APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1997, p. 553–560 Vol. 63, No. 2
0099-2240/97/$04.00+0

Changes in the Size and Composition of Intracellular Pools of Nonesterified Coenzyme A and Coenzyme A Thioesters in Aerobic and Facultatively Anaerobic Bacteria

SHIGERU CHOHNAN, HIDEKI FURUKAWA, TOORU FUJIO, HIROFUMI NISHIHARA, AND YOSHICHIKA TAKAMURA*

Department of Bioresource Sciences, School of Agriculture, Ibaraki University, Ami-machi, Ibaraki 300-03, Japan

Received 11 July 1996/Accepted 7 November 1996

Intracellular levels of three coenzyme A (CoA) molecular species, i.e., nonesterified CoA (CoASH), acetyl-CoA, and malonyl-CoA, in a variety of aerobic and facultatively anaerobic bacteria were analyzed by the acyl-CoA cycling method developed by us. It was demonstrated that there was an intrinsic difference between aerobes and facultative anaerobes in the changes in the size and composition of CoA pools. The CoA pools in the aerobic bacteria hardly changed and were significantly smaller than those of the facultatively anaerobic bacteria. On the other hand, in the facultatively anaerobic bacteria, the size and composition of the CoA pool drastically changed within minutes in response to the carbon and energy source provided. Acetyl-CoA was the major component of the CoA pool in the facultative anaerobes grown on sufficient glucose, although CoASH was dominant in the aerobes. Therefore, the acetyl-CoA/CoASH ratios in facultatively anaerobic bacteria were 10 times higher than those in aerobic bacteria. In *Escherichia coli* K-12 cells, the addition of reagents to inhibit the respiratory system led to a rapid decrease in the amount of acetyl-CoA with a concomitant increase in the amount of CoASH, whereas the addition of cerulenin, a specific inhibitor of fatty acid synthase, triggered the intracellular accumulation of malonyl-CoA. The acylation and deacylation of the three CoA molecular species coordinated with the energy-yielding systems and the restriction of the fatty acid-synthesizing system of cells. These data suggest that neither the accumulation of acetyl-CoA nor that of malonyl-CoA exerts negative feedback on pyruvate dehydrogenase and acetyl-CoA carboxylase, respectively.

Since adenine nucleotides and adenine nucleotide cofactors regulate the activities of a large number of enzyme reactions and probably all metabolic sequences, numerous investigators have attempted to define the pool sizes of cellular nucleotides (for a review, see reference 21) such as ATP (3), cyclic AMP (26), NAD, and NADP (25). Thus, "adenylate energy charge" (3, 4, 8, 9) and "reduction charge" (1) parameters have been established. Compared with the data on adenine nucleotides and nicotinamide nucleotides in microbial cells, relatively few reports are available on the intracellular pools of nonesterified coenzyme A (CoASH) and its thioesters, even though they are essential intermediates in numerous biosynthetic and energy-yielding metabolic pathways as well as regulators of several key metabolic reactions.

Jackowski and her coworkers (20, 40, 41) demonstrated that the coenzyme A (CoA) pools in *Escherichia coli* usually grown on glucose were composed of acetyl-CoA, succinyl-CoA, malonyl-CoA, and CoASH, by using a radioisotopic method with a β-alanine auxotroph of *E. coli* that requires β-alanine for growth to manipulate the specific cellular CoASH and its derivative contents. This method is highly specific and sensitive, but its application was limited exclusively to β-alanine auxotrophs. Recently, Boynton et al. (6) analyzed intracellular levels of CoA and its derivatives involved in the metabolic pathways of *Clostridium acetobutylicum* ATCC 824 by using reverse-phase high-performance liquid chromatography.

Takamura et al. (37) and Chohnan and Takamura (10) have developed a specific micromethod (acyl-CoA cycling method) for the measurement of acetyl-CoA, malonyl-CoA, and CoASH by using malonate decarboxylase (EC 2.8.3.3) as an amplification apparatus of the CoA molecular species mentioned above. This nonisotopic method is sufficiently sensitive to detect the intracellular level of CoASH and short-chain acyl-CoA thioesters in both prokaryotic and eukaryotic cells (10, 38). Then we revealed the following events by using this simple micromethod (10). Intracellular CoASH in *E. coli* K-12 cells was rapidly acylated to acetyl-CoA when assimilable carbon sources were provided, and acetyl-CoA was deacylated to CoASH when carbon sources in the medium were depleted. There is an inverse relationship between CoASH and short-chain acyl-CoAs, particularly acetyl-CoA. Furthermore, it was demonstrated that the thiolactomycin [(4S)(2E,5E)-2,4,6-trimethyl-3-hydroxy-2,5,7-octatriene-4-thiolide] resistance of *E. coli* CDM5 is associated with the multidrug resistance efflux pump (24), by defining the changes in the malonyl-CoA pool in the presence of the drugs together with performing a genetic analysis of *E. coli* CDM5 (14).

These findings with intracellular concentrations of CoASH and its thioesters were obtained exclusively in a study of *E. coli* cells, and there have been few reports on the size and composition of the CoA pools of other facultatively anaerobic and aerobic bacteria. In this article, we show that there is an intrinsic difference in the size and composition of the CoA pools between facultatively anaerobic and aerobic bacteria and that they change drastically within minutes in response to environmental stresses in *E. coli* K-12 cells.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* K-12 IAM 1246, *E. coli* ME6200, and *Klebsiella pneumoniae* subsp. *pneumoniae* 19-35 were used as representative strains of facultatively anaerobic bacteria, and *Pseudomonas putida* IAM 1177, *Alcaligenes faecalis* IB-14, *Micrococcus luteus* IFO 3242,

* Corresponding author. Mailing address: Department of Bioresource Sciences, School of Agriculture, Ibaraki University, 3-21-1, Ami-machi, Ibaraki 300-03, Japan. Phone: (81) 298 88 8672. Fax: (81) 298 88 8672. E-mail: takamura@ami.ipc.ibaraki.ac.jp.

Corynebacterium glutamicum IAM 12435, *Corynebacterium ammoniagenes* IAM 1641, *Brevibacterium helvolum* IFO 12073, *Bacillus subtilis* IAM 1069, *Arthrobacter globiformis* IFO 12137, and *Flavobacterium aquatile* IAM 12316 were used as representative strains of aerobic bacteria. As representative strains of aerotolerant anaerobic bacteria, *Lactobacillus homohiochii* 55 and *Lactobacillus fructivorans* 56 were used. For cultivation of *E. coli* K-12, *K. pneumoniae*, *P. putida*, *A. globiformis*, and *F. aquatile*, a glucose-mineral salts medium was used. This medium contained 5 g of KH_2PO_4, 13 g of K_2HPO_4, 0.2 g of $MgCl_2$, 2 g of $(NH_4)_2SO_4$, and 5 g of glucose as the sole carbon source in 1 liter of distilled water (pH 7.0). Cultivation was at 30°C with reciprocal shaking in 500-ml flasks containing 100 ml of medium. *E. coli* ME6200, which required biotin for growth, was cultivated aerobically at 30°C in medium containing 5 g of KH_2PO_4, 13 g of K_2HPO_4, 0.2 g of $MgCl_2$, 2 g of $(NH_4)_2SO_4$, 1 mg of thiamine, 2 g of Casamino Acids (vitamin free), 5 g of glucose, and 25 μg of biotin in 1 liter of distilled water (pH 7.0). For cultivation of *M. luteus*, *C. glutamicum*, *C. ammoniagenes*, *B. helvolum*, and *B. subtilis*, the medium contained 1.5 g of KH_2PO_4, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, 10 g of $(NH_4)Cl$, 10 g of glucose, 1 g of yeast extract, and 5 g of peptone in 1 liter of distilled water (pH 7.0). *A. faecalis* was grown aerobically in the above medium containing 10 g of DL-malic acid instead of glucose. For both strains of *Lactobacillus*, the medium contained 5 g of yeast extract, 5 g of polypeptone, 10 g of glucose, 0.5 g of KCl, 0.2 g of KH_2PO_4, 0.1 g of $CaCl_2 \cdot 6H_2O$, 0.1 g of $MgSO_4 \cdot 7H_2O$, 1 mg of $FeCl_3 \cdot 6H_2O$, 1 mg of $MnSO_4 \cdot 5H_2O$, 20 g of sodium acetate, 5 mg of DL-mevalolactone, and 60 ml of ethanol in 1 liter of distilled water (pH 5.0). For the cultivation of strain 55, 1 g of sodium ascorbate was added to this medium. The cultivation was carried out at 30°C in 500-ml Erlenmeyer flasks containing 300 ml of medium, anaerobically. Growth was monitored by measuring the absorbance at 660 nm (A_{660}).

Transfer experiment. To define the short-term changes in the CoA pool, transfer experiments were conducted as follows. The bacteria were grown aerobically in the glucose-mineral salts medium. Cells of the late stationary growth phase were harvested by centrifugation at 12,000 × *g* at 20°C for 10 min and transferred to 20 ml of the fresh medium prewarmed at 30°C without glucose in 100-ml Erlenmeyer flasks at a cell density giving an A_{660} of 1.0 for *E. coli* K-12 and an A_{660} of 2.0 for *K. pneumoniae*, *P. putida*, and *F. aquatile*. Replacement culture was started by the addition of glucose with reciprocal shaking at 30°C.

Extraction of CoASH, acetyl-CoA, and malonyl-CoA. To extract the intracellular pools of CoASH, acetyl-CoA, and malonyl-CoA, the cells were collected by filtering a few milliliters of culture broth through a cellulose nitrate filter (Sartorious; pore size, 0.45 μm) (10, 14, 29). The cells on the filter were immediately immersed in 4 ml of ice-cold 0.3 M sulfuric acid in a centrifuge tube. For complete extraction of the intracellular CoASH and its thioesters in the cells, the cells in the centrifuge tube were vortex mixed (2,500 rpm) at 4°C for 20 min. When the culture broth was viscous and filtration through the cellulose nitrate filter was difficult, the cells were collected by centrifugation at 12,000 rpm (17,000 × *g*) at 4°C for 10 min and suspended in 4 ml of ice-cold 0.3 M sulfuric acid. The cell suspension was subjected to sonic disintegration at 0°C for 4 min at 50 W and then centrifuged at 12,000 rpm (17,000 × *g*) for 5 min. The supernatant was carefully adjusted to pH 6.0 with sodium hydroxide on ice. CoASH, acetyl-CoA, and malonyl-CoA in the neutralized extract were stable for at least 50 h at 4°C (29).

The acyl-CoA cycling assay. The reaction mixture for the acyl-CoA cycling assay (37) contained 50 mM Tris-HCl (pH 7.2), 1 mM 2-mercaptoethanol, 10 mM magnesium sulfate, 50 mM malonate, 10 mM ATP, 1.0 U of malonate decarboxylase, and cell extracts containing acetyl-CoA and/or malonyl-CoA (2.5 to 80 pmol). The cycling reaction was initiated by the addition of malonate decarboxylase, and the reaction mixture was incubated at 30°C for 20 min, followed by the addition of 1.0 U of acetate kinase (EC 2.7.2.1). After 20 min of incubation, 0.2 ml of 2.5 M neutralized hydroxylamine was added, and the incubation was continued for an additional 20 min at 30°C. The reaction was terminated by adding 0.6 ml of 10 mM ferric chloride dissolved in 25 mM trichloroacetic acid–1 M HCl. The A_{540} of the acetylhydroxamate which had formed was measured. Under the conditions described above, the moles of acetylhydroxamate formed per mole of acetyl-CoA added corresponded to 97,500 cycles per h. A separate determination of malonyl-CoA in the cell extract was conducted after the enzymatic conversion of acetyl-CoA to citrate and CoASH by using citrate synthase (EC 4.1.3.7) (37). The reaction mixture contained 50 mM Tris-HCl (pH 7.2), 10 mM magnesium sulfate, 1 mM 2-mercaptoethanol, 2 mM oxaloacetate, and 1.32 U of citrate synthase in a total volume of 1.0 ml. The reaction mixture was incubated for 20 min at 30°C. The reaction was terminated by placing the reaction tube in an ice slush. Free CoASH was measured following its enzymatic conversion to acetyl-CoA by using phosphoacetyltransferase (EC 2.3.1.8) (10). The reaction mixture contained 50 mM Tris-HCl (pH 7.2), 10 mM magnesium sulfate, 1 mM 2-mercaptoethanol, 10 mM ammonium sulfate, 0.1 mM acetylphosphate, 1.0 U of phosphoacetyltransferase, and cell extract containing CoASH in a total volume 1.0 ml. The mixture was incubated at 30°C for 20 min, and the reaction was terminated by filtration through a Millipore UFP1 LGC ultrafilter to remove the phosphoacetyltransferase. Every assay was conducted in duplicate, and all results were expressed as means of two samples. The intracellular concentrations of CoASH, acetyl-CoA, and malonyl-CoA were expressed in nanomoles per milligram of dry cell weight, which was calculated on the assumption that the volume of internal water in the organisms was 2.7 μl/mg of dry cell weight (43).

Measurement of glucose. The residual glucose in the culture broth was measured by the phenol-sulfuric acid method (13).

Chemicals and enzymes. CoASH, acetyl-CoA, and malonyl-CoA were obtained from Sigma Chemical Co. Phosphoacetyltransferase, acetate kinase, and citrate synthase were obtained from BMY Chemicals. Malonate decarboxylase was purified from *P. putida* IAM 1177 as described previously (36). All other materials were reagent grade or better.

RESULTS

Changes in the size and composition of the CoA pool in *K. pneumoniae* subsp. *pneumoniae* and *P. putida* during growth. Changes in the size and composition of CoASH and short acyl-CoA in the cells of *K. pneumoniae* and *P. putida* during growth in the glucose-mineral salts medium were examined. As shown in Fig. 1A, the *K. pneumoniae* cells harvested at the early stationary growth phase (12 h) had a maximal level of acetyl-CoA, i.e., 1.88 nmol/mg of dry cell weight. When the organisms had completely entered the stationary growth phase (20 h) and glucose in the medium was exhausted, the acetyl-CoA level fell rapidly and the CoASH level increased until CoASH was the predominant component of the CoA pool, at a maximal level of 0.96 nmol/mg of dry cell weight. Malonyl-CoA was a minor component throughout the growth period. The profile of changes in the CoA pool observed in *K. pneumoniae* was very similar to that of *E. coli* K-12 as reported previously (10). These results indicate that acetyl-CoA was the major component of the CoA pool in facultatively anaerobic bacteria when the cells were grown on sufficient glucose, whereas CoASH was the predominant component when the cells were starved for carbon sources.

In contrast to facultatively anaerobic bacteria, the size and composition of the CoA pool in cells of *P. putida*, an aerobic bacterium, were hardly changed during growth in the same medium (Fig. 1B). Although glucose in the medium was rapidly assimilated by *P. putida* cells in the logarithmic phase of growth, intracellular CoASH and acetyl-CoA were at low levels, and drastic changes such as those in the *K. pneumoniae* cells were not observed. When the organism entered the stationary growth phase (20 h), CoASH, not acetyl-CoA, was the major component of the CoA pool; maximal levels of CoASH and acetyl-CoA were 0.55 and 0.15 nmol/mg of dry cell weight, respectively. After prolonged cultivation, the amounts of acetyl-CoA and CoASH decreased gradually and the size of the CoA pool in the cells harvested after 72 h of cultivation reached a very low level of 0.10 nmol/mg of dry cell weight. Similar results were also obtained with another aerobic bacterium, *F. aquatile*. In the *F. aquatile* cells grown on the glucose-mineral salts medium, CoASH was the major component of the CoA pool, and its maximal level was 0.14 nmol/mg of dry cell weight (data not shown). There was a striking difference between the aerobic and facultatively anaerobic bacteria in the profile of changes in the CoA pool during growth. In addition, the CoA pool in facultatively anaerobic bacteria was about three times as large as that in aerobic bacteria.

Changes in CoA pools in short-term transfer experiments. To further define the differences in the size and composition of CoA pools between facultatively anaerobic and aerobic bacteria, short-term transfer experiments were used. Both the facultatively anaerobic bacteria, *E. coli* K-12 and *K. pneumoniae*, which had been cultured for 48 h in the glucose-mineral salts medium, were transferred to the fresh medium and incubated aerobically at 30°C. As shown in Fig. 2A and B, at the beginning of incubation, CoASH was the major component (72 to 75%) of the CoA pool in both organisms, since the 48-h-cultured cells had been starved for glucose. After 10 min of incubation, the acetyl-CoA level rapidly increased and reached

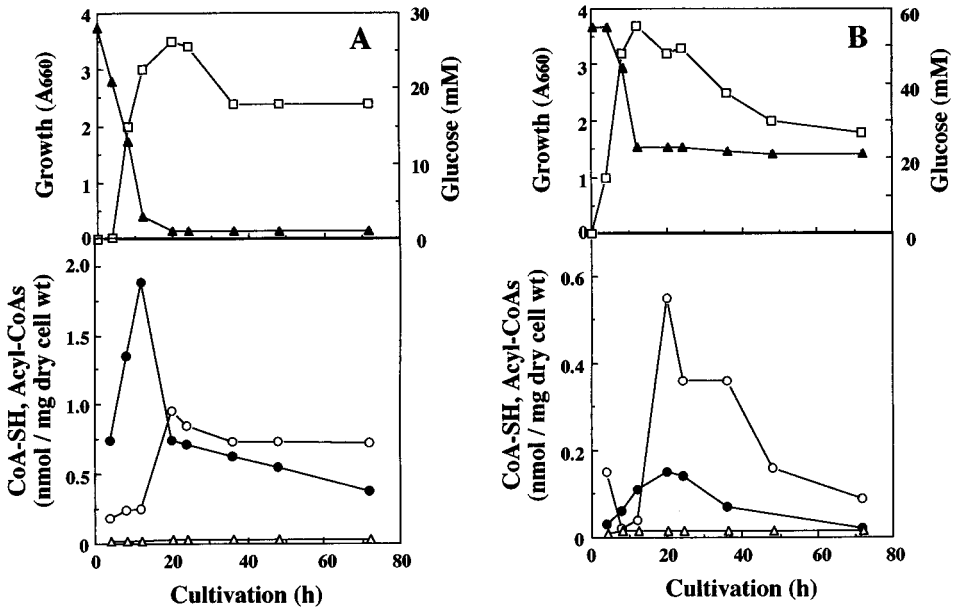

FIG. 1. Changes in the size and composition of the CoA pool of *K. pneumoniae* cells (A) and *P. putida* cells (B). The organisms were grown aerobically in glucose-mineral salts medium with shaking at 30°C. The contents of CoASH (○), acetyl-CoA (●), and malonyl-CoA (△) of the cells harvested at the indicated times were determined by the acyl-CoA cycling method (see Materials and Methods). Residual glucose in the medium (▲) and cell growth (□) were measured by the phenol-sulfuric acid method and by monitoring the A_{660}, respectively.

a maximal value of 0.82 nmol/mg of dry cell weight in *E. coli* K-12 and 0.90 nmol/mg of dry cell weight in *K. pneumoniae*, to become the predominant component of the CoA pools (67%) in both bacteria. The CoASH level decreased inversely in proportion to the increase in the acetyl-CoA level, from 0.63 to 0.23 nmol/mg of dry cell weight in *E. coli* K-12 and from 0.96 to 0.45 nmol/mg of dry cell weight in *K. pneumoniae* in 30 min. Malonyl-CoA was a minor CoA thioester in the two facultative anaerobes, although a slight increase in its level was observed in *E. coli* K-12. These short-term transfer experiments showed that the size and composition of the CoA pools in the two facultatively anaerobic bacteria changed drastically within minutes in response to the addition of the carbon and energy substrate and that there was an inverse relationship between the CoASH and acetyl-CoA levels in vivo, similar to that observed in growing cultures (Fig. 1A).

As for the short-term transfer experiments of the aerobic bacteria *P. putida* and *F. aquatile*, cells at the stationary growth phase were used similarly to the facultatively anaerobic bacteria. The size and composition of the CoA pools of both aerobic bacteria changed little during incubation (Fig. 2C and D). In *P. putida* cells, CoASH was the major component of the CoA

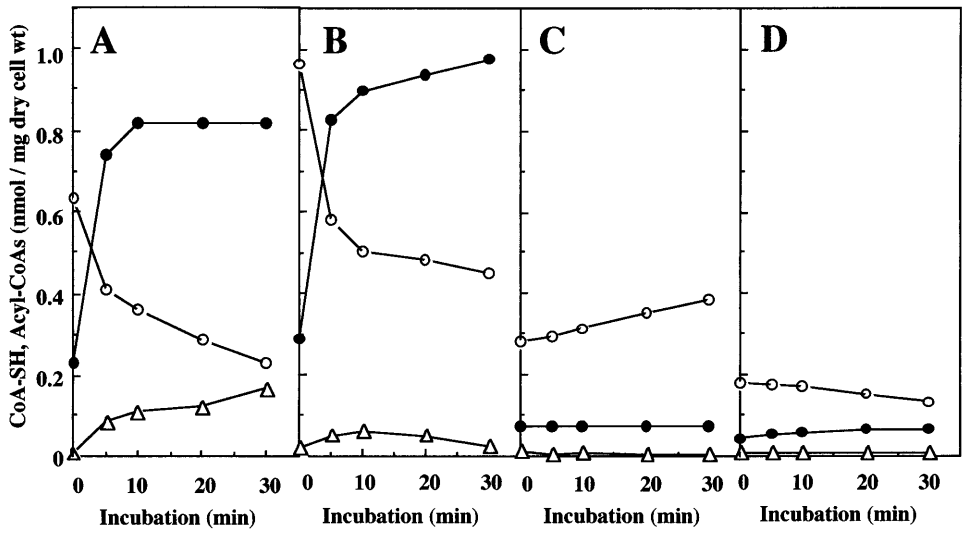

FIG. 2. Changes in the size and composition of the CoA pool of facultatively anaerobic and aerobic bacteria in a transfer experiment. *E. coli* K-12 (A), *K. pneumoniae* (B), and *F. aquatile* (D) were grown for 48 h, and *P. putida* (C) was grown for 18 h in the glucose-mineral salts medium at 30°C 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 times, the intracellular CoASH (○), acetyl-CoA (●), and malonyl-CoA (△) contents were determined (see Materials and Methods).

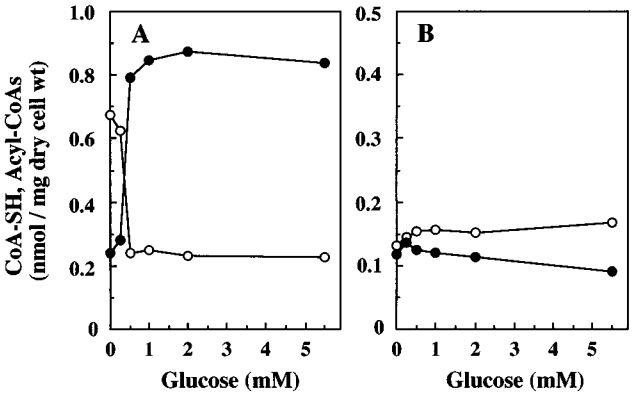

FIG. 3. Effects of glucose concentration on the intracellular CoA pools of *E. coli* K-12 (A) and *F. aquatile* (B). *E. coli* K-12 (A) and *F. aquatile* (B) were grown aerobically in the glucose-mineral salts medium for 48 h at 30°C. The cells harvested were transferred to fresh medium, and the indicated concentrations of glucose were added. After the cells were aerobically incubated for 10 min at 30°C, intracellular CoASH (○) and acetyl-CoA (●) levels were assayed (see Materials and Methods).

pool, and its level increased gradually from an initial value of 0.28 nmol/mg of dry cell weight to 0.38 nmol/mg in 30 min. The acetyl-CoA level remained unchanged at lower values of 0.07 nmol/mg of dry cell weight (Fig. 2C). In *F. aquatile* cells, CoASH was also the predominant component of the CoA pool, and the CoA pool composition was hardly changed (Fig. 2D). In either case, the size and composition of the CoA pool of aerobic bacteria changed little during incubation and the CoA pool size of aerobic bacteria was significantly smaller than those of the facultatively anaerobic bacteria. These phenomena were also observed in the growing-culture experiments (Fig. 1).

Response of the CoA pool to glucose concentrations. Figure 3 shows the effect of the glucose concentration on the size and composition of the CoA pools of *E. coli* K-12 and *F. aquatile*. For *E. coli* K-12, the cells with an initial CoASH level of 0.67 nmol/mg of dry cell weight and an acetyl-CoA level of 0.24 nmol/mg of dry cell weight were transferred to fresh media containing different concentrations of glucose, as indicated in Fig. 3A. The CoA pool composition in the *E. coli* cells was altered by incubation with different concentrations of glucose for 10 min. With more than 0.5 mM glucose, the acetyl-CoA level increased and reached a saturated value of 0.79 to 0.88 nmol/mg of dry cell weight, and in turn CoASH level decreased to a minimum value of 0.22 to 0.25 nmol/mg of dry cell weight.

On the other hand, when *F. aquatile* cells with an initial CoASH level of 0.13 nmol/mg of dry cell weight and an acetyl-CoA level of 0.12 nmol/mg of dry cell weight were transferred, the acetyl-CoA level was not altered by incubation with glucose but, rather, decreased even when more than 2 mM glucose was provided. There was a clear difference in changes of the CoA pool in *E. coli* and *F. aquatile* in response to the glucose concentration.

Effect of anaerobic incubation on the CoA pool. The effects of anaerobic incubation on the CoA pool were examined with both facultatively anaerobic and aerobic bacteria. *E. coli* K-12 and *P. putida* cells were anaerobically incubated by bubbling N_2 gas into the cell suspension for 10 min before the addition of glucose, and the vessels were tightly sealed after the addition of glucose. As shown in Fig. 4B, in *E. coli* K-12, anaerobic incubation only slightly influenced the size and composition of the CoA pool. The results indicate that the enteric bacterium

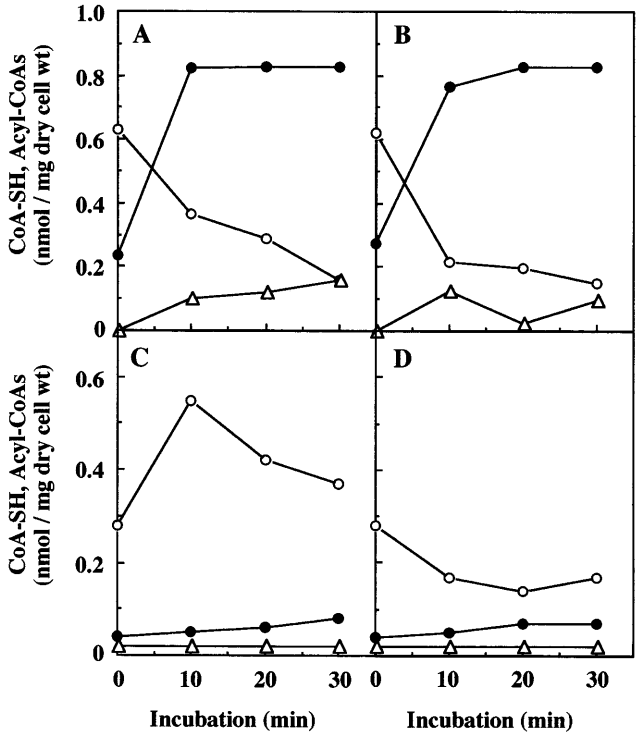

FIG. 4. Changes in the intracellular CoA pools of *E. coli* K-12 and *P. putida* under aerobic or anaerobic conditions. Cells of *E. coli* K-12 and *P. putida* were grown in glucose-mineral salts medium at 30°C with shaking for 48 and 18 h, respectively. The harvested cells were transferred to fresh medium supplemented with glucose at a cell density giving an A_{660} of 1.0 for *E. coli* (A and B) and an A_{660} of 2.0 for *P. putida* (C and D). (A and C) The cells were aerobically incubated with shaking at 30°C. (B and D) The cells were bubbled with N_2 gas for 10 min and anaerobically incubated with standing. Symbols: ○, CoASH; ●, acetyl-CoA; △, malonyl-CoA.

is very slightly sensitive to the aerobic-anaerobic transition and that the organism lives largely by anaerobic glycolysis even when aerobically growing on glucose. The accumulation of acetyl-CoA in *E. coli* K-12 suggests that pyruvate dehydrogenase might not be regulated by acetyl-CoA under the conditions used in vivo, although it is well known that acetyl-CoA exerts negative feedback on pyruvate dehydrogenase in *E. coli* K-12 (34). Furthermore, another possibility considered is that the flux of acetyl-CoA toward citrate by citrate synthase reaction is rather small in facultative anaerobes. On the other hand, in *P. putida* cells, anaerobic incubation decreased the CoASH levels remarkably (Fig. 4D): the initial CoASH level of 0.28 nmol/mg of dry cell weight decreased to 0.14 nmol/mg. A similar decrease in the CoASH level brought on by the aerobic-to-anaerobic transition was observed with *F. aquatile* (data not shown). Although the composition of the CoA pool in aerobic bacteria did not change dramatically as observed in the facultative bacteria, anaerobic incubation of aerobic bacteria resulted in a decrease of the CoA pool size.

Size and composition of CoA pools of various aerobic and facultatively anaerobic bacteria. The size and composition of CoA pools of various aerobic, facultatively anaerobic, and aerotolerant anaerobic bacteria which were harvested during the middle phase of logarithmic growth were measured (Table 1). In aerobic bacteria such as *Alcaligenes faecalis* IB-14, *Pseudomonas putida* IAM 1177, *Micrococcus luteus* IFO 3242, *Corynebacterium glutamicum* IAM 12435, *Corynebacterium ammoniagenes* IAM 1641, *Brevibacterium helvolum* IFO 12073, *Bacillus*

TABLE 1. Size and composition of CoA pools in various bacteria[a]

Strain	Amt (pmol/mg of dry cell wt) of:			Acetyl-CoA/ CoASH ratio
	CoASH	Acetyl-CoA	Malonyl-CoA	
Aerobes				
Alcaligenes faecalis IB-14	1,003	51	Trace	0.05
Pseudomonas putida IAM 1177	553	217	9	0.39
Micrococcus luteus IFO 3242	411	43	4	0.10
Corynebacterium glutamicum IAM 12435	128	51	Trace	0.40
Corynebacterium ammoniagenes IAM 1641	278	132	11	0.47
Brevibacterium helvolum IFO 12073	449	347	Trace	0.77
Bacillus subtilis IAM 1069	213	71	Trace	0.33
Arthrobacter globiformis IFO 12137	231	32	Trace	0.14
Facultative anaerobes				
Escherichia coli ME6200	182	680	11	3.74
Escherichia coli K-12 IAM 1246	113	588	Trace	5.20
Klebsiella pneumoniae subsp. *pneumoniae* 19-35[c]	458	655	13	1.43
Aerotolerant anaerobes				
Lactobacillus homohiochii 55[d]	ND[b]	1,300	75	
Lactobacillus fructivorans 56[d]	ND	760	130	

[a] The bacteria were grown aerobically at 30°C. Intracellular CoASH, acetyl-CoA, and malonyl-CoA in cells harvested at the early stationary phase were extracted and assayed by 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, 2-6-30 Takinogawa, Kita-ku, Tokyo 114, Japan.

subtilis IAM 1069, and *Arthrobacter globiformis* IFO 12137, CoASH was the predominant CoA species in the CoA pool, although the size and composition differed considerably among the strains examined. On the other hand, acetyl-CoA is the predominant component of the CoA pool in facultatively anaerobic bacteria such as *E. coli* K-12, *E. coli* ME 6200, and *K. pneumoniae* subsp. *pneumoniae* 19-35 and in aerotolerant anaerobes such as *Lactobacillus homohiochii* 55 and *Lactobacillus fructivorans* 56. It was clearly demonstrated that the pools of the CoA species in aerobic bacteria were significantly smaller than those in the facultatively anaerobic and aerotolerant bacteria, except for *A. faecalis* IB-14, which was grown on DL-malate, and that large acetyl-CoA pools were not formed in aerobic bacteria under any given conditions. Therefore, the acetyl-CoA/CoASH ratios in aerobic bacteria were 10 times lower than those of facultatively anaerobic bacteria; the mean values of the acetyl-CoA/CoASH ratio of aerobic and facultatively anaerobic bacteria were calculated to be 0.33 and 3.46, respectively. These results indicate that there are intrinsic differences between aerobic and facultatively anaerobic bacteria with regard to the size and composition of their CoA pools, their responses to environmental stresses, and their acetyl-CoA/CoASH ratios.

Effect of metabolic inhibitors on the size and composition of the CoA pool of *E. coli* K-12. Since the size and composition of CoA pools drastically change in facultatively anaerobic bacteria, the effects of various metabolic inhibitors on the CoA pool in *E. coli* K-12 cells were examined by using the transfer experiment. When cells from the stationary growth phase were transferred to fresh media containing various metabolic inhibitors, their specific cellular contents were remarkably altered by several metabolic inhibitors of respiration and fatty acid synthesis. In the presence of 20 μM carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) or 1 mM KCN, both of which are respiratory inhibitors, the increase in the level of acetyl-CoA was strongly inhibited and CoASH was the predominant component of the CoA pool even if 28 mM glucose was provided. The profiles of changes in the CoA pool of cells incubated with CCCP or KCN were almost the same as those of glucose-depleted cells. Cerulenin [(2R)(3S)-2,3-epoxy-4-oxo-7,10-dodecadienolyamide] and thiolactomycin, specific antibiotics that inhibit the fatty acid synthases (11, 15, 16, 27, 28, 30–33, 42), triggered the accumulation of intracellular malonyl-CoA, normally not detected within cells; the malonyl-CoA level increased to 0.80 nmol/mg of dry cell weight after the addition of 45 μM cerulenin and 0.93 nmol/mg of dry cell weight after the addition of 0.2 mM thiolactomycin (data not shown). Then, the intracellular malonyl-CoA became the predominant component of the CoA pool. The size and composition of the CoA pools of *E. coli* K-12 were not altered at all by the addition of other metabolic inhibitors such as penicillin, chloramphenicol, hydroxyurea, and rifampin, indicating that inhibition of the biosynthesis of the cell wall, protein, and RNA did not affect the size and composition of the CoA pools (data not shown).

Figure 5 shows the results of experiments on the effects of KCN and cerulenin concentrations on the size and composition of CoA pools in *E. coli* K-12. The cells with an initial CoASH level of 0.62 nmol/mg of dry cell weight and an acetyl-CoA level of 0.24 nmol/mg of dry cell weight were transferred to fresh media containing one of them and 28 mM glucose, and intracellular CoASH, acetyl-CoA, and malonyl-CoA levels were measured after 10 min of incubation. The addition of KCN at concentrations of more than 0.25 mM inhibited the acetyl-CoA accumulation, and the CoASH level remained unchanged (Fig. 5A). When 4 mM KCN was added after a 20-min incubation with 28 mM glucose, the acetyl-CoA content drastically decreased in 2 min from 0.78 to 0.39 nmol/mg of dry cell weight and, in turn, the CoASH content increased almost stoichiometrically from 0.16 to 0.57 nmol/mg of dry cell weight (Fig. 6). Treatment of cells with cerulenin (Fig. 5B) resulted in a dose-dependent decrease in acetyl-CoA levels and a concomitant increase in the content of malonyl-CoA; cerulenin at concentrations of more than 0.01 mM dramatically increased the level of malonyl-CoA, and the intracellular malonyl-CoA level reached a maximal value of 1.05 nmol/mg of dry cell weight at 0.09 mM cerulenin. The CoASH level did not change

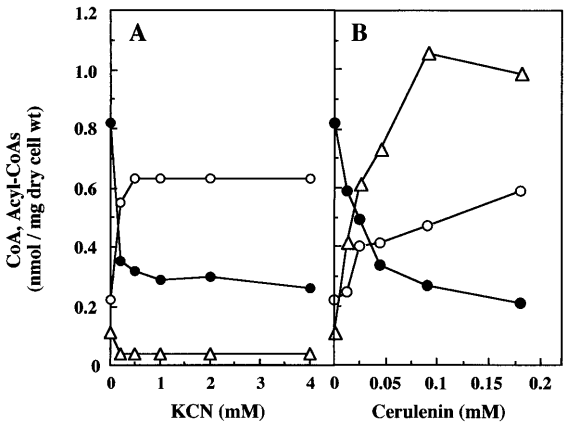

FIG. 5. Effects of various concentrations of KCN (A) and cerulenin (B) on the intracellular CoA pool in *E. coli* K-12. Cells of *E. coli* K-12 was grown aerobically in glucose-mineral salts medium for 48 h at 30°C. The harvested cells were transferred to fresh medium containing the indicated concentrations of KCN (A) or cerulenin (B) at a cell density giving an A_{660} of 1.0. After a 10-min incubation at 30°C with shaking, 28 mM glucose was added to each reaction mixture. After an additional 10-min incubation, the intracellular CoASH (○), acetyl-CoA (●), and malonyl-CoA (△) contents were assayed (see Materials and Methods).

appreciably following exposure to the inhibitor of β-keto-acyl acyl carrier protein (ACP) synthase. The unusual accumulation of malonyl-CoA indicates that malonyl-CoA formation by acetyl-CoA carboxylase might not be regulated by events in the fatty acid synthetic pathway.

DISCUSSION

Intracellular levels of three CoA molecular species, i.e., free CoASH, acetyl-CoA, and malonyl-CoA, in a variety of aerobic and facultatively anaerobic bacteria were analyzed by using the acyl-CoA cycling method developed by us. It was demonstrated that there was a remarkable difference in the size and composition of intracellular pools of CoASH and CoA thioesters between aerobic bacteria and facultatively anaerobic bacteria.

The CoA pools in facultatively anaerobic bacteria are considerably larger than those in aerobic bacteria, and the composition of the CoA pools drastically changes in response to environmental stresses such as the quantity and quality of carbon sources in the medium, growth phase, and antibiotics that inhibit energy-yielding systems and fatty acid biosynthesis. It is shown to be a peculiar characteristic of facultatively anaerobic bacteria that a large acetyl-CoA pool is formed when the cells are normally grown on sufficient glucose and that CoASH is a predominant component in the cells starved for a carbon source. The conversion of pyruvate to acetyl-CoA was the central metabolic step in the facultatively anaerobic bacteria. Our results are consistent with the results obtained with a β-alanine auxotroph of *E. coli* K-12 (20), which showed that the CoA pool in cells exponentially growing in glucose-mineral salts medium was composed of four compounds; acetyl-CoA (79.8%), CoASH (13.8%), succinyl-CoA (5.9%), and malonyl-CoA (0.5%). Acetyl-CoA is also the major CoA derivative in *Salmonella typhimurium* LT2 grown in glucose-mineral salts medium (5). Because of the low ATP yield from carbon sources by fermentation, facultatively anaerobic bacteria are obliged to consume large amounts of carbon sources to fulfill ATP demands for cell growth, resulting in the excretion of large amounts of metabolites such as organic acids and alcohols as waste into the medium (for a review, see reference 23). Thus, for the fermentative degradation of carbon sources, acylated intermediates of CoASH, especially acetyl-CoA, are more prevalent than CoASH in facultatively anaerobic bacteria. Although it has been reported that pyruvate dehydrogenase from *E. coli* is inhibited by acetyl-CoA (34), the abundant acetyl-CoA formation indicates that this might not be valid in vivo in facultative anaerobes. Furthermore, the accumulation of acetyl-CoA in response to glucose in facultatively anaerobic bacteria grown aerobically suggests that the metabolic flux from acetyl-CoA to citrate via citrate synthase might not be so functional in those bacteria. The inhibition of the activity of citrate synthase in *E. coli* by NADH and 2-oxoglutarate is generally believed to occur in vivo (44). Accordingly, *E. coli* and the related facultative anaerobes utilize glucose to form acetyl-CoA in a wasteful way.

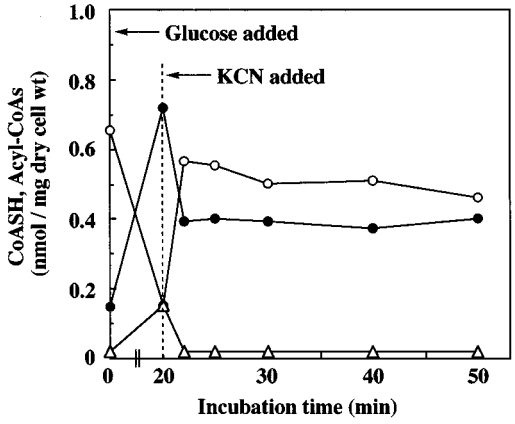

FIG. 6. Effect of KCN on the CoA pool of *E. coli* K-12 cells. Cells of *E. coli* K-12 cultivated for 48 h was transferred to fresh medium containing 28 mM glucose at a cell density giving an A_{660} of 1.0. After aerobic incubation at 30°C for 20 min, 4 mM KCN was added to the cell suspension. At the times indicated in the figure, the intracellular contents of CoASH (○), acetyl-CoA (●), and malonyl-CoA (△) were assayed (see Materials and Methods).

When KCN was added to the cells grown aerobically on glucose, the acetyl-CoA level rapidly decreased within minutes and there was a concomitant increase in the CoASH level. It is significant that the addition of KCN provoked acetyl-CoA depletion but not CoASH of the CoA pool. This result indicates that the acylation of free CoASH coordinates with the energy state of the cell (41) and a restriction of the energy-yielding system. Part or all of the acetyl-CoA formed by pyruvate dehydrogenase might be consumed for the synthesis of ATP, which proceeds via phosphotransacetylase (EC 2.3.1.8) and acetate kinase (EC 2.7.2.1) reactions when ATP synthesis via electron transport phosphorylation was inhibited by KCN. Although the intracellular CoA pool in facultatively anaerobic bacteria drastically changed, the sum of the three CoA molecular species was confined within a narrow range. Decreases or increases in the level of acetyl-CoA correlated almost stoichiometrically with those of CoASH or malonyl-CoA. Our results were consistent with the results reported by Jackowski and her coworkers that the biosynthetic sequence of CoASH in *E. coli* was tightly regulated at the pantothenate kinase step (18, 35, 40, 41).

Up to the present, little information has been available regarding the intracellular CoA pool of aerobic bacteria. This investigation clearly demonstrates that the CoA pool in aerobic bacteria was small and the composition of the CoA pool hardly changed, even when sufficient glucose was provided in the medium. A profile of changes in the CoA pool in the

aerobic bacteria is similar to that of changes in the pool in the facultatively anaerobic bacteria starved for carbon and energy sources. In aerobic bacteria, a large acetyl-CoA pool was not formed and CoASH was the major component of the CoA pool. Furthermore, CoASH was significantly decreased by the anaerobic incubation of the cells. This phenomenon might result from the depression of the ATP level due to anaerobic transition, because the biosynthesis of CoA is an energy-requiring metabolic sequence, i.e., 4 mol of ATP is needed for the synthesis of 1 mol of CoASH (7, 18, 19, 40). In aerobic bacteria, the acetyl-CoA produced seemed to be immediately converted to free CoASH by the citrate synthase reaction, and therefore the CoASH was the predominant component of CoA pool. The flux of acetyl-CoA toward citrate in aerobic bacteria seems to be larger than that in facultative anaerobes, although the flux from glucose toward pyruvate is smaller in the former bacteria. It was suggested by Atkinson (2) that the entry of acetyl-CoA into the citric cycle may be regulated by ATP. Our data indicate that this regulation might be valid for aerobic bacteria but not for facultative anaerobes. 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.

As shown in Fig. 5B, it is noteworthy that the level of malonyl-CoA, which is a minor species of the CoA pool in normally grown cells, dramatically increased so that malonyl-CoA became the dominant component in the cells treated with cerulenin or thiolactomycin (14), the antibiotics that inhibit β-keto-acyl ACP synthase and acetyl-CoA:ACP transacylase (11, 12, 17, 22, 28, 39, 42) of fatty acid synthase. This result suggests that malonyl-CoA does not exert negative feedback on acetyl-CoA carboxylase and that malonyl-CoA was formed wastefully in spite of blocking the supply of the CoA thioester to the fatty acid-synthesizing system. Acetyl-CoA carboxylase is well known to be the first enzyme of the biosynthetic sequence of fatty acid synthesis. However, it is probable that the enzyme is not the pacemaker of fatty acid synthesis.

Further experiments are required to clearly demonstrate the in vivo roles of these three CoA molecular species in enzyme regulation.

ACKNOWLEDGMENTS

We thank Charles O. Rock and Suzanne Jackowski, Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tenn., for useful discussions.

REFERENCES

1. **Andersen, K. B., and K. von Meyenburg.** 1977. Changes of nicotinamide adenine nucleotides and adenylate energy charge as regulatory parameters of the metabolism in *Escherichia coli*. J. Biol. Chem. **252:**4151–4156.
2. **Atkinson, D. E.** 1965. Biological feedback control at the molecular level. Science **150:**851–857.
3. **Atokinson, D. E., and G. M. Walton.** 1967. Adenosine triphosphate conservation in metabolic regulation. Rat liver citrate cleavage enzyme. J. Biol. Chem. **242:**3239–3241.
4. **Ball, W. J., Jr., and D. E. Atkinson.** 1975. Adenylate energy charge in *Saccharomyces cerevisiae* during starvation. J. Bacteriol. **121:**975–982.
5. **Bochner, B. R., and B. N. Ames.** 1982. Complete analysis of cellular nucleotides by two dimensional thin-layer chromatography. J. Biol. Chem. **257:** 9759–9769.
6. **Boynton, Z. L., G. N. Bennet, and F. B. Roudolph.** 1994. Intracellular concentrations of coenzyme A and its derivatives from *Clostridium acetobutylicum* ATCC 824 and their roles in enzyme regulation. Appl. Environ. Microbiol. **60:**39–44.
7. **Brown, G. M.** 1959. The metabolism of pantothenic acid. J. Biol. Chem. **234:**370–378.
8. **Chapman, A. G., and D. E. Atkinson.** 1977. Adenine nucleotide concentrations and turnover rates. Their correlation with biological activity in bacteria and yeast. Adv. Microb. Physiol. **15:**253–306.
9. **Chapman, A. G., L. Fall, and D. E. Atkinson.** 1971. Adenylate energy charge in *Escherichia coli* during growth and starvation. J. Bacteriol. **108:**1072–1086.
10. **Chohnan, S., and Y. Takamura.** 1991. A simple micromethod for measurement of CoASH and its use in measuring intracellular levels of CoASH and short chain acyl-CoAs in *Escherichia coli* K12 cells. Agric. Biol. Chem. **55:**87–94.
11. **D'Agnolo, G., I. S. Rosenfeld, J. Awaya, S. Omura, and P. R. Vagelos.** 1973. Inhibition of fatty acid biosynthesis by the antibiotic cerulenin. Specific inactivation of β-ketoacyl-acyl carrier protein synthetase. Biochim. Biophys. Acta **326:**155–166.
12. **de Mendoza, D., A. Klages Ulrich, and J. E. Cronan, Jr.** 1983. Thermal regulation of membrane fluidity in *Escherichia coli*. Effects of overproduction of β-ketoacyl-acyl carrier protein synthase. I. J. Biol. Chem. **258:**2098–2101.
13. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28:**350–356.
14. **Furukawa, H., J. T. Tsay, S. Jackowski, Y. Takamura, and C. O. Rock.** 1993. Thiolactomycin resistance in *Escherichia coli* is associated with the multidrug resistance efflux pump encoded by *emrAB*. J. Bacteriol. **175:**3723–3729.
15. **Hayashi, T., O. Yamamoto, H. Sasaki, A. Kawaguchi, and H. Okazaki.** 1983. Mechanism of action of the antibiotic thiolactomycin inhibition of fatty acid synthesis of *Escherichia coli*. Biochem. Biophys. Res. Commun. **115:**1108–1113.
16. **Hayashi, T., O. Yamamoto, H. Sasaki, and H. Okazaki.** 1984. Inhibition of fatty acid synthesis by the antibiotic thiolactomycin. J. Antibiot. **37:**1456–1461.
17. **Jackowski, S., C. M. Murphy, J. E. Cronan, Jr., and C. O. Rock.** 1989. Acetoacetyl-acyl carrier protein synthase. A target for the antibiotic thiolactomycin. J. Biol. Chem. **264:**7624–7629.
18. **Jackowski, S., and C. O. Rock.** 1981. Regulation of coenzyme A biosynthesis. J. Bacteriol. **148:**926–932.
19. **Jackowski, S., and C. O. Rock.** 1984. Metabolism of 4′-phosphopantetheine in *Escherichia coli*. J. Bacteriol. **158:**115–120.
20. **Jackowski, S., and C. O. Rock.** 1986. Consequences of reduced intracellular coenzyme A content in *Escherichia coli*. J. Bacteriol. **166:**866–871.
21. **Karl, D. M.** 1980. Cellular nucleotide measurements and applications in microbial ecology. Microbiol. Rev. **44:**739–796.
22. **Kauppinen, S., M. Siggaard-Anderson, and P. van Wettstein-Knowles.** 1988. β-Ketoacyl-ACP synthase I of *Escherichia coli*: nucleotide sequence of the *febB* gene and identification of the cerulenin binding residue. Carlsberg Res. Commum. **53:**357–370.
23. **Knappe, J.** 1987. Anaerobic dissimilation of pyruvate, p. 151–155. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology. American Society for Microbiology, Washington, D.C.
24. **Lomovskoya, O., and K. Lewis.** 1992. *emr*, an *Escherichia coli* locus for multidrug resistance. Proc. Natl. Acad. Sci. USA **89:**8938–8942.
25. **Lowry, O. H., J. V. Passoneau, D. W. Schulz, and M. K. Rock.** 1961. The measurement of pyridine nucleotides by enzymatic cycling. J. Biol. Chem. **236:**2746–2755.
26. **Makman, R. S., and E. W. Sutherland.** 1965. Adenosine 3′,5′-phosphate in *Escherichia coli*. J. Biol. Chem. **240:**1309–1314.
27. **Miyakawa, S., K. Suzuki, T. Noto, Y. Harada, and H. Okazaki.** 1982. Thiolactomycin, a new antibiotic. IV. Biological properties and chemotherapeutic activity in mice. J. Antibiot. **35:**411–419.
28. **Nishida, I., A. Kawaguchi, and M. Yamada.** 1986. Effect of thiolactomycin on the individual enzymes of the fatty acid synthase system in *Escherichia coli*. J. Biochem. **99:**1447–1454.
29. **Nomura, G., Y. Yoshida, and Y. Takamura.** 1988. Measurement of intracellular acetyl-CoA and malonyl-CoA in bacteria and yeasts using acyl-CoA cycling. Agric. Biol. Chem. **52:**843–844.
30. **Noto, T., S. Miyakawa, H. Oishi, H. Endo, and H. Okazaki.** 1982. Thiolactomycin, a new antibiotic. III. *In vitro* antibacterial activity. J. Antibiot. **35:**401–410.
31. **Oishi, H., T. Noto, H. Sasaki, K. Suzuki, T. Hayashi, H. Okazaki, K. Ando, and M. Sawada.** 1982. Thiolactomycin, a new antibiotic. I. Taxonomy of the producing organism, fermentation and biological properties. J. Antibiot. **35:**391–395.
32. **Omura, S.** 1976. The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol. Rev. **40:**681–697.
33. **Sasaki, H., H. Oishi, T. Hayashi, I. Matsuura, K. Ando, and M. Sawada.** Thiolactomycin, a new antibiotic. II. Structure elucidation. J. Antibiot. **35:** 396–400.
34. **Schwartz, E. R., L. O. Old, and L. J. Reed.** 1968. Regulatory properties of pyruvate dehydrogenase from *Escherichia coli*. Biochem. Biophys. Res. Commun. **31:**495–500.
35. **Song, W.-J., and S. Jackowski.** 1992. Cloning, sequencing, and expression of the pantothenate kinase (*coaA*) gene of *Escherichia coli*. J. Bacteriol. **174:**6411–6417.
36. **Takamura, Y., and Y. Kitayama.** 1981. Purification and some properties of malonate decarboxylase from *Pseudomonas ovalis*, an oligomeric enzyme with bifunctional properties. Biochem. Int. **3:**483–491.
37. **Takamura, Y., Y. Kitayama, A. Arakawa, S. Yamanaka, M. Tosaki, and Y.**

Ogawa. 1985. Malonyl-CoA:acetyl-CoA cycling. A new micromethod for determination of acyl-CoAs with malonate decarboxylase. Biochim. Biophys. Acta **834:**1–7.
38. **Takamura, Y., and G. Nomura.** 1988. Changes in the intracellular concentration of acetyl-CoA and malonyl-CoA in relation to the carbon and energy metabolism of *Escherichia coli* K12. J. Gen. Microbiol. **134:**2249–2253.
39. **Tsay, J. T., C. O. Rock, and S. Jackowski.** 1992. Overproduction of β-ketoacyl-acyl carrier protein synthase I imparts thiolactomycin resistance to *Escherichia coli* K-12. J. Bacteriol. **174:**508–513.
40. **Vallari, D. S., and S. Jackowski.** 1988. Biosynthesis and degradation both contribute to the regulation of coenzyme A content in *Escherichia coli*. J. Bacteriol. **170:**3961–3966.
41. **Vallari, D. S., S. Jackowski, and C. O. Rock.** 1987. Regulation of pantothenate kinase by coenzyme A and its thioester. J. Biol. Chem. **262:**2468–2471.
42. **Vance, D. E., I. Goldberg, O. Mitsuhashi, K. Bloch, S. Omura, and S. Nomura.** 1972. Inhibition of fatty acid synthetases by the antibiotic cerulenin. Biochem. Biophys. Res. Commun. **48:**649–656.
43. **Winkler, H. H., and T. H. Wilson.** 1966. The role of energy coupling in the transport of β-galactosides by *Escherichia coli*. J. Biol. Chem. **241:**2200–2211.
44. **Wright, J. A., P. Maeba, and B. D. Sanwal.** 1967. Allosteric regulation of the activity of citrate synthetase of *Escherichia coli* by α-ketoglutarate. Biochem. Biophys. Res. Commun. **29:**34–38.