J. Dairy Sci. 97:5975–5982
http://dx.doi.org/10.3168/jds.2014-8047


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

**Liang Li*† and Ying Ma*[1]**
*School of Food Science and Engineering, Harbin Institute of Technology, 202 Haihe Road, Harbin, Heilongjiang 150090, China
†College of Food Science, Northeast Agricultural University, 59 Gongbin Road, Harbin, Heilongjiang 150030, China

## 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 generation 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-

Received February 13, 2014.
Accepted May 20, 2014.
[1]Corresponding author: maying@hit.edu.cn

Aldrich Co. Ltd. (Shanghai, China). 5,5′-Dithiobis-(2-nitrobenzoicacid) (**DNTB**), HEPES, NADH, phenylmethanesulfonyl 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 for18 h. Cell-free extracts were prepared essentially as described by Engelvin et al. (2000) with modifications. Cells were harvested at 5,000 × *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 × *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-Protean 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 containing 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 μmol of 2,6-dichlorophenolindophenol (DCPIP) was monitored at 600 nm in the presence of 50 μmol of phenazine metosulfate, 72 nmol of acyl CoA, and 200 μg 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 μmol/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 μmol/L ace-