

新制

農

905

**Production of conjugated fatty acids  
by lactic acid bacteria**

**Shigenobu Kishino**

**2005**



**Production of conjugated fatty acids  
by lactic acid bacteria**

**Shigenobu Kishino**

**2005**

<b>CONTENTS</b>	
<b>GENERAL INTRODUCTION</b>	1
<b>CHAPTER I Transformation of Linoleic Acid by Lactic Acid Bacteria</b>	
<b>Section 1</b> Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by <i>Lactobacillus acidophilus</i>	2
<b>Section 2</b> Production of conjugated linoleic acid from linoleic acid by lactic acid bacteria	13
<b>CHAPTER II Ricinoleic Acid and Castor Oil as Substrates for Conjugated Linoleic Acid Production by Washed Cells of <i>Lactobacillus plantarum</i></b>	24
<b>CHAPTER III Transformation of Polyunsaturated Fatty Acids by Lactic Acid Bacteria</b>	
<b>Section 1</b> Polyunsaturated fatty acids transformation to conjugated fatty acids by <i>Lactobacillus plantarum</i> AKU 1009a	32
<b>Section 2</b> Conjugated $\alpha$ -linolenic acid production from $\alpha$ -linolenic acid by <i>Lactobacillus plantarum</i> AKU 1009a	49
<b>Section 3</b> Conjugated $\gamma$ -linolenic acid production from $\gamma$ -linolenic acid by <i>Lactobacillus plantarum</i> AKU 1009a	56
<b>CONCLUSION</b>	62
<b>REFERENCES</b>	63
<b>ACKNOWLEDGMENTS</b>	67
<b>PUBLICATIONS</b>	68

## GENERAL INTRODUCTION

Conjugated linoleic acid (CLA), an octadecadienoic acid with conjugated double bonds, has a variety of positional and geometric isomers. Recently, CLA has attracted considerable attention because of its potentially beneficial effects. It was reported that CLA inhibits the initiation of mouse skin carcinogenesis [1,2], and mouse forestomach [3] and rat mammary tumorigenesis [4]. In addition, CLA has been reported to be effective in preventing the catabolic effects of immune stimulation [5,6] and to alter the low-density lipoprotein/high-density lipoprotein cholesterol ratio in rabbits [7]. Of the individual isomers of CLA, *cis*-9,*trans*-11-octadecadienoic acid (18:2) has been suggested to be the most important in terms of biological activity because this is the major isomer in nature and is incorporated into the phospholipid fraction of tissues of animals fed a mixture of CLA isomers [3].

The dietary sources of CLA are mainly food products derived from ruminants such as beef, lamb and milk products [8,9]. Dairy products are among the major dietary sources of CLA, of which *cis*-9,*trans*-11-18:2 is the main isomer. CLA has been shown to be produced from polyunsaturated fat by certain rumen microorganisms such as *Butyrivibrio* species [10]. *cis*-9,*trans*-11-18:2 has been suggested to be the first intermediate in the biohydrogenation of linoleic acid by the anaerobic rumen bacterium, *Butyrivibrio fibrisolvens* [11]. More recently, it was reported that *Propionibacterium freudenreichii*, commonly used as a dairy starter culture, could produce CLA from free linoleic acid [12]. The existence of CLA in the lyophilized bacterial samples of several *Lactobacillus* sp. was also reported [13]. These earlier results suggested that CLA is derived from linoleic acid, but little is known about the precise structures of the produced CLA, or the mechanisms of CLA production. Also, the amounts of CLA produced are very low.

To clarify the mechanism of microbial CLA formation from linoleic acid and to establish practical processes for conjugated fatty acid production, the author investigated the ability to produce CLA from linoleic acid in lactic acid bacteria, analyzed the reactions involved, and applied the obtained information to conjugated fatty acid production.

In CHAPTER I, CLA production from linoleic acid by lactic acid bacteria is described.

In CHAPTER II, CLA production from ricinoleic acid or castor oil as a substrate by lactic acid bacteria is described.

In CHAPTER III, polyunsaturated fatty acids transformation to conjugated fatty acids by lactic acid bacteria is described.

## CHAPTER I

### Transformation of Linoleic Acid by Lactic Acid Bacteria

#### Section 1. Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by *Lactobacillus acidophilus*

#### INTRODUCTION

Conjugated linoleic acid (CLA), an octadecadienoic acid (18:2) with conjugated double bonds, has a variety of positional and geometric isomers. Among these isomers, *cis*-9,*trans*-11-18:2 and *trans*-10,*cis*-12-18:2 have attracted attention because of their unique physiological effects such as inhibition of carcinogenesis and reduction of the body fat content [1,4,8,14]. These isomers act both independently and together to produce the multitude of physiological effects that attribute to CLA. Decreased body fat gain is an example of a single-isomer effect caused by *trans*-10,*cis*-12-18:2. Various specific effects of isomers are being identified in a number of laboratories.

The author found that resting cells of *Lactobacillus acidophilus* AKU 1137 produce CLA from linoleic acid under microaerobic conditions preceded by the accumulation of hydroxy fatty acids. In CHAPTER I, the author reports the chemical structures of CLA and hydroxy fatty acids produced by *L. acidophilus* and discusses the role of hydroxy fatty acids as intermediates in the transformation of linoleic acid to CLA.

#### MATERIALS AND METHODS

**Chemicals.** Linoleic acid and fatty acid-free (<0.02%) bovine serum albumin (BSA), were purchased from Wako Pure Chemicals (Osaka, Japan) and Nacalai tesque (Kyoto, Japan), respectively. All other chemicals used were of analytical grade and are commercially available.

**Microorganism cultivation and preparation of washed cells.** *Lactobacillus acidophilus* AKU 1137 (AKU Culture Collection, Faculty of Agriculture, Kyoto University, Kyoto, Japan) was used. It was cultivated in MRS medium comprised of 1.0% tryptone, 1.0% meat extract, 0.5% yeast extract, 2.0% glucose, 0.1% Tween 80, 0.2% K<sub>2</sub>HPO<sub>4</sub>, 0.5% sodium acetate, 0.2% diammonium citrate, 0.02% MgSO<sub>4</sub>·7H<sub>2</sub>O, and 0.005% MnSO<sub>4</sub>·H<sub>2</sub>O (pH 6.5). The strain was

inoculated in 15 ml of liquid medium in screw-cap tubes (16.5 x 125 mm) and cultivated microaerobically for 3 days at 28°C with shaking (120 strokes/min). The cells were harvested by centrifugation (14,000 x *g*, 30 min), washed twice with 0.85% NaCl, centrifuged again, and then used as the washed cells.

**Reaction conditions.** Reactions were carried out at 28°C with gentle shaking (120 strokes/min) in screw-cap tubes (16.5 x 125 mm) filled with N<sub>2</sub>. The reaction mixture contained, in 1 ml of 100 mM potassium phosphate buffer (pH 6.5), 5 mg of linoleic acid in a complex with BSA (0.2 mg BSA/mg of linoleic acid) and washed cells from 15 ml culture broth (approximately 20 mg as dry-weight).

**Lipid analyses.** Lipids were extracted from the reaction mixture with chloroform-methanol (1:2, by vol.) according to the procedure of Bligh-Dyer [15], methylated with diazomethane in diethylether for 15 min, and further methylated with 0.5 M sodium methoxide in methanol for 30 min at 50°C. The resultant fatty acid methyl esters were extracted with *n*-hexane and analyzed by gas-liquid chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-17A gas chromatograph equipped with a flame ionization detector and a split injection system and fitted with a capillary column (HR-SS-10, 50 m x 0.25 mm I.D., Shinwa Kako, Kyoto, Japan). The column temperature, initially 180°C, was raised to 220°C at a rate of 2°C/min and maintained at that temperature for 5 min. The injector and detector were operated at 250°C. Helium was used as a carrier gas at 225 kPa/cm<sup>2</sup>. Extraction and fractionation into lipid classes were carried out essentially as described previously [16,17].

**Isolation of reaction products.** The methyl esters of the reaction products were separated by reverse-phase high-performance liquid chromatography (HPLC) using a Shimadzu LC-10A system equipped with a Cosmosil column (5C<sub>18</sub>-AR, 20 x 250 mm, Nacalai Tesque). The mobile phase was acetonitrile-H<sub>2</sub>O (8:2, by vol.) at a flow rate of 3.0 ml/min, and the effluent was monitored by ultraviolet detection (205 nm). The chemical structures of purified fatty acids were determined by mass spectroscopy (MS), infrared spectroscopy (IR), proton nuclear magnetic resonance (<sup>1</sup>H-NMR) and <sup>1</sup>H-<sup>1</sup>H correlation spectroscopy (COSY).

**Preparation of free fatty acids.** Free fatty acids were prepared by heating the fatty acid methyl esters (50 mg) in a mixture of 50 µl of 7.0 N sodium hydroxide and 50 µl of methanol in screw-cap tubes. After heating in a boiling water bath for 1 h, the solution was acidified to pH 2.0 with 10% (w/v) sulfuric acid in water. The free fatty acids were extracted with diethylether. The organic extract was washed with water and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and the solvent was removed under vacuum in a rotary evaporator.

**Preparation of pyrrolidide fatty acids.** Pyrrolidide derivatives were prepared by direct treatment of the isolated methyl esters with pyrrolidine-acetic acid (10:1, by vol.) in screw-cap tubes for 1 h at 115°C followed by extraction according to the method of Andersson and Holman [18]. The organic extract was washed with water and dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and then the solvent was removed under vacuum in a rotary evaporator.

**GC-MS analysis.** GC-MS QP5050 (Shimadzu) with a GC-17A gas chromatograph was used for mass spectral analyses. The GC separation of fatty acid methyl esters was performed on an HR-SS-10 column as described above at the same temperature. The GC separation of fatty acid pyrrolidide derivatives was performed on the HR-1 column (25 m x 0.5 mm I.D., Shinwa Kako) at 300°C. MS was used in the electron impact mode at 70 eV with a source temperature of 250°C. Split injection was employed with the injector port at 250°C.

**MS-MS analysis.** MS-MS analyses were performed on the free acids of the fatty acids with a JEOL-HX110A/HX110A tandem mass spectrometer. The ionization method was fast atom bombardment (FAB) and the acceleration voltage was 3 kV. Glycerol was used for the matrix.

**Infrared spectroscopy.** IR analysis of fatty acid methyl esters was performed with infrared spectrophotometer IR-420 (Shimadzu) in a chloroform solution.

**<sup>1</sup>H-NMR and <sup>1</sup>H-<sup>1</sup>H COSY analyses.** All NMR experiments were performed on a JEOL EX-400 (400 MHz at <sup>1</sup>H), and chemical shifts were assigned relative to the solvent signal. Fatty acid methyl esters were dissolved in dichloromethane-d<sub>2</sub> and the diameter of the tube was 5 mm.

## RESULTS

### **Transformation of linoleic acid by washed cells of *L. acidophilus*.**

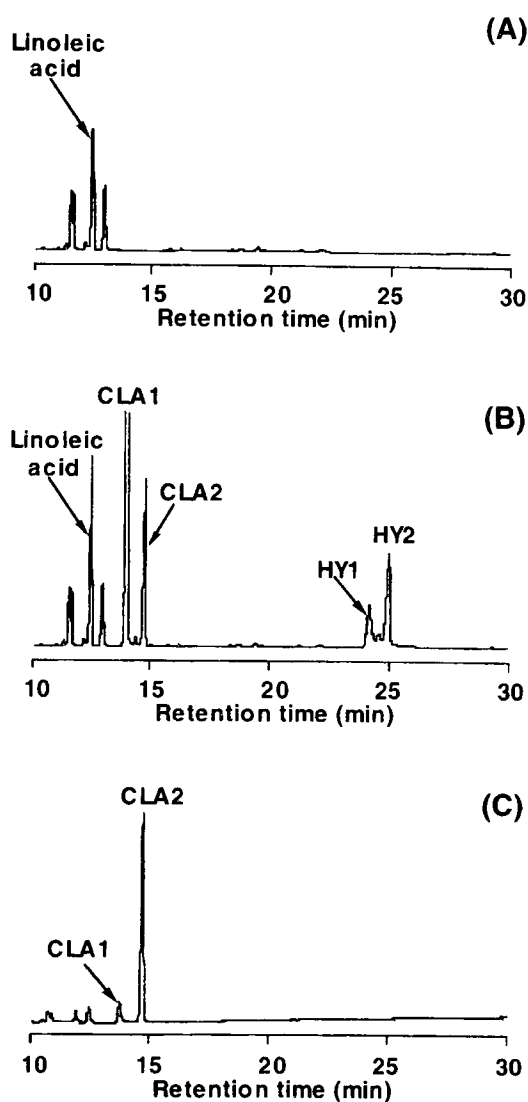
When the reaction was carried out under aerobic conditions, linoleic acid was decomposed by washed cells of *L. acidophilus* without generation of detectable amounts of fatty acids (Fig. 1A). When the reaction was carried out under microaerobic conditions, four major, newly generated fatty acids, designated CLA1, CLA2, HY1, and HY2, were found on the GC chromatograms of the methylated fatty acids (Fig. 1 B). The peaks for CLA1 and CLA2, with retention times slightly greater than that of linoleic acid, were shown to have the same retention times as those from the CLA mixture purchased from Nu-Chek-Prep, Inc.

(Minnesota, U.S.A.). The peaks for HY1 and HY2 indicated that HY1 and HY2 were relatively polar fatty acids such as hydroxylated ones acids because of their far greater retention times.

### Identification of CLA1 and CLA2.

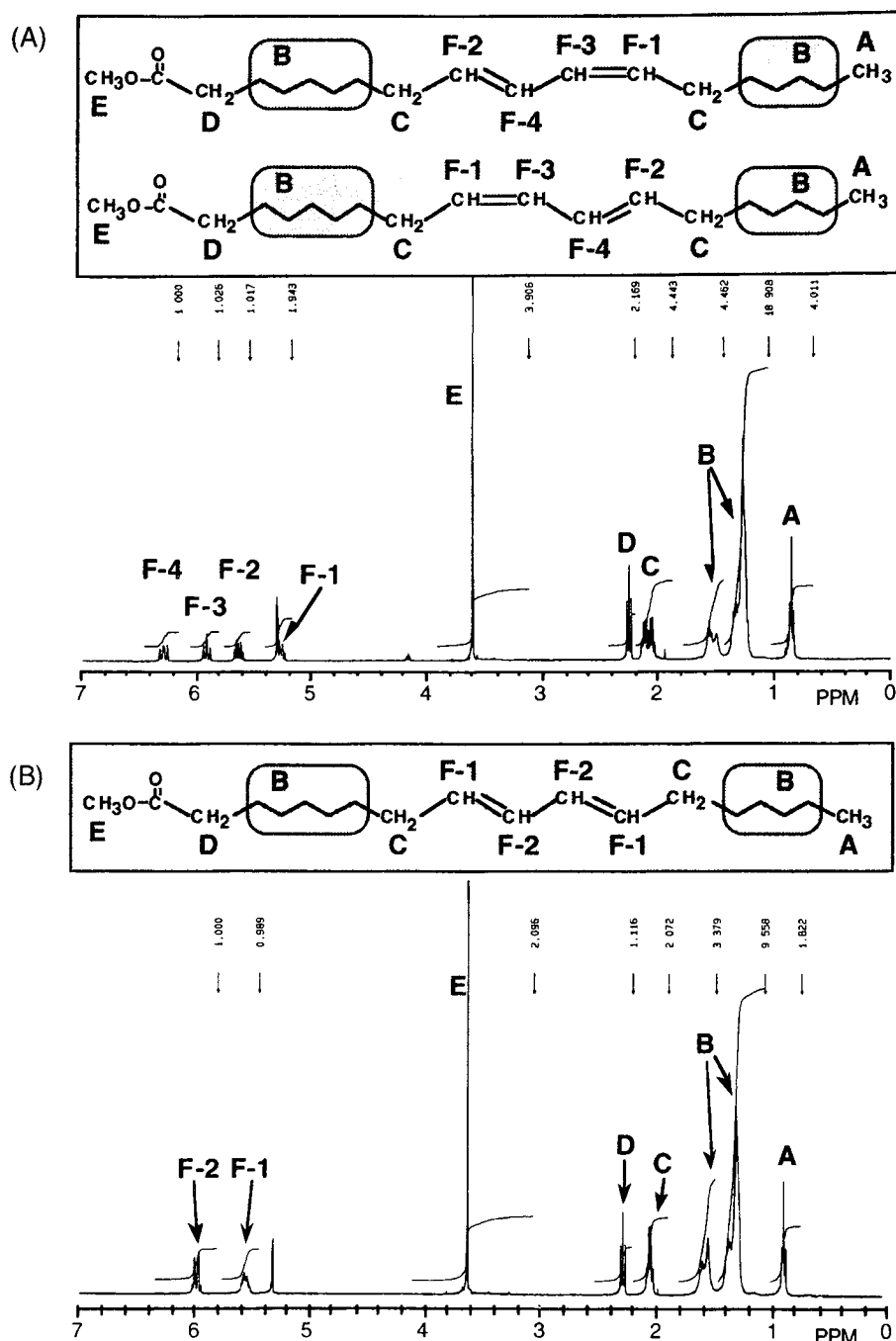
Mass spectra of the isolated methyl esters of both CLA1 and CLA2 exhibited molecular weights of  $m/z$  294, and those of pyrrolidide derivatives showed molecular weights of  $m/z$  333. These results suggested that CLA1 and CLA2 are C18 fatty acids containing two double bonds. FAB-MS data of the free fatty acids of both CLA1 and CLA2 exhibited molecular weights of  $m/z$  280 ( $[M-H]^+$ , 279). These peaks ( $m/z$  279) were fragmented again by MS-MS. Typical fragments ( $m/z$ ) for both CLA1 and CLA2 were 127, 141, 167, 193, 207 and 208. The  $m/z$  141, 167 and 193 were derived from cleavage between single bonds 8-9 and 10-11 and 12-13, numbered from the carboxyl group. The  $m/z$  127 and 207 derived from the cleavage of a single bond between the  $\alpha$  and  $\beta$  positions from the double bond, were clearly detected. Hence, CLA1 and CLA2 were identified as 9,11 positional isomers of octadecadienoic acid.

Furthermore,  $^1\text{H-NMR}$  analysis was carried out to identify the geometric configurations of CLA1 and CLA2 (Fig. 2). With respect to CLA1, the signals F-1 (5.28 ppm,  $m$ , 1H), F-2 (5.64 ppm,  $m$ , 1H), F-3 (5.92 ppm,  $t$ , 1H) and F-4 (6.29 ppm,  $m$ , 1H) suggested the existence of double bonds. Other signals were identified as shown in Fig. 2A. When the methyl ester was irradiated at 2.17 ppm ( $m$ , 4H, signal C), the coupling constant between F-1 and F-3 was 10.26 Hz, which suggests that the double bond between F-1 and F-3 is in the *cis* configuration. When irradiated at 2.02 ppm ( $m$ , 4H, signal C), the coupling constant between F-2 and F-4 was 14.65 Hz, indicating the *trans* configuration. These results indicated that CLA1 is *cis/trans*-conjugated octadecadienoic acid. With regard to CLA2, the signals F-1 (5.53 ppm,  $m$ , 2H) and F-2 (6.00 ppm,  $m$ , 2H), suggesting the existence of double bond, were mixtures of two signals (Fig. 2B).



**Fig. 1.** GC chromatograms of fatty acid methyl esters produced by washed cells of *L. acidophilus*. (A) Reaction with linoleic acid as a substrate under aerobic conditions. (B) Reaction with linoleic acid as a substrate under microaerobic conditions. (C) Reaction with HY2 as a substrate under microaerobic conditions.



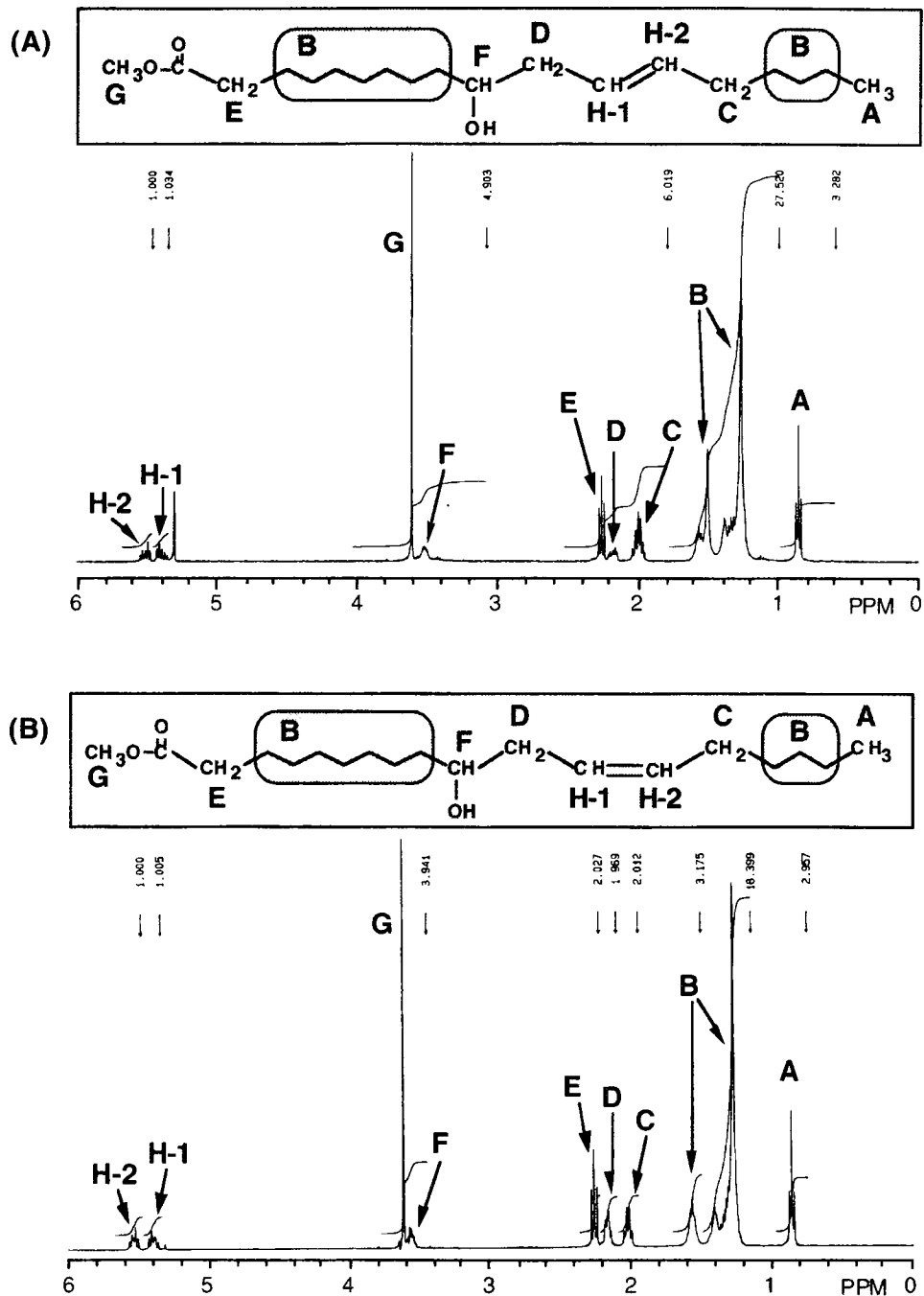


**Fig. 2.** <sup>1</sup>H-NMR spectra and structures of CLA1 (A) and CLA2 (B). The letters indicate the positions of protons and their corresponding signals.

It was not possible to determine the coupling constant of the double bond.

IR analysis was performed to confirm the geometric configuration. The major differences in IR spectra were in the 800-1000  $\text{cm}^{-1}$  range. In the spectrum of CLA1, sharp absorption peaks at 990 and 944  $\text{cm}^{-1}$  were observed, indicating that it is a *cis/trans* isomer [19]. CLA2 showed sharp absorption at 990  $\text{cm}^{-1}$ , indicating that it is a *trans/trans* isomer [19].

On the basis of the results of spectral analyses, CLA1 and CLA2 were identified as



**Fig. 3.** <sup>1</sup>H-NMR spectra and structures of HY1 (A) and HY2 (B). The letters indicate the positions of protons and their corresponding signals.

*cis*-9,*trans*-11- or *trans*-9,*cis*-11-octadecadienoic acid and *trans*-9,*trans*-11-octadecadienoic acid, respectively.

### Identification of HY1 and HY2.

FAB-MS analysis of the isolated methyl esters of HY1 and HY2 revealed molecular weights of  $m/z$  312 ( $[M+H]^+$ , 313). On MS analyses of methyl esters of both HY1 and HY2,

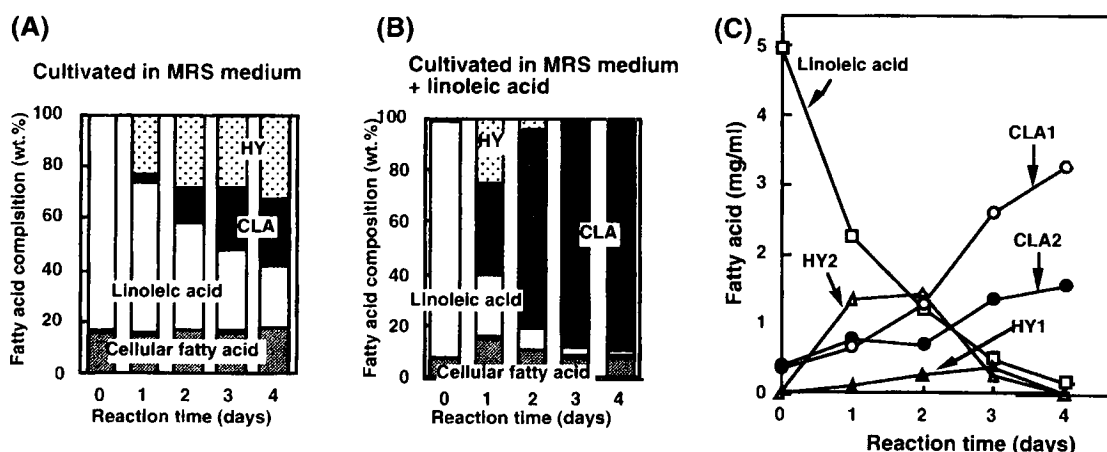
typical fragments of  $m/z$  169, 201 and 294 were found. The fragment  $m/z$  294 (M-18) was thought to indicate cleavage between the hydroxyl group and carbon and the existence of one double bond in the hydrocarbon chain. Moreover, cleavage between the  $\alpha$  and  $\beta$  positions from the hydroxyl group yielded  $m/z$  201. This suggested that the hydroxyl group is located at carbon 10, numbered from the carboxyl group.

$^1\text{H-NMR}$  and  $^1\text{H-}^1\text{H COSY}$  analyses were carried out to identify the positions and geometric configurations of double bonds in HY1 and HY2.  $^1\text{H-NMR}$  spectra of methyl esters of HY1 and HY2 are shown in Fig. 3. The signal at 3.5 ppm suggested the existence of a hydroxyl group, and the signals H-1 (5.4 ppm, *m*, 1H) and H-2 (5.5 ppm, *m*, 1H) were identified as the protons on the double bond. The  $^1\text{H-}^1\text{H COSY}$  spectra of the methyl esters of HY1 and HY2 showed that there is one methylene group between the double bond and the carbon bonding to the hydroxyl group (data not shown). Therefore, a double bond was thought to be located at the  $\Delta_{12}$  position. Coupling constants between H-1 and H-2 of HY1 and HY2 determined by irradiation at 2.0 ppm (*m*, 3H, signal C) were 15.14 Hz and 11.23 Hz, indicating that the double bonds are in *trans* and *cis* configurations, respectively.

From these results, HY1 and HY2 were identified as 10-hydroxy-*trans*-12-octadecaenoic acid and 10-hydroxy-*cis*-12-octadecaenoic acid, respectively.

#### Time course of linoleic acid transformation by washed cells of *L. acidophilus* under microaerobic conditions.

The time course of changes in fatty acid composition during linoleic acid transformation by washed cells of *L. acidophilus* under microaerobic conditions was studied using the washed cells obtained by cultivated in MRS medium with or without linoleic acid [0.1% (w/v)]. In



**Fig. 4.** Time courses of changes in fatty acid composition during the reaction with washed cells obtained by cultivation in MRS medium without (A) or with (B) linoleic acid [0.1% (w/v)], and of changes in levels of CLA and HY production from linoleic acid (C). The results are averages of three separate determinations that were reproducible within  $\pm 10\%$ .

comparison with the results shown in Fig. 4A and B, the CLA productivity of the washed cells obtained by cultivation with linoleic acid was much higher than that of the cells obtained by cultivation without linoleic acid. This may have been due to induction of the enzymes catalyzing CLA formation by linoleic acid during cultivation. The amounts of cellular fatty acids (myristic acid, palmitic acid, palmitoleic acid, oleic acid, vaccenic acid and 2-hexyl-1-cyclopropane-octanoic acid) did not significantly change for 4 days after the reaction. With the washed cells obtained by cultivation with linoleic acid, CLA (sum of CLA1 and CLA2) levels reached 36% (w/w) of the total fatty acids on the first day and exceeded 80% (w/w) on the fourth day (Fig. 4B). The ratio HY (sum of HY1 and HY2) was 25% (w/w) on the first day and rapidly decreased, followed by an increase in CLA level (Fig. 4B). These results suggested that HY produced by the washed cells may be converted to CLA and that HY may be the intermediate in CLA formation. The time courses of CLA and HY production from linoleic acid are presented in Fig. 4C. The final level of CLA was 4.9 mg/ml (CLA1, 3.3 mg/ml; CLA2, 1.6 mg/ml; molar conversion yield from linoleic acid, 98%).

#### **Production of CLA from hydroxy fatty acid.**

HY2 was isolated by preparative high-performance liquid chromatography and used as the substrate for the reaction instead of linoleic acid to determine whether HY is converted to CLA by washed cells of *L. acidophilus*. The GC chromatogram of the fatty acids obtained after reaction under microaerobic conditions is shown in Fig. 1C. No HY2 was detected after the reaction, and CLA1 and CLA2 were found. These observations suggested that HY2 may be a substrate for CLA and an intermediate in the formation of CLA from linoleic acid. Preparative isolation of HY1 resulted in contamination with a small amount of HY2, so that it was difficult to determine whether HY1 was indeed an intermediate. However, during linoleic acid transformation, HY1 accumulated, while CLA was being produced, until the third day; then the level of HY1 decreased, followed by an increase in the level of CLA (Fig. 4C), indicating that HY1 is also an intermediate of CLA formation.

#### **Distribution and lipid classes of the fatty acids produced by washed cells of *L. acidophilus*.**

The reaction mixture of linoleic acid transformation was centrifuged after 3 days of reaction and separated into supernatant and cells. The distribution and lipid classes of the fatty acids produced in the both supernatant and cells were analyzed (Table 1). Most of the fatty acids (92.0%) were found in the cells or associated with the cells as free fatty acids, with CLA the most abundant. The fatty acids found in the cells (or associated with cells) consisted of free fatty acids (86.4%), acylglycerols (5.6%) and trace amounts of phospholipids. Most of the CLA produced was found as free fatty acid in the cells (or associated with cells).

**TABLE 1. Distribution and lipid classes of fatty acids produced from linoleic acid by *L. acidophilus* under microaerobic conditions<sup>a</sup>**

Fatty acid	Fatty acid found after reaction (mg/ml reaction mixture)	Lipid composition (mol%) in						
		Supernatant			Cells			Total
		FA <sup>c</sup>	AG <sup>d</sup>	PL <sup>e</sup>	FA	AG	PL	
Linoleic acid	0.24	1.9	0.3		2.7	0.3	5.2	
CLA	3.76	3.5	0.6		74.5	3.9	82.5	
HY	0.04	f			0.6	0.3	0.9	
Other fatty acids <sup>b</sup>	0.52	1.4	0.3		8.6	1.1	11.4	
Total	4.56	6.8	1.2		86.4	5.6	tr	
			8.0			92.0	100	

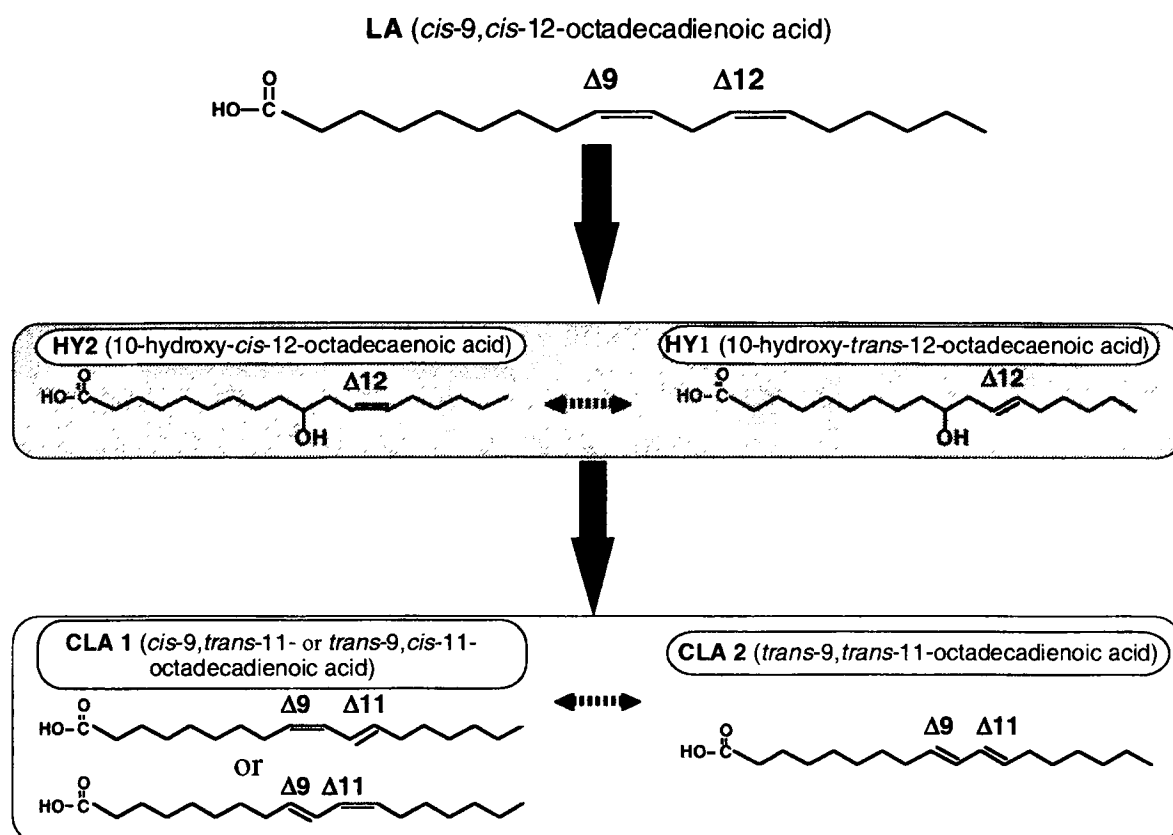
<sup>a</sup>*L. acidophilus* was cultivated in MRS medium with linoleic acid (0.1%) for 3 days. The reaction was carried out with 5 mg/ml of linoleic acid as the substrate for 3 days under the conditions described in MATERIALS AND METHODS. <sup>b</sup>Other lipids were myristic acid (0.1 mol%), palmitic acid (1.5), palmitoleic acid (0.1), oleic acid (7.4), vaccenic acid (1.7) and 2-hexy-1-cyclopropane-octanoic acid (0.7). <sup>c</sup>FA, free fatty acids; <sup>d</sup>AG, acylglycerols; <sup>e</sup>PL, phospholipids; <sup>f</sup>-, not detected; <sup>g</sup>tr, trace (less than 0.05 mol%).

## DISCUSSION

Some anaerobic bacteria have been reported to produce CLA. The rumen bacterium *Butyrivibrio fibrisolvens* produces *cis*-9,*trans*-11-octadecadienoic acid as an intermediate of biohydrogenation of linoleic acid to oleic acid [11]. The lyophilized cells of some lactobacilli have been found to contain small amounts of CLA [13]. However, the mechanism of CLA formation has not been elucidated in detail. The results reported here indicated that the transformation of linoleic acid to CLA is not a one-step isomerization of a nonconjugated diene to a conjugated diene. Rather, the transformation involves the production of hydroxy fatty acids, i.e., 10-hydroxy-*trans*-12-octadecaenoic acid and 10-hydroxy-*cis*-12-octadecaenoic acid. The following findings obtained here using resting cells of *L. acidophilus*, i.e., (i) accumulation of HY prior to CLA formation and its decrease concomitant with increased formation of CLA and (ii) conversion of exogenously added 10-hydroxy-*cis*-12-octadecaenoic acid to CLA, strongly support the above suggestion. It is not yet clear whether the generation of geometric isomers of the hydroxy fatty acid and CLA is a biological or chemical process occurring during analysis or whether the *trans* isomer of the hydroxy fatty acid is involved as an intermediate. However, the pathway involving hydroxylation at carbon 10, numbered from carboxyl group, as the first step in the reaction, could be proposed for isomerization of linoleic acid to CLA (Fig. 5).

A study of production of CLA was performed with *Propionibacterium freudenreichii*, a bacterium commonly used in dairy starter cultures, and showed the extracellular production of CLA (265 µg/ml) mainly consisting of *cis*-9,*trans*-11- or *trans*-9,*cis*-11-octadecadienoic acid [12]. This previous study revealed the potential of lactic acid bacteria to produce CLA. The transformation of linoleic acid into CLA with washed cells of *L. acidophilus* under





**Fig. 5.** Proposed pathway of CLA production from linoleic acid by washed cells of *L. acidophilus* under microaerobic conditions.

microaerobic conditions is a promising system for the following reason: (i) specific isomers of CLA, i.e., *cis*-9,*trans*-11- or *trans*-9,*cis*-11-octadecadienoic acid and *trans*-9,*trans*-11-octadecadienoic acid, are obtained as reaction products; (ii) CLA accumulates at high concentrations (nearly 5 mg/ml); (iii) CLA content in the recovered fatty acids reaches nearly 90% (w/w); (iv) CLA is accumulated as intracellular or cell-associated lipids in free form, making it easy to recover by centrifugation, and cells themselves could be used as sources of CLA; and (v) the reaction requires only microaerobic conditions and no energy input.

## SUMMARY

Conjugated linoleic acid (CLA) was produced from linoleic acid by washed cells of *Lactobacillus acidophilus* AKU 1137 under microaerobic conditions. The CLA produced was identified as *cis*-9,*trans*-11- or *trans*-9,*cis*-11-octadecadienoic acid and *trans*-9,*trans*-11-octadecadienoic acid. Preceding the production of CLA, hydroxy fatty acids identified as 10-hydroxy-*cis*-12-octadecaenoic acid and 10-hydroxy-*trans*-12-octadecaenoic acid were accumulated. The isolated 10-hydroxy-*cis*-12-octadecaenoic acid was transformed into CLA

during incubation with washed cells of *L. acidophilus*, suggesting that this hydroxy fatty acid is one of the intermediates of CLA production from linoleic acid. The washed cells of *L. acidophilus* producing high levels of CLA were obtained by cultivation in a medium containing linoleic acid, indicating that the enzyme system for CLA production is induced by linoleic acid. After 4 days of reaction with these washed cells, more than 95% of the linoleic acid (5 mg/ml) was transformed into CLA, and the CLA content in the total fatty acids recovered exceeded 80% (w/w). Almost all of the CLA produced was in the cells or associated with the cells as the free fatty acid.

## Section 2. Production of conjugated linoleic acid from linoleic acid by lactic acid bacteria

### INTRODUCTION

Interest in conjugated linoleic acid (CLA), an octadecadienoic acid (18:2) with conjugated double bonds, has increased in the last two decades because of its unique physiological effects. It was reported that dietary CLA reduced carcinogenesis [1,3,4,20], atherosclerosis [7], and body fat [21], and had several other beneficial effects [22-24].

The author investigated biological systems for CLA production and found that the washed cells of *Lactobacillus acidophilus* AKU 1137 produced CLA isomers from linoleic acid [25]. They efficiently produced CLA from linoleic acid with 10-hydroxy-12-octadecaenoic acid (18:1) as a possible intermediate under microaerobic reaction conditions. Systems using lactic acid bacteria for CLA production were found to be advantageous for the following reasons: (i) specific isomers of CLA, i.e., *cis*-9,*trans*-11 or *trans*-9,*cis*-11-18:2 (CLA1) and *trans*-9,*trans*-11-18:2 (CLA2), are obtained, whereas chemical synthesis produces a mixture of CLA isomers [26,27]; (ii) CLA is accumulated in washed cells as the free fatty acid form, making it easy to recover, and the cells themselves can be used as the CLA source. These merits prompt the author to search potential strains for practical production of CLA. Moreover, some studies found that linoleic acid is converted to 9,11-18:2 by several species of rumen bacteria [28-35] and by dairy starter cultures [12]. However, to the author's knowledge, exact identification of the geometric configuration of the 9,11-18:2 produced has not been done. The author reports here that *L. plantarum* AKU 1009a, which was selected through screening a wide range of lactic acid bacteria, produces large amount of CLA even under aerobic conditions. The produced CLA was identified as *cis*-9,*trans*-11-18:2 and *trans*-9,*trans*-11-18:2. The former is one of the physiologically active CLA isomers. Investigation of culture conditions to obtain active catalysts, optimization of reaction conditions for practical CLA productions, and some factors affecting isomer production using *L. plantarum* AKU 1009a are also described.

### MATERIALS AND METHODS

**Chemicals.** Standard samples of *cis*-9,*trans*-11- (or *trans*-9,*cis*-11-) 18:2 (CLA1), *trans*-9,*trans*-11-18:2 (CLA2), 10-hydroxy-*trans*-12-18:1 (HY1) and 10-hydroxy-*cis*-12-18:1 (HY2) were prepared as described in CHAPTER I, section 1. Linoleic acid and fatty acid-free (<0.02%) bovine serum albumin (BSA) were purchased from Wako Pure Chemical (Osaka, Japan) and

Sigma (MO, U.S.A.), respectively. All other chemicals used were of analytical grade and were commercially available.

**Microorganisms, cultivation and preparation of washed cells.** Lactic acid bacteria preserved in the author's laboratory (AKU Culture Collection, Faculty of Agriculture, Kyoto University, Kyoto, Japan) and those obtained from other culture collections (IAM, Institute of Molecular and Cellular Bioscience, The University of Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; and JCM, Japan Collection of Microorganisms, Wako, Japan) were subjected to screening. For screening, strains were cultivated in MRS medium [25] supplemented with 0.06% linoleic acid. Each strain was inoculated into 15 ml of medium in screw-cap tubes (16.5 x 125 mm) and then incubated under O<sub>2</sub>-limited conditions in sealed tubes for 24-72 h at 28°C with shaking (120 strokes/min). For optimization of culture conditions for *L. plantarum* AKU 1009a, cultivation was carried out essentially under the same conditions as described above. For optimization of reaction conditions and preparative CLA production, cultivation was carried out aerobically with 550 ml of MRS medium containing 0.06% linoleic acid in 600-ml flasks for 24 h at 28°C with shaking (120 strokes/min). Cells were harvested by centrifugation (14,000 x g, 30 min), washed twice with 0.85% NaCl, and centrifuged again, then used as the washed cells for the reactions.

**Reaction conditions.** For screening and optimization of culture and reaction conditions, the reaction mixture, 1 ml, in test tubes (16.5 x 125 mm) was composed of 0.4% (w/v) linoleic acid complexed with BSA [0.08% (w/v)], 0.1 M potassium phosphate buffer (KPB, pH 6.5), and 22.5% (wet cells, w/v) washed cells [corresponding to 3.2% (dry cells, w/v)]. The reactions were carried out microaerobically in an O<sub>2</sub>-adsorbed atmosphere in a sealed chamber with O<sub>2</sub>-absorbent (AnaeroPack "Kenki"; Mitsubishi Gas Chemical Co, Inc., Tokyo, Japan), and gently shaken (120 strokes/min) at 37°C for 24 to 72 h. For investigation of the effects of linoleic acid concentration and cell concentration on the reaction, and for preparative CLA production, the reactions were carried out essentially under the same conditions as described above except that the volume of the reaction mixtures was 5 ml. All experiments were carried out in triplicate, and the averages of three separate experiments, which were reproducible within ±10%, are presented in figures and tables, except for Fig. 4, where exact error limits are provided.

**Lipid analyses.** Lipids were extracted from the reaction mixture with chloroform-methanol (1:2, by vol.) according to the procedure of Bligh-Dyer [15], and transmethylated with 10% methanolic HCl at 50°C for 20 min. The resultant fatty acid methyl esters were extracted with *n*-hexane and analyzed by gas-liquid chromatography (GC) as described in CHAPTER I, section

1. Extraction and fractionation into lipid classes were carried out essentially as described in CHAPTER I, section 1 [16,17].

**Purification and structural analysis of fatty acids.** Fatty acids in the reaction mixtures were isolated by high-pressure liquid chromatography and their chemical structures were identified by MS as described in CHAPTER I, section 1, and by  $^1\text{H-NMR}$ . All NMR experiments were done with a Bruker Biospin DMX-750 (750 MHz for  $^1\text{H}$ ), and chemical shifts were assigned relative to the solvent signal. Fatty acid methyl esters were dissolved in  $\text{CDCl}_3$ , and analyzed by two-dimensional NMR techniques of  $^1\text{H-}^1\text{H}$  double quantum filtered chemical shift correlation spectroscopy (DQF-COSY),  $^1\text{H}$  clean-total correlation spectroscopy (clean-TOCSY), and two-dimensional nuclear Overhauser effect spectroscopy (NOESY).

## RESULTS AND DISCUSSION

### Screening of lactic acid bacteria producing CLA from linoleic acid.

The ability to produce CLA from linoleic acid was investigated using washed cells of lactic acid bacteria. The following observations obtained in the author's previous study using *L. acidophilus* AKU 1137 [25] were taken into consideration: (i) washed cells of *L. acidophilus* with high levels of CLA production were obtained by cultivation in a medium with linoleic acid, and (ii) production of CLA was only observed under microaerobic conditions ( $\text{O}_2$  concentration was less than 1%). More than 250 strains were tested from the genera of *Lactobacillus*, *Streptococcus*, *Pediococcus*, *Leuconostoc*, *Propionibacterium*, *Bifidobacterium*, *Weissella*, *Aquaspirillum*, *Enterococcus*, *Tetragenococcus*, *Aerococcus*, *Butyrivibrio*, *Lactococcus*, and *Weissella*. Of these, strains belonging to the genera *Enterococcus*, *Pediococcus*, *Propionibacterium*, and *Lactobacillus* produced considerable amounts of two CLA isomers, i.e. *cis-9,trans-11* or *trans-9,cis-11-18:2* (CLA1) and *trans-9,trans-11-18:2* (CLA2). Table 1 summarizes the results with strains that produced more than 0.07 mg CLA/ml reaction mixture, most of which were lactobacilli. Either 10-hydroxy-*trans-12-18:1* (HY1) or 10-hydroxy-*cis-12-18:1* (HY2), possible intermediates of CLA biosynthesis from linoleic acid [25], were also found in all of these reaction mixtures. *Pediococcus acidilactici* AKU 1059 and *L. rhamnosus* AKU 1124 showed almost some level of CLA production as *L. acidophilus* AKU 1137 (about 1.5 mg/ml reaction mixture). *L. plantarum* AKU 1009a and *L. plantarum* JCM 1551 were found to produce CLA (sum of CLA1 and CLA2) at more than 1.5 mg/ml reaction mixture. Notably, *L. plantarum* AKU 1009a produced the highest amounts of CLA (3.41 mg/ml), and was used for further optimization of culture and reaction conditions.



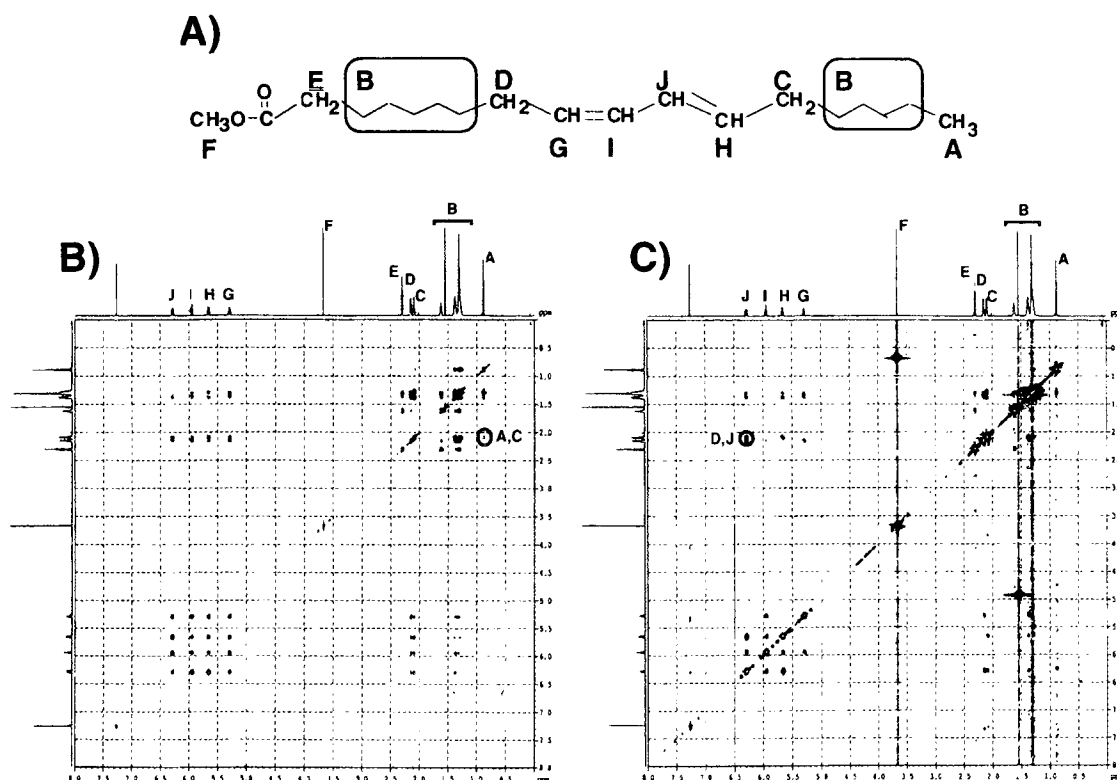
TABLE 1. Potential strains for CLA production from linoleic acid

Strain	Origin	Fatty acid (mg/ml reaction mixture)					
		Other fatty acids	LA	Total CLA	(CAL1:CLA2)	HY1	HY2
<i>Enterococcus faecium</i>	AKU 1021	0.09	0.72	0.10	(0.04 : 0.06)	0.02	0.06
<i>Pediococcus acidilactici</i>	AKU 1059	0.14	1.29	1.40	(1.00 : 0.40)	0.30	0.43
<i>Propionibacterium shermanii</i>	AKU 1254	0.11	1.42	0.11	(0.09 : 0.02)		0.07
<i>Lactobacillus acidophilus</i>	AKU 1137	0.14	0.24	1.50	(0.85 : 0.65)	0.11	0.07
<i>Lactobacillus acidophilus</i>	IAM 10074	0.25	0.22	0.60	(0.18 : 0.42)	0.60	0.18
<i>Lactobacillus acidophilus</i>	AKU 1122	0.09	0.91	0.12	(0.02 : 0.10)	0.00	0.02
<i>Lactobacillus brevis</i>	IAM 1082	0.10	0.16	0.55	(0.23 : 0.32)	0.79	
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	IFO 12004	0.18	0.83	0.20	(0.05 : 0.15)	0.22	0.45
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	JCM 1109	0.17	0.76	0.07	(0.02 : 0.05)		0.57
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	AKU 1142	1.08	0.90	0.07	(0.04 : 0.03)	0.05	1.00
<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>	IFO 3533	0.32	0.93	0.09	(0.05 : 0.04)	0.06	0.68
<i>Lactobacillus pentosus</i>	AKU 1148	0.10	1.24	0.08	(0.05 : 0.03)	0.08	0.05
<i>Lactobacillus pentosus</i>	IFO 12011	0.09	0.89	0.13	(0.10 : 0.03)	0.13	0.74
<i>Lactobacillus plantarum</i>	AKU 1138	0.11	0.10	0.45	(0.10 : 0.35)	1.21	
<i>Lactobacillus plantarum</i>	AKU 1009a	0.07	0.06	3.41	(0.25 : 3.16)	0.11	0.16
<i>Lactobacillus plantarum</i>	JCM 8341	0.18	0.43	0.19	(0.04 : 0.15)	0.27	0.40
<i>Lactobacillus plantarum</i>	JCM 1551	0.36	0.02	2.02	(0.10 : 1.92)	0.02	0.46
<i>Lactobacillus rhamnosus</i>	AKU 1124	0.10	0.22	1.41	(0.69 : 0.72)	0.13	0.15

Reactions were carried out in 72 h as described in MATERIALS AND METHODS. Other fatty acids included myristic acid, palmitic acid, palmitoleic acid, oleic acid, vaccenic acid, and 2-hexy-1-cyclopropane-octanoic acid. LA, linoleic acid; CLA1, *cis*-9,*trans*-11- or *trans*-9,*cis*-11-18:2; CLA2, *trans*-9,*trans*-11-18:2; HY1, 10-hydroxy-*trans*-12-18:1; HY2, 10-hydroxy-*cis*-12-18:1; -, not detected.

### Identification of CLA1.

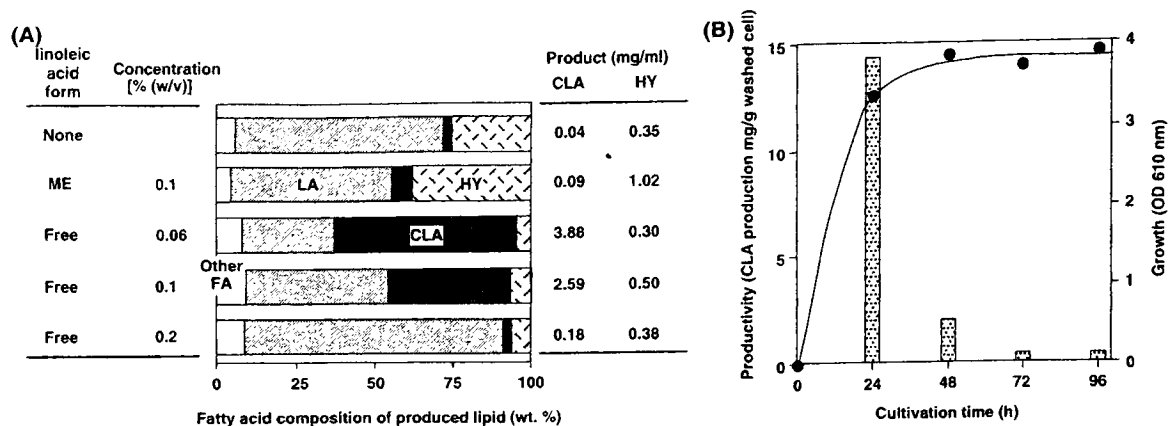
The CLA1 produced by *L. plantarum* AKU 1009a was isolated as the methyl ester form and further was transformed into its pyrrolidide derivative [25]. The mass spectrum of the pyrrolidide derivative showed a molecular weight at  $m/z$  333. This result suggested that CLA1 was a C18 fatty acid containing two double bonds. The FAB-MS data for the free fatty acid of CLA1 [25] showed a molecular weight at 280 ( $m/z$  279 [M-H]<sup>+</sup>). The material ( $m/z$  279) was fragmented again by MS-MS [ $m/z$  (FAB<sup>-</sup>, 8.00 kV), 263 (6), 249 (5), 235 (5), 221 (16), 208 (11), 207 (11), 193 (5), 181 (4), 167 (3), 141 (12), 127 (71), 113 (10), 100 (8), 98 (10), 86 (18), 72 (11), 71 (57), 58 (100)]. Typical fragments ( $m/z$ ) for CLA1 were 127, 141, 167, 193, 207, and 208. The  $m/z$  141, 167 and 193 fragments were derived through cleavage at single bonds 8-9, 10-11, and 12-13, as numbered from the carboxyl group. The  $m/z$  127 and 207 fragments, derived through the cleavage of the single bond between the  $\alpha$  and  $\beta$  positions from the double bond, were detected clearly. Hence, CLA1 was identified as a 9,11 positional isomer of octadecadienoic acid. Results of IR analysis of CLA1 methyl esters were as follows: IR  $\nu_{\max}$  (CHCl<sub>3</sub>) cm<sup>-1</sup>: 2923, 2846, 1742, 1461, 1435, 1196, 1170, 990, 944. The peaks at 990 and 944 indicated that CLA1 was a *cis/trans* isomer. <sup>1</sup>H-NMR analysis also suggested that CLA1 was an isomer of 18:2 (Fig. 1) [NMR  $\delta_{\text{H}}$  (CDCl<sub>3</sub>): 6.29 (1H, dd,  $J$  = 15.0, 10.5 Hz, =CH-CH=), 5.94 (1H, dd,  $J$  = 11.2, 10.5 Hz, =CH-CH=), 5.66 (1H, dt,  $J$  = 15.8, 6.8 Hz, -CH=CH-),



**Fig. 1.**  $^1\text{H}$ -NMR analysis of CLA1 and structure of CLA1 identified. (A) Structure of CLA1. (B)  $^1\text{H}$  clean-total correlation spectroscopic (clean-TOCSY) spectrum of the methyl ester of CLA1. (C) Two-dimensional nuclear Overhauser effect spectroscopy (NOESY) of the methyl ester of CLA1.

5.29 (1H, dt,  $J = 10.5, 7.5$  Hz,  $-\text{CH}=\text{CH}-$ ), 3.67 (3H, s,  $-\text{OCH}_3$ ), 2.30 (2H, t,  $J = 7.5$  Hz,  $\text{COCH}_2-$ ), 2.14 (2H, dt,  $J = 7.5, 6.8$  Hz,  $-\text{CH}_2-\text{CH}=\text{}$ ), 2.09 (2H, dt,  $J = 7.5, 6.8$  Hz,  $=\text{CH}-\text{CH}_2-$ ), 1.62 (2H, m,  $-\text{CH}_2\text{CH}_2\text{CH}_2-$ ), 1.39 (4H, m,  $-\text{CH}_2\text{CH}_2\text{CH}_2-$ ), 1.30 (12H, m,  $-\text{CH}_2\text{CH}_2\text{CH}_2-$ ), 0.88 (3H, t,  $J = 7.1$  Hz,  $-\text{CH}_3$ ]. Signals G (5.29 ppm), H (5.66 ppm), I (5.94 ppm), and J (6.29 ppm) indicated the existence of two double bonds in CLA1 (Fig. 1). The sequence of the protons from the methyl end of the molecule was deduced to be A-B-C-H-J-I-G-D-B-E or A-B-D-G-I-J-H-C-B-E on the basis of the signal pattern of the interaction between adjacent protons observed by DQF-COSY. The sequence was confirmed to be the former one by the appearance of an interaction signal between A and C but not A and D on clean-TOCSY analysis (Fig. 1B), indicating that C was near to A, but that D was far from A.

NOESY was done to identify the geometric configurations of double bonds. The appearance of a cross-peak between D and J suggested that the double bond between G and I had the *cis* configuration (close enough to interact) (Fig. 1C). This conclusion was confirmed by analysis of the spin-spin coupling constants between the G and I protons (11.2 and 10.5 Hz); the spin-spin coupling constants between the J and H protons (15.0 and 15.8 Hz) suggested that the double bond between J and H was in the *trans* configuration. On the basis of the results of



**Fig. 2.** Optimization of culture conditions. (A), Effects of linoleic acid on CLA production by *Lactobacillus plantarum* AKU 1009a. Cultivations were carried out in MRS medium with or without linoleic acid at the indicated concentrations. (B), Time course of cultivation and CLA productivity of washed cells of *L. plantarum* AKU 1009a. The bars and line represent CLA productivity and OD 610 nm, respectively. Reactions were carried out with linoleic acid as the substrate for 72 h as described in the MATERIALS AND METHODS section. ME, linoleic acid methyl ester; Free, free linoleic acid; LA, linoleic acid; HY, hydroxylated octadecenoic acid, HY1+HY2.

spectral analyses, CLA1 was identified as *cis*-9,*trans*-11-18:2 (Fig. 1A). These results together with previous results showed that the bacterium produced two CLA isomers, *cis*-9,*trans*-11- and *trans*-9,*trans*-11-octadecadienoic acid (CLA1 and CLA2, respectively), from linoleic acid.

### Optimization of culture conditions for the preparation of washed cells of *L. plantarum* with high CLA productivity.

*L. plantarum* AKU 1009a was easy to cultivate and showed high a growth rate even under aerobic conditions. To obtain washed cells with high CLA productivity, culture conditions were examined using MRS medium under aerobic conditions. When free linoleic acid was added to the MRS medium, CLA production increased markedly (Fig. 2A). On the other hand, addition of linoleic acid methyl ester resulted in accumulation of the intermediate, HY. CLA production was the highest with addition of 0.06% linoleic acid to the medium (Fig. 2A). Below 0.06%, linoleic acid did not affect cell growth, but higher concentrations of linoleic acid (0.2%) inhibited the growth and decreased CLA productivity. The changes in CLA productivity during cultivation in MRS medium supplemented with 0.06% linoleic acid were monitored (Fig. 2B). The cells at late log-phase showed significant productivity, but further cultivation resulted in a decrease in productivity (Fig. 2B). Washed cells obtained from late log-phase culture (24-h cultivation) were used for further optimization of reaction conditions.

**Fig. 3.** Effects of oxygen on CLA production. Reactions were carried out in 24 h as described in MATERIALS AND METHODS in an O<sub>2</sub>-adsorbed atmosphere or under air. LA, linoleic acid; CLA1, *cis*-9,*trans*-11-18:2; CLA2, *trans*-9,*trans*-11-18:2; HY1, 10-hydroxy-*trans*-12-18:1; HY2, 10-hydroxy-*cis*-12-18:1.

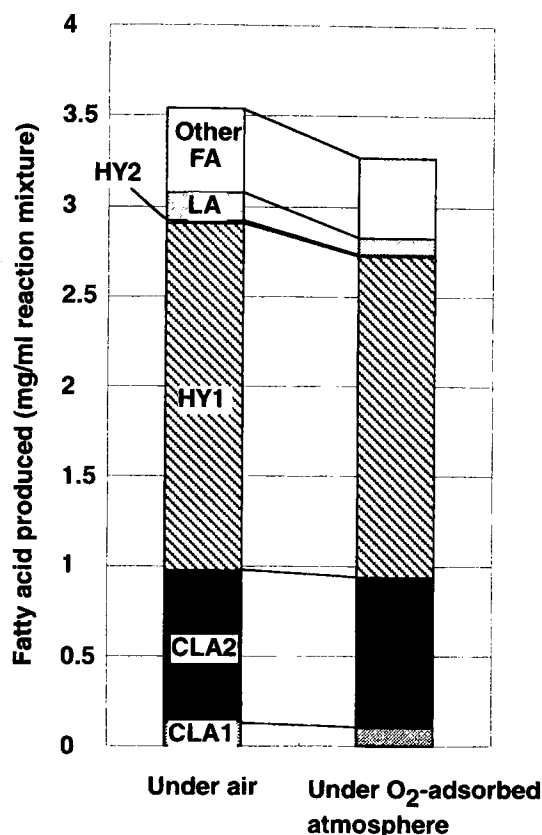
### Optimization of reaction conditions.

(i) Effects of reaction pH: Reactions were carried out for 72 h in buffer systems of 0.1, 0.5 or 1.0 M of acetate/sodium acetate buffer (pH 5.0, 6.0) or KPB (pH 6.5, 7.0, 7.5). CLA was most efficiently produced with 0.1 M KPB, pH 6.5.

(ii) Effect of reaction temperature: Reactions were carried out for 72 h at different temperatures in the range of 20 to 52°C. CLA production increased with increasing temperature from 20 to 37°C, but decreased with higher temperature. At 52°C, neither CLA nor HY were produced. At 20°C, HY was produced in good yield, but CLA was not.

(iii) Effects of substrate form: Free or methyl ester forms of linoleic acid were tested as substrates [0.5% (w/v)] after treatment with BSA [0.5% (w/v)]. BSA is a free fatty acid carrier dispersing the fatty acid in the reaction mixture. After 72-h reaction, the free form of linoleic acid was well converted to CLA (2.59 mg/ml), while methyl ester was not (<0.05 mg/ml). The effect of the ratio of BSA to linoleic acid were also examined. The amounts of CLA produced in 72-h reaction was not markedly changed with ratios of linoleic acid/BSA between 5:2.5 and 5:1 (by weight), but decreased with higher and lower ratios.

(iv) Effects of oxygen: Reactions were carried out in an O<sub>2</sub>-adsorbed atmosphere in test tubes in a sealed chamber with O<sub>2</sub>-absorbent, or under air in open test tubes. The amounts of CLA produced and the fatty acid compositions of the lipids produced were almost the same under both conditions. The results of 24-h reactions are presented in Fig. 3. In the author's previous study using *L. acidophilus* AKU 1137, the presence of oxygen promoted oxidative metabolism, e.g.,  $\beta$ -oxidation, and resulted in lower CLA production [25]. Based on these findings, screening under microaerobic conditions was conducted in this study. However, the presence of oxygen did not affect CLA production from linoleic acid by *L. plantarum* AKU 1009a, resulting in easy control of the reaction conditions. *L. plantarum* AKU 1009a may lack oxidative linoleic acid degradation activity. Strains with such high CLA-producing activity are promising for efficient production of CLA.



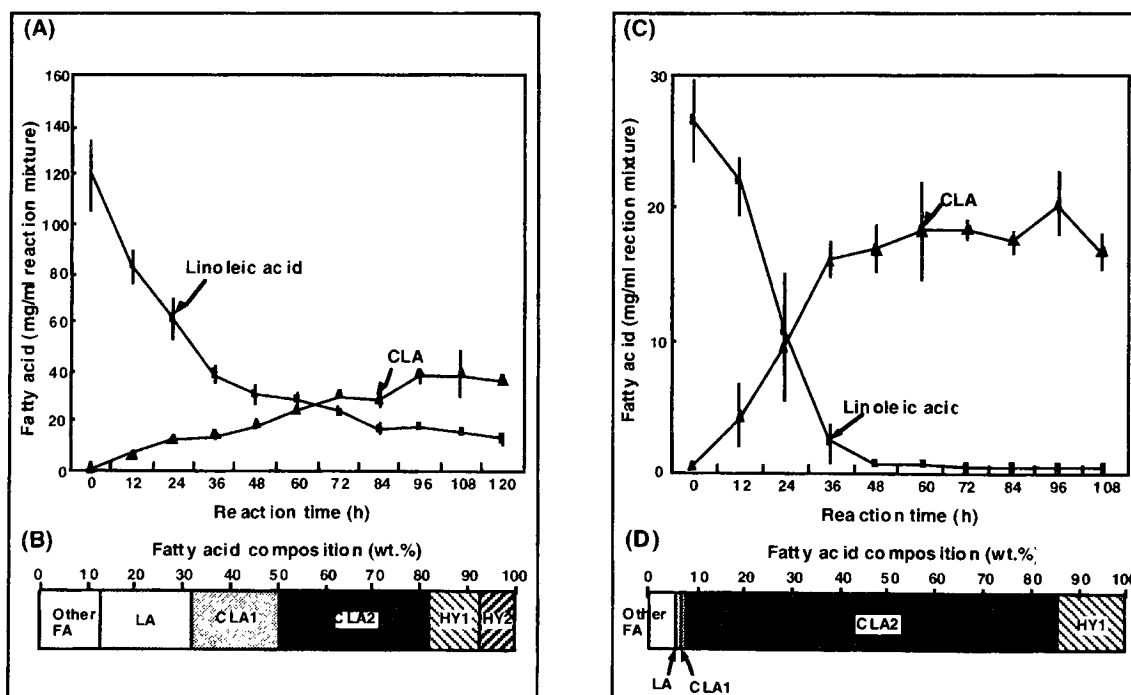
### Effects of concentrations of linoleic acid and washed cells.

Reactions were carried out for 48 h with 20% [wet cells (w/v)] washed cells and different concentrations of linoleic acid in 5-ml reaction mixtures with a fixed ratio of linoleic acid/ BSA, 5:1 (by weight). CLA production increased with increasing concentration of linoleic acid up to 2% (w/v) and reached a plateau (8.9 mg/ml) with higher concentrations, while HY production increased up to 5% (w/v) linoleic acid and reached a plateau (18.5 mg/ml) at higher concentrations.

Reactions were carried out for 48 h with 6.9% (w/v) linoleic acid and different amounts of washed cells in 5 ml reaction mixtures. CLA production increased to 23.9 mg/ml with increasing amount of washed cells up to 33% (wet cells, w/v), which corresponded to 5% (dry cells, w/v), but decreased slightly with greater amounts of washed cells.

### Time course of preparative CLA production.

The time course of CLA production from linoleic acid was monitored under two different conditions. With 12% (w/v) linoleic acid as the substrate and 33% (wet cells, w/v) washed cells as the catalyst, the production of CLA reached a maximum (40 mg/ml) at 108 h and then gradually decreased (Fig. 4A). On the other hand, with 2.6% (w/v) linoleic acid as the



**Fig. 4.** Time course of preparative CLA production with 12 (A and B) or 2.6% (w/v) (C and D) linoleic acid as the substrate with 33 or 23% (wet cells, w/v) washed cells, respectively, under the conditions described in MATERIALS AND METHODS. (A) and (C), time course of the reaction. (■), linoleic acid; (▲), CLA (*cis*-9,*trans*-11- and *trans*-9,*trans*-11-18:2). (B) and (D), fatty acid composition (wt.%) of the lipid produced in 108 or 96 h reaction, respectively. For abbreviations, see Fig. 3.



substrate and 23% (wet cells, w/v) washed cells as the catalyst, 80% (mol%) of the linoleic acid added was converted to CLA (20 mg/ml) in 96 h (Fig. 4C). Fatty acid compositions of the produced lipids are also presented in Fig. 4. With high substrate concentration (Fig. 4B), large amount of CLA1 (15 mg/ml) was found, while with low substrate concentration (Fig. 4D) significantly lesser in amount of CLA1 (0.6 mg/ml) was observed and CLA2 became dominant (19.5 mg/ml). The proportions of CLA isomers changed depending on reaction conditions. Lower substrate concentration and longer reaction tended to increase CLA2 production.

### Factors affecting isomer production.

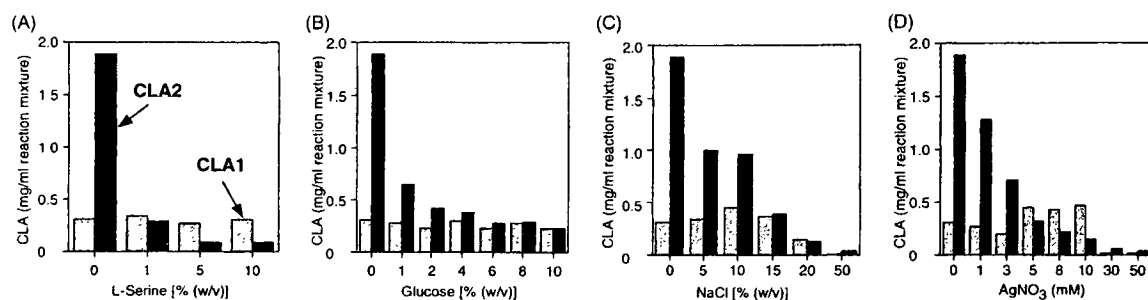
CLA2 can be produced at more than 90% purity by *L. plantarum* AKU 1009a, if the reaction is done long enough with a low linoleic acid concentration as described above. However, selective production of CLA1 has never been achieved, whatever the reaction conditions. Hence, the effects of various compounds on isomer production were investigated. Among nine sugars (10%, w/v), 37 amino acids (1%, w/v), 31 metal ions (1 to 10 mM), 10 salts (10%, w/v), four enzyme cofactors (40 mM), and 43 enzyme inhibitors (1 to 10 mM) added one by one to the reaction mixture with linoleic acid and washed cells of *L. plantarum* as the substrate and catalysts, respectively, the several compounds listed in Table 2 affected isomer production. These compounds reduced CLA2 production, by which the apparent selectivity for CLA1 was increased. Effects of the concentration of L-serine, glucose, NaCl, and AgNO<sub>3</sub> were examined

TABLE 2. Effects of various compounds on CLA isomer production

Compound	Concentration	Fatty acid (mg/ml of reaction mixture)			CLA1/CLA2
		CLA1	CLA2	Total CLA	
Control		0.32	1.89	2.21	0.17
Amino acid					
L-Serine	1 % (w/v)	0.34	0.30	0.64	1.13
Sugar					
Glucose	10 % (w/v)	0.23	0.23	0.46	1.00
Maltose	10 % (w/v)	0.28	0.37	0.65	0.76
Fluctose	10 % (w/v)	0.33	0.50	0.83	0.66
Salt					
NaCl	10 % (w/v)	0.45	0.96	1.41	0.47
Metal					
AgNO <sub>3</sub>	10 mM	0.47	0.15	0.62	3.13
Inhibitor					
2,3,5-Triphenyltetrazolium chloride	10 mM	0.34	0.10	0.44	3.40
Phenylhydrazine	5 mM	0.45	0.23	0.68	1.96
Dinitrophenol	10 mM	0.45	0.30	0.75	1.50

Reactions were done as described in MATERIALS AND METHODS with 0.4 mg/ml linoleic acid and 22.5% (w/v) wet washed cells of *L. plantarum* AKU 1009a as the substrate and catalyst, respectively, at 37°C for 24 h, except for the addition of the indicated concentrations of a compound. Control experiments were done without additions. Fatty acids in the reaction mixtures were extracted, methyl-esterified, and analyzed by gas-liquid chromatography as described in MATERIALS AND METHODS.

(Fig. 5). Additions of 5 to 10% (w/v) L-serine or 5 to 10 mM AgNO<sub>3</sub> were effective for selective production of CLA1. Interestingly, D-serine did not have such an effect. The additions of 5 to 15% (w/v) NaCl or 5 to 10 mM AgNO<sub>3</sub> slightly increased CLA1 production and reduced CLA2 production. Above all, the production of CLA1 and CLA2 were independently controlled by these compounds. These results indicated that there were different pathways for biosynthesis of these isomers.



**Fig. 5.** Effects of concentrations of L-serine (A), glucose (B), NaCl (C), and AgNO<sub>3</sub> (D) on CLA isomer production. Reactions were carried out as described in MATERIALS AND METHODS with 4.0 mg/ml linoleic acid and 22.5% (w/v) wet washed cells of *L. plantarum* AKU 1009a as the substrate and catalysts, respectively, at 37°C for 24 h, except for the addition of the indicated concentrations of a compound. Fatty acids in the reaction mixtures were extracted, methyl-esterified, and analyzed on gas-liquid chromatography as described in MATERIALS AND METHODS.

### Distribution and lipid classes of the fatty acid produced by *L. plantarum*.

The reaction mixture with 2.6% (w/v) linoleic acid as the substrate and 23% (wet cells, w/v) washed cells as the catalyst was centrifuged after 108-h reaction, and separated into supernatant and cells. The distribution and lipid classes of the fatty acid produced in both supernatant and cells were analyzed (Table 3). Most of the fatty acid (98.9%) were found in the cells (or associated with the cells), in which CLA was found as the most abundant fatty acid. Of the CLA in the cells (or associated with cells), 53% and 41% were found in free fatty acid and nonpolar lipid fractions, respectively.

Biological systems are promising for the selective preparation of CLA isomers. Natural dietary sources of CLA are the meat and milk of ruminants and products made from them. In these materials, the predominant isomer is *cis*-9,*trans*-11-18:2, which accounts for over 75% of the total CLA [8,36]. The *cis*-9,*trans*-11-18:2 is also produced from linoleic acid as an intermediate of biohydrogenation by rumen bacteria [11] and dairy starter cultures [12]. However, the amounts of CLA in these materials are very low. The results presented here clearly showed that *L. plantarum* AKU 1009a is a promising biocatalyst for CLA production. The produced CLA accumulated in the cells, reaching approximately 38% (w/w) of the dry cells obtained after the reaction.

**TABLE 3. Distribution and lipid classes of fatty acids produced from linoleic acid by washed cells of *L. plantarum*<sup>a</sup>**

Fatty acid	Fatty acid conc. (mg/ml reaction mixture) after reaction	Distribution of fatty acids in indicated lipid class (mol%) in.						Total
		Supernatant			Cells			
		FA <sup>b</sup>	NL <sup>c</sup>	PL <sup>d</sup>	FA	NL	PL	
Linoleic acid	0.19	0.2	- <sup>e</sup>		0.1	0.1	0.4	0.8
CLA1	0.38	0.1			0.8	0.9		1.8
CLA2	16.33	0.5			39.7	30.4	4.4	75.0
HY1	1.32	tr <sup>f</sup>	tr		0.2	5.8		6.0
HY2	2.20	tr			9.6	0.2	0.3	10.1
Other fatty acids	1.33	0.1	0.1	0.1	5.2	0.6	0.2	6.3
Total	21.75	0.9	0.1	0.1	55.6	38.0	5.3	100

<sup>a</sup>*L. plantarum* was cultivated in MRS medium with linoleic acid [0.06% (w/v)] for 24 h. The reaction was carried out with 2.6% (w/v) linoleic acid as the substrate for 108 h under the conditions described in MATERIALS AND METHODS. For abbreviations, see Table 1. Other fatty acids (mg/ml reaction mixture) were palmitic acid (0.02), oleic acid (0.75), vaccenic acid (0.43) and 2-hexyl-1-cyclopropane-octanoic acid (0.13). <sup>b</sup>FA, free fatty acids. <sup>c</sup>NL, nonpolar lipids. <sup>d</sup>PL, polar lipids. <sup>e</sup>-, not detected. <sup>f</sup>tr, trace (<0.05 mol%).

## SUMMARY

After screening 14 genera of lactic acid bacteria, *Lactobacillus plantarum* AKU 1009a was selected as a potential strain for conjugated linoleic acid (CLA) production from linoleic acid. Washed cells of *L. plantarum* with high levels of CLA production were obtained by cultivation in a nutrient medium with 0.06% (w/v) linoleic acid. Under the optimum reaction conditions with the free form of linoleic acid as the substrate, washed cells of *L. plantarum* produced 40 mg CLA/ml reaction mixture (33% molar yield) from 12% (w/v) linoleic acid in 108 h. The resulting CLA was a mixture of two CLA isomers, *cis*-9,*trans*-11-octadecadienoic acid (CLA1, 38% of total CLA) and *trans*-9,*trans*-11-octadecadienoic acid (CLA2, 62% of total CLA), and accounted for 50% of total fatty acid obtained. A higher yield (80% molar yield to linoleic acid) was attained with 2.6% (w/v) linoleic acid as the substrate in 96 h, resulting in CLA production of 20 mg/ml reaction mixture [consisting of CLA1 (2%) and CLA2 (98%)] and accounting for 80% of total fatty acid obtained. Most of the CLA produced was obtained being associated with the washed cells (approximately 380 mg CLA/g dry cells), mainly as free fatty acid form. The addition of L-serine, glucose, AgNO<sub>3</sub>, or NaCl to the reaction mixture specifically reduced production of CLA2.

## CHAPTER II

### **Ricinoleic Acid and Castor Oil as Substrates for Conjugated Linoleic Acid Production by Washed Cells of *Lactobacillus plantarum***

#### INTRODUCTION

Conjugated linoleic acid (CLA) is a collective term for isomers of linoleic acid with conjugated double bonds. Specific CLA isomers such as *cis*-9,*trans*-11-octadecadienoic acid (18:2) and *trans*-10,*cis*-12-18:2 may have beneficial physiological and anticarcinogenic effects [1,4,8,14]. In this CHAPTER, the author presents the first example of the biosynthesis of CLA from ricinoleic acid and castor oil. The author's previous studies showed that two CLA isomers, CLA1 (*cis*-9,*trans*-11-18:2) and CLA2 (*trans*-9,*trans*-11-18:2), were efficiently produced from linoleic acid on incubation with washed cells of lactic acid bacteria. Analysis of the pathway of CLA production from linoleic acid by lactic acid bacteria indicated the participation of two hydroxy fatty acids, 10-hydroxy-*cis*- and 10-hydroxy-*trans*-12-octadecaenoic acid (18:1), as possible intermediates [25].

In this CHAPTER, the author evaluated a hydroxy fatty acid, ricinoleic acid (12-hydroxy-*cis*-9-18:1), the chemical structure of which is similar to that of 10-hydroxy-12-18:1, as an alternative substrate for CLA production by the lactic acid bacterium *Lactobacillus plantarum*. Ricinoleic acid is readily available from castor oil and would be a practical substrate for microbial CLA production.

#### MATERIALS AND METHODS

**Chemicals.** Ricinoleic acid and fatty acid-free (<0.02%) bovine serum albumin (BSA) were purchased from Wako Pure Chemical (Osaka, Japan) and Sigma (MO, U.S.A.), respectively. Lipases were obtained from Amano Enzyme Co. (Nagoya, Japan). Castor oil [triacylglycerol of fatty acids (ricinoleic acid 88.2%, linoleic acid 4.8%, and others 7.0%)] was obtained from Itoh Oil Chemicals Co. (Yokkaichi, Japan). All other chemicals used were of analytical grade and were commercially available.

**Microorganisms, cultivation, and preparation of washed cells.** Washed cells of *L. plantarum* AKU 1009a [37] (AKU culture collection, Faculty of Agriculture, Kyoto University, Kyoto, Japan), selected as a potential catalyst for CLA production from linoleic acid via hydroxy fatty acids, were given a preliminary examination. *L. plantarum* AKU 1009a was cultivated in MRS medium [10 g of Polypepton (Nihon-pharm. Co., Tokyo, Japan), 10 g of meat extract (Mikuni Co., Tokyo), 5 g of yeast extract (Difco, MD, USA), 20 g of glucose, 1 g of Tween 80, 2 g of  $K_2HPO_4$ , 5 g of sodium acetate, 2 g of diammonium citrate, 0.2 g of  $MgSO_4 \cdot 7H_2O$ , 0.05 g of  $MnSO_4 \cdot 5H_2O$  in 1 liter, pH 6.5 by NaOH] containing various fatty acids (0.6 g/l as free fatty acids). After cultivation in 550 ml of liquid medium in 600-ml flasks for 24 h at 28°C with shaking (120 strokes/min), the cells were harvested by centrifugation (12,000 x g, 10 min), washed twice with 0.85% NaCl, centrifuged again, then used as the washed cells for the reactions.

**Reaction conditions with linoleic acid or ricinoleic acid as a substrate .** The reactions were done microaerobically in an  $O_2$ -adsorbed atmosphere in a sealed chamber with  $O_2$ -absorbent (AnaeroPack “Kenki”, Mitsubishi Gas Chemical Co., Inc., Tokyo), and gently shaken (120 strokes/min) at 37°C for 24 h. The reaction mixture, 1 ml, in a test tube (16.5 x 125 mm) contained 4.0 mg/ml ricinoleic acid or linoleic acid mixed with 0.8 mg/ml BSA to disperse the fatty acid in the reaction mixture, 0.1 M potassium phosphate buffer (KPB, pH 6.5), and 22.5% (wet cells, w/v) washed cells [corresponding to 3.2% (dry cells, w/v)] cultivated with various fatty acids. In the microaerobic assays, the oxygen concentrations, monitored by an oxygen indicator (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan), were kept under 1%. All experiments were carried out in triplicate, and the averages of three separate experiments, which were reproducible within  $\pm 10\%$ , are presented in figures and tables.

**Screening of lipases for ricinoleic acid production from castor oil.** Production of free-form ricinoleic acid from castor oil by lipase was monitored in reaction mixtures containing 4.0 mg/ml castor oil, 0.1 M KPB, pH 6.5, and 100 U/ml each lipase. Reactions were done at 37°C for 24 h and the reaction mixtures were analyzed by thin-layer chromatography (TLC) on silica gel 60 F<sub>254</sub> (Merck, Darmstadt, Germany) with *n*-hexane-diethyl ether-acetic acid (60:40:1, by vol.) and 5% 12-molybdo(VI)phosphoric acid *n*-hydrate in ethanol as the developing solvent and detection reagent, respectively.

**Lipid analyses.** Lipids were extracted from the reaction mixture with chloroform-methanol (1:2, by vol.) according to the procedure of Bligh-Dyer [15], and methylated with 10% methanolic HCl at 50°C for 20 min. The resultant fatty acid methyl esters were extracted with *n*-hexane and analyzed by gas-liquid chromatography (GC) using a Shimadzu (Kyoto, Japan)



GC-1700 gas chromatograph equipped with a flame ionization detector and a split injection system and fitted with a capillary column (HR-SS-10, 50 m x 0.25 mm I.D., Shinwa Kako, Kyoto, Japan). The column temperature, initially 180°C, was raised to 220°C at a rate of 2°C/min and maintained at that temperature for 20 min. The injector and detector were operated at 250°C. Helium was used as a carrier gas at 225 kPa/cm<sup>2</sup>

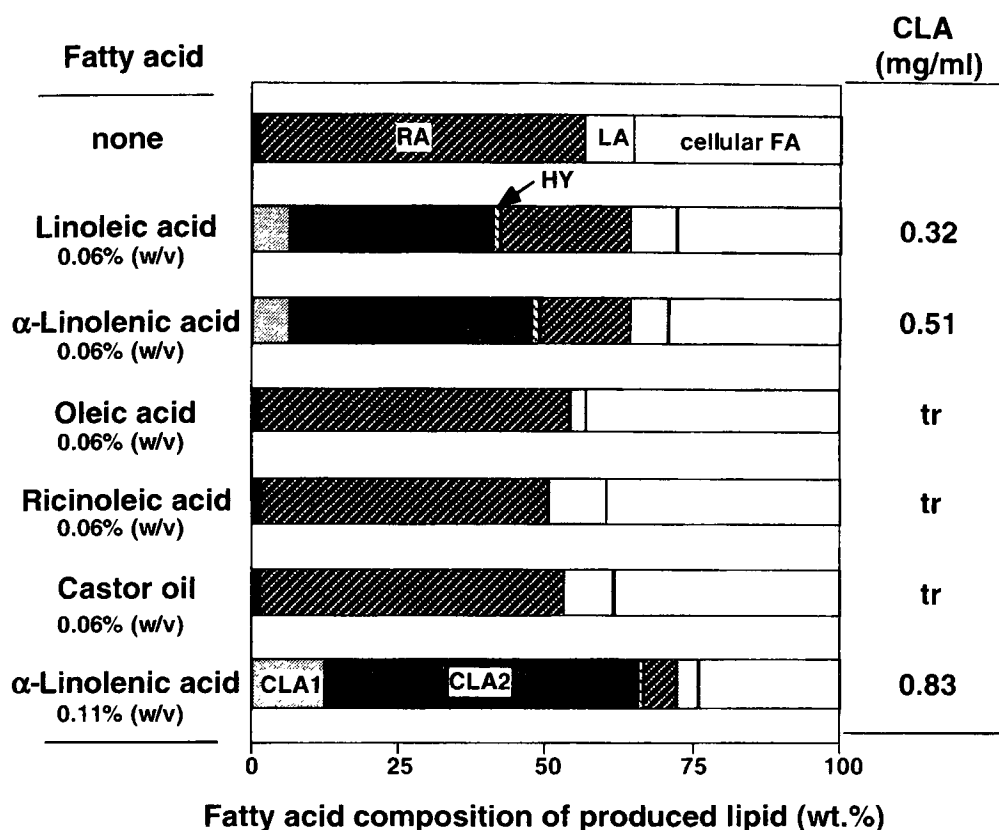
## RESULTS

### **Evaluation of ricinoleic acid as a substrate for CLA production.**

The washed cells of *L. plantarum* AKU 1009a prepared under the optimum culture conditions for CLA production from linoleic acid were used (see CHAPTER I). Under the reaction conditions optimized for CLA production from linoleic acid [37], the washed cells produced 0.32 mg/ml CLA [sum of *cis*-9,*trans*-11-18:2 (CLA1) and *trans*-9,*trans*-11-18:2 (CLA2)] from 4.0 mg/ml ricinoleic acid. Small amounts of linoleic acid and 10-hydroxy-12-18:1 (HY) were also detected in the reaction mixture with ricinoleic acid as the substrate. On the other hand, 1.66 mg/ml CLA (sum of CLA1 and CLA2) was produced from 4.0 mg/ml linoleic acid by the same washed cells.

### **Optimization of culture conditions for the preparation of washed cells of *L. plantarum* with high CLA productivity from ricinoleic acid.**

As the washed cells of lactic acid bacteria cultivated in medium containing 0.6 g/l linoleic acid produced much CLA production from linoleic acid [25], the washed cells of *L. plantarum* cultivated in the MRS medium containing various fatty acids were evaluated for CLA productivity from ricinoleic acid or linoleic acid. Among the fatty acids tested as additives (0.6 g/l) in the medium (linoleic acid,  $\alpha$ -linolenic acid, oleic acid, ricinoleic acid, and castor oil),  $\alpha$ -linolenic acid increased the CLA productivity of the washed cells (Fig. 1). The effects of  $\alpha$ -linolenic acid concentration was examined, and the highest CLA productivity was found in the washed cells cultivated with 0.11% (w/v)  $\alpha$ -linolenic acid. By using the cells cultivated in the medium supplemented with  $\alpha$ -linolenic acid (0.11%), CLA production from ricinoleic acid reached 0.83 mg/ml (molar conversion yield, 21%). The CLA produced was identified as a mixture of CLA1 (0.16 mg/ml) and CLA2 (0.67 mg/ml). Linoleic acid, which increases the CLA production from linoleic acid (0.6 g/l), was less effective on the CLA production from ricinoleic acid (CLA production, 0.32 mg/ml), and the combination of linoleic acid (0.6 g/l) and  $\alpha$ -linolenic acid (0.6 g/l) also decreased the CLA production from ricinoleic acid (CLA production, 0.58 mg/ml).



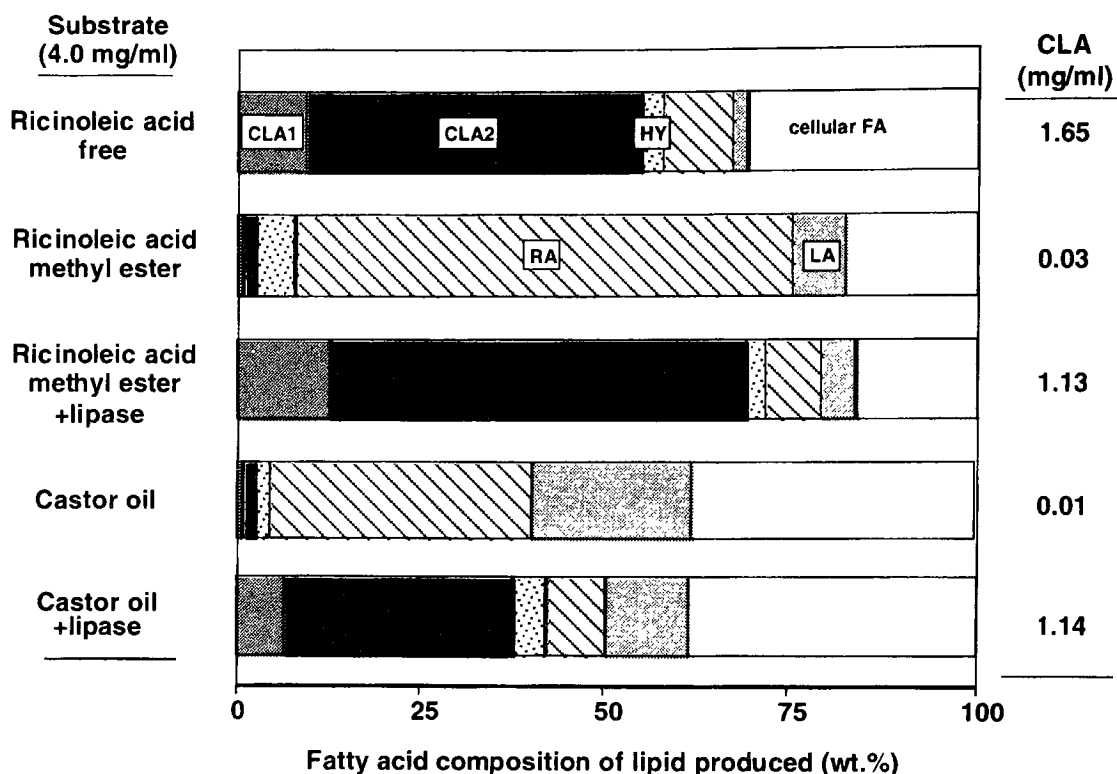
**Fig. 1.** Effects of fatty acid supplementation on CLA productivity of the washed cells of *L. plantarum* AKU 1009a. Cellular FA included myristic acid, palmitic acid, palmitoleic acid, oleic acid, *trans*-vaccenic acid, and 2-hexy-1-cyclopropane-octanoic acid. LA, linoleic acid; RA, ricinoleic acid; HY, 10-hydroxy-12-octadecanoic acid.

The changes in CLA productivity during cultivation in the medium with 0.6 g/l  $\alpha$ -linolenic acid were investigated. The cells after 36-h cultivation (midlog-phase;  $OD_{610}$ , 3.25) produced 1.52  $\mu$ g of CLA per mg of dry cells per h, but prolonged cultivation did not further increase the productivity. Washed cells obtained after 36-h cultivation were used for further experiments.

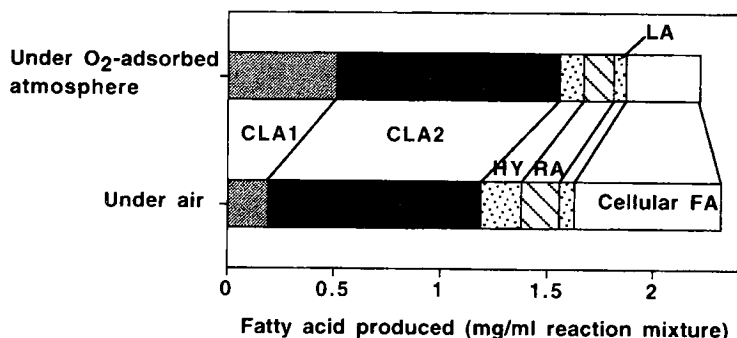
### Optimization of reaction conditions.

(i) Evaluation of substrate form: Free and methyl ester forms of ricinoleic acid and castor oil, in which the major fatty acid component is ricinoleic acid, were tested as substrates (4.0 mg/ml) for CLA production after they were mixed with 0.8 mg/ml BSA. Reactions were done as described above except for the substrates. The free form of ricinoleic acid was converted to CLA at 1.65 mg/ml, but little CLA was produced from the methyl ester or castor oil (Fig. 2).

(ii) Effects of oxygen: Reactions were carried out in an  $O_2$ -adsorbed atmosphere in test tubes in a sealed chamber with  $O_2$ -absorbent, or under air in open test tubes. The amounts of CLA produced under air were lower than that in an  $O_2$ -adsorbed atmosphere. The results of



**Fig. 2.** CLA production from different forms of ricinoleic acid and castor oil with or without lipase (lipase M "Amano" 10). CLA1, *cis*-9,*trans*-11-octadecadienoic acid; CLA2, *trans*-9,*trans*-11-octadecadienoic acid; HY, 10-hydroxy-*trans*-12-octadecaenoic acid and 10-hydroxy-*cis*-12-octadecaenoic acid; RA, ricinoleic acid; LA, linoleic acid. Cellular fatty acid (cellular FA) includes myristic acid, palmitic acid, palmitoleic acid, oleic acid, *trans*-vaccenic acid, and 2-hexy-1-cyclopropane-octanoic acid.

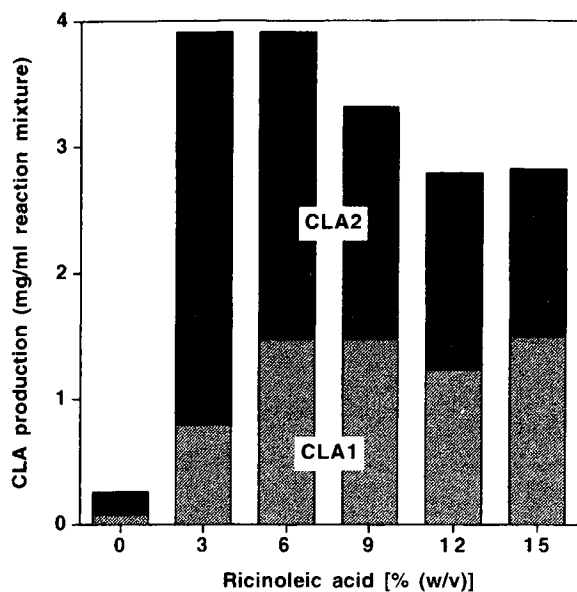


**Fig. 3.** Effect of oxygen on CLA production from ricinoleic acid. Reactions were carried out in 24 h as described in MATERIALS AND METHODS in an O<sub>2</sub>-adsorbed atmosphere or under air. For abbreviations, see Fig. 2.

24-h reactions are presented in Fig. 3. In CHAPTER I, though the presence of oxygen did not affect CLA production from linoleic acid by *L. plantarum* AKU 1009a, the presence of oxygen resulted in reduced CLA production from ricinoleic acid.

**Effects of concentrations of ricinoleic acid and washed cells.**

Reactions were carried out for 24 h with 22% (wet cells, w/v) washed cells and different concentrations of ricinoleic acid with a fixed ratio of ricinoleic acid/ BSA, 5:1 (by weight).



**Fig. 4.** Effect of ricinoleic acid concentration as the substrate on CLA production. Reactions were carried out with 22% (wet cells, w/v) washed cells.

CLA production increased with increasing concentration of ricinoleic acid up to 3% (w/v) and reached a plateau (3.9 mg/ml) with higher concentrations up to 6%, but decreased slightly with greater concentrations of ricinoleic acid (Fig. 4).

Reactions were carried out for 24 h with 3% (w/v) ricinoleic acid and different amounts of washed cells. CLA production increased with increasing amount of washed cells.

#### **Production of CLA from castor oil in the presence of lipases.**

Castor oil and ricinoleic acid methyl ester could be the substrates if the lipases converted it to the free form, ricinoleic acid. Eight lipases obtained from Amano Enzyme Co. (Nagoya, Japan) (Bioenzyme M, Lipase AH-S, Lipase GC “Amano” 4, Lipase PS-C “Amano” I, Pancreatin F, Lipase AY “Amano” 30, Lipase F-AP 15, and Lipase M “Amano” 10) were tested for their ability to produce free-form ricinoleic acid from castor oil. TLC analysis of the reaction mixtures revealed that Lipase M “Amano” 10 produced the most free-form ricinoleic acid (data not shown). To produce CLA from castor oil or ricinoleic acid methyl ester by the washed cells of *L. plantarum*, reactions were done with 4.0 mg/ml castor oil or ricinoleic acid methyl ester in the presence of Lipase M “Amano” 10 (100 U/ml). As shown in Fig. 2, 1.14 and 1.13 mg/ml CLA was produced from castor oil and ricinoleic acid methyl ester, respectively, in the presence of the lipase (molar conversion yield, 28.5% and 28.3%, respectively). The CLA produced from castor oil was a mixture of CLA1 (0.19 mg/ml) and CLA2 (0.95 mg/ml) and that from ricinoleic acid methyl ester was a mixture of CLA1 (0.20 mg/ml) and CLA2 (0.93 mg/ml).

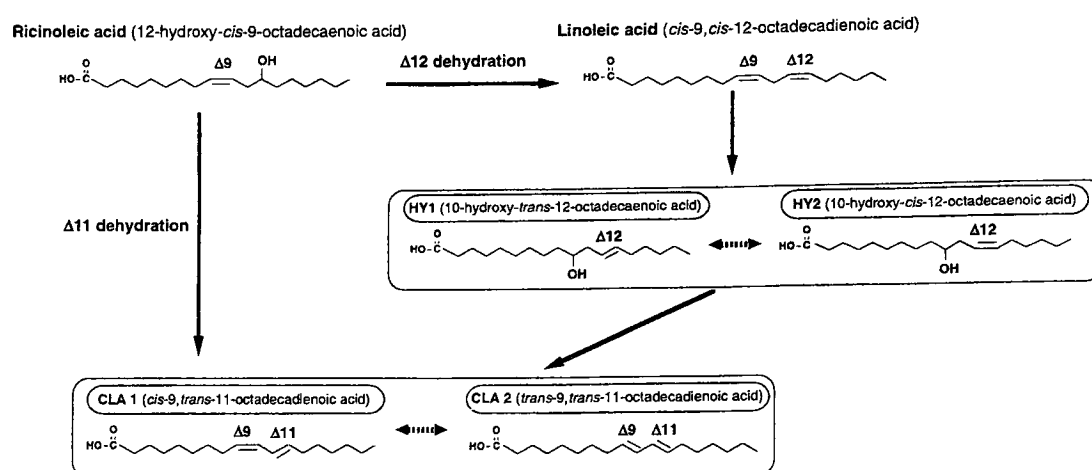


Fig. 5. Proposed pathway of CLA production from ricinoleic acid by *L. plantarum* AKU 1009a.

## DISCUSSION

Chemical syntheses of CLA from ricinoleic acid and castor oil were reported previously [38,39]. The author presented here the biosynthesis of CLA from ricinoleic acid and castor oil. Although only the free form of ricinoleic acid is a suitable substrate for this reaction, CLA was produced from castor oil also with the help of lipases. These results suggest the possibility of development of a new process for CLA production from readily available castor oil with *L. plantarum*.

There are two possible pathways for CLA synthesis from ricinoleic acid by *L. plantarum*; i) direct transformation of ricinoleic acid to CLA through dehydration at the  $\Delta 11$  position, and ii) dehydration of ricinoleic acid at the  $\Delta 12$  position to linoleic acid, which is a potential substrate for CLA production by lactic acid bacteria (Fig. 5). The observation that the cells cultivated in the medium containing  $\alpha$ -linolenic acid produced much CLA from ricinoleic acid, although those cultivated in the medium containing linoleic acid produced little, suggested the significance of the former pathway. However, the existence of linoleic acid and 10-hydroxy-12-18:1 in the reaction mixture with ricinoleic acid as a substrate also indicated the participation of the latter pathway.

## SUMMARY

Ricinoleic acid (12-hydroxy-cis-9-octadecaenoic acid) was an effective substrate for conjugated linoleic acid (CLA) production by washed cells of *Lactobacillus plantarum* AKU 1009a. The CLA produced was a mixture of cis-9,trans-11- and trans-9,trans-11-octadecadienoic

acids. Addition of  $\alpha$ -linolenic acid to the culture medium increased the CLA productivity of the washed cells. In the presence of lipase, castor oil, in which the main fatty acid component is ricinoleic acid, also was a substrate for CLA. Under optimized conditions, 3.9 mg/ml and 1.14 mg/ml CLA were produced from 30 mg/ml ricinoleic acid and 4.0 mg/ml castor oil, respectively.

## CHAPTER III

### Transformation of Polyunsaturated Fatty Acids by Lactic Acid Bacteria

#### Section 1. Polyunsaturated fatty acids transformation to conjugated fatty acids by *Lactobacillus plantarum* AKU 1009a

#### INTRODUCTION

There are several reports on the occurrence of conjugated fatty acids in nature, especially in plants, for example,  $\alpha$ -eleostearic acid [*cis*-9,*trans*-11,*trans*-13-octadecatrienoic acid (18:3)] in *Momordica charantia* [40],  $\beta$ -eleostearic acid (*trans*-9,*trans*-11,*trans*-13-18:3) in paulownia oil, punicic acid (*cis*-9,*trans*-11,*cis*-13-18:3) in *Punica granatum* and *Cayaponia africana*, jarcenic acid (*cis*-8,*trans*-10,*cis*-12-18:3) in *Jacaranda mimosifolia* [41], calendic acid (*trans*-8,*trans*-10,*cis*-12-18:3) in *Calendula officinalis* [42], and  $\alpha$ -parinaric acid [*cis*-9,*trans*-11,*trans*-13,*cis*-15-octadecatetraenoic acid (18:4)] in *Impatiens balsamina* seeds [43]. They are C18 fatty acids considered to be derived from oleic acid [*cis*-9-octadecaenoic acid (18:1)], linoleic acid [*cis*-9,*cis*-12-octadecadienoic acid (18:2)],  $\alpha$ -linolenic acid (*cis*-9,*cis*-12,*cis*-15-18:3) or stearidonic acid (*cis*-6,*cis*-9,*cis*-12,*cis*-15-18:4). Secondary metabolism of fatty acids by the marine algae involves numerous polyunsaturated fatty acids containing conjugated olefin systems, for example, *cis*-6,*trans*-8,*trans*-10,*cis*-12-18:4 produced from  $\gamma$ -linolenic acid by the coralline red alga *Lithothamnion corallioides* and bosseopentaenoic acid [*cis*-5,*cis*-8,*trans*-10,*trans*-12,*cis*-14-eicosapentaenoic acid (20:5)] produced from arachidonic acid [*cis*-5,*cis*-8,*cis*-11,*cis*-14-eicosatetraenoic acid (20:4)] by the marine red alga *Bossiella orbigniana*.

Interest in such conjugated fatty acids as a novel class of functional lipids has increased in the last two decades, along with the discovery of unique biological/physiological effects of conjugated linoleic acid (CLA). Dietary CLA has been reported to reduce carcinogenesis [1,4,20,44], atherosclerosis [7] and body fat [21]. Recently, similar effects were found in conjugated trienoic acids. The conjugated trienoic acid produced from  $\alpha$ -linolenic acid by alkali-isomerization showed cytotoxicity toward human tumor cells [45]. 9,11,13-Octadecatrienoic acid isomers in pomegranate, tung and catalpa oils were also found to be cytotoxic toward mouse tumor and human monocytic leukemia cells [46]. These findings led the author to develop a novel microbial method for conjugated trienoic acid production.

In this CHAPTER III, the author reports that *Lactobacillus plantarum* AKU 1009a, which was selected as a potential strain producing CLA from linoleic acid, transforms various polyunsaturated fatty acids to a variety of conjugated fatty acids.

## MATERIALS AND METHODS

**Chemicals.**  $\gamma$ -Linolenic acid and fatty acid-free (<0.02%) bovine serum albumin (BSA) were purchased from Sigma (MO, U.S.A.). The  $\alpha$ -linolenic acid (Wako Pure Chemical, Osaka, Japan) used in this study was of 76% purity, and its fatty acid composition was: 76%  $\alpha$ -linolenic acid, 19% linoleic acid, and 5% oleic acid. Standard samples of CLA isomers, *i.e.*, *cis*-9,*trans*-11-octadecadienoic acid (CLA1) and *trans*-9,*trans*-11-octadecadienoic acid (CLA2), and 10-hydroxy-12-octadecanoic acid (HY) were prepared as described previously [25]. All other chemicals used were of analytical grade and were commercially available.

**Microorganisms, cultivation, and preparation of washed cells.** The washed cells of *L. plantarum* AKU 1009a (AKU culture collection, Faculty of Agriculture, Kyoto University, Kyoto Japan), were used as the catalysts for fatty acid transformation [37]. The strain was cultivated in MRS medium comprised of 1.0% tryptone, 1.0% meat extract, 0.5% yeast extract, 2.0% glucose, 0.1% Tween 80, 0.2%  $K_2HPO_4$ , 0.5% sodium acetate, 0.2% diammonium citrate, 0.02%  $MgSO_4 \cdot 7H_2O$ , 0.005%  $MnSO_4 \cdot 5H_2O$ , and 0.06% linoleic acid (pH 6.5). The strain was inoculated into 550 ml of medium in 600-ml flasks and then incubated at 28°C with shaking (120 strokes/min) for 24 h. Growth was monitored by optical density (OD) at 610 nm. Cells were harvested by centrifugation (12,000 x g, 10 min), washed twice with 0.85% NaCl, centrifuged again, and then used as the washed cells for the reactions.

**Reaction conditions.** The reaction mixture, 1 ml, in test tubes (16.5 x 125 mm) was composed of 0.4% (w/v) fatty acid complexed with BSA [0.08% (w/v)], 0.1 M potassium phosphate buffer (KPB, pH 6.5), and 22.5% (wet cells, w/v) washed cells [corresponding to 3.2% (dry cells, w/v)]. The fatty acids used as the substrates were *cis*-6-18:1, *cis*-9-18:1 (oleic acid), *trans*-9-18:1 (elaidic acid), *cis*-11-18:1 (*cis*-vaccenic acid), *trans*-11-18:1 (*trans*-vaccenic acid), *cis*-12-18:1, *trans*-12-18:1, *cis*-9,*cis*-12-18:2 (linoleic acid), *cis*-9,*cis*-12,*cis*-15-18:3 ( $\alpha$ -linolenic acid), *cis*-6,*cis*-9,*cis*-12-18:3 ( $\gamma$ -linolenic acid), *trans*-9,*trans*-12,*trans*-15-18:3 (linolenelaidic acid), *trans*-5,*cis*-9,*cis*-12-18:3 (columbinic acid), *cis*-6,*cis*-9,*cis*-12,*cis*-15-18:4 (stearidonic acid), *cis*-8,*cis*-11,*cis*-14-eicosatrienoic acid (20:3) (dihomo- $\gamma$ -linolenic acid), *cis*-5,*cis*-8,*cis*-11,*cis*-14-20:4 (arachidonic acid), *cis*-5,*cis*-8,*cis*-11,*cis*-14,*cis*-17-20:5 (EPA), *cis*-13-



docosaenoic acid (22:1) and *cis*-15-tetracosanoic acid (24:1). The reactions were carried out microaerobically in an O<sub>2</sub>-adsorbed atmosphere in a sealed chamber with O<sub>2</sub>-absorbent (AnaeroPack “Kenki”, Mitsubishi Gas Chemical Co, Inc., Tokyo, Japan), and gently shaken (120 strokes/min) at 37°C for 24 to 72 h. In the microaerobic conditions, the oxygen concentrations, monitored by an oxygen indicator (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan), were kept under 1%. All experiments were done in triplicate, and the averages of three separate experiments, which were reproducible within ±10%, are presented in the figures.

**Lipid analyses.** Lipids were extracted from the reaction mixture with chloroform-methanol (1:2, by vol.) according to the procedure of Bligh-Dyer [15], and methylated with 10% methanolic HCl at 50°C for 20 min. The resultant fatty acid methyl esters were extracted with *n*-hexane and analyzed by gas-liquid chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-1700 gas chromatograph equipped with a flame ionization detector and a split injection system and fitted with a capillary column (HR-SS-10, 50 m x 0.25 mm I.D., Shinwa Kako, Kyoto, Japan) as described previously [25].

**Isolation, derivatization, and identification of reaction products.** The fatty acid methyl esters of the reaction products were separated at 30°C by high-performance liquid chromatography (HPLC, monitored at 205 and 233 nm) using a Shimadzu LC-VP system fitted with a Cosmosil 5C<sub>18</sub>-AR-II-packed column (20 x 250 mm, Nacalai Tesque, Kyoto, Japan). The mobile phase was acetonitrile-H<sub>2</sub>O (8:2, by vol.) at a flow rate of 3.0 ml/min. The separated fatty acid methyl esters were further purified by HPLC fitted with ChromSpher 5 Lipids-packed column (4.6 x 250 mm, Chrompack, NJ, U.S.A.) [47]. The mobile phase was hexane-acetonitrile (99.9:0.1, by vol.) at a flow rate of 1.0 ml/min. Free fatty acids and pyrrolidide derivatives were prepared by saponification with sodium hydroxide and direct treatment with pyrrolidine-acetic acid, respectively, as described previously [25]. The isolated fatty acid methyl esters were dissolved in CDCl<sub>3</sub> and analyzed by proton nuclear magnetic resonance (<sup>1</sup>H-NMR), <sup>1</sup>H-<sup>1</sup>H chemical shift correlation spectroscopy (DQF-COSY), two-dimensional nuclear Overhauser enhancement spectroscopy (NOESY) and <sup>1</sup>H clean-total correlation spectroscopy (clean-TOCSY) [48] with a Bruker Biospin CMX-750 (750 MHz for <sup>1</sup>H). The chemical shifts were assigned relative to the solvent signal. The free fatty acids and pyrrolidide derivatives were subjected to mass spectroscopy (MS)-MS analysis and GC-MS analysis, respectively, as described previously [25].

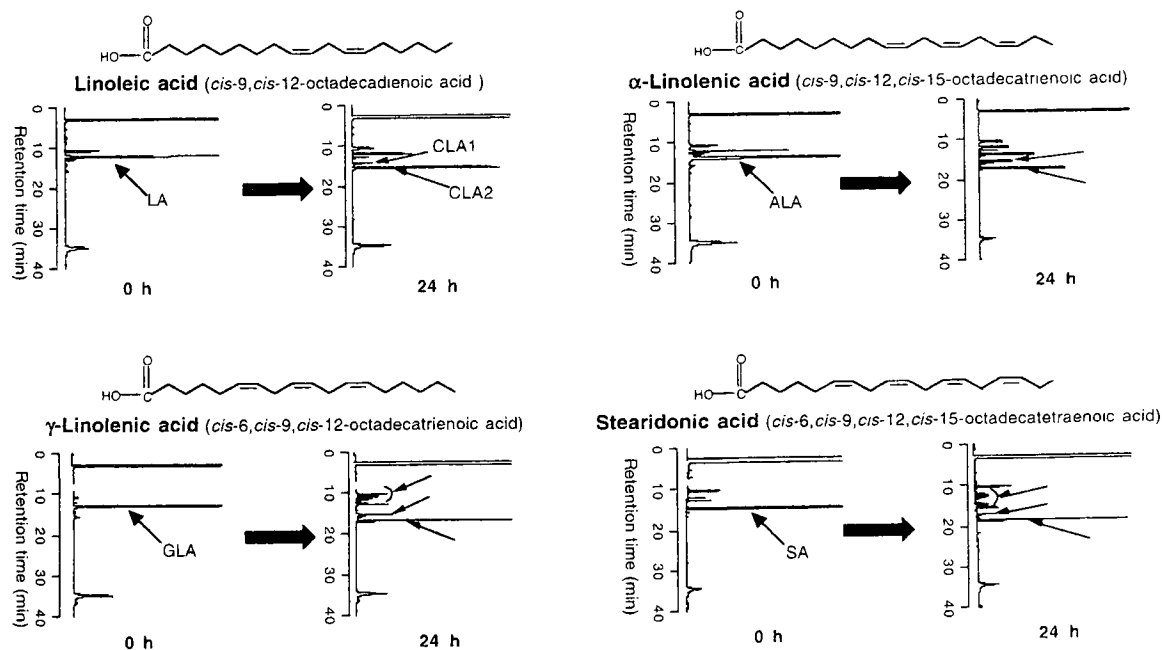
## RESULTS

**Transformation of polyunsaturated fatty acids by washed cells of *L. plantarum* AKU 1009a.**

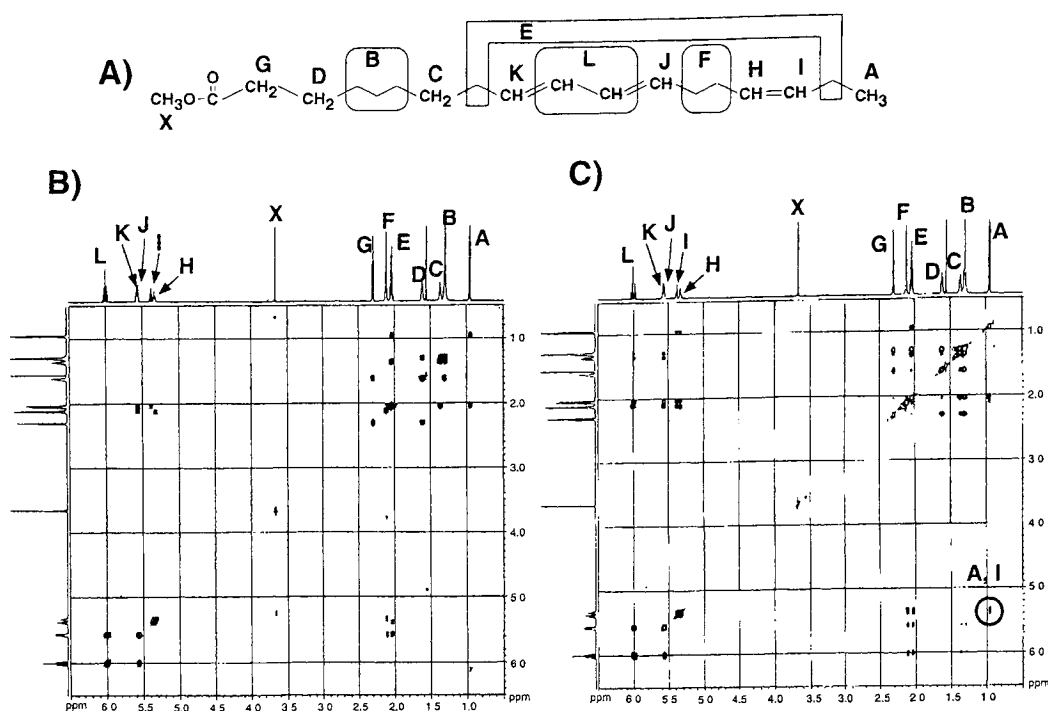
The washed cells of *L. plantarum* AKU 1009a prepared under the optimum culture conditions for CLA production from linoleic acid were used as the catalysts for fatty acid transformation (see CHAPTER I). Free fatty acids of *cis*-6-18:1, oleic acid, elaidic acid, *cis*-vaccenic acid, *trans*-vaccenic acid, *cis*-12-18:1, *trans*-12-18:1, linoleic acid,  $\alpha$ -linolenic acid,  $\gamma$ -linolenic acid, linolenelaidic acid, columbinic acid, stearidonic acid, dihomo- $\gamma$ -linolenic acid, arachidonic acid, EPA, *cis*-13-22:1, and *cis*-15-24:1 were used as the substrates. When linoleic acid,  $\alpha$ -linolenic acid,  $\gamma$ -linolenic acid, columbinic acid, or stearidonic acid was used as the substrate, newly generated fatty acids were found on the GC chromatograms of the methylated fatty acid products (Fig. 1). The fatty acids recognized as the substrate were C18 free fatty acids having *cis*-9,*cis*-12-diene system. The other fatty acids tested were not transformed by the washed cells of *L. plantarum*.

**Fatty acids produced from  $\alpha$ -linolenic acid by washed cells of *L. plantarum* AKU 1009a.**

GC chromatogram of the methylated fatty acids produced from  $\alpha$ -linolenic acid by washed cells of *L. plantarum* AKU 1009a is shown in Fig. 2A. Three major newly generated fatty acids designated as A1, CALA1 and CALA2 were found on the GC chromatogram of the



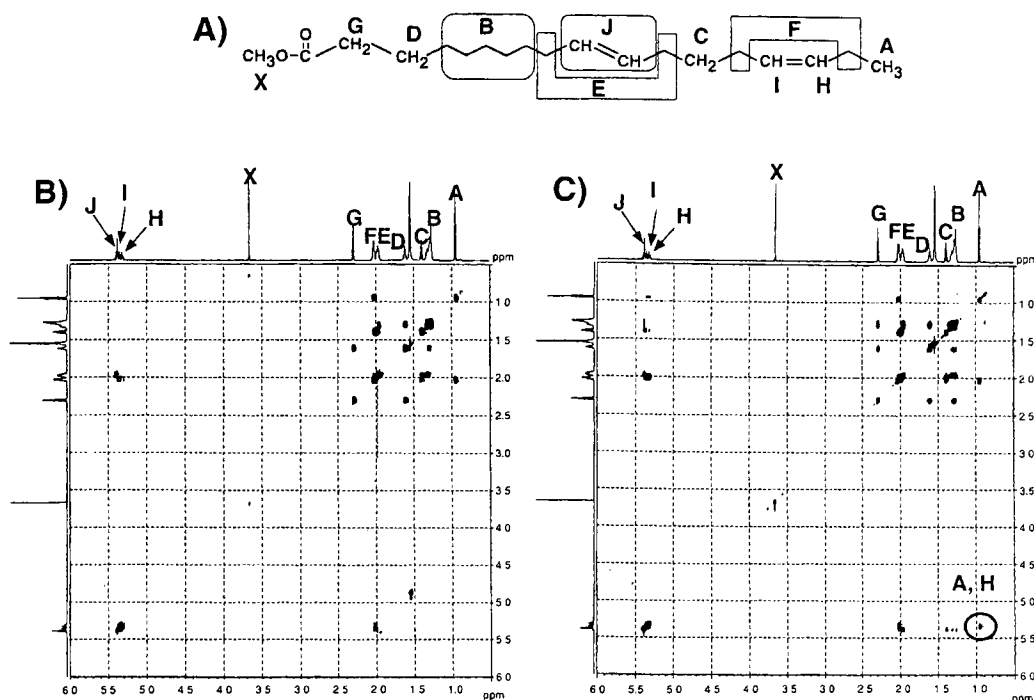
**Fig. 1.** GC chromatograms of methylated fatty acids produced from various polyunsaturated fatty acids by *L. plantarum* AKU 1009a. The peaks indicated by arrows are the newly generated fatty acids. 0 h and 24 h represent the reaction times. LA, linoleic acid; CLA1, *cis*-9,*trans*-11-octadecadienoic acid; CLA2, *trans*-9,*trans*-11-octadecadienoic acid; ALA,  $\alpha$ -linolenic acid; GLA,  $\gamma$ -linolenic acid; SA, stearidonic acid.



**Fig. 4.** <sup>1</sup>H-NMR analysis of CALA2 and structure of CALA2 identified. A), Structure of CALA2; B), <sup>1</sup>H-<sup>1</sup>H chemical shift correlation spectroscopic spectrum of the methyl ester of CALA2; C), <sup>1</sup>H clean-total correlation spectroscopic spectrum of the methyl ester of CALA2.

The DQF-COSY signal pattern of CALA2 indicated fragment proton sequences of A-E-I, K or C, I-H-F, K-L-J, and E-C-B-D-G (Fig. 4B). The signal pattern of clean-TOCSY showed clear interaction between A and I but not A and J or A and C (Fig. 4C). Decoupled <sup>1</sup>H-NMR spectra irradiated at 2.04 ppm (signal E) and 2.11 ppm (signal F) resulted in disappearances of signal K and J, respectively. These results confirmed that the proton sequence from the methyl of CALA2 is A-E-I-H-F-J-L-K-E-C-B-D-G. <sup>1</sup>H-NMR coupling constant between J and L obtained by irradiation at 2.11 ppm (signal F) was 14.3 Hz, and those of H and I, and K and L obtained by irradiation at 2.04 ppm (signal E) were 10.7 Hz and 14.3 Hz, respectively. These results indicated that the double between J and L, H and I, and K and L are in *trans*, *cis*, and *trans* configuration, respectively. On the basis of the results of above spectral analyses, CALA2 was identified as *trans*-9,*trans*-11,*cis*-15-18:3 (Fig. 4A).

**Identification of A1:** Mass spectrum of pyrrolidide derivative of A1 showed molecular weight of  $m/z$  333. This result suggested that compound A1 is C18 fatty acid containing two double bonds. The molecular ion peak (Li-complex,  $m/z$  301 [M+Li]<sup>+</sup>) obtained by FAB-MS analysis (FAB<sup>+</sup>) of free fatty acid of A1 was fragmented again by MS-MS [ $m/z$  (FAB<sup>+</sup>, 8.00 kV), 285(4), 271(1), 245(1), 231(19), 218(7), 217(12), 203(3), 177(2), 163(23), 149(11), 135(18), 121(11), 107(36), 94(30), 93(100), 80(87)]. The  $m/z$  177, 203, 217, 231, 245, and 271 were

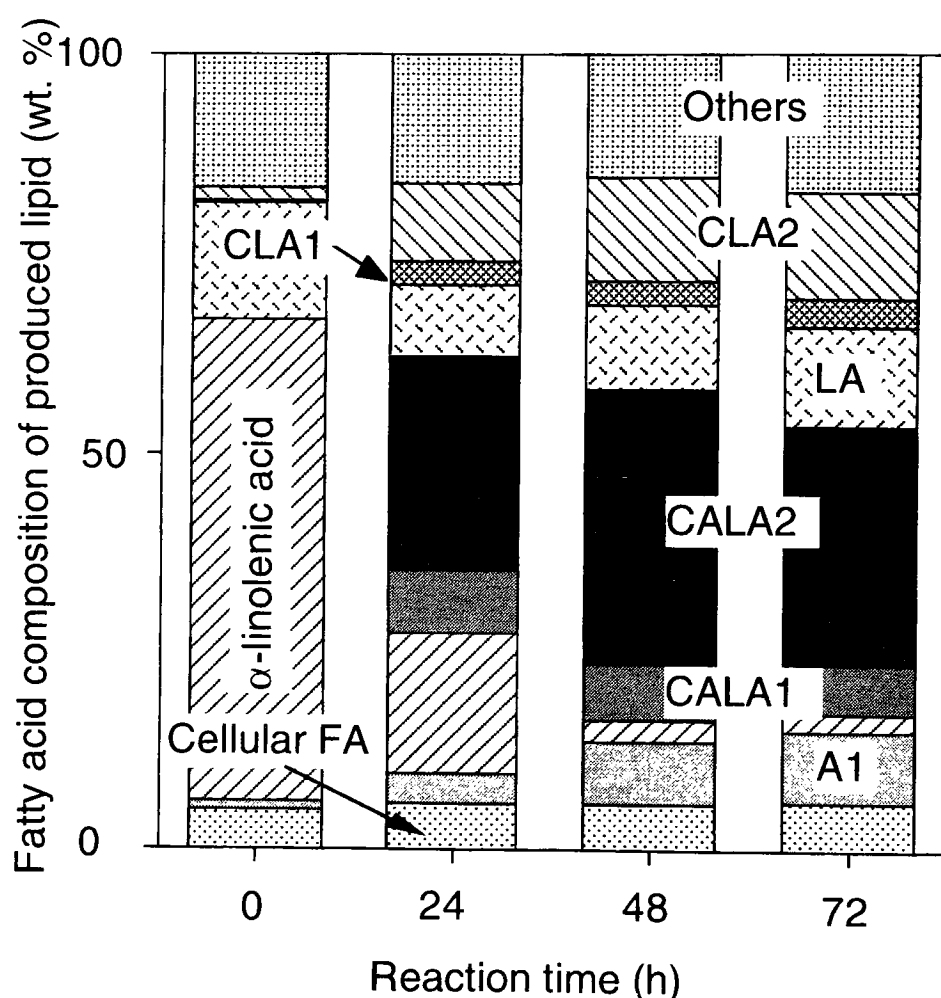


**Fig. 5.** <sup>1</sup>H-NMR analysis of A1 and structure of A1 identified. A), Structure of A1; B), <sup>1</sup>H-<sup>1</sup>H chemical shift correlation spectroscopic spectrum of the methyl ester of A1; C), <sup>1</sup>H clean-total correlation spectroscopic spectrum of the methyl ester of A1.

derived from cleavage between single bonds 9-10, 11-12, 12-13, 13-14, 14-15, and 16-17, numbered from the carboxyl group. The  $m/z$  163, 217, 231, and 285, derived from the cleavage of a single bond between the  $\alpha$  and  $\beta$  positions from the double bond were clearly detected. On the basis of the results of spectral analyses, A1 was identified as 10,15-18:2. This deduced structure was further confirmed by the results of <sup>1</sup>H-NMR [NMR  $\delta_{\text{H}}$  (CDCl<sub>3</sub>): 5.40 (2H, m, CH<sub>2</sub>-CH=), 5.37 (1H, dt,  $J = 10.8, 7.0$  Hz, -CH<sub>2</sub>-CH=), 5.32 (1H, dt,  $J = 10.8, 7.2$  Hz, =CH-CH<sub>2</sub>-), 3.67 (3H, s, -OCH<sub>3</sub>), 2.30 (2H, t,  $J = 7.5$  Hz, -COCH<sub>2</sub>-), 2.03 (4H, m, -CH<sub>2</sub>-CH=), 1.98 (4H, m, -CH<sub>2</sub>-CH=), 1.61 (2H, tt,  $J = 7.5, 7.4$  Hz, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 1.40 (2H, tt,  $J = 8.2, 7.3$  Hz, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 1.29 (10H, m, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 0.97 (3H, t,  $J = 7.5$  Hz, -CH<sub>3</sub>)]. The sequence of the protons from the methyl end of A1 was deduced to be A-F-H-I-F-C-E-J-E-B-D-G or A-F-I-H-F-C-E-J-E-B-D-G on the basis of the signal pattern of DQF-COSY (Fig. 5B). The signal pattern of clean-TOCSY showed clear interaction between A and H but not A and I (Fig. 5C). These results confirmed that the proton sequence from the methyl of A1 is A-F-H-I-F-C-E-J-E-B-D-G. <sup>1</sup>H-NMR coupling constant between H and I obtained by irradiation at 2.03 ppm (signal F) was 10.8 Hz, and that of J and J obtained by irradiation at 1.98 ppm (signal E) were 15.3 Hz. These results indicated that the double between H and I, and J and J are in *cis*, and *trans* configuration, respectively. On the basis of the results of above spectral analyses, A1 was identified as *trans*-10,*cis*-15-18:2 (Fig. 5A).

### Time course of $\alpha$ -linolenic acid transformation by washed cells of *L. plantarum* AKU 1009a.

The time course of changes in fatty acid composition during  $\alpha$ -linolenic acid [0.3% (w/v)] transformation by washed cells [22.5% (wet cells, w/v)] of *L. plantarum* AKU 1009a was studied. CALA (sum of CALA1 and CALA2) reached 41.7% (wt%) of total fatty acids after 48-h reaction (Fig. 6). The proportion of A1 in total fatty acids was 3.8% (wt%) after 24-h reaction, and it gradually increased followed by a decrease in the proportion of CALA. These results suggest that CALA was further converted to A1. The amount of CALA produced after 48-h reaction was 1.59 mg/ml (CALA1, 0.27 mg/ml; CALA2, 1.32 mg/ml; molar conversion yield to  $\alpha$ -linolenic acid, 47%).



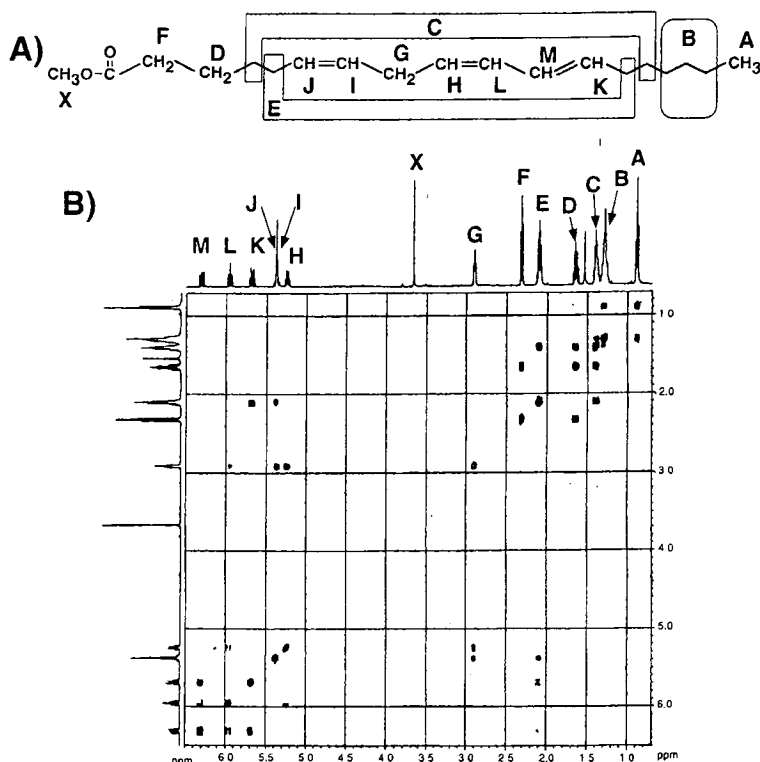
**Fig. 6.** Time course of  $\alpha$ -linolenic acid transformation by *L. plantarum* AKU 1009a. Cellular FA included myristic acid, palmitic acid, palmitoleic acid, oleic acid, *trans*-vaccenic acid, and 2-hexy-1-cyclopropane-octanoic acid. CALA1, *cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid; CALA2, *trans*-9,*trans*-11,*cis*-15-octadecatrienoic acid; A1, *trans*-10,*cis*-15-octadecadienoic acid; LA, linoleic acid; CLA1, *cis*-9,*trans*-11-octadecadienoic acid; CLA2, *trans*-9,*trans*-11-octadecadienoic acid.

**Fatty acids produced from  $\gamma$ -linolenic acid by washed cells of *L. plantarum* AKU 1009a.**

GC chromatogram of the methylated fatty acids produced from  $\gamma$ -linolenic acid by washed cells of *L. plantarum* AKU 1009a is shown in Fig. 2B. Five major newly generated fatty acids designated as G1, G2, G3, CGLA1, and CGLA2 were found on the GC chromatogram. G1, G2, CGLA1, and CGLA2 were purified by HPLC from the mixture of fatty acid methyl esters and subjected to structure analysis.

**Identification of CGLA1 and CGLA2:** Mass spectra of pyrrolidide derivatives of CGLA1 and CGLA2 showed molecular weights of  $m/z$  331. These results suggested that CGLA1 and CGLA2 are C18 fatty acids containing three double bonds. The molecular ion peak ( $[M-H]^+$ , 277) obtained by FAB-MS analysis (FAB<sup>-</sup>) of free fatty acid of CGLA1 was fragmented again by MS-MS [ $m/z$  (FAB<sup>-</sup>, 8.00 kv), 261(11), 247(6), 233(13), 219(12), 205(11), 191(25), 179(5), 177(10), 167(2), 165(4), 153(13), 139(13), 127(12), 125(11), 113(12), 100(7), 99(45), 86(32), 85(7), 72(23), 71(91), 58(100), 44(48)]. The  $m/z$  99, 125, 139, 165, and 191 were derived from cleavage between single bounds 5-6, 7-8, 8-9, 10-11, and 12-13, numbered from the carboxyl group. The  $m/z$  85/86 and 205, derived from the cleavage of a single bond between the  $\alpha$  and  $\beta$  positions from the double bond, were detected. The molecular ion peak (Li-complex,  $m/z$  299  $[M+Li]^+$ ) obtained FAB-MS analysis (FAB<sup>+</sup>) of free acid of CGLA2 was fragmented again by MS-MS [ $m/z$  (FAB<sup>+</sup>, 8.00 kv), 283(4), 269(2), 255(5), 241(15), 228(13), 227(11), 213(4), 201(2), 187(1), 175(11), 162(8), 161(38), 147(9), 133(1), 121(1), 108(12), 107(27), 94(24), 93(50), 80(100)]. The  $m/z$  121, 147, 161, 187, and 213 were derived from cleavage between single bounds 5-6, 7-8, 8-9, 10-11, and 12-13, numbered from the carboxyl group. The  $m/z$  107 and 227, derived from the cleavage of a single bond between the  $\alpha$  and  $\beta$  positions from the double bond were detected. On the basis of the results of MS analyses, CGLA1 and CGLA2 were identified as the geometrical isomers of 6,9,11-18:3.

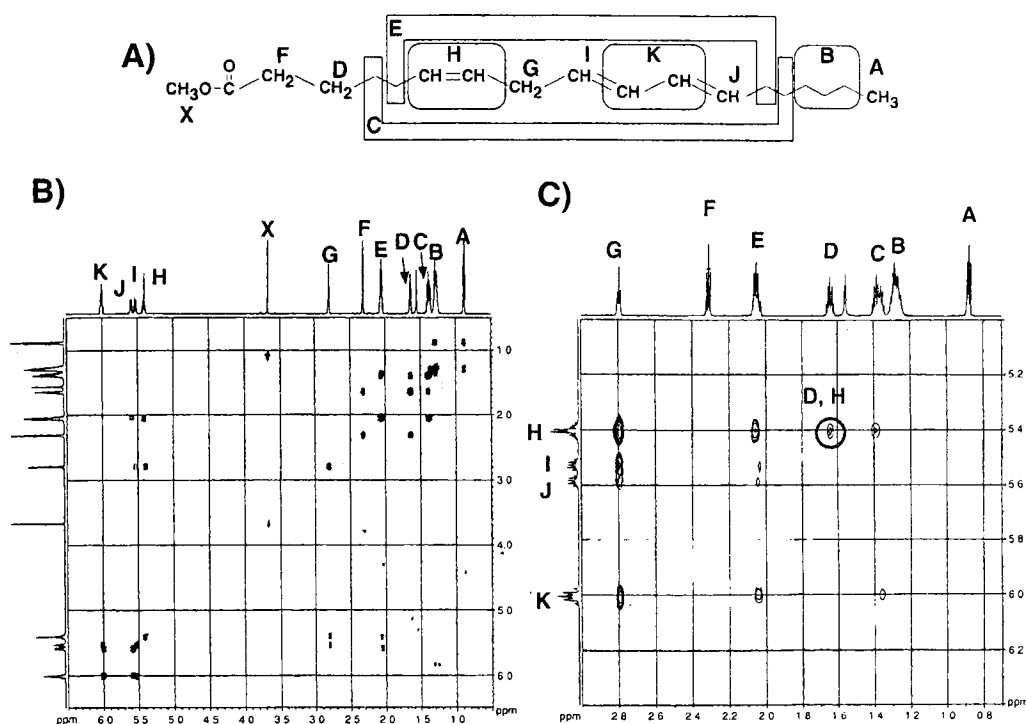
<sup>1</sup>H-NMR analysis also suggested that CGLA1 is an isomer of octadecatrienoic acid [ $NMR \delta_H$  (CDCl<sub>3</sub>): 6.31 (1H, dd,  $J = 15.0, 9.6$  Hz, =CH-CH=), 5.96 (1H, dd,  $J = 11.0, 10.7$  Hz, =CH-CH=), 5.69 (1H, dt,  $J = 15.0, 7.3$  Hz, =CH-CH<sub>2</sub>-), 5.40 (1H, dt,  $J = 11.0, 5.9$  Hz, -CH<sub>2</sub>-CH=), 5.37 (1H, dt,  $J = 11.0, 6.0$  Hz, =CH-CH<sub>2</sub>-), 5.25 (1H, dt,  $J = 10.7, 7.4$  Hz, -CH<sub>2</sub>-CH=), 3.67 (3H, s, -OCH<sub>3</sub>), 2.90 (2H, dd,  $J = 6.0, 5.9$  Hz, =CH-CH<sub>2</sub>-CH=), 2.32 (2H, t,  $J = 7.5$  Hz, COCH<sub>2</sub>-), 2.10 (4H, dt,  $J = 14.4, 7.2$  Hz, -CH<sub>2</sub>-CH=), 1.65 (2H, tt,  $J = 7.8, 7.5$  Hz, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 1.39 (4H, m, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 1.28 (6H, m, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 0.88 (3H, t,  $J = 6.9$  Hz, -CH<sub>3</sub>)]. The sequence of the protons from the methyl ester of the molecule was deduced to be A-B-C-E-K-M-L-H-G-I-J-E-C-D-F, or A-B-C-E-J-I-G-H-L-M-K-E-C-D-F based on the signal pattern of interaction between adjacent protons observed by DQF-COSY analysis (Fig. 7B). The sequence was confirmed as the former one based on the results of MS analyses of free fatty acid that make clear that C8 carbon numbered from the carboxyl group is interposed by saturated bounds, and the results of <sup>1</sup>H-NMR analysis showing that the signals H (5.25 ppm), I (5.37



**Fig. 7.**  $^1\text{H}$ -NMR analysis of CGLA1 and structure of CGLA1 identified. A), Structure of CGLA1; B),  $^1\text{H}$ - $^1\text{H}$  chemical shift correlation spectroscopic spectrum of the methyl ester of CGLA1.

ppm), J (5.40 ppm), K (5.69 ppm), L (5.96 ppm), M (6.31 ppm) are on the double bonds.  $^1\text{H}$ -NMR coupling constant between H and L obtained by irradiation at 2.90 ppm (signal G) was 10.7 Hz, and those of I and J, and K and M obtained by irradiation at 2.10 ppm (signal E) were 11.0 Hz and 15.0 Hz, respectively. These results indicated that the double between H and L, I and J, and K and M are in *cis*, *cis*, and *trans* configuration, respectively. On the basis of the results of above spectral analyses, CGLA1 was identified as *cis*-6,*trans*-9,*trans*-11-18:3 (Fig. 7A).

$^1\text{H}$ -NMR analysis also suggested that CGLA2 is an isomer of octadecatrienoic acid [NMR  $\delta_{\text{H}}$  ( $\text{CDCl}_3$ ): 6.01 (2H, m, =CH-CH=), 5.59 (1H, dt,  $J = 14.1, 7.0$  Hz, =CH-CH<sub>2</sub>-), 5.52 (1H, dt,  $J = 14.2, 7.1$  Hz, -CH<sub>2</sub>-CH=), 5.40 (2H, m, -CH<sub>2</sub>-CH=), 3.67 (3H, s, -OCH<sub>3</sub>), 2.79 (2H, dd,  $J = 7.4, 5.3$  Hz, =CH-CH<sub>2</sub>-CH=), 2.32 (2H, t,  $J = 7.5$  Hz, -COCH<sub>2</sub>-), 2.05 (4H, dt,  $J = 15.4, 7.3$  Hz, -CH<sub>2</sub>-CH<sub>2</sub>-CH=), 1.64 (2H, tt,  $J = 7.7, 7.6$  Hz, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 1.38 (4H, m, CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 1.27 (6H, m, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 0.89 (3H, t,  $J = 7.0$  Hz, -CH<sub>3</sub>)]. The sequence of the protons from the methyl end of CGLA2 was deduced to be A-B-C-E-J-K-I-G-H-E-C-D-F or A-B-C-E-H-G-I-K-J-E-C-D-F on the basis of the signal pattern of the interaction between adjacent protons observed by DQF-COSY (Fig. 8B). The sequence was confirmed to be the former one by the appearance of an interaction signal between D and H but not D and J on

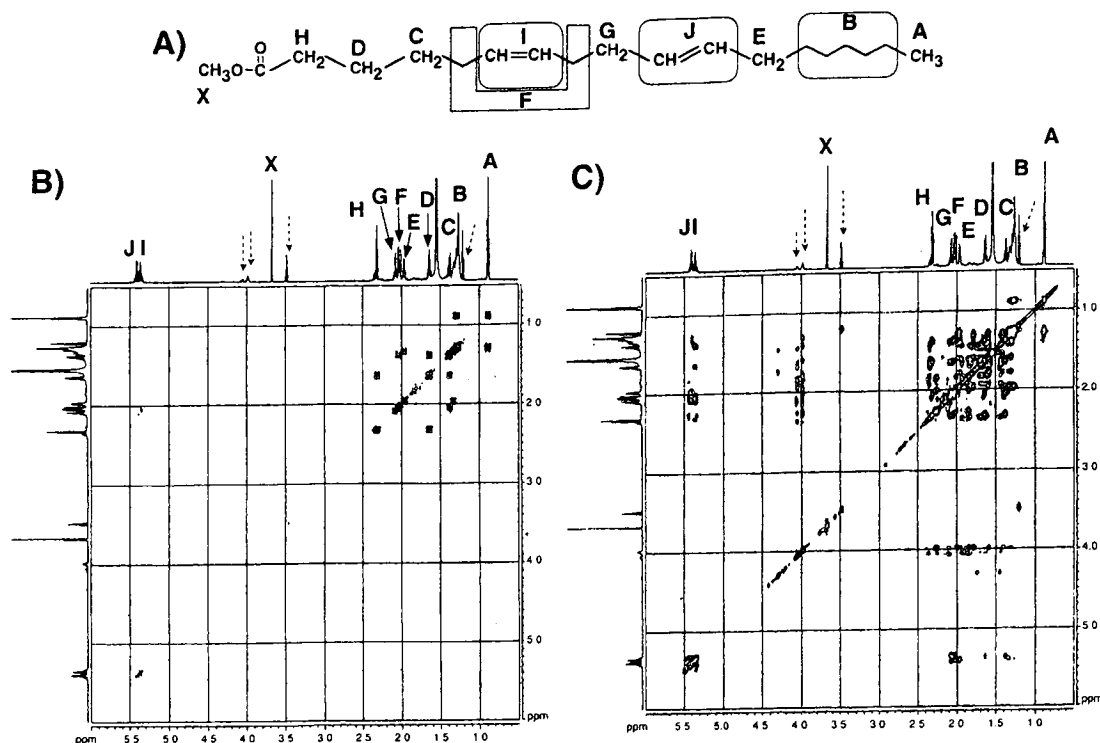


**Fig. 8.** <sup>1</sup>H-NMR analysis of CGLA2 and structure of CGLA2 identified. A), Structure of CGLA2; B), <sup>1</sup>H-<sup>1</sup>H chemical shift correlation spectroscopic spectrum of the methyl ester of CGLA2; C), <sup>1</sup>H clean-total correlation spectroscopic spectrum of the methyl ester of CGLA2.

clean-TOCSY analysis (Fig. 8C), indicating that D was near to H, but that D was far from J. <sup>1</sup>H-NMR coupling constant between I and K obtained by irradiation at 2.79 ppm (signal G) was 14.2 Hz, and those of H and H, and J and K obtained by irradiation at 2.05 ppm (signal E) were 10.9 Hz and 14.1 Hz, respectively. These results indicated that the double between I and K, H and H, and J and K are in *trans*, *cis*, and *trans* configuration, respectively. On the basis of the results of above spectral analyses, CGLA2 was identified as *cis*-6,*trans*-9,*trans*-11-18:3 (Fig. 8A).

**Identification of G2:** Mass spectrum of the pyrrolidide derivative of G2 showed molecular weight of  $m/z$  333. This result suggested that compound G2 is C18 fatty acid containing two double bonds. FAB-MS data of the free fatty acid of G2 exhibited molecular weight of  $m/z$  280 ( $[M-H]^+$ , 279). The molecular ion peak ( $[M-H]^+$ , 279) obtained by FAB-MS analysis (FAB<sup>+</sup>) of free fatty acid of G2 was fragmented again by MS-MS [ $m/z$  (FAB<sup>+</sup>, 8.00 kv), 263(9), 249(7), 235(13), 221(22), 207(15), 194(9), 193(10), 179(7), 153(3), 140(36), 139(36), 127(1), 125(1), 113(2), 100(1), 99(2), 86(29), 71(66), 58(100), 44(18)]. The  $m/z$  99, 125, 139, 153, and 179 were derived from cleavage between single bounds 5-6, 7-8, 8-9, 9-10, and 11-12, numbered from the carboxyl group. The  $m/z$  86, 139 and 193, derived from the

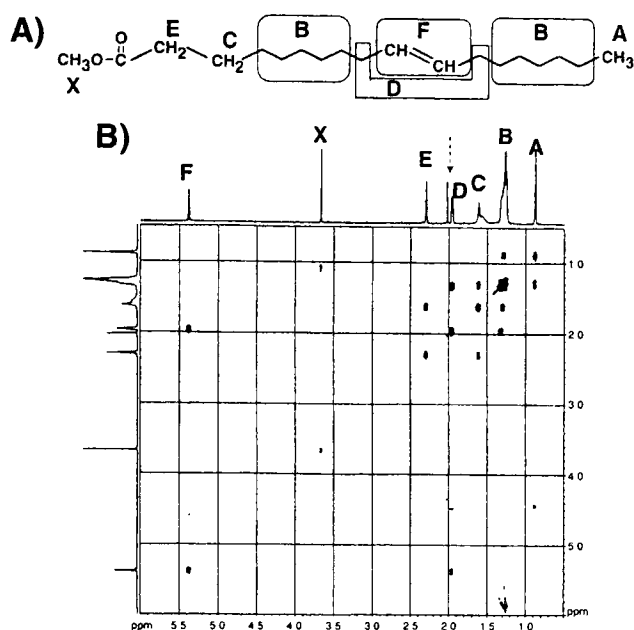




**Fig. 9.**  $^1\text{H-NMR}$  analysis of G2 and structure of G2 identified. A), Structure of G2; B),  $^1\text{H-}^1\text{H}$  chemical shift correlation spectroscopic spectrum of the methyl ester of G2; C),  $^1\text{H}$  clean-total correlation spectroscopic spectrum of the methyl ester of G2. The peaks indicated by broken arrows derived from minor impurities.

cleavage of a single bond between the  $\alpha$  and  $\beta$  positions from the double bond, were clearly detected. On the basis of the results of MS analyses, G2 was identified as 6,10-18:2.

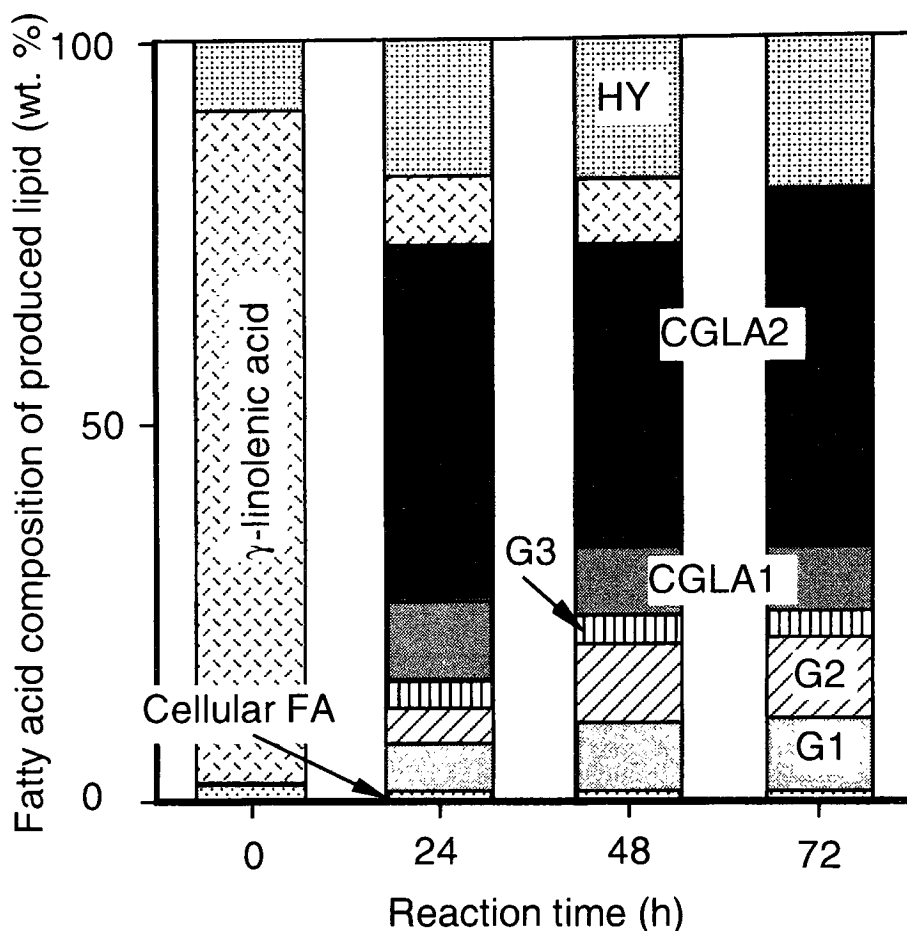
This structure was further confirmed by the results of  $^1\text{H-NMR}$  analysis of the fatty acid methyl ester [ $\text{NMR } \delta_{\text{H}}$  ( $\text{CDCl}_3$ ): 5.40 (2H, dt,  $J = 14.6, 6.0$  Hz,  $=\text{CH-CH}_2-$ ), 5.36 (2H, dt,  $J = 11.4, 7.0$  Hz,  $-\text{CH}_2-\text{CH}=\text{}$ ), 3.67 (3H, s,  $-\text{OCH}_3$ ), 2.31 (2H, t,  $J = 7.5$  Hz,  $-\text{COCH}_2-$ ), 2.08 (2H, m,  $-\text{CH}_2-\text{CH}_2-\text{CH}=\text{}$ ), 2.04 (4H, m,  $-\text{CH}_2-\text{CH}_2-\text{CH}=\text{}$ ), 1.97 (2H, m,  $=\text{CH}_2-\text{CH}_2-\text{CH}-$ ), 1.64 (2H, tt,  $J = 7.7, 7.6$  Hz,  $-\text{CH}_2\text{CH}_2\text{CH}_2-$ ), 1.38 (2H, m,  $-\text{CH}_2\text{CH}_2\text{CH}_2-$ ), 1.28 (10H, m,  $-\text{CH}_2\text{CH}_2\text{CH}_2-$ ), 0.88 (3H, t,  $J = 6.9$  Hz,  $-\text{CH}_3$ )]. On the  $^1\text{H-NMR}$  analysis, signals around 2.9 ppm, which indicate the existence of proton of methylene interposed by double bonds, were not observed. This results also supported that G2 is 6,10-18:2. The sequence of the protons from the methyl end of G2 was deduced to be A-B-E-J-G-F-I-F-C-D-H on the basis of the signal pattern of the interaction between adjacent protons observed by DQF-COSY (Fig. 9B).  $^1\text{H-NMR}$  coupling constant between I and I was 11.4 Hz, and that of J and J was 14.6 Hz. These results indicated that the double between I and I, and J and J are in *cis*, and *trans* configuration, respectively. The absence of interaction signal between G and E on clean-TOCSY analysis (Fig. 9C), also indicated that the double bond between J and J is in *trans* configuration. On the basis of the results of above spectral analyses, G2 was identified as *cis*-6,*trans*-10-18:2 (Fig. 9A).



**Fig. 10.** <sup>1</sup>H-NMR analysis of G1 and structure of G1 identified. A), Structure of G1; B), <sup>1</sup>H-<sup>1</sup>H chemical shift correlation spectroscopic spectrum of the methyl ester of G1. The peak indicated by the broken arrow derived from minor impurity.

**Identification of G1:** Mass spectrum of the pyrrolidide derivative of G1 showed molecular weight of  $m/z$  335. This result suggested that G1 is C18 fatty acid containing one double bond. The molecular ion peak (Li-complex,  $m/z$  303 [M+Li]<sup>+</sup>) obtained FAB-MS analysis (FAB<sup>+</sup>) of free acid of G1 was fragmented again by MS-MS [ $m/z$  (FAB<sup>+</sup>, 8.00 kv), 287(4), 273(2), 259(4), 245(9), 231(21), 218(8), 217(12), 203(3), 201(3), 177(4), 163(26), 149(15), 135(20), 121(15), 107(35), 94(31), 93(100), 80(83)]. The  $m/z$  177 and 203 were derived from cleavage between single bounds 9-10, and 11-12, numbered from the carboxyl group. The  $m/z$  163 and 217, derived from the cleavage of a single bond between the  $\alpha$  and  $\beta$  positions from the double bond were clearly detected. On the basis of the results of MS analyses, G1 was identified as 10-18:1.

This deduced structure was further confirmed by the results of <sup>1</sup>H-NMR [NMR  $\delta_H$  (CDCl<sub>3</sub>): 5.38 (2H, m, -CH<sub>2</sub>-CH=), 3.67 (3H, s, -OCH<sub>3</sub>), 2.30 (2H, t,  $J = 7.6$  Hz, -COCH<sub>2</sub>-), 1.96 (4H, m, -CH<sub>2</sub>-CH<sub>2</sub>-CH=), 1.62 (2H, m, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 1.30 (20H, m, -CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>-), 0.88 (3H, t,  $J = 6.9$  Hz, -CH<sub>3</sub>)]. The sequence of the protons from the methyl end of G1 was deduced to be A-B-D-F-D-B-C-E on the basis of the signal pattern of the interaction between adjacent protons observed by DQF-COSY (Fig. 10B). The results of chemical shifts were simulated by gNMR to estimate the configuration of  $\Delta$  10 double bonds. The simulation results supported that the structure of G1 is *trans*-10-18:1 (Fig. 10A).



**Fig. 11.** Time course of  $\gamma$ -linolenic acid transformation by *L. plantarum* AKU 1009a. CGLA1, *cis*-6,*cis*-9,*trans*-11-octadecatrienoic acid; CGLA2, *cis*-6,*trans*-9,*trans*-11-octadecatrienoic acid; G2, *cis*-6,*trans*-10-octadecadienoic acid; G1, *trans*-10-octadecaenoic acid; HY, hydroxy fatty acids. For other abbreviations, see the legend to Fig. 6.

#### Time course of $\gamma$ -linolenic acid transformation by washed cells of *L. plantarum* AKU 1009a.

The time course of changes in fatty acid composition during  $\gamma$ -linolenic acid [0.4% (w/v)] transformation by washed cells [22.5% (wet cells, w/v)] of *L. plantarum* AKU 1009a was studied. CGLA (sum of CGLA1 and CGLA2) reached 56.8% (wt%) of total fatty acids after 24-h reaction (Fig. 11). The proportions of G2 and G1 in total fatty acids were 4.7% (wt%) and 6.1% (wt%) after 24-h reaction, respectively, and they gradually increased followed by a decrease in the proportion of CGLA. These results suggest that CGLA was converted to G2 and further converted to G1. The amount of CGLA produced after 24-h reaction was 1.94 mg/ml (CGLA1, 0.36 mg/ml; CGLA2, 1.58 mg/ml; molar conversion yield to  $\gamma$ -linolenic acid, 46%).

## DISCUSSION

The fatty acids recognized as the substrate by the washed cells of *L. plantarum* AKU 1009a had a common structure of C18 fatty acid with *cis*-9,*cis*-12 diene system. The diene system of *cis*-9,*cis*-12 is converted to *cis*-9,*trans*-11- and *trans*-9,*trans*-11-diene systems and further saturated to the monoene system of *trans*-10 by washed cells of *L. plantarum* AKU 1009a.

Fig. 12 shows proposed pathways for  $\alpha$ -linolenic acid and  $\gamma$ -linolenic acid transformation by the washed cells of *L. plantarum* AKU 1009a.  $\alpha$ -Linolenic acid is isomerized to CALA1 and CALA2, and further saturated *trans*-10,*cis*-15-18:2. Similarly,  $\gamma$ -linolenic acid is isomerized to CGLA1 and CGLA2, and further saturated to *trans*-10-18:1 via *cis*-6,*trans*-10-18:2.

Although the products from stearidonic acid and columbinic acid were not identified because of their insufficient amounts, on the basis of above results, three major fatty acids produced from stearidonic acid are supposed to be *cis*-6,*cis*-9,*trans*-11,*cis*-15-18:4, *cis*-6,*trans*-9,*trans*-11,*cis*-15-18:4 and *cis*-6,*trans*-10,*cis*-15-18:3, and three major fatty acids produced from columbinic acid are supposed to be *trans*-5,*cis*-9,*trans*-11-18:3, *trans*-5,*trans*-9,*trans*-11-18:3 and *trans*-5,*trans*-10-18:2. Further purification and identification of these fatty acids were required.

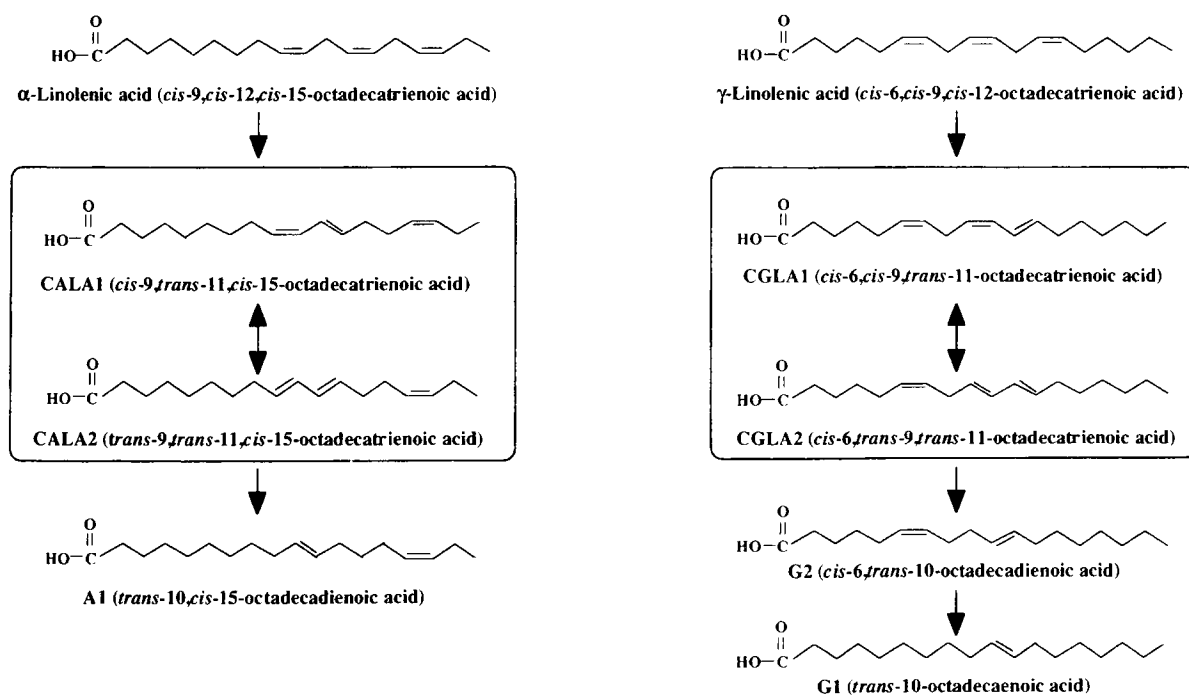


Fig. 12. Putative pathway of  $\alpha$ - and  $\gamma$ -linolenic acid transformation by *L. plantarum* AKU 1009a.

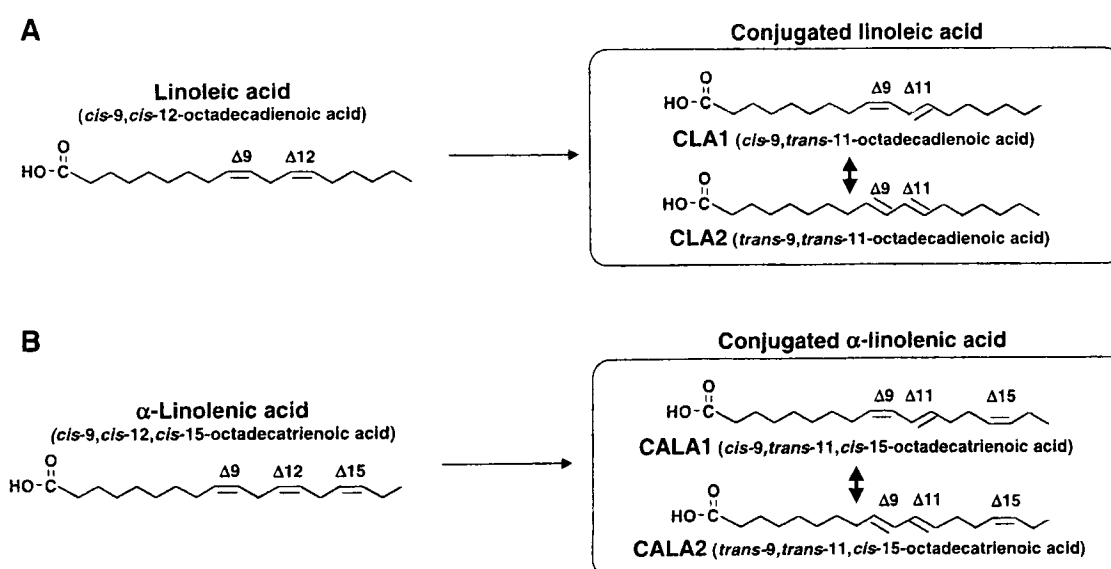
## SUMMARY

The substrate specificity of fatty acid transformation by the washed cells of *Lactobacillus plantarum* AKU 1009a was investigated. Among various polyunsaturated fatty acids tested  $\alpha$ -linolenic acid [*cis*-9,*cis*-12,*cis*-15-octadecatrienoic acid (18:3)],  $\gamma$ -linolenic acid (*cis*-6,*cis*-9,*cis*-12-18:3), columbinic acid (*trans*-5,*cis*-9,*cis*-12-18:3), and stearidonic acid [*cis*-6,*cis*-9,*cis*-12,*cis*-15-octadecatetraenoic acid (18:4)] were found to be transformed. The fatty acids transformed by the strain had a common structure of C18 fatty acid with *cis*-9,*cis*-12 diene system. Three major fatty acids were produced from  $\alpha$ -linolenic acid, and they were identified as *cis*-9,*trans*-11,*cis*-15-18:3, *trans*-9,*trans*-11,*cis*-15-18:3, and *trans*-10,*cis*-15-18:2. Five major fatty acids were produced from  $\gamma$ -linolenic acid, and four of them were identified as *cis*-6,*cis*-9,*trans*-11-18:3, *cis*-6,*trans*-9,*trans*-11-18:3, *cis*-6,*trans*-10-octadecadienoic acid (18:2), and *trans*-10-octadecaenoic acid (18:1). The strain transformed *cis*-9,*cis*-12 diene system of C18 fatty acids to conjugated diene systems of *cis*-9,*trans*-11- and *trans*-9,*trans*-11. These conjugate dienes were further saturated by the strain to monoene of *trans*-10.

## Section 2. Conjugated $\alpha$ -linolenic acid production from $\alpha$ -linolenic acid by *Lactobacillus plantarum* AKU 1009a

### INTRODUCTION

The author established methods for CLA production from linoleic acid using washed cells of lactic acid bacteria as the catalysts [25,37,48,49] (Fig. 1A). Lactic acid bacteria produced two CLA isomers, *i.e.*, *cis*-9,*trans*-11-octadecadienoic acid (CLA1) and *trans*-9,*trans*-11-octadecadienoic acid (CLA2), together with 10-hydroxy-12-octadecanoic acid (HY) as an intermediate. In CHAPTER III, section 1, the author found that the same strategy is applicable for the production of a conjugated trienoic acid, conjugated  $\alpha$ -linolenic acid (CALA), from  $\alpha$ -linolenic acid. The washed cells of *Lactobacillus plantarum* AKU 1009a transformed  $\alpha$ -linolenic acid into two CALA isomers, *i.e.*, *cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (CALA1) and *trans*-9,*trans*-11,*cis*-15-octadecatrienoic acid (CALA2) (Fig. 1B). In this section, the author describes the culture conditions to obtain active catalysts and an optimization of reaction conditions for practical CALA production from  $\alpha$ -linolenic acid using washed cells of *L. plantarum* AKU 1009a.



**Fig. 1.** Transformation of linoleic acid to conjugated linoleic acid (A) and  $\alpha$ -linolenic acid to conjugated  $\alpha$ -linolenic acid (B) by lactic acid bacteria.

## MATERIALS AND METHODS

**Chemicals.** The  $\alpha$ -linolenic acid (Wako Pure Chemical, Osaka, Japan) used in this study was of 76% purity, and its fatty acid composition was: 76%  $\alpha$ -linolenic acid, 19% linoleic acid, and 5% oleic acid. Standard samples of CLA isomers, *i.e.*, CLA1 and CLA2, and HY were prepared as described previously [25]. Fatty acid-free (<0.02%) bovine serum albumin (BSA) was purchased from Sigma Chemicals (MO, USA). All other chemicals used were of analytical grade and were commercially available.

**Microorganism, cultivation, and preparation of washed cells.** *L. plantarum* AKU 1009a (AKU Culture Collection, Faculty of Agriculture, Kyoto University) was used for all experiments [37]. The strain was cultivated in MRS medium comprised of 1.0% tryptone, 1.0% meat extract, 0.5% yeast extract, 2.0% glucose, 0.1% Tween 80, 0.2%  $K_2HPO_4$ , 0.5% sodium acetate, 0.2% diammonium citrate, 0.02%  $MgSO_4 \cdot 7H_2O$ , and 0.005%  $MnSO_4 \cdot H_2O$  (pH 6.5) [25]. For investigation of culture conditions, the strain was cultivated in 15 ml of the medium in screw-cap tubes (16.5 x 125 mm) at 28°C for 24 h under  $O_2$ -limited conditions in sealed tubes with shaking (120 strokes/min). The seed culture (15ml) was transferred into 550 ml of the medium in a 600-ml flask and then incubated at 28°C with shaking (120 strokes/min) to prepare a large amount of the cells for optimization of reaction conditions. Growth was monitored by optical density (OD) at 610 nm. Cells were harvested by centrifugation (12,000 x g, 10 min), washed twice with 0.85% NaCl, centrifuged again, and then used as the washed cells for the reactions.

**Reaction conditions.** 1 ml of reaction mixture, composed of 4 mg/ml  $\alpha$ -linolenic acid complexed with BSA [0.08% (w/v)], 0.1 M potassium phosphate buffer (KPB, pH 6.5), and 22.5% (wet cells, w/v) washed cells [corresponding to 3.2% (dry cells, w/v)], was filled into a 16.5 x 125 mm test tubes. The reaction mixture was incubated microaerobically in an  $O_2$ -adsorbed atmosphere in a sealed chamber with  $O_2$ -absorbent (AnaeroPack "Kenki", Mitsubishi Gas Chemical Co, Inc., Tokyo, Japan), and gently shaken (120 strokes/min) at 37°C for 24 h. For investigation of the effects of  $\alpha$ -linolenic acid and cell concentrations, and for the preparative CALA production, the reaction mixture was incubated essentially under the same conditions as described above except that the volume of the reaction mixture was 5 ml. All experiments were done in triplicate, and the averages of three separate experiments, which were reproducible within  $\pm 10\%$ , are presented in the figures.

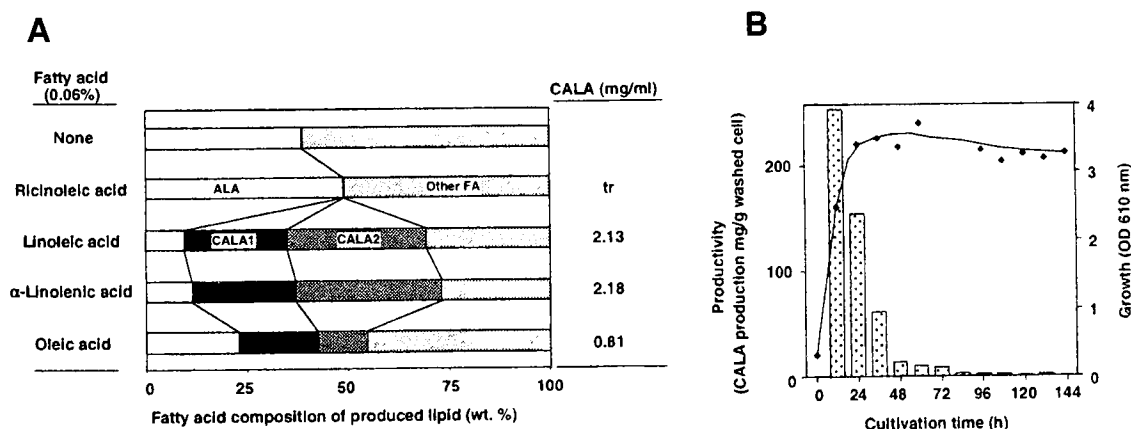
**Lipid analyses.** Lipids were extracted from the reaction mixture with chloroform-methanol (1:2, by vol.) according to the procedure of Bligh-Dyer [15], and transmethylated with 10% methanolic HCl at 50°C for 20 min. The resultant fatty acid methyl esters were extracted with

n-hexane and analyzed by gas-liquid chromatography (GC) as described in CHAPTER I, section 1. Extraction and fractionation into lipid classes were carried out essentially as described in CHAPTER I, section 1 [16,17].

## RESULTS

### Optimization of culture conditions.

To obtain washed cells with high CALA productivity, the author examined the culture conditions using MRS medium [25] as the basal medium. Effects of fatty acid supplementation (0.06% w/v) into the medium were investigated. Among the tested fatty acids presented in Fig. 2A, linoleic acid and  $\alpha$ -linolenic acid markedly increased the CALA productivity of the washed cells. The concentration of these fatty acids were examined, and the highest CALA productivity was obtained in the washed cells cultivated with 0.01%  $\alpha$ -linolenic acid. The changes in CALA productivity during cultivation in MRS medium supplemented with 0.01%  $\alpha$ -linolenic acid were monitored. The cells at late log-phase showed significantly high productivity (Fig. 2B). The washed cells obtained from late log-phase culture were used for optimization of reaction conditions.

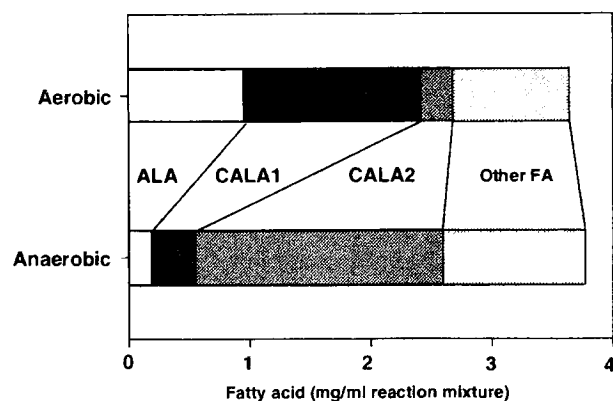


**Fig. 2.** Optimization of culture conditions. (A), Effects of fatty acid supplementation on CALA productivity of the washed cells of *L. plantarum* AKU 1009a. (B), Time course of cultivation and CALA productivity of washed cells of *L. plantarum* AKU 1009a. The bars and line represent CALA productivity and OD 610 nm, respectively. ALA,  $\alpha$ -linolenic acid; CALA1, *cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid; CALA2, *trans*-9,*trans*-11,*cis*-15-octadecatrienoic acid; CALA, sum of CALA1 and CALA2. Other fatty acid (FA) includes palmitic acid, oleic acid, *trans*-vaccenic acid, 2-hexy-1-cyclopropan-octanoic acid, linoleic acid, *cis*-9,*trans*-11-octadecadienoic acid, *trans*-9,*trans*-11-octadecadienoic acid, *trans*-10,*cis*-15-octadecadienoic acid, and 10-hydroxy-12-octadecadienoic acid.



### Optimization of reaction conditions.

(i) Effects of oxygen: Reactions were carried out in an O<sub>2</sub>-adsorbed atmosphere in test tubes in a sealed chamber with O<sub>2</sub>-absorbent (anaerobic) or under air in open test tubes (aerobic). Higher production of CALA was observed under anaerobic conditions with a much higher proportion of CALA2 (Fig. 3). The reaction conditions were further optimized under anaerobic conditions.



**Fig. 3.** Effects of oxygen on CALA production by the washed cells of *L. plantarum* AKU 1009a. For abbreviations, see the legend to Fig. 2.

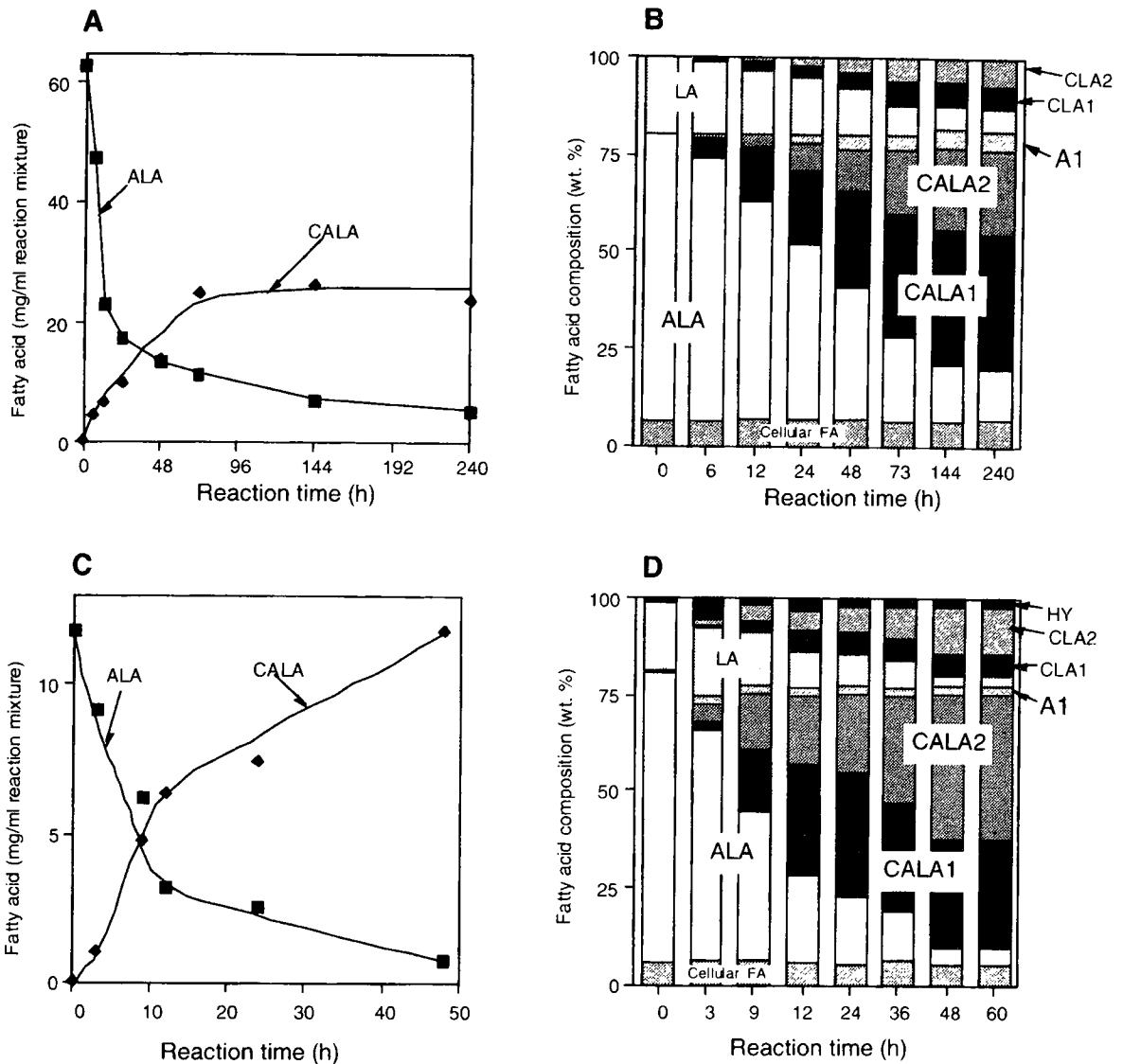
(ii) Effects of reaction temperature: Reactions were carried out at various temperatures in the range of 24 to 50°C. CALA production increased with increasing temperature from 24 to 37°C, but decreased at higher temperatures.

(iii) Effects of concentrations of  $\alpha$ -linolenic acid and washed cells: Reactions were carried out with 15% (wet cells, w/v) washed cells and various concentrations of  $\alpha$ -linolenic acid in 5-ml reaction mixtures with a fixed ratio of  $\alpha$ -linolenic acid/BSA, 5:1 (by vol.). BSA was used to disperse the  $\alpha$ -linolenic acid in the reaction mixture. CALA production increased with increasing concentration of  $\alpha$ -linolenic acid up to 23 mg/ml and reached a plateau (14 mg CALA/ml) at higher concentrations.

Reaction mixtures were incubated for 24 h with 28 mg/ml  $\alpha$ -linolenic acid and different amounts of washed cells in 5 ml reaction mixtures. CALA production increased to 25 mg/ml with increasing amount of washed cells up to 18% (wet cells, w/v), which corresponded to 2.6% dry cells (w/v), but decreased slightly with greater amounts of washed cells.

### Time course analysis of preparative CALA production.

The production of CALA from  $\alpha$ -linolenic acid with the passage of time was monitored under 2 conditions. With 63 mg/ml  $\alpha$ -linolenic acid as the substrate and 33% (wet cells, w/v) washed cells as the catalyst, the production of CALA reached a maximum (25 mg/ml, molar yield to  $\alpha$ -linolenic acid, 39.7%) at 73 h reaction (Fig. 4A). On the other hand, with 12 mg/ml  $\alpha$ -linolenic acid and 20% (wet cells, w/v) washed cells, almost all of the  $\alpha$ -linolenic acid added was converted to CALA (12 mg/ml) in 48 h (Fig. 4C). The fatty acid composition of the

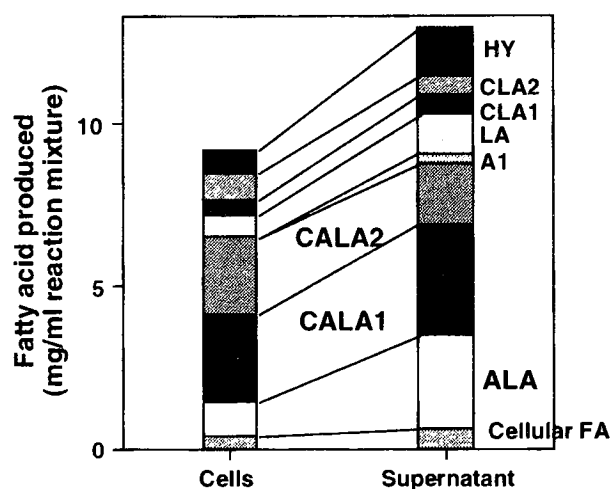


**Fig. 4.** Time course of preparative CALA production with 63 (A and B) or 12 mg/ml (C and D)  $\alpha$ -linolenic acid as the substrate with 33 or 20% (wet cells, w/v) washed cells, respectively. (A) and (C), time courses of the reactions; (B) and (D), fatty acids compositions (wt%) of the lipids produced. LA, linoleic acid; CLA1, *cis*-9,*trans*-11-octadecadienoic acid; CLA2, *trans*-9,*trans*-11-octadecadienoic acid; HY, 10-hydroxy-12-octadecadienoic acid; A1, *trans*-10,*cis*-15-octadecadienoic acid. Cellular fatty acid (FA) includes the fatty acids (palmitic acid, oleic acid, *trans*-vaccenic acid, 2-hexy-1-cyclopropan-octanoic acid) synthesized de novo by the bacterium. For other abbreviations, see the legend to Fig. 2.

produced lipids was also examined. The ratio of CALA1/CALA2 was about 2 at the higher substrate concentration (63 mg/ml, Fig. 4B), but decreased to about 0.75 at the lower substrate concentration (12 mg/ml, Fig. 4D) and CALA2 became dominant. CLA1, CLA2 and HY, which are derived from contaminated linoleic acid in  $\alpha$ -linolenic acid, were found in the lipids produced. Another product found was *trans*-10,*cis*-15-octadecadienoic acid, which was produced through further saturation of CALA1 and CALA2.

### Distribution and lipid classes of the fatty acids produced.

The reaction mixture with 29 mg/ml  $\alpha$ -linolenic acid as the substrate and 20% (wet cells, w/v) washed cells as catalysts was centrifuged after 24 h reaction time, and separated into supernatant and cells. The lipid classes of the fatty acids produced in both the supernatant and the cells, were analyzed by thin-layer chromatography. Almost all the lipids were obtained as free fatty acids (data not shown) and 58% of the fatty acids produced were found in the supernatant. The fatty acid composition of the lipids produced in the supernatant resembled that of the cells (Fig. 5).



**Fig. 5.** Fatty acid compositions of the lipid produced in the reaction supernatant and the cells. The reaction was done with 29 mg/ml  $\alpha$ -linolenic acid and 20% (wet cells, w/v) washed cells of *L. plantarum* AKU 1009a. For abbreviations, see the legend to Fig. 4.

## DISCUSSION

Many researches on CLA are progressing from standpoints of their nutritional and physiological effects. On the other hand, because of lack of appropriate sources, only few reports describe the effects of conjugated trienoic acids in spite of its potential cytotoxicity toward mammalian tumor cells [45,46]. The author has presented here the first example of preparative production of conjugated trienoic acids, CALA1 and CALA2, from  $\alpha$ -linolenic acid by a lactic acid bacterium. The conjugated trienoic acids produced by the methods presented here are good sources to evaluate their physiological and nutritional effects and chemical characteristics such as stability against oxygen.

CALA was produced mostly in the free fatty acid form by the methods presented here. The free fatty acid form of CALA, as well as CLA, could be transformed to the acylglycerol or ester forms by lipase-catalyzing reactions [50]. The physiological and nutritional effects of such materials derived from CALA produced by lactic acid bacteria are of future interests.

## SUMMARY

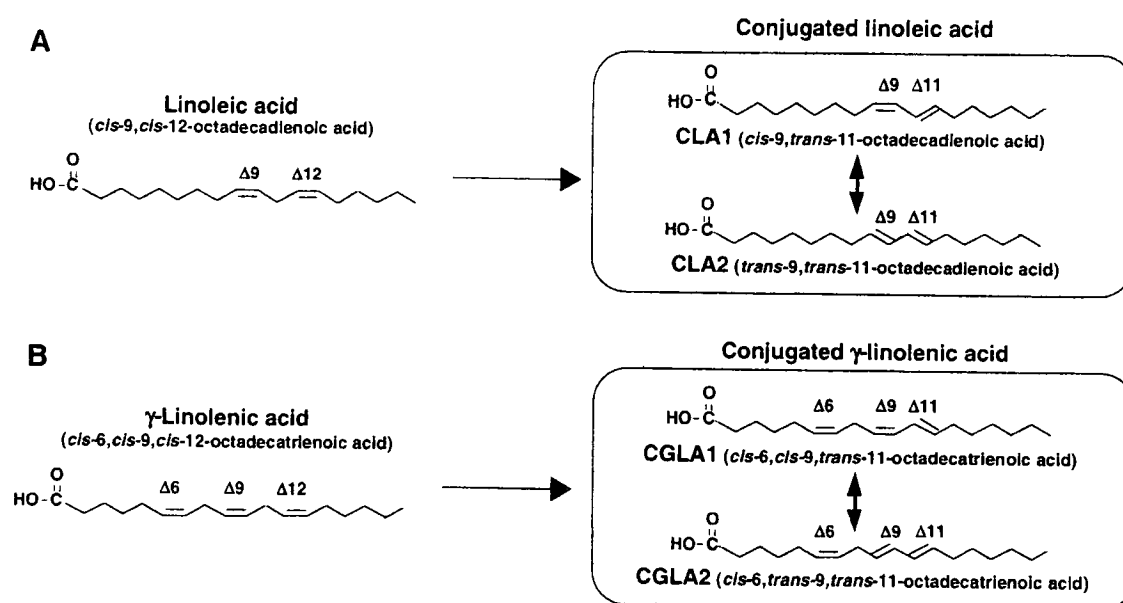
Conjugated  $\alpha$ -linolenic acid (CALA) was produced by incubation of  $\alpha$ -linolenic acid with the washed cells of *Lactobacillus plantarum* AKU 1009a. The washed cells expressing high levels of CALA productivity were obtained by cultivation in a nutrient medium supplemented with 0.01% (w/v)  $\alpha$ -linolenic acid as an inducer. Under the optimum reaction conditions with 63 mg/ml  $\alpha$ -linolenic acid as the substrate, the washed cells produced 25 mg CALA/ml reaction mixture (40% molar yield) in 72 h. The produced CALA was a mixture of the two isomers, *i.e.*, *cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (CALA1, 67% of total CALA) and *trans*-9,*trans*-11,*cis*-15-octadecatrienoic acid (CALA2, 33% of total CALA), and accounted for 48% of total fatty acid obtained. Almost stoichiometric conversion was attained with 12 mg/ml  $\alpha$ -linolenic acid as the substrate in 48 h. It resulted in 12 mg CALA/ml reaction mixture consisting of 43% CALA1 and 57% CALA2 accounting for 66% of total fatty acid obtained. The CALA produced was obtained as the free fatty acid.

### Section 3. Conjugated $\gamma$ -linolenic acid production from $\gamma$ -linolenic acid by *Lactobacillus plantarum* AKU 1009a

#### INTRODUCTION

The author established the methods for CLA production from linoleic acid using washed cells of lactic acid bacteria as the catalysts [25,37,48,49] (Fig. 1A). In CHAPTER III section 1, the author found that the same strategy is applicable for the production of a conjugated trienoic acid, conjugated  $\gamma$ -linolenic acid (CGLA), from  $\gamma$ -linolenic acid. The washed cells of *Lactobacillus plantarum* AKU 1009a transformed  $\gamma$ -linolenic acid into two CGLA isomers, *i.e.*, *cis*-6,*cis*-9,*trans*-11-octadecatrienoic acid (CGLA1) and *cis*-6,*trans*-9,*trans*-11-octadecatrienoic acid (CGLA2) (Fig. 1B).

In this section, the author reports the culture conditions to obtain active catalysts and the optimized reaction conditions for practical CGLA production from  $\gamma$ -linolenic acid using washed cells of *L. plantarum* AKU 1009a.



**Fig. 1.** Transformation of linoleic acid to conjugated linoleic acid (A) and  $\gamma$ -linolenic acid to conjugated  $\gamma$ -linolenic acid (B) by lactic acid bacteria.

## MATERIALS AND METHODS

**Chemicals.**  $\gamma$ -Linolenic acid and fatty acid-free (<0.02%) bovine serum albumin (BSA) were purchased from Sigma (MO, U.S.A.). The  $\alpha$ -linolenic acid (Wako Pure Chemical, Osaka, Japan) used in this study was of 76% purity, and its fatty acid composition was: 76%  $\alpha$ -linolenic acid, 19% linoleic acid, and 5% oleic acid. Standard samples of CLA isomers, *i.e.*, *cis*-9,*trans*-11-octadecadienoic acid (CLA1) and *trans*-9,*trans*-11-octadecadienoic acid (CLA2), and 10-hydroxy-12-octadecanoic acid (HY) were prepared as described previously [25]. All other chemicals used were of analytical grade and were commercially available.

**Microorganisms, cultivation, and preparation of washed cells.** *L. plantarum* AKU 1009a (AKU Culture Collection, Faculty of Agriculture, Kyoto University) was used for all experiments [37]. The strain was cultivated in MRS medium comprised of 1.0% tryptone, 1.0% meat extract, 0.5% yeast extract, 2.0% glucose, 0.1% Tween 80, 0.2%  $K_2HPO_4$ , 0.5% sodium acetate, 0.2% diammonium citrate, 0.02%, and  $MgSO_4 \cdot 7H_2O$ , 0.005%  $MnSO_4 \cdot H_2O$  (pH 6.5) [25]. For investigation of culture conditions, the strain was cultivated in 15 ml of the medium in screw-cap tubes (16.5 x 125 mm) at 28°C for 24 h under  $O_2$ -limited conditions in sealed tubes with shaking (120 strokes/min). The seed culture (15 ml) was transferred into 550 ml of the medium in a 600-ml flask and incubated at 28°C with shaking (120 strokes/min) to prepare a large amount of the cells for optimization of reaction conditions. Growth was monitored by optical density (OD) at 610 nm. Cells were harvested by centrifugation (12,000 x g, 10 min), washed twice with 0.85% NaCl, centrifuged again, and then used as the washed cells for the reactions.

**Reaction conditions.** The reaction mixture, 1 ml, in test tubes (16.5 x 125 mm) was composed of 4 mg/ml  $\gamma$ -linolenic acid complexed with BSA [0.08% (w/v)], 0.1 M potassium phosphate buffer (KPB, pH 6.5), and 22.5% (wet cells, w/v) washed cells [corresponding to 3.2% (dry cells, w/v)]. The reaction mixture was incubated microaerobically in an  $O_2$ -adsorbed atmosphere in a sealed chamber with  $O_2$ -absorbent (AnaeroPack "Kenki", Mitsubishi Gas Chemical Co, Inc., Tokyo, Japan), and gently shaken (120 strokes/min) at 37°C for 6 h to 42 h. In the microaerobic conditions, the oxygen concentrations, monitored by an oxygen indicator (Mitsubishi Gas Chemical Co. Inc., Tokyo, Japan), were kept under 1%. For investigation of the effects of  $\gamma$ -linolenic acid and cell concentrations, and for the preparative CGLA production, the reaction mixture was incubated essentially under the same conditions as described above except that the volume of the reaction mixture was 5 ml. All experiments were done in triplicate, and the averages of three separate experiments, which were reproducible within  $\pm 10\%$ , are presented in the figures.

**Lipid analyses.** Lipids were extracted from 1 ml of reaction mixture with 3 ml of chloroform-methanol (1:2, by vol.) according to the procedure of Bligh-Dyer and concentrated by evaporation under reduced pressure [15]. The resultant lipids were dissolved in 1 ml of dichloromethane and then methylated with 2 ml of 10% methanolic HCl at 50°C for 20 min. After addition of 1 ml of water, the resultant fatty acid methyl esters were extracted with 5 ml of *n*-hexane and concentrated by evaporation under reduced pressure. The fatty acid methyl ester solutions were analyzed by gas-liquid chromatography (GC) using a Shimadzu (Kyoto, Japan) GC-1700 gas chromatograph equipped with a flame ionization detector and a split injection system and fitted with a capillary column (HR-SS-10, 50 m x 0.25 mm I.D., Shinwa Kako, Kyoto, Japan). The column temperature was initially 180°C and was raised to 200°C at a rate of 1°C/min and maintained at that temperature for 20 min and was raised to 220°C at a rate of 5°C/min and maintained at that temperature for 10 min. The injector and detector were operated at 250°C. Helium was used as a carrier gas at 225 kPa/cm<sup>2</sup>. Fractionation into lipid classes and lipid class analysis on thin-layer chromatography (TLC) were carried out as described previously [16,17].

## RESULTS

### Optimization of culture conditions.

To obtain washed cells with high CGLA productivity, the author examined the culture conditions using MRS medium [25] as the basal medium. Effects of fatty acid supplementation (0.06% w/v) into the medium were investigated. Among the tested fatty acids (oleic acid, linoleic acid,  $\alpha$ -linolenic acid, and  $\gamma$ -linolenic acid), linoleic acid and  $\alpha$ -linolenic acid markedly increased the CGLA productivity of the washed cells. The concentrations of these fatty acids were examined, and the washed cells with highest CGLA productivity was obtained by cultivation with 0.03%  $\alpha$ -linolenic acid. The changes in CGLA productivity during cultivation in MRS medium supplemented with 0.03%  $\alpha$ -linolenic acid were monitored. The cells at late log-phase showed significantly high productivity (Fig. 2). The washed cells obtained from late log-phase culture were used for optimization of reaction conditions.

### Optimization of reaction conditions.

(i) Effects of reaction pH: Reactions were carried out for 6 h in different buffer systems of 0.1, 0.5 or 1.0 M of sodium citrate/NaOH buffer (pH 4.0, 5.0, 6.0, 7.0), KPB (pH 6.0, 6.5, 7.0, 7.5) or Tris/HCl (pH 7.0, 8.0, 9.0). CGLA was most efficiently produced with 0.1 M KPB, pH 6.5.

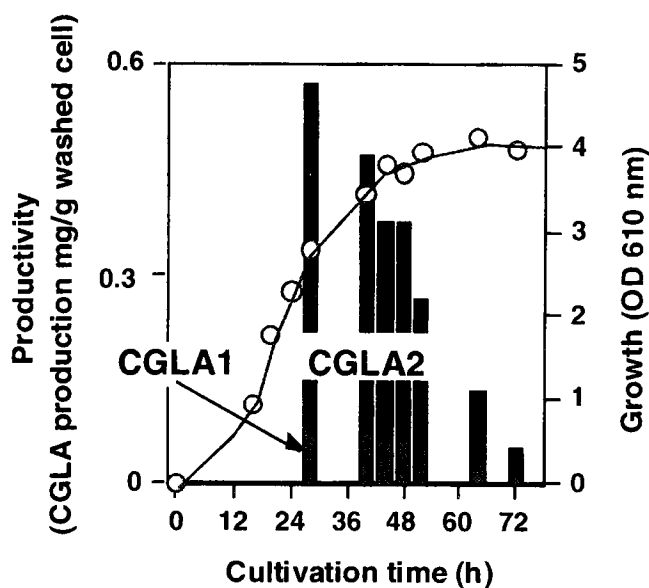


Fig. 2. Time course of cultivation and CGLA productivity of washed cells of *L. planatarum* AKU 1009a. The bars and line represent CGLA productivity and OD 610 nm, respectively. CGLA1, *cis*-6,*cis*-9,*trans*-11-octadecatrienoic acid; CALA2, *cis*-6,*trans*-9,*trans*-11-octadecatrienoic acid.

(ii) Effects of detergents: The effects of detergents, which disperse  $\gamma$ -linolenic acid in the reaction mixture like BSA, were examined.  $\gamma$ -linolenic acid was complexed with various detergents (0.1% w/v) instead of BSA, and then used for the reaction under the standard conditions. The  $\gamma$ -linolenic acid treated with *N*-heptyl- $\beta$ -D-thiogluconide was most efficiently transformed to CGLA. *N*-heptyl- $\beta$ -D-thiogluconide was thus used in the following experiments.

(iii) Effects of reaction temperature: Reactions were carried out at various temperatures in the range of 15 to 45°C. CGLA production increased with increasing temperature from 15 to 34°C, but decreased at higher temperatures.

(iv) Effects of oxygen: Reactions were carried out in an O<sub>2</sub>-adsorbed atmosphere in test tubes in a sealed chamber with O<sub>2</sub>-absorbent (anaerobic) or under air in open test tubes (aerobic). Higher production of CGLA was observed under anaerobic conditions with a much higher proportion of CGLA2 (CGLA1, 0.35 mg/ml; CGLA2, 1.4 mg/ml). On the other hand, higher production of CGLA1 was observed under aerobic conditions (CGLA1, 0.74 mg/ml; CGLA2, 0.40 mg/ml). The reaction conditions were further optimized under anaerobic conditions.

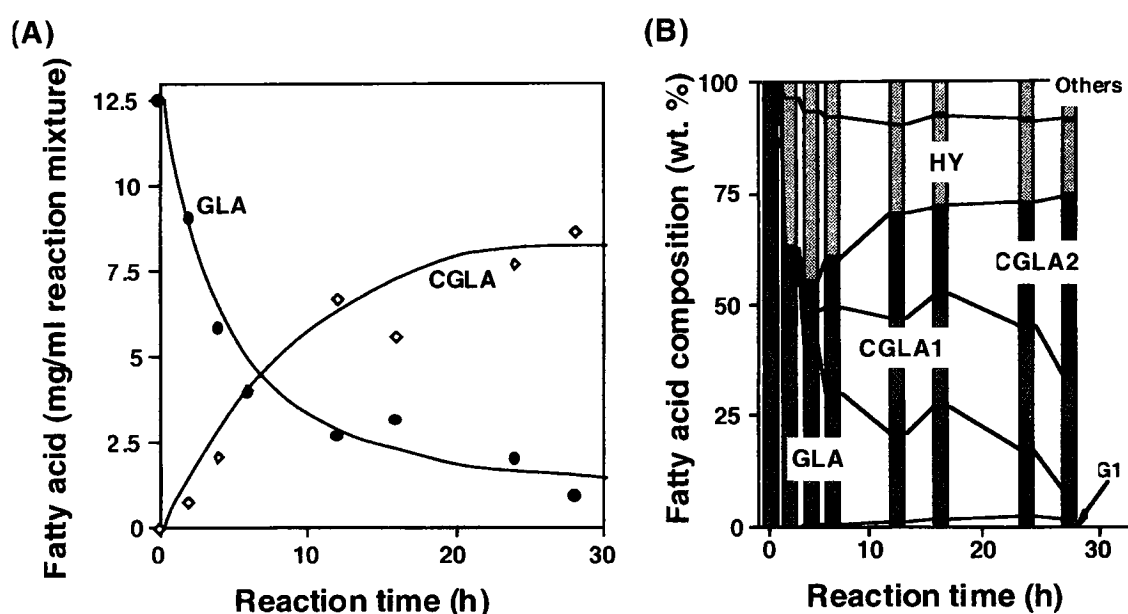
(v) Effects of concentrations of  $\gamma$ -linolenic acid and washed cells: Reactions were carried out with 13% (wet cells, w/v) washed cells and various concentrations of  $\gamma$ -linolenic acid in 5 ml reaction mixtures with a fixed ratio of  $\gamma$ -linolenic acid/*N*-heptyl- $\beta$ -D-thiogluconide, 5:1 (by weight). CGLA production increased with increasing concentration of  $\gamma$ -linolenic acid up to 10 mg/ml and reached a plateau (2.3 mg CGLA/ml) at higher concentrations.

Reaction mixtures were incubated for 6 h with 3.2 mg/ml  $\gamma$ -linolenic acid and different amounts of washed cells in 5-ml reaction mixtures. CGLA production increased to 2.8 mg/ml with increasing amount of washed cells up to 32% (wet cells, w/v), which corresponded to 4.6% dry cells (w/v), but decreased slightly with greater amounts of washed cells.



### Time course analysis of preparative CGLA production.

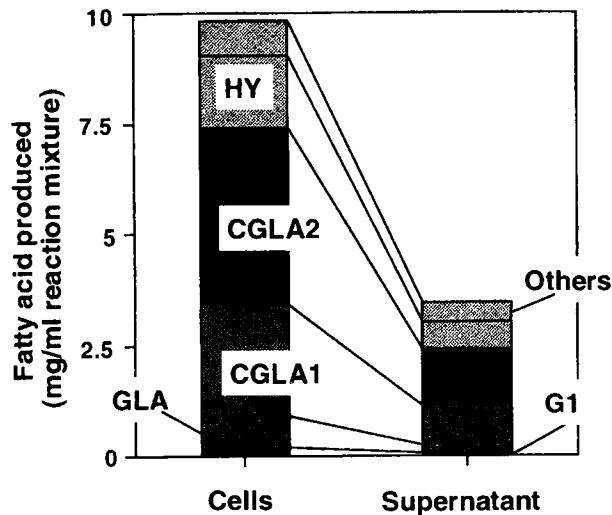
The production of CGLA from  $\gamma$ -linolenic acid with the passage of time was monitored. With 13 mg/ml  $\gamma$ -linolenic acid as the substrate and 32% (wet cells, w/v) washed cells as the catalyst, the production of CGLA reached a maximum (8.8 mg/ml, molar yield to  $\gamma$ -linolenic acid, 68%) at 27 h reaction time (Fig. 3A). The fatty acid composition of the produced lipids was also examined (Fig. 3B). The ratio of CGLA1/CGLA2 was in the range of 2/3 to 2/1 through the reaction time. In the initial stage of the reaction, hydroxy fatty acids, possible intermediates of CGLA production, were accumulated, and reduced followed by CGLA accumulation. A small amount of further saturated product, *cis*-6,*trans*-10-octadecadienoic acid, was also produced in the latter stage of the reaction.



**Fig. 3.** Time course of preparative CGLA production with 13 mg/ml  $\gamma$ -linolenic acid as the substrate and 32% (wet cells, w/v) washed cells as the catalysts. (A), time course of the reaction, GLA (closed circle), CGLA (open square); (B), fatty acids compositions (wt%) of the lipids produced. GLA, *cis*-6,*cis*-9,*cis*-12-octadecatrienoic acid; CGLA, sum of CGLA1 (*cis*-6,*cis*-9,*trans*-11-octadecatrienoic acid) and CGLA2 (*cis*-6,*trans*-9,*trans*-11-octadecatrienoic acid), HY, hydroxy fatty acids; G1, *cis*-6,*trans*-10-octadecadienoic acid.

### Distribution and lipid classes of the fatty acids produced.

The reaction mixture with 13 mg/ml  $\gamma$ -linolenic acid as the substrate and 32% (wet cells, w/v) washed cells as the catalyst was centrifuged after 27 h reaction time, and separated into supernatant and cells. The lipid classes of the fatty acids produced in both the supernatant and the cells were analyzed by TLC. Almost all the lipids were obtained as free fatty acids (data not shown) and 74% of the fatty acids produced were found in the cells (or associated with cells). The fatty acid composition of the lipids produced in the supernatant resembled that of the cells (Fig. 4).



**Fig. 4.** Fatty acid compositions of the lipid produced in the reaction supernatant and the cells. The reaction was done with 13 mg/ml  $\gamma$ -linolenic acid and 32% (wet cells, w/v) washed cells of *L. plantarum* AKU 1009a. For abbreviations, see the legend to Fig.3.

## DISCUSSION

The author has presented here the first example of preparative production of conjugated trienoic acids, CGLA, from  $\gamma$ -linolenic acid by a lactic acid bacterium. CLA is a fatty acid found in the meat and dairy products of ruminant animals. CLA is produced by rumen bacteria including lactic acid bacteria, and its content of milk fat can be increased by offering diets rich in linoleic acid, which is accessible to rumen organisms. Increased level of CLA was also obtained with the diet rich in linolenic acid, but the content of CGLA in milk was not reported [51]. The author's results suggest that CGLA is produced from  $\gamma$ -linolenic acid in a similar fashion with CLA production from linoleic acid by lactic acid bacteria [25]. These results provide a clue to increase CGLA amounts in dairy products. The physiological and nutritional effects of such dairy products and CGLA itself are of further interests.

## SUMMARY

Conjugated  $\gamma$ -linolenic acid (CGLA) was produced by incubation of  $\gamma$ -linolenic acid with the washed cells of *Lactobacillus plantarum* AKU 1009a. The washed cells expressing high levels of CGLA productivity were obtained by cultivation in a nutrient medium supplemented with 0.03% (w/v)  $\alpha$ -linolenic acid as an inducer. Under the optimum reaction conditions with 13 mg/ml  $\gamma$ -linolenic acid as the substrate, the washed cells produced 8.8 mg CGLA/ml reaction mixture (68% molar yield) in 27 h. The produced CGLA was a mixture of the two isomers, i.e., *cis*-6,*cis*-9,*trans*-11-octadecatrienoic acid (CGLA1, 40% of total CGLA) and *cis*-6,*trans*-9,*trans*-11-octadecatrienoic acid (CGLA2, 60% of total CGLA), and accounted for 66% of total fatty acid obtained. The CGLA produced was obtained as the free fatty acid.

## CONCLUSION

Conjugated fatty acids have attracted many attentions as a novel type of biologically beneficial functional lipids. Some isomers of conjugated linoleic acid (CLA) reduce carcinogenesis, atherosclerosis, and body fat. Considering the uses of CLA for medicinal and nutraceutical purposes, isomer-selective and safety process are required. Introduction of biological reactions could be an answer. The author screened various biological systems useful for conjugated fatty acid synthesis and found some unique reactions in lactic acid bacteria. The one is isomerization of nonconjugated double bonds to form conjugated double bonds, and the other is dehydration of a hydroxy group to form conjugated double bonds.

The author applied the isomerization reaction to the production of CLA, conjugated  $\alpha$ -linolenic acid (CALA), and conjugated  $\gamma$ -linolenic acid (CGLA). Through the intensive screening, *Lactobacillus plantarum* AKU 1009a was selected as a potential catalyst. Under optimized reaction conditions using washed cells of *L. plantarum* AKU 1009a as the catalysts, 40 mg/ml CLA, 24.4 mg/ml CALA, and 8.7 mg/ml CGLA were produced from linoleic acid,  $\alpha$ -linolenic acid, and  $\gamma$ -linolenic acid, respectively. The produced CLA, CALA, and CGLA were consisted of *cis*-9,*trans*-11-octadecadienoic acid (18:2, CLA1) and *trans*-9,*trans*-11-18:2 (CLA2), *cis*-9,*trans*-11,*cis*-15-octadecatrienoic acid (18:3, CALA1) and *trans*-9,*trans*-11,*cis*-15-18:3 (CALA2), and *cis*-6,*cis*-9,*trans*-11-18:3 (CGLA1) and *cis*-6,*trans*-9,*trans*-11-18:3 (CGLA2), respectively. The isomer proportion was controlled by changing the reaction conditions. For examples, the addition of L-serine, glucose, AgNO<sub>3</sub>, or NaCl to the reaction mixture reduced the production of CLA2, resulted in selective production of CLA1. CLA2 can be produced at more than 97% selectivity, if the reaction is done long enough with a low linoleic acid concentration. The isomerization reactions were revealed to be consisted of hydration to 10-hydroxy fatty acids (HY) and subsequent dehydrating isomerization of HY to  $\Delta$ 9,  $\Delta$ 11-conjugated fatty acids. Same reactions were observed with the fatty acids of C18 with *cis*-9 and *cis*-12 double bonds such as columbinic acid and stearidonic acid.

The author applied the dehydration reaction to the production of CLA from ricinoleic acid (12-hydroxy-*cis*-9-octadecaenoic acid). Under optimized reaction conditions using washed cells of *L. plantarum* AKU 1009a as the catalysts, 1.7 mg/ml CLA was produced from ricinoleic acid. The produced CLA was consisted of CLA1 and CLA2. Two possible pathways for CLA synthesis from ricinoleic acid are proposed. The one is direct dehydration to CLA, and the other is dehydration to linoleic acid that is subsequently isomerized to CLA. Castor oil, a triacylglycerol rich in ricinoleic acid, was also found as a substrate for CLA production with the help of lipase-catalyzing triacylglycerol hydrolysis.

## REFERENCES

- [1] **Y.L. Ha, N.K. Grimm, and M.W. Pariza**, Anticarcinogens from fried ground beef: heat-altered derivatives of linoleic acid. *Carcinogenesis*, **8**:1881-1887 (1987).
- [2] **M.W. Pariza, and W.A. Hargraves**, A beef-derived mutagenesis modulator inhibits initiation of mouse epidermal tumors by 7,12-dimethylbenz [a] anthracene. *Carcinogenesis*, **6**:591-593 (1985).
- [3] **Y.L. Ha, J. Storkson, and M.W. Pariza**, Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Res.*, **50**:1097-1101 (1990).
- [4] **C. Ip, S.F. Chin, J.A. Scimeca, and M.W. Pariza**, Mammary cancer prevention by conjugated dienoic derivative of linoleic acid. *Cancer Res.*, **51**:6118-6124 (1991).
- [5] **M.E. Cook, C.C. Miller, Y. Park, and M.W. Pariza**, Immune modulation by altered nutrient metabolism: nutritional control of immune-induced growth depression. *Poult. Sci.*, **72**:1301-1305 (1993).
- [6] **C.C. Miller, Y. Park, M.W. Pariza, and M.E. Cook**, Feeding conjugated linoleic acid to animals partially overcomes catabolic responses due to endotoxin injection. *Biochem. Biophys. Res. Commun.*, **198**:1107-1112 (1994).
- [7] **K.N. Lee, D. Kritchevsky, and M.W. Pariza**, Conjugated linoleic acid and atherosclerosis in rabbits. *Atherosclerosis*, **108**:19-25 (1994).
- [8] **S.F. Chin, W. Liu, J.M. Storkson, Y.L. Ha, and M.W. Pariza**, Dietary sources of conjugated dienoic isomers of linoleic acid, a newly recognized class of anticarcinogens. *J. Food Compos. Anal.*, **5**:185-197 (1992).
- [9] **J. Fritsche, and H. Steinhart**, Amounts of conjugated linoleic acid (CLA) in German foods and evaluation of daily intake. *Z. Lebensm.-Unters. Forsch.*, **206**:77-82 (1998).
- [10] **A.C. Fogerty, G.L. Ford, and D. Svoronos**, Octadeca-9,11-decenoic acid in the lipids of human blood and breast milk. *Nutr. Reports Int.*, **38**:937-944 (1988).
- [11] **C.R. Kepler, K.P. Hiron, J.J. Mcneill, and S.B. Tove**, Intermediates and products of the biohydrogenation of linoleic acid by *Butyrivibrio fibrisolvens*. *J. Biol. Chem.*, **241**:1350-1354 (1966).
- [12] **J. Jiang, L. Bjorck, and R. Fonden**, Production of conjugated linoleic acid by dairy starter cultures. *J. Appl. Microbiol.*, **85**:95-102 (1998).
- [13] **D. Fabiola, G. Pierre-Alain, E. Marina, and B.F. Laurent**, Stability of cyclopropane and conjugated linoleic acid during fatty acid quantification in lactic acid bacteria. *Lipids*, **34**:1107-1115 (1999).

- [14] **R. J. Nicolosi, E. J. Rogers, D. Kritchevsky, J. A. Scimeca, and P. J. Huth**, Dietary conjugated linoleic acid reduces plasma lipoproteins and early aortic atherosclerosis in hypercholesterolemic hamsters. *Artery*, **22**:266-277 (1997).
- [15] **E.G. Bligh, and W.J. Dyer**, A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, **37**:911-917 (1959).
- [16] **S. Jareonkitmongkol, S. Shimizu, and H. Yamada**, Fatty acid desaturation-defective mutants of an arachidonic-acid-producing fungus, *Mortierella alpina* 1S-4. *J. Gen. Microbiol.*, **138**:997-1002 (1992).
- [17] **S. Shimizu, S. Jareonkitmongkol, H. Kawashima, K. Akimoto, and H. Yamada**, Production of a novel  $\omega$ 1-eicosapentaenoic acid by *Mortierella alpina* 1S-4 grown on 1-hexadecene. *Arch. Microbiol.*, **156**:163-166 (1991).
- [18] **B.A. Andersson, and R.T. Holman**, Pyrrolidides for mass spectrometric determination of the position of the double bond in monounsaturated fatty acids. *Lipids*, **9**:185-190 (1974).
- [19] **W.E. Tolberg, and D.H. Wheeler**, *Cis, trans* isomerization of conjugated linoleates by iodine and light. *J. Am. Oil Chem. Soc.*, **35**:385-388 (1958).
- [20] **M.W. Pariza, and Y.L. Ha**, Newly recognized anticarcinogenic fatty acids. In *Antimutagenesis and Anticarcinogenesis Mechanism II*, edited by Y. Kuroda, D. Shankel, and M.D. Waters, Plenum Press, New York, pp.167-170. (1990).
- [21] **Y. Park, K.J. Albright, W. Liu, J.M. Storkson, M.E. Cook, and M.W. Pariza**, Effect of conjugated linoleic acid on body composition in mice. *Lipids*, **32**:853-858 (1997).
- [22] **M.A. Belury**, Conjugated dienoic linoleate: a polyunsaturated fatty acid with unique chemoprotective properties. *Nutr. Rev.*, **53**:83-89 (1995).
- [23] **P.W. Parodi**, Cows' milk fat components as potential anticarcinogenic agents. *J. Nutr.*, **127**:1055-1060 (1997).
- [24] **M.P. Yurawecz, N. Sehat, M.M. Mossoba, J.A.G. Roach, and Y. Ku**, Oxidation products of conjugated linoleic acid and furan fatty acids. In *New Techniques and Applications in Lipid Analysis*, edited by R.E. McDonald, and M.M. Mossoba, AOCS Press, Champaign, pp.183-215 (1997).
- [25] **J. Ogawa, K. Matsumura, S. Kishino, Y. Omura, and S. Shimizu**, Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.*, **67**:1246-1252 (2001).
- [26] **M.H. Haas, J.K.G. Kramer, G. McNeill, K. Scott, T.A. Foglia, N. Sehat, J. Fritsche, M.M. Mossoba, and M.P. Yurawecz**, Lipase-catalyzed fractionation of conjugated linoleic acid. *Lipids*, **34**:979-987 (1999).

- [27] **T.L. Mounts, H.J. Dutton, and D. Glover**, Conjugation of polyunsaturated acids. *Lipids*, **5**:997-1005 (1970).
- [28] **C. R. Kepler, W. P. Tucker, and S. B. Tove**, Biohydrogenation of unsaturated fatty acids. IV. Substrate specificity and inhibition of linoleate delta-12-*cis*, delta-11-*trans*-isomerase from *Butyrivibrio fibrisolvens*. *J. Biol. Chem.*, **245**:3612-3620 (1970).
- [29] **H. Eyssen, and A. Verhulst**, Biotransformation of linoleic acid and bile acids by *Eubacterium lentum*. *Appl. Environ. Microbiol.*, **47**:39-43 (1984).
- [30] **A. Verhulst, G. Semjen, U. Meerts, G. Janssen, G. Parmentier, S. Asselberghs, H. Van Hespen, and H. Eyssen**, Biohydrogenation of linoleic acid by *Clostridium sporogenes*, *Clostridium bifermentans*, *Clostridium sordellii* and *Bacteroides* sp. *FEMS Microbiol. Ecol.*, **31**:255-259 (1985).
- [31] **S. C. Mills, T. W. Scott, G. R. Russell, and R. M. Smith**, Hydrogenation of C18 unsaturated fatty acids by pure cultures of a rumen *Micrococcus*. *Aust. J. Biol. Sci.*, **23**:1109-1113 (1970).
- [32] **P. Kemp, R.W. White, and D.J. Lander**, The hydrogenation of unsaturated fatty acids by five bacterial isolates from the sheep rumen, including a new species. *J. Gen. Microbiol.*, **90**:100-114 (1975).
- [33] **K. Fujumoto, H. Kimoto, M. Shishikura, Y. Endo, and K. Ogimoto**, Biohydrogenation of linoleic acid by anaerobic bacteria isolated from rumen. *Biosci. Biotechnol. Biochem.*, **57**:1026-1027 (1993).
- [34] **Y. J. Kim, R. H. Liu, D. R. Bond, and J. B. Russell**, Effect of linoleic acid concentration on conjugated linoleic acid production by *Butyrivibrio fibrisolvens* A38. *Appl. Environ. Microbiol.*, **66**:5226-5230 (2000).
- [35] **A. Rainio, M. Vahvaselka, T. Suomalainen, and S. Laakso**, Reduction of linoleic acid inhibition in production of conjugated linoleic acid by *Propionibacterium freudenreichii* ssp. *shermanii*. *Can. J. Microbiol.*, **47**:735-740 (2001).
- [36] **H. Lin, T.D. Boylston, M.J. Chang, L.O. Luedecke, and T.D. Shultz**, Survey of the conjugated linoleic acid contents of dairy products. *J. Dairy Sci.*, **78**:2358-2365 (1995).
- [37] **S. Kishino, J. Ogawa, Y. Omura, K. Matsumura, and S. Shimizu**, Conjugated linoleic acid production from linoleic acid by lactic acid bacteria. *J. Am. Oil Chem. Soc.*, **79**:159-163 (2002).
- [38] **O. Berdeaux, W. W. Christie, F. D. Gunstone, and J. L. Sebedio**, Large-scale synthesis of methyl *cis*-9,*trans*-11-octadecadienoate from methyl ricinoleate. *J. Am. Oil Chem. Soc.*, **74**:1011-1015 (1997).
- [39] **M. S. Jie, M. K. Pasha, and M. S. Alam**, Synthesis and nuclear magnetic resonance properties of all geometrical isomers of conjugated linoleic acids. *Lipids*, **32**:1041-1044 (1997).

- [40] **L. Liu, E.G. Hammond, and B.J. Nikolau**, In vivo studies of the biosynthesis of  $\alpha$ -eleostearic acid in the seed of *Momordica charantia*. *L. Plant Physiol.*, **113**:1343-1349 (1997).
- [41] **M.J. Chisholm, and C.Y. Hopkins**, Conjugated fatty acids in some *Cucurbitaceae* seed oils. *Can. J. Biochem.*, **45**:1081-1086 (1967).
- [42] **M.J. Chisholm, and C.Y. Hopkins**, Calendic acid in seed oils of the genus *Calendula*. *Can. J. Biochem.*, **45**:251-255 (1967).
- [43] **C.Y. Hopkins, and M.J. Chisholm**, A survey of the conjugated fatty acids of seed oils. *J. Am. Oil Chem. Soc.*, **45**:176-182 (1968).
- [44] **Y.L. Ha, J. Storkson, and M.W. Pariza**, Inhibition of benzo(a)pyrene-induced mouse forestomach neoplasia by conjugated dienoic derivatives of linoleic acid. *Cancer Res.*, **50**:97-1101 (1990).
- [45] **M. Igarashi, and T. Miyazawa**, Newly recognized cytotoxic effect of conjugated trienoic fatty acids on cultured human tumor cells. *Cancer Lett.*, **148**:173-179 (2000).
- [46] **R. Suzuki, R. Noguchi, T. Ota, M. Abe, K. Miyashita, and T. Kawada**, Cytotoxic effect of conjugated trienoic fatty acids on mouse tumor and human monocytic leukemia cells. *Lipids*, **36**:477-482 (2001).
- [47] **E. Ostrowska, F.R. Dunshea, M. Muralitharan, and R.F. Cross**, Comparison of silver-ion high-performance liquid chromatographic quantification of free and methylated conjugated linoleic acids. *Lipids*, **35**:1147-1153 (2000).
- [48] **S. Kishino, J. Ogawa, A. Ando, T. Iwashita, T. Fujita, H. Kawashima, and S. Shimizu**, Structural analysis of conjugated linoleic acid production by *Lactobacillus plantarum*, and factors affecting isomer production. *Biosci. Biotechnol. Biochem.*, **67**:179-182 (2003).
- [49] **S. Kishino, J. Ogawa, A. Ando, Y. Omura, and S. Shimizu**, Ricinoleic acid and castor oil as substrates for conjugated linoleic acid production by washed cells of *Lactobacillus plantarum*. *Biosci. Biotechnol. Biochem.*, **66**:2283-2286 (2002).
- [50] **T. Nagao, Y. Shimada, Y. Yamaguchi-Sato, T. Yamamoto, M. Kasai, K. Tsutsumi, A. Sugihara, and Y. Tominaga**, Fractionation and enrichment of CLA isomers by selective esterification with *Candida rugosa* lipase. *J. Am. Oil Chem. Soc.*, **79**:303-308 (2002).
- [51] **T.R. Dhiman, L.D. Satter, M.W. Pariza, M.P. Galli, K. Albright, and M.X. Tolosa**, Conjugated linoleic acid (CLA) content of milk from cows offered diets rich in linoleic and linolenic acid. *J. Dairy Sci.*, **83**: 1016-1027 (2000).

## ACKNOWLEDGMENTS

The author wishes to express his deepest gratitude and sincere appreciation to Professor, Dr. Sakayu Shimizu, Kyoto University, for his valuable guidance, warm encouragement, and kind support during the course of this work.

The author is greatly indebted to Assistant Professor, Dr. Jun Ogawa, Kyoto University, for his valuable discussion, support, critical reading of the manuscripts, and constructive suggestions throughout the course of this study.

The author is greatly indebted to Emeritus Professor, Dr. Hideaki Yamada, Kyoto University, for his kind encouragements in carrying out this work.

The author is also very grateful to Associate Professor, Dr. Michihiko Kataoka, and Assistant Professor, Dr. Eiji Sakuradani, Kyoto University, for their warm supports, encouragements, and thoughtful advice during the course of this study.

The author is deeply indebted to Professor, Dr. Michihiko Kobayashi, Tsukuba University, for his silent encouragement in carrying out this work.

The author is deeply indebted Dr. Hiroshi Kawashima, Dr. Tsuyoshi Fujita, Dr. Takashi Iwashita, Suntory Ltd., for their supports in NMR and MS analyses.

The author is deeply indebted Mr. Kenji Matsumura, Ms. Yoriko Omura for their collaborations.

Special thanks are due to Mr. Akinori Ando, Mr. Satoshi Sugimoto, Mr. Kousuke Mihara, Kyoto University, for their helpful suggestion.

The author greatly appreciated all the member of the laboratory of Fermentation Physiology and Applied Microbiology, Division of Applied Life Sciences, Graduate School of Agriculture, Kyoto University, for their help, cooperation and valuable information.

Finally, but not the least, the author would like to acknowledge his family who have been understanding and supporting during the course of this study. Special thanks to Ms. Ayumi Kishino for her commitment and unwavering moral support and sharing her affection.

Kyoto, 2005

*Shigenobu Kishino*

Shigenobu Kishino



## PUBLICATIONS

## CHAPTER I

- 1) **J. Ogawa, K. Matsumura, S. Kishino, Y. Omura, and S. Shimizu**, Conjugated linoleic acid accumulation via 10-hydroxy-12-octadecaenoic acid during microaerobic transformation of linoleic acid by *Lactobacillus acidophilus*. *Appl. Environ. Microbiol.*, **67**:1246-1252 (2001).
- 2) **S. Kishino, J. Ogawa, Y. Omura, K. Matsumura, and S. Shimizu**, Conjugated linoleic acid production from linoleic acid by lactic acid bacteria. *J. Am. Oil Chem. Soc.*, **79**:159-163 (2002).
- 3) **S. Kishino, J. Ogawa, A. Ando, T. Iwashita, T. Fujita, H. Kawashima, and S. Shimizu**, Structural analysis of conjugated linoleic acid production by *Lactobacillus plantarum*, and factors affectig isomer production. *Biosci. Biotechnol. Biochem.*, **67**:179-182 (2003).

## CHAPTER II

- 4) **S. Kishino, J. Ogawa, A. Ando, Y. Omura, and S. Shimizu**, Ricinoleic acid and castor oil as substrates for conjugated linoleic acid production by washed cells of *Lactobacillus plantarum*. *Biosci. Biotechnol. Biochem.*, **66**:2283-2286 (2002).

## CHAPTER III

- 5) **S. Kishino, J. Ogawa, A. Ando, and S. Shimizu**, Conjugated  $\alpha$ -linolenic acid production from  $\alpha$ -linolenic acid by *Lactobacillus plantarum* AKU 1009a. *Eur. J. Lipid Sci. Technol.*, **105**:572-577 (2003).
- 6) **S. Kishino, J. Ogawa, and S. Shimizu**, Conjugated  $\gamma$ -linolenic acid production from  $\gamma$ -linolenic acid by *Lactobacillus plantarum* AKU 1009a. (in preparation).
- 7) **S. Kishino, J. Ogawa, and S. Shimizu**, Conjugated fatty acid production from polyunsaturated acid by *Lactobacillus plantarum* AKU 1009a. (in preparation).