent membrane enzyme and the other is a biotin-independent cytosolic enzyme. In the former from a microaerotolerant anaerobic bacterium, *Malonomonas rubra*, biotin carrier protein is responsible for the decarboxylation of malonyl-S-ACP[6][7,20,28-31,78]. The enzymes from aerobic and facultatively anaerobic bacteria, *Pseudomonas putida*[11,13,14,83], *P. fluorescens*[8,24,44], *Acinetobacter calcoaceticus*[8,24,43,49,50], and *Klebsiella pneumoniae*[20,32-35,78] belong to the latter category. However, the prosthetic group is common to both categories of malonate decarboxylase, i.e., every enzyme contains ACP with a covalently attached 2-(5'-phosphoribosyl)-3'-dephospho-CoA[6,20,50,78] (Fig. 1B). The ACP in malonate decarboxylase is clearly different from the ACP in the fatty acid synthase system in amino acid sequence as well as chemical structure of the prosthetic group.

There are also differences in subunit composition between malonate decarboxylases from *K. pneumoniae*[20,78], *A. calcoaceticus*[83], and *P. fluorescens*[8] and the enzyme from *P. putida*. The former consist of four subunits α, β, γ, and δ, and do not possess a malonyl-CoA:ACP transacylase corresponding to the ε subunit of the *P. putida* enzyme. In these bacteria, malonyl-CoA:ACP transacylase independently exists in the cytoplasm[20,50,78] or part of the proteins stoichiometrically associate with the four subunits[32], and participates in the initial step of malonate decarboxylation (Fig. 1A). On the other hand, the *Pseudomonas putida* enzyme is composed of five subunits including the ε subunit that is indispensable for initiation of the enzyme reaction. The ε subunit of the *P. putida* enzyme is endowed with an
essential function, specific affinity for short chain acyl-CoAs such as acetyl-CoA, malonyl-CoA, and acetocacetyl-CoA. With the aim of elucidating the characteristics of the \( \varepsilon \) subunit and cyclic reaction of this enzyme, the acyl-CoA cycling method for the determination of intracellular levels of acyl-CoAs and CoASH has been developed by us as described later.

**Malonate decarboxylase gene clusters in bacteria**

As shown in Fig. 2, the malonate decarboxylase (mdc) gene cluster of *P. putida* is composed of nine genes, *mdc-ABCDEGHLM* (Genbank accession no. AB017138\(^{13}\)). The five subunits \( \alpha, \beta, \gamma, \delta, \) and \( \varepsilon \) are encoded by *mdcA, mdcD, mdcE, mdcC*, and *mdcH*, respectively. Sequences with certain homology to the *E. coli* promoter sequence\(^{74}\) and a possible hairpin loop structure are located upstream of *mdcA* and downstream of *mdcM*, respectively. The *mdc* gene cluster, therefore, would form a transcriptional unit as an operon that is regulated by malonate. The *Klebsiella* mdc operon is regulated by MdcR belonging to the LysR family\(^{77}\) that functions as an activator for malonate decarboxylase transcription and as a repressor for its own transcription\(^{69}\), although the gene encoding such a regulatory protein has not been found in the region flanking the *Pseudomonas putida mdc* genes yet.

The first ORF, *mdcA*, encodes a protein of 553 amino acids with a predicted molecular mass of 61,386 Da, identified based on the N-terminal amino acid sequence of the purified protein as the \( \varepsilon \) subunit of malonate decarboxylase, acetyl-S-ACP:malonate ACP transferase. The putative protein sequence is 78, 58, and 56% identical to that of the \( \varepsilon \) subunit of *K. pneumoniae* (U95087)\(^{33}\) and *A. calcoaceticus* (U89347)\(^{49,50}\), and MadA of *M. rubra* (U87980)\(^{7}\), respectively (Fig. 2).

The *mdcC* gene codes for a 99-amino-acid polypeptide (10,728 Da), which is 51, 31, and 36% identical to the sequences of \( \delta \) subunits (ACPs) of *K. pneumoniae*\(^{33}\), *A. calcoaceticus*\(^{49,50}\), and MadE of *M. rubra*\(^{7}\), respectively. The most conserved region of the \( \delta \) subunits is located at the position 21-GCVSSGDLEVL-31. This region very likely corresponds to the attachment site of the prosthetic group, 2’-(5’-phosphoribosyl)-3’-dephospho-CoA, which is covalently attached via a phosphodiester linkage to serine of the \( \delta \) subunit\(^{6,7,20,33,49,50,78}\).

MdcD consists of 282 amino acids (29,966 Da). The deduced amino acid sequence of *mdcD* displays similarity with that of MdcD protein from *K. pneumoniae* (65% identical residues), MdcD from *A. calcoaceticus* (40%), and MadC from *M. rubra* (38%), which are malonyl-S-ACP decarboxylase subunits (\( \beta \) subunit). The protein encoded by the *mdcE* gene consists of 263 amino acid residues (27,861 Da) and represents the \( \gamma \) subunit of malonate decarboxylase. The protein is similar to MdcE from *K. pneumoniae* (63% identical residues), MdcE from *A. calcoaceticus* (27%), and MadD from *M. rubra* (31%), which act as a malonyl-S-ACP decarboxylase together with MdcD (\( \beta \) subunit). The deduced amino acid sequences of the subunits \( \beta \) and \( \gamma \) are homologous to various enzymes that catalyze the carboxylation or decarboxylation of acyl-CoA in a biotin-dependent system, although the malonate decarboxylase from *P. putida* malonate decarboxylase does not possess biotin. The \( \beta \) subunit (MdcD) conserves the flanking regions of the putative carboxybiont-binding sites\(^{47}\) in the \( \beta \) subunit of acetyl-CoA carboxylase from *E. coli*\(^{33,54,66}\), acetyl-CoA

![Fig. 2. Comparison of physical maps of malonate decarboxylase genes from *Pseudomonas putida*, *Klebsiella pneumoniae*, *Acinetobacter calcoaceticus*, and *Malonomonas rubra*. aa, Amino acid. %, Amino acid identities of gene products.](image-url)
carboxylase from rat(51), α subunit of methylmalonyl-CoA decarboxylase from *Archaeoglobus fulgidus*(52), and β subunit of propionyl-CoA carboxylase from rat(51). On the other hand, the region proposed to be a CoA-binding domain in the α subunit of *E. coli* acetyl-CoA carboxylase(53) is observed in MdcE (γ subunit), also existing in rat acetyl-CoA carboxylase(54), *A. fulgidus* methylmalonyl-CoA decarboxylase(55), and rat propionyl-CoA carboxylase(51). Based on these findings together with the enzyme activity mentioned above, the subunits β and γ are assigned as the malonyl-S-ACP decarboxylase subunits that would possess specific binding sites for the carboxyl group and ACP moiety of malonyl-S-ACP, respectively.

MdcH consists of 306 amino acids (32,623 Da) in the ε subunit, which catalyzes the transfer of the malonyl residue of malonyl-CoA to ACP, and is easily dissociated from the other subunits(44). The amino acid sequence alignment of the gene product shows high similarities with various malonyl-CoA-ACP transacylases (EC 2.3.1.39), e.g., MdcHs from *K. pneumoniae* (57%) and *A. calcoaceticus* (23%) and FabDs from *Bacillus subtilis* (30%, U59433)(56), *E. coli* (36%, Z11565 and M87040)(57,58,59), and *Haemophilus influenzae* (36%, U32701)(60). However, the histidine residue responsible for binding the malonyl moiety of malonyl-CoA in FabDs from *B. subtilis*, *E. coli*, and *H. influenzae* is not conserved in *Pseudomonas* and *Klebsiella* MdcHs. This substitution, therefore, may distinguish the ACP used in malonate metabolism from the ACP in fatty acid metabolism for substrate recognition.

MdcL (163 aa, 16,956 Da) and MdcM (254 aa, 25,707 Da) are very hydrophobic and certainly membrane proteins, being similar to counterparts from *M. rubra* and *A. calcoaceticus*(71,72). The malonate uptake activity of the mdcLM gene disruptant of *P. putida* into the cells is remarkably reduced, i.e., at most 15% of that accumulated in cells of the wild-type strain. Furthermore, this strain is unable to grow on malonate at all, although the wild-type strain can grow well. These results clearly show that MdcL and MdcM proteins together function as a malonate transporter, closely related to the functions of MadL and MadM reported in *M. rubra*(73,74).

The residual genes of the mdc gene cluster, *mdcB* and *mdcG*, are similar to counterparts from *K. pneumoniae*, showing 56% and 42% identity at the amino acid level, respectively. Recently, the MdcB and MdcG of *K. pneumoniae* were successfully expressed in *E. coli* and the functions were identified by Dimroth and his coworkers(34,35). Both proteins are accessory gene products required for the biosynthesis of the prosthetic group and its attachment to the apo-ACP (MdcC). MdcB catalyzes the condensation of dephospho-CoA with ATP to 2’-(5''-triphosphoribosyl)-3’-dephospho-CoA, ATP:dephospho-CoA 5’-triphosphoribosyl transferase. Subsequently, the prosthetic group precursor generated is attached to the apo-ACP by MdcG, 2’-(5’-triphosphoribosyl)-3’-dephospho-CoA:apo-ACP 2’-(5’-phosphoribosyl)-3’-dephospho-CoA transferase, and finally the synthesis of the functional δ subunit (holo-ACP) to allow malonate decarboxylation in a cyclic manner is completed.

The genes encoding the malonate decarboxylase of *P. putida* are clustered in one operon. Not only the homology of deduced amino acid sequences but also the mdc gene order from *P. putida* resembles that from *K. pneumoniae*. However, the mdcF gene observed in *K. pneumoniae*, which is the gene encoding a malonate transporter(26,33), does not exist in the *P. putida* mdc gene cluster and is replaced by *mdcL* and *M* genes following the mdcH gene. The sequences of the gene products of *mdcL* and *mdcM* are similar to

Table 1. Correlation of the Mdc proteins of the malonate decarboxylase gene cluster from *P. putida* with the Mdc proteins of *K. pneumoniae* and *A. calcoaceticus* and Mad proteins of *M. rubra*

<table>
<thead>
<tr>
<th><em>P. putida</em></th>
<th><em>K. pneumoniae</em></th>
<th><em>A. calcoaceticus</em></th>
<th><em>M. rubra</em></th>
<th>Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>MdcA</td>
<td>MdcA</td>
<td>MdcA</td>
<td>MadA</td>
<td>acetyl-S-ACP:malonate ACP transferase</td>
</tr>
<tr>
<td>MdcB</td>
<td>MdcB</td>
<td>MdcB</td>
<td>MadG</td>
<td>ATP:dephospho-CoA 5’-triphosphoribosyl transferase</td>
</tr>
<tr>
<td>MdcC</td>
<td>MdcC</td>
<td>MdcC</td>
<td>MadE</td>
<td>acyl carrier protein (ACP)</td>
</tr>
<tr>
<td>MdcD, E</td>
<td>MdcD, E</td>
<td>MdcD, E</td>
<td>MadC, D</td>
<td>malonyl-S-ACP decarboxylase</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td></td>
<td></td>
<td>carboxyltransferase</td>
</tr>
<tr>
<td>MdcG</td>
<td>MdcG</td>
<td>MdcG</td>
<td>2’-(5’-triphosphoribosyl)-3’-dephospho-CoA:apo-ACP</td>
<td>2’-(5’-phosphoribosyl)-3’-dephospho-CoA transferase</td>
</tr>
<tr>
<td>MdcH</td>
<td>MdcH</td>
<td>MdcH</td>
<td>MadH</td>
<td>malonyl-CoA:ACP transferase</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td></td>
<td>MadL, M</td>
<td>ACP:acetate ligase</td>
</tr>
<tr>
<td>MdcL, M</td>
<td>MdcF</td>
<td>MdcL, M</td>
<td>MadL, M</td>
<td>malonate transporter</td>
</tr>
</tbody>
</table>
those of madL and madM from M. rubra, respectively. The gene cluster encoding the malonate decarboxylase of P. putida, therefore, seems to be a fusion of mdc genes of K. pneumoniae and mad genes of M. rubra. On the other hand, the gene species are completely identical with Acinetobacter mdc genes. Table 1 summarizes the functions of the mdc and mad gene products, and correlation of these proteins to each other.

**Application of P. putida malonate decarboxylase to the measurement of intracellular acyl-CoA levels**

**Acyl-CoA cycling method**

Based on a specific property of the cyclic reaction, we applied P. putida malonate decarboxylase to the measurement of short chain acyl-CoAs\(^{16,84}\). The enzymatic analysis is simple and sufficiently sensitive to detect pmol levels of the acyl-CoAs. The reaction mixture for the acyl-CoA cycling method contains one unit of malonate decarboxylase\(^{12,15,16,23,65,84,86}\), corresponding to approximately 300 pmol of the enzyme, i.e., 300 pmol of nonesterified ACP (δ subunit). The free ACP is acylated by the ε subunit in proportion to the amount of short chain acyl-CoAs given, the acyl residue of acyl-S-ACP formed is replaced with malonate to generate malonyl-S-ACP. Then, malonyl-S-ACP was decarboxylated by the subunits β and γ to yield acetyl-S-ACP, which in turn is used as the substrate for acetyl-S-ACP:malonate ACP transferase (α subunit) in a cyclic manner. One cycle of this reaction generates one molecule of acetate. After a sufficient reaction time, the acetate amplified is spectrophotometrically measured by an indicator reaction; phosphorylation by acetate kinase (EC 2.7.2.1) to acetyl phosphate, followed by conversion to acetohydroxamate. Under the reaction conditions we employed, the cyclic reaction rate is 97,500 cycle/h when malonyl-CoA was used as an acyl donor. The log-log plots between the amount of malonyl-CoA given and acetohydroxamate formed are linear within 2.5–80 pmol/tube (Fig. 3). Besides malonyl-CoA, the ε subunit recognizes acetyl-CoA, acetoacetyl-CoA, propionyl-CoA, butyryl-CoA, and methylmalonyl-CoA as active acyl donors to free ACP.

Since the apparent \(K_m\) values against malonyl-CoA and acetyl-CoA are particularly low (10\(^{-8}\) M), the method is available to determine in vivo concentrations of acetyl-CoA and malonyl-CoA. Separate assays are also possible to differentiate among acetyl-CoA, malonyl-CoA, and CoASH following pretreatment of the samples with citrate synthase (EC 4.1.3.7)\(^{84}\) and phosphate acetyltransferase (EC 2.3.1.8)\(^{18}\).

**Changes in size and composition of the intracellular CoA pool in aerobic and facultatively anaerobic bacteria**

Intracellular levels of three CoA molecular species in a variety of aerobic and facultatively anaerobic bacteria were analyzed using the acyl-CoA cycling method\(^{12,15,16,23,65,84,86}\). It has been demonstrated that there is a remarkable difference in the size and composition of intracellular pools of CoASH and CoA thioesters between aerobic bacteria and facultatively anaerobic bacteria. The intracellular pools of CoASH and CoA thioesters are significantly larger in facultatively anaerobic bacteria (Table 2 and Fig. 4). Also, the composition of CoA pools changes drastically within minutes in response to environmental stress such as the carbon source in the medium, growth phase, pH, incubation temperature, osmotic stress, and antibiotics to inhibit energy yielding systems and fatty acid biosynthesis\(^{12,15}\). Generally in facultatively anaerobes, a large acetyl-CoA pool is formed when cells are grown on sufficient glucose, while CoASH is a predominant component of cells starved for a carbon source. The total CoA pool (the sum of the concentrations of CoASH, acetyl-CoA, and malonyl-CoA) of E. coli, remains within the limits of 0.83–1.40 nmol/mg of dry cell weight (0.30–0.52 mM). Therefore, the increase in the level of acetyl-CoA correlates almost stoichiometrically with the decrease of CoASH, and vice versa (Fig. 4). Inter-
Interestingly, when fatty acid biosynthesis is inhibited by antibiotics, the increase in malonyl-CoA accompanies a decrease in acetyl-CoA\(^{15}\). Our results are consistent with the reports of Jackowski and her coworkers that the biosynthetic sequence of CoASH in *E. coli* is tightly regulated at the pantothenate kinase step\(^{38–40,72,73,80,89,90,91}\). On the other hand, the CoA pools in aerobic bacteria are smaller and the composition hardly changes, even when sufficient glucose is

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**Table 2. Size and composition of CoA pools in various bacteria**

<table>
<thead>
<tr>
<th>Strain</th>
<th>CoASH</th>
<th>Acetyl-CoA</th>
<th>Malonyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Alcaligenes faecalis</em> IB-14(^c)</td>
<td>1,003</td>
<td>51</td>
<td>Trace</td>
</tr>
<tr>
<td><em>Pseudomonas ovalis</em> IAM 1177</td>
<td>553</td>
<td>217</td>
<td>9</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em> IFO 3242</td>
<td>411</td>
<td>43</td>
<td>4</td>
</tr>
<tr>
<td><em>Corynebacterium glutamicum</em> IAM 12435</td>
<td>128</td>
<td>51</td>
<td>Trace</td>
</tr>
<tr>
<td><em>Brevibacterium ammoniagenes</em> IAM 1641</td>
<td>278</td>
<td>132</td>
<td>11</td>
</tr>
<tr>
<td><em>Brevibacterium helvolum</em> IFO 12073</td>
<td>449</td>
<td>347</td>
<td>Trace</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> IAM 1069</td>
<td>213</td>
<td>71</td>
<td>Trace</td>
</tr>
<tr>
<td><em>Arthrobacter globiformis</em> IFO 12137</td>
<td>231</td>
<td>32</td>
<td>Trace</td>
</tr>
</tbody>
</table>

**Facultative anaerobes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>CoASH</th>
<th>Acetyl-CoA</th>
<th>Malonyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> ME6200</td>
<td>182</td>
<td>680</td>
<td>11</td>
</tr>
<tr>
<td><em>Escherichia coli</em> K-12 IAM 1246</td>
<td>113</td>
<td>588</td>
<td>Trace</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em> subsp. pneumoniae strain 19–35(^c)</td>
<td>458</td>
<td>655</td>
<td>13</td>
</tr>
</tbody>
</table>

**Aerotolerant anaerobes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>CoASH</th>
<th>Acetyl-CoA</th>
<th>Malonyl-CoA</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Lactobacillus homohiochii</em> 55(^d)</td>
<td>ND(^b)</td>
<td>1,300</td>
<td>75</td>
</tr>
<tr>
<td><em>Lactobacillus heterohiochii</em> 56(^d)</td>
<td>ND(^b)</td>
<td>760</td>
<td>130</td>
</tr>
</tbody>
</table>

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\(^a\) The bacteria were grown aerobically at 30°C. Intracellular CoASH, acetyl-CoA, and malonyl-CoA in cells harvested at an early stationary phase were extracted and assayed using the acyl-CoA cycling method.

\(^b\) ND, not determined.

\(^c\) This strain was isolated by our laboratory.

\(^d\) This strain was obtained from National Research Institute of Brewing, 3–7–1 Kagamiyama, Higashihiroshima, Hiroshima, 739–0046, Japan.

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Fig. 4. Changes in the size and composition of the CoA pool of facultatively anaerobic and aerobic bacteria in a transfer experiment. Facultative anaerobes, *Escherichia coli* K-12 (A) and *Klebsiella pneumoniae* subsp. *pneumoniae* strain 19–35 isolated by our laboratory (B), and aerobes, *Pseudomonas putida* IAM 1177 (C) and *Flabobacterium aquatile* IAM 12316 (D), were grown on glucose mineral salts medium at 30°C aerobically, and the cells were transferred to fresh medium supplemented with glucose at a cell density giving an *A*\(_{660}\) of 1.0 for *E. coli* and an *A*\(_{660}\) of 2.0 for *K. pneumoniae*, *P. putida*, and *F. aquatile*. After the indicated period of time, intracellular levels of CoASH (open circle), acetyl-CoA (closed circle), and malonyl-CoA (open triangle) were determined by the acyl-CoA cycling method (see reference 15).
provided in the medium (Fig. 4). The profile of changes in the CoA pool in aerobic bacteria is very similar to that of facultatively anaerobic bacteria starved for glucose.

The acylation of CoASH and deacylation of acetyl-CoA and/or malonyl-CoA to CoASH coordinate with in vivo conditions of the energy-yielding systems and fatty acid synthesis. In aerobic bacteria, a large acetyl-CoA pool is not formed because it would be rapidly converted to citrate and free CoASH by citrate synthase of the TCA cycle. This explains why CoASH is the predominant component of the intracellular CoA pool in aerobic bacteria. On the other hand, because of the low ATP yield by fermentation, facultatively anaerobic bacteria are obliged to consume large amounts of carbon sources to fulfill ATP demands for cell growth, and consequently, acylated intermediates of CoASH, especially acetyl-CoA, are more prevalent than CoASH in facultative anaerobes. It has been reported that pyruvate dehydrogenase from E. coli is inhibited by acetyl-CoA in vitro. This might not be true in vivo, since abundant acetyl-CoA was formed in E. coli cells provided with sufficient glucose. Even during the aerobic growth of E. coli with vigorous shaking, a large amount of acetyl-CoA was accumulated, suggesting that the metabolic flux of acetyl-CoA to citrate via citrate synthase might not be so functional in facultative anaerobes. Accordingly, E. coli and related facultative anaerobes utilize glucose to form acetyl-CoA in a wasteful way leading to excretion of the metabolite into the medium. Thus, the differences in the behavior of intracellular CoA pools between aerobes and facultative anaerobes might be due primarily to the difference in the energy-yielding systems.

Malonyl-CoA is a minor component of the CoA pool in normally grown cells, but it dramatically increases to being systems.

**Conclusions**

Malonate is not extensively distributed in nature. This compound, however, is produced industrially in Japan, the Republic of Korea, Switzerland, and Germany. In particular, its dimethyl and diethyl esters are of technical importance and the total capacity is estimated to be about 12,000 tons per year. Furthermore, producers are also reported in the People’s Republic of China and in Romania. Therefore, as more malonate appears in the environment, its degradation by the bacteria possessing malonate decarboxylase, especially *Pseudomonas putida*, is of interest.

Malonate decarboxylase has a rational reaction mechanism for the decarboxylation of malonate. Once free ACP (δ subunit) is acylated with acetyl-CoA or malonyl-CoA via the function of the ε subunit, the reactions of acetyl-S-ACP:malonate ACP transferase (α subunit) and malonyl-S-ACP decarboxylase (subunits β and γ) proceed in a cyclic manner, and then acetate is formed. Every bacterium able to grow on malonate possesses malonate decarboxylase, the synthesis of which is induced at 10 to 100-fold higher by malonate or its homologues. Analyses of flanking regions of the genes encoding the malonate decarboxylases have clearly elucidated the induction mechanism. However, the properties of the gene products of MdcE (δ subunit) identified as malonyl-S-ACP decarboxylase and MdcLM as the malonate transporter remain unclear. Furthermore, it is of interest whether the ε subunit (malonyl-CoA:ACP transacylase) loosely associated with the *Pseudomonas* malonate decarboxylase is complementary to the malonylation of ACP in the fatty acid synthesis system. The significance of the enzyme in intracellular metabolism may be to link the β-alanine metabolic pathway to an energy-yielding pathway, because *Pseudomonas* possesses a unique metabolic pathway leading to malonate from β-alanine through malonate semialdehyde (3-oxopropanoate).

The acyl-CoA cycling method developed on the basis of the cyclic reaction of malonate decarboxylase is sufficiently sensitive and specific for monitoring in vivo levels of acetyl-CoA, malonyl-CoA, and CoASH in prokaryotic and eukaryotic cells. Most recently, malonyl-CoA was identified as a possible mediator of the hypothalamic signaling pathway that monitors energy status in mammals, and the “malonyl-CoA hypothesis” has been demonstrated using this assay method. In another field, pantothenate kinase being a key enzyme of CoA biosynthesis has attracted our attention concerning the development of novel drugs against antibiotic-resistant pathogens.
Pseudomonas aeruginosa, Staphylococcus aureus, and Bacillus anthracis were missing from their genomes, although the other genes involved in the CoA biosynthetic pathway were found by BLAST search using related sequences. This meant that the pantothenate kinases of the above pathogens were completely different from known enzymes. In 2003, the gene encoding the Staphylococcus aureus enzyme was cloned, and actually its inhibitor was synthesized with the intention of using it as a novel and specific antibiotic. Thus the in vivo regulation of metabolic pathways involving CoASH and/or CoA thioesters is now actively under investigation in cell physiology and medical sciences, and the acyl-CoA cycling method has received much attention as a useful tool in these fields.

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References

Malonate Decarboxylase

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