Effects of metal ions on growth, β -oxidation system, and thioesterase activity of *Lactococcus lactis*

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ABSTRACT

The effects of divalent metal ions (Ca^{2+} , Mg^{2+} , Fe^{2+} , and Cu^{2+}) on the growth, β -oxidation system, and thioesterase activity of *Lactococcus lactis* were investigated. Different metal ions significantly influenced the growth of *L. lactis*: Ca^{2+} and Fe^{2+} accelerated growth, whereas Cu^{2+} inhibited growth. Furthermore, Mg^{2+} inhibited growth of *L. lactis* at a low concentration but stimulated growth of *L. lactis* at a high concentration. The divalent metal ions had significant effects on activity of the 4 key enzymes of the β -oxidation system (acyl-CoA dehydrogenase, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and thiolase) and thioesterase of *L. lactis*. The activity of acyl-CoA dehydrogenases increased markedly in the presence of Ca^{2+} and Mg^{2+} , whereas it decreased with 1 mmol/L Fe^{2+} or 12 mmol/L Mg^{2+} . All the metal ions could induce activity of enoyl-CoA hydratase. In addition, 12 mmol/L Mg^{2+} significantly stimulated activity of L-3-hydroxyacyl-CoA dehydrogenase, and all metal ions could induce activity of thiolase, although thiolase activity decreased significantly when 0.05 mmol/L Cu^{2+} was added into M17 broth. Inhibition of thioesterase activity by all 4 metal ions could be reversed by 2 mmol/L Ca^{2+} . These results help us understand the effect of metal ions on the β -oxidation system and thioesterase activity of *Lactococcus lactis*.

Key words: metal ion, β -oxidation system, thioesterase, *Lactococcus lactis*

INTRODUCTION

Lactococcus lactis strains are the predominant lactic acid bacteria components of commercial starter cultures used by the dairy industry for the manufacture and ripening of cheese and fermented milk (Alegría et al., 2010). Starter *L. lactis* have an important effect on flavor precursor development, especially in the genera-

tion of methyl ketones, which is related to incomplete β -oxidation in cheese and fermented milk (Hannon et al., 2007; Li and Ma, 2013). The β -oxidation pathway is a cycle of 4 sequential reactions in which the FA substrate is shortened by 2 carbons with each cycle (Kurtz et al., 1998). Fatty acyl-CoA is first oxidized to enoyl-CoA, enoyl-CoA is then hydrated to hydroxyacyl-CoA, which is in turn oxidized to ketoacyl-CoA (Maggio-Hall et al., 2008), but β -ketoacyl-CoA may not be metabolized to acetyl-CoA units via β -oxidation. β -Ketoacyl-CoA can be deacylated into β -ketoacids under the action of the thioesterases, which can catalyze the hydrolysis of acyl-CoA to FFA and CoA (Hunt and Alexson, 2002), and the ketoacid is then decarboxylated to methyl ketone (Engelvin et al., 2000). Incomplete β -oxidation is associated with 4 key enzymes in the β -oxidation system (acyl-CoA dehydrogenases, enoyl-CoA hydratase, L-3-hydroxyacyl-CoA dehydrogenase, and thiolase) and thioesterase.

In addition, the ionic environment may interfere with bacterial cell walls, especially in gram-positive bacteria, and modify electron flow in a substrate or enzyme, thus effectively controlling an enzyme-catalyzed reaction (Ellwood and Tempest, 1972; Glusker et al., 1999). From the literature, many researchers have studied the effects of metal ions on the growth of lactic acid bacteria and enzymes in metabolic processes; however, little information has been reported on the effect of divalent metal ions on the β -oxidation system and thioesterase activity of *L. lactis* (Eades and Womack, 1953; Boyaval, 1989; Imbert and Blondeau, 1998).

The objective of this study was to investigate the effects of Ca^{2+} , Mg^{2+} , Fe^{2+} , and Cu^{2+} on the growth, β -oxidation system, and thioesterase activity of *L. lactis*. These results will provide a new way of regulating FA metabolism and, in turn, methyl ketone synthesis related to FA metabolism.

MATERIALS AND METHODS

Chemicals

CoA, crotonoyl coenzyme A, acetoacetyl coenzyme A and palmitoyl-CoA were purchased from Sigma-

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Aldrich Co. Ltd. (Shanghai, China). 5,5'-Dithiobis-(2-nitrobenzoic acid) (**DNTB**), HEPES, NADH, phenyl-methanesulfonyl fluoride (**PMSF**), BSA, phenazine metosulfate, and EDTA were purchased from Aladdin Chemistry Co. Ltd. (Shanghai, China). The Enhanced BCA Protein Assay kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China).

Microorganism

Lactococcus lactis ssp. *lactis* was isolated from a traditional dairy product in China, identified by morphological and genetic methods, and grown in M17 broth medium (Hope Bio-Technology Co., Qingdao, China) at 37°C (Terzaghi and Sandine, 1975). The strain was maintained at -20°C. The cultures were activated at least 3 times before use.

Ferric sulfate, calcium chloride, copper sulfate, and magnesium sulfate were used as metal ion supplements in M17 broth for growth tests. Cell density was measured by using a spectrophotometer (TU-1800 Pgeneral Instrument Co. Ltd., Beijing, China) at 600 nm after 18 h of growth (Papagianni et al., 2007). All enzyme activities were measured after 18 h of growth of *L. lactis* ssp. *lactis*.

Preparation of Cell-Free Extracts

For preparation of the cell-free extracts, cells were grown in 1 L of medium for 18 h. Cell-free extracts were prepared essentially as described by Engelvin et al. (2000) with modifications. Cells were harvested at $5,000 \times g$ for 10 min at 4°C, washed with K_2HPO_4 - KH_2PO_4 (100 mmol/L, pH 7.5), and sonicated in HEPES buffer (20 mmol/L, pH 7.5) containing 1 mmol/L EDTA and 1 mmol/L PMSF using an ultrasonic homogenizer (JY92-II, Ningbo Scientz Biotechnology Co. Ltd., Ningbo, China), with 30 cycles of 10 s on and 10 s off at 300 W. Unbroken bacteria were removed by centrifugation ($5,000 \times g$, 10 min, 4°C). The supernatant fraction was designated the crude cell-free extract (**CFE**) and was used immediately or stored at -80°C. The content of protein in the CFE was determined by using the Enhanced BCA Protein Assay Kit.

SDS-PAGE Analysis

Sodium dodecyl sulfate-PAGE was performed in a Mini-Protein 3 Cell apparatus (Bio-Rad Laboratories, Hercules, CA) according to the modified procedure of Laemmli (1970), which used 12% separating gels (0.375 mol/L Tris-HCl, pH 8.8, and 0.1% SDS) and 4% stacking gels (0.125 mol/L Tris-HCl, pH 6.8, and 0.1% SDS), respectively, and a buffer system contain-

ing 0.025 mol/L Tris-HCl, 0.192 mol/L Gly, and 0.1% SDS, pH 8.3. Samples were mixed with reducing sample buffer (10% SDS, 2.5% β -mercaptoethanol) to give a concentration of 2 mg/mL and were heated at 95°C for 5 min. Ten microliters of each sample was loaded per lane. Electrophoresis was run at 10 mA before the sample was into the separating gel, and run at 20 mA after the sample was into the separating gel. The gel was stained with a mixed solution of 0.1% Coomassie Brilliant Blue R-250 in 40% methanol and 10% acetic acid, and destained with a solution of 40% methanol and 10% acetic acid. Protein molecular weights were estimated using a protein molecular weight marker (SM0431, Fermentas/Thermo Scientific, Waltham, MA), including β -galactosidase (116 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa), REase Bsp981 (25.0 kDa), β -LG (18.4 kDa), and lysozyme (14.4 kDa). The densitometric analyses of the bands were done using the Biorad ChemiDoc XR system and software (Bio-Rad Laboratories).

Acyl-CoA Dehydrogenase Activity

Acyl-CoA dehydrogenase (EC 1.3.99.3) was determined according to the procedure of Baltazar et al. (1999) and Feron et al. (2005). Total activity was determined in 1 mL of HEPES/KOH buffer (50 mmol/L, pH 8.0), and the reduction of 100 μ mol of 2,6-dichlorophenolindophenol (DCPIP) was monitored at 600 nm in the presence of 50 μ mol of phenazine metosulfate, 72 nmol of acyl CoA, and 200 μ g of CFE. An absorption coefficient of $21,500 M^{-1} \cdot cm^{-1}$ was used for DCPIP at pH 8.0.

Enoyl-CoA Hydratase Activity

Enoyl-CoA hydratase (crotonase; EC 4.2.1.17) was assayed by following the decrease in absorbance at 263 nm due to the hydration of the Δ -2,3 double bond of the substrate (Binstock and Schulz, 1981). The assay mixture contained 0.2 mol/L potassium phosphate, pH 8, BSA (0.2 mg/mL), and 30 μ mol/L crotonyl-CoA. The reaction was started by addition of the enzyme. An extinction coefficient (ϵ) of $6,700 M^{-1} \cdot cm^{-1}$ was used to calculate rates.

L-3-Hydroxyacyl-CoA Dehydrogenase Activity

L-3-Hydroxyacyl-CoA dehydrogenase (EC 1.1.1.35) was routinely assayed by measuring the decrease in absorbance at 340 nm due to the dehydrogenation of NADH (Binstock and Schulz, 1981). The assay mixture contained 0.1 mol/L potassium phosphate, pH 7, BSA (0.2 mg/mL), 0.1 mmol/L NADH, and 30 μ mol/L ace-

toacetyl-CoA. The assay was begun by addition of the enzyme. An extinction coefficient (ϵ) of $6,220\text{ M}^{-1}\cdot\text{cm}^{-1}$ was used to calculate rates.

Thiolase Activity

3-Ketoacyl-CoA thiolase (EC 2.3.1.16) was assayed by measuring the decrease in absorbance at 303 nm due to the disappearance of the Mg^{2+} enolate complex of the substrate (Binstock and Schulz, 1981). The assay mixture contained 0.1 mol/L HEPES, pH 8.1, 25 mmol/L MgCl_2 , BSA (0.2 mg/mL), 2 mmol/L mercaptoethanol, 5% (vol/vol) glycerol, 0.1 mmol/L coenzyme A, and 30 $\mu\text{mol/L}$ acetoacetyl-CoA. The reaction was begun by addition of the enzyme. Extinction coefficient for acetoacetyl-CoA in HEPES buffer was $12,000\text{ M}^{-1}\cdot\text{cm}^{-1}$.

Thioesterase Activity

Thioesterase activity was determined according to the procedure of Engelman et al. (2000). Thioesterase activity was measured by the release of CoASH, which was assayed continuously by its reaction with DTNB. The assay contained, in a final volume of 1 mL, $\text{K}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$ (300 mmol/L, pH 8.0), 250 nmol of DTNB, 72 nmol of palmitoyl-CoA, and 280 μg of CFE protein. Activity was measured by following the increase in absorbance at 412 nm against a control without substrates [$\epsilon_{\text{DTNB}}(412) = 13,600\text{ M}^{-1}\cdot\text{cm}^{-1}$]. Activity was expressed as nanomoles of palmitoyl-CoA deacylated per milligram of protein per minute.

Statistical Analysis

All statistical analyses, including one-way ANOVA and the Duncan multiple-range test, were performed by using SPSS 17.0 software (SPSS Inc., Chicago, IL). All experiments were performed at least in triplicate, and mean values $\pm$ standard deviations were used for analyses.

RESULTS AND DISCUSSION

Whole-Cell Protein Analysis by SDS-PAGE

The whole-cell protein profiles showed that the molecular weight of major subunits ranged from 25 to 50 kDa (Figure 1). These results were in accordance with previous descriptions: acyl-CoA dehydrogenase had a subunit with mass of approximately 43 kDa (Zeng et al., 2006; Maher et al., 2010); enoyl-CoA hydratase is composed of 6 identical subunits of 26 kDa each; 3-hydroxyacyl-CoA dehydrogenase consists of 2 identi-

cal subunits of 32 kDa each (Yokota and Hashimoto, 1984); and thiolase has a subunit of 41 kDa (Mannaerts et al., 2000). Thioesterase showed a band at 38 kDa consistent with the 33 kDa component that is a degradation product generated during purification (Hellyer et al., 1992). Based on the previous studies and our results, we predict that *L. lactis* has these active subunits of the 5 enzymes.

Effect of Metal Ions on the Growth of *L. lactis*

The effects of different concentrations of metal ions on the growth of *L. lactis* are shown in Figure 2. The addition of Mg^{2+} to M17 could stimulate (at 8–20 mmol/L) the growth of *L. lactis* or inhibit it (at 4 mmol/L; Figure 2A). This result was similar to that found in previous research (Wright and Klaenhammer, 1983), which reported that supplementation of Chelex-exchanged basal broth with magnesium (1–30 mmol/L) resulted in a proportional growth response in *Lactobacillus bulgaricus* 1243. In contrast, the growth of *L. lactis* ssp. *lactis* CNRZ 1076 was unchanged when Mg^{2+} and Mn^{2+} were added to reconstituted skim milk (Bellengier et al., 1997). Growth rates of *L. lactis* ssp. *lactis* NCDO 2118 were reported to be affected when Mg^{2+} concentrations $<15\text{ }\mu\text{mol/L}$ (Loubiere et al., 1997), and Mg^{2+} resulted in a significant increase in viable count of *Lactococcus rhamnosus* FTDC 8313 when cultivated in reconstituted skimmed milk with Mg at 0 to 2 mg/mL (Lew et al., 2013).

As shown in Figure 2B, we found no significant change in growth rate with Ca^{2+} concentrations $<12\text{ mmol/L}$ ($P > 0.05$) but growth decreased dramatically when Ca^{2+} concentration increased from 12 to 20 mmol/L. Our results differ from other works. Eades and Womack (1953) claimed that calcium when added to medium at 0.025 to 12.5 mmol/L stimulated the early growth of *Lactobacillus casei* ATCC 7469. Wright and Klaenhammer (1983) noted that *Lactobacillus bulgaricus* 1243 had a proportional growth response in Chelex-exchanged basal broth supplementation with calcium at 0.001 to 1 mmol/L.

As shown in Figure 2C, Fe^{2+} inhibited the growth of *L. lactis*. Data presented by Efstathiou and McKay (1977) showed that lactose-metabolizing strains of *Streptococcus lactis* were more sensitive to copper compared with the lactose-negative derivatives, and Mrvčić et al. (2013) showed that added Cu^{2+} inhibited the growth of lactic acid bacteria. In the current study, copper ions stimulated the growth of *L. lactis* (Figure 2D).

In this study, we examined the influence of some divalent cations on the growth of *L. lactis*. The relationships between the growth of *L. lactis* and metal ions are variable: sometimes they have no effects (Bellengier

Figure 1. Whole-cell protein profiles of *Lactococcus lactis* ssp. *lactis* by using SDS-PAGE. Lane M = marker; lane 1 = whole-cell protein; numbers 1 through 7 in lane 1 indicate the major subunits of whole-cell protein.

et al., 1997) but sometimes they have inhibitory effects (Fe^{2+} , Ca^{2+}) or positive effects (Mg^{2+} , Cu^{2+} ; Figure 2). Our results indicated that metal ions had an important influence on the growth of *L. lactis*.

Both Mg^{2+} and Cu^{2+} had positive effects on the growth of *L. lactis*. The essential requirement for Mg^{2+} in growth is explained by the fact that it acts as an activator of different metabolic reactions, cell division, and stabilization of DNA and RNA (Boyaval, 1989). Although Cu^{2+} can be highly toxic to microorganisms at high concentrations, the presence of copper may provide selection pressure that causes the microbial population to adapt to environmental conditions (Trevors and Cotter, 1990); *L. lactis* (in this study) and *Lactobacillus delbrueckii* (Rodríguez and Alatossava, 2008) are most resistant to copper compared with other lactobacilli.

Both Fe^{2+} and Ca^{2+} had inhibitory effects on the growth of *L. lactis* (Figure 2). However, researchers have rarely studied the effects of iron on the growth of lactic acid bacteria, because lactic acid bacteria are thought to be an exception among living organisms in that they have no requirement for iron (Bruyneel et al., 1989). However, genome sequences of *Lactobacillus sakei* 23 K as well as *L. lactis* MG1363 reveal that lactic acid bacteria have genes that code for proteins involved in iron transport (Mrvčić et al., 2012). Imbert and Blondeau (1998) confirmed that iron did not induce any significant growth in lactobacilli. A few reports have shown that Fe^{2+} inhibits growth of *Lactobacillus johnsonii* (Elli et al., 2000). Dunning et al. (1998) described the damage (including lethal damage) induced by Fe^{2+} to oral streptococci but not to *Lactobacillus casei* ATCC 4646. Therefore, the inhibitory effects of Fe^{2+} on lactic acid bacteria must be further studied. From our results, we can conclude that variations in metal ion sensitivity and resistance may reflect variability in species, strains, media, cation contents, and different culture conditions.

Influence of Mg^{2+} on Enzyme Activity

Activities of 5 enzymes of incomplete β -oxidation were detected in the extracts of *L. lactis* grown in M17 broth with different concentrations of Mg^{2+} (Table 1). Levels of acyl-CoA dehydrogenase activity were enhanced 1.8-fold when the concentration of Mg^{2+} in M17 broth was 16 mmol/L and were reduced 3-fold when the concentration of Mg^{2+} was 12 mmol/L. Levels of enoyl-CoA hydratase activity and L-3-hydroxyacyl-CoA dehydrogenase activity exhibited different degrees of increase. Thiolase activity was increased 23-fold with 8 mmol/L Mg^{2+} . This result was similar to that of other thiolases: the activity of rat mitochondrial 3-ketoacyl-CoA thiolase was stimulated by Mg^{2+} in Tris-HCl buffer (Zeng and Li, 2004), but the activity of acetoacetyl-CoA thiolase from *Halobacterium* strain ZP-6 was inhibited by 20 mmol/L Mg^{2+} (Liu et al., 2002). Activity of thioesterase is decreased by different concentrations of Mg^{2+} . The activity of the enzyme located in pig heart muscle mitochondria was not stimulated by Mg^{2+} (Lee and Schulz, 1979), but 2.5 mmol/L Mg^{2+} had marked stimulatory effects on activities of the short- and medium-chain thioesterases in brown adipose tissue mitochondria (Alexson and Nedergaard, 1988). Long-chain fatty acyl-CoA thioesterase activity was stimulated by 0.5 mmol/L Mg^{2+} and inhibited at high concentrations (Berge and Døssland, 1979). From these results, we can conclude that Mg^{2+} stimulated activity of acyl-CoA dehydrogenase, enoyl-CoA hydra-

Figure 2. Effect of concentration of metal ions on the growth of *Lactococcus lactis*. OD = optical density.

tase, L-3-hydroxyacyl-CoA dehydrogenase, and thiolase but did not stimulate activity of thioesterase.

Influence of Ca²⁺ on Enzyme Activity

Calcium ions stimulated activity of acyl-CoA dehydrogenase, enoyl-CoA hydratase, and thiolase when the

concentration was <10 mmol/L. However, for L-3-hydroxyacyl-CoA dehydrogenase and thioesterase, the role of stimulating or inhibiting enzyme activities depended on calcium concentration (Table 2). Thioesterase activity was stimulated by Ca²⁺ only at 2 mmol/L in this study, which differs from the results of Lee and Schulz (1979). The finding of the influence of Ca²⁺ on enzyme

Table 1. Influence of Mg²⁺ concentration on enzyme activity (nmol/min per mg of protein)

Mg ²⁺ (mmol/L)	Acyl-CoA dehydrogenase	Enoyl-CoA hydratase	L-3-Hydroxyacyl-CoA dehydrogenase	Thiolase	Thioesterase
0	9.302 ± 2.270 ^b	ND ¹	119.373 ± 7.335 ^b	3.540 ± 0.955 ^c	16.176 ± 0.318 ^a
4	9.418 ± 1.047 ^b	ND	149.116 ± 17.067 ^{ab}	46.667 ± 31.290 ^b	11.765 ± 1.148 ^{ab}
8	12.442 ± 2.710 ^{ab}	55.597 ± 23.649 ^a	141.479 ± 16.459 ^{ab}	82.708 ± 5.907 ^a	13.971 ± 3.989 ^{ab}
12	3.023 ± 1.066 ^c	41.418 ± 11.633 ^a	163.987 ± 9.571 ^a	78.958 ± 9.382 ^a	15.625 ± 4.457 ^a
16	16.511 ± 1.846 ^a	82.836 ± 58.209 ^a	143.087 ± 10.040 ^{ab}	41.250 ± 5.962 ^b	7.353 ± 1.773 ^c
20	12.558 ± 2.287 ^{ab}	18.657 ± 9.117 ^a	48.633 ± 18.458 ^c	2.500 ± 1.250 ^c	7.721 ± 0.955 ^c

^{a-c}Values with different superscripts in the same column differ significantly ($P < 0.05$).

¹Enzyme activity was not detected.

Table 2. Influence of Ca^{2+} concentration on enzyme activity (nmol/min per mg of protein)

Ca^{2+} (mmol/L)	Acyl-CoA dehydrogenase	Enoyl-CoA hydratase	L-3-Hydroxyacyl-CoA dehydrogenase	Thiolase	Thioesterase
0	10.814 $\pm$ 3.540 ^b	ND ¹	119.373 $\pm$ 7.335 ^a	3.540 $\pm$ 0.955 ^b	16.176 $\pm$ 0.318 ^b
2	45.349 $\pm$ 9.820 ^a	316.418 $\pm$ 164.554 ^{ab}	96.865 $\pm$ 20.225 ^{ab}	21.667 $\pm$ 2.366 ^a	19.301 $\pm$ 1.102 ^a
4	58.721 $\pm$ 10.124 ^a	103.731 $\pm$ 19.592 ^b	64.711 $\pm$ 1.842 ^c	16.458 $\pm$ 0.361 ^a	5.882 $\pm$ 0.842 ^d
6	62.209 $\pm$ 19.114 ^a	85.597 $\pm$ 43.914 ^b	83.601 $\pm$ 5.569 ^{bc}	7.500 $\pm$ 5.340 ^b	10.294 $\pm$ 1.773 ^c
8	41.512 $\pm$ 16.878 ^a	382.024 $\pm$ 54.053 ^a	123.794 $\pm$ 15.504 ^a	6.667 $\pm$ 0.361 ^b	3.309 $\pm$ 0.637 ^e
10	72.326 $\pm$ 10.844 ^a	469.245 $\pm$ 123.94 ^a	62.701 $\pm$ 6.265 ^c	4.167 $\pm$ 1.573 ^b	4.044 $\pm$ 0.550 ^{de}

^{a-c}Values with different superscripts in the same column differ significantly ($P < 0.05$).

¹Enzyme activity was not detected.

activity in this study was in common with other thioesterases: Ca^{2+} had a stimulatory effect on short-chain thioesterase activity in brown adipose tissue mitochondria at 2.5 mmol/L (Alexson and Nedergaard, 1988). Long-chain fatty acyl-CoA thioesterase activity was stimulated at low concentrations and inhibited at high concentrations of Ca^{2+} (Berge and Døssland, 1979).

Influence of Fe^{2+} on Enzyme Activity

The effects of Fe^{2+} ions on β -oxidation enzymes of *L. lactis* are shown in Table 3, where 1 mmol/L Fe^{2+} inhibited acyl-CoA dehydrogenase activity. When the concentration of Fe^{2+} increased to 5 mmol/L, Fe^{2+} induced activities of enoyl-CoA hydratase and thiolase but inhibited activities of L-3-hydroxyacyl-CoA dehydrogenase and thioesterase. Moreover, Fe^{2+} had an inhibitory effect on thioesterase activity, which was not consistent with a previous report (Lee and Schulz, 1979).

Influence of Cu^{2+} on Enzyme Activity

The influence of Cu^{2+} on enzyme activity is shown in Table 4. Copper ion induced enoyl-CoA hydratase activity and inhibited activities of L-3-hydroxyacyl-CoA dehydrogenase and thioesterase. This result was not inconsistent with previous reports, in which Cu^{2+} significantly inhibited thioesterase at 1 and 1.2 mmol/L (Berge and Døssland, 1979; Lee and Schulz, 1979).

To our knowledge, there are no published studies reporting the effect of metal ions on the β -oxidation

system and thioesterase activity of *L. lactis*. In this study, we investigated the influence of some divalent cations on 5 enzymes of incomplete β -oxidation of *L. lactis*. The activity of acyl-CoA dehydrogenase could be induced by Ca^{2+} (0–10 mmol/L) and Mg^{2+} (0–20 mmol/L) and separately inhibited by 1 mmol/L Fe^{2+} or 12 mmol/L Mg^{2+} . Four of the metal divalent ions in this experiment induced activity of enoyl-CoA hydratase, and 4 inhibited L-3-hydroxyacyl-CoA dehydrogenase activity; Mg^{2+} strongly stimulated activity of L-3-hydroxyacyl-CoA dehydrogenase at 12 mmol/L. All metal ions induced activity of thiolase but its activity decreased significantly when 0.05 mmol/L Cu^{2+} was added to M17. Four ions inhibited activity of thioesterase in this study but activity decreased significantly with Ca^{2+} at 2 mmol/L. From these data, we can conclude that activity levels of the enzymes of β -oxidation of *L. lactis* are induced by metal ions. Weeks et al. (1969) reported that carbon sources (palmitate or oleate) could affect enzyme activities of β -oxidation of *Escherichia coli*. Culture conditions could affect the activity of β -oxidation and thioesterases of bacteria.

CONCLUSIONS

We investigated the effects of divalent metal ions (Ca^{2+} , Mg^{2+} , Fe^{2+} , and Cu^{2+}) on the growth, β -oxidation system, and thioesterase activity of *L. lactis*. Different ions significantly influenced the growth of *L. lactis* and significantly affected activity of the 4 key enzymes of the β -oxidation system (acyl-CoA dehydrogenase,

Table 3. Influence of Fe^{2+} concentration on enzyme activity (nmol/min per mg of protein)

Fe^{2+} (mmol/L)	Acyl-CoA dehydrogenase	Enoyl-CoA hydratase	L-3-Hydroxyacyl-CoA dehydrogenase	Thiolase	Thioesterase
0	10.814 $\pm$ 3.540	ND ¹	119.373 $\pm$ 7.335 ^a	3.540 $\pm$ 0.955 ^c	16.176 $\pm$ 0.318 ^a
1	4.651 $\pm$ 4.132	126.493 $\pm$ 115.461 ^b	52.251 $\pm$ 1.842 ^b	15.000 $\pm$ 3.802 ^{bc}	7.537 $\pm$ 2.296 ^{bc}
2	ND	144.776 $\pm$ 101.183 ^b	125.000 $\pm$ 31.681 ^a	21.875 $\pm$ 11.456 ^b	9.559 $\pm$ 1.148 ^b
3	ND	285.448 $\pm$ 63.835 ^{ab}	65.916 $\pm$ 8.723 ^b	51.042 $\pm$ 3.442 ^a	5.515 $\pm$ 0.955 ^c
4	ND	103.731 $\pm$ 28.787 ^b	78.376 $\pm$ 18.796 ^b	25.833 $\pm$ 14.662 ^b	8.640 $\pm$ 2.088 ^b
5	ND	521.268 $\pm$ 294.24 ^a	114.148 $\pm$ 18.537 ^a	27.708 $\pm$ 1.443 ^b	6.801 $\pm$ 1.773 ^{bc}

^{a-c}Values with different superscripts in the same column differ significantly ($P < 0.05$).

¹Enzyme activity was not detected.

Table 4. Influence of Cu²⁺ concentration on enzyme activity (nmol/min per mg of protein)

Cu ²⁺ (mmol/L)	Acyl-CoA dehydrogenase	Enoyl-CoA hydratase	L-3-Hydroxyacyl-CoA dehydrogenase	Thiolase	Thioesterase
0	10.814 ± 3.540	ND ¹	119.373 ± 7.335 ^a	3.540 ± 0.955 ^b	16.176 ± 0.318 ^a
0.025	ND	30.970 ± 9.651 ^a	50.643 ± 2.088 ^c	8.958 ± 0.361 ^a	10.110 ± 3.588 ^b
0.05	ND	73.507 ± 13.996 ^a	73.553 ± 0 ^b	1.458 ± 0.955 ^c	5.699 ± 1.148 ^c
0.075	ND	74.254 ± 9.520 ^a	68.730 ± 8.354 ^b	ND	6.066 ± 2.866 ^c
0.1	ND	101.866 ± 124.606 ^a	44.614 ± 3.190 ^c	ND	5.882 ± 1.148 ^c
0.125	ND	59.701 ± 6.165 ^a	44.614 ± 2.412 ^c	ND	8.272 ± 0.551 ^{bc}

^{a-c}Values with different superscripts in the same column differ significantly ($P < 0.05$).

¹Enzyme activity was not detected.

enoyl-CoA hydratase, L-3-hydroxyacyl CoA dehydrogenase, and thiolase) and thioesterase in *L. lactis*. Because divalent metal ions have some important effects on enzyme activity, we could control the metabolic pathways through the regulation of metal ion concentrations and ultimately regulate the outputs and varieties of methyl ketones.

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