A derivative of *Lactococcus lactis* strain H61 with less interleukin-12 induction has a different cell wall

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ABSTRACT

Lactococcus lactis H61 can increase the cellular immune responses of aged (14-mo-old) senescence-accelerated mice. The aim of this study was to investigate the factors contributing to IL-12 induction by strain H61 by analyzing strains derived from it. Strain H61 derivative no. 13 was obtained by growing the parent strain at 37°C. This derivative induced significantly lower production of IL-12 from J774.1 macrophage cells than did the parent strain H61. The 2 strains differed in the resistance of their whole cells or cell walls to lysozyme, a cell wall-degrading enzyme. Sodium hydroxide treatment to de-*O*-acetylate muramic acid in the cell walls of the 2 strains reduced the lysozyme resistance, compared with untreated cell walls: at 3 h after adding lysozyme, the lysozyme resistance of untreated and NaOH treated cell wall from strain H61 was 55.4% and 11.7%, respectively. The values of untreated and NaOH-treated cell walls from strain no.13 were 73.7 and 42.8%, respectively. The reduction was higher in strain H61, indicating that the cell walls of strain H61 were highly *O*-acetylated. Trichloroacetic acid treatment to remove wall-associated polymers such as teichoic acids made the lysozyme resistance of the cell walls of both strains similar. The sugar content of cell walls prepared from strain H61 was significantly higher than that of strain no. 13 cell wall. A derivative with less activity for inducing IL-12 by macrophage cells had less *O*-acetylation and had lower sugar content in the cell wall than did strain H61. Modifying the cell wall of strain H61 may be a useful way to regulate its ability to induce IL-12. Strain H61 has been used as a starter bacterium in the dairy industry. This study could lead to enhancing the value of dairy products made by strain H61 by characterizing the key factor(s) responsible for its stimulation of immunity.

Key words: *Lactococcus lactis*, interleukin-12, cell wall, lysozyme resistance

INTRODUCTION

As the elderly population increases, the prevalence of aging-related diseases will also increase, and functional foods that provide health benefits to control aging and prolong health span will become more desirable. Recently, we showed that administration of *Lactococcus lactis* H61 to aged (14-mo-old) senescence-accelerated mice can suppress some of the manifestations associated with aging (Kimoto-Nira et al., 2007). The anti-aging properties of compounds are associated with their effects on biological systems, such as their antioxidant activity, immunomodulatory activity, and ability to improve the balance of intestinal microflora. We previously showed that orally administered strain H61 had a slight effect on counts of viable intestinal microflora including lower counts for only *Staphylococcus* spp. and no effect on oxidation levels in serum; however, spleen cells from mice fed strain H61 produced more interferon- γ and IL-12 than those from control mice (i.e., without feeding strain H61) by using aged senescence-accelerated mice (Kimoto-Nira et al., 2007). Thus, it is possible that the anti-aging activity of strain H61 is associated with its activity to stimulate immunity. Cytokines produced by immunocompetent cells, such as antigen-presenting cells and helper T cells, play important roles in the immune system. For example, IL-12 can induce the development of cellular immune responses (Trinchieri, 1994). Certain lactic acid bacteria have immunomodulatory activity (Daniel et al., 2006; Bermúdez-Humarán et al., 2007) and can stimulate cytokine production by immunocompetent cells (Kimoto et al., 2004). In the present study, we investigated the ability of strain H61 and its variants to induce a cellular immune response by using cell line. Strain H61 used in this study is a starter bacterium in the dairy industry. If the factors concerning its stimulation of immunity are clarified, which lead to clarification of the mechanisms involved, the value of the strain could be enhanced.

Lactic acid bacteria cellular components that stimulate vertebrate host immunity have been studied previously by several investigators. Tejada-Simon and Pestka (1999) reported that the cell wall and cytoplasm fraction of some lactic acid bacteria could induce the

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production of cytokines such as IL-6 and tumor necrosis factor- α in macrophage RAW264.7 cells to the same extent as whole bacterial cells. Kitazawa et al. (1992) reported that slime products produced by *L. lactis* ssp. *cremoris* are potent B-cell-dependent mitogens. Furthermore, the CpG (Krieg et al., 1995) motif in bacterial DNA (e.g., *Bifidobacterium* species; Ménard et al., 2010) enhances immune system responses. The AT motif in *Lactobacillus gasseri* (Kitazawa et al., 2001) has also been reported.

Comparison of a bacterial strain that has high activity in stimulating immunity with a strain that has low or no activity can help in identification of the factors that affect this activity. Comparison within the same strain modified by certain treatments (e.g., UV irradiation or heat treatment) could be also effective. In the present study, we subjected strain H61 to growth at high temperature to obtain strains with varied levels of ability to stimulate more measureable immune responses than the parent strain H61, as indicated by their ability to induce IL-12 production from immunocompetent cells. We then compared the cell lysis by lysozyme and autolysis, and the cellular components, including the chemical composition and lysis of the cell wall, between strain H61 and one of its derivatives.

MATERIALS AND METHODS

Strain and Growth Conditions

Lactococcus lactis ssp. *cremoris* H61 was maintained at the International Patent Organism Depository (FERM P-18415; No. 400007; Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Japan). The strain was maintained by subculture of 1% inocula in de Man, Rogosa, and Sharpe (MRS) broth (Becton, Dickinson and Co., Sparks, MD) with incubation for 18 h at 30°C. The culture was stored at 4°C between transfers and was subcultured once before experimental use.

Under certain conditions of stress, such as low or high temperature and the presence of some agents like acridine orange, the bacterial properties of lactococci are altered (Fujita and Okamoto, 1999; Kim et al., 2001). In this experiment, we used a higher temperature for altering the bacterial properties of strain H61. Most *Lactococcus lactis* ssp. *lactis* can grow well at 40°C, but most ssp. *cremoris* strains do not (Holt et al., 1994). However, according to Nomura et al. (2006), some *cremoris* strains can grow well at 40°C. In our previous study, strain H61 grew slightly in broth at 37°C (Kimoto et al., 2002), and we first investigated the growth of strain H61 at a limited number of higher temperatures (38, 39, and 40°C). However, strain H61 did not grow at temperatures over at 37°C. When

strain H61 was subcultured 3 times at 37°C, its growth was low. We spread the culture on an MRS agar plate, randomly picked 22 colonies after 1 d of incubation at 30°C, and examined the colonies for their ability to induce IL-12 by macrophages. A derivative of strain H61 was defined as an isolate that had altered ability to induce IL-12 production compared with strain H61. Fermentation and biological tests of strain H61 and its derivative were conducted with an API 20 STREP kit (API-bioMérieux, Marcy L'Étoile, France).

To prepare bacterial cells for the assay of cytokine production by macrophage cells, overnight cultures were collected and washed twice with 0.85% NaCl. To prepare heat-killed bacterial cells, resuspended cells were incubated at 95°C for 15 min. Then, they were resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma Chemical Co., St. Louis, MO) to 1 mg wet weight/mL.

J774.1 Macrophage Culture and Assay for Cytokine Production

A murine macrophage-like cell line (J774.1) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Cells were routinely grown at 37°C in a 5% CO₂/95% air atmosphere in RPMI 1640 medium supplemented with 10% fetal calf serum (Sigma Chemical Co.) inactivated at 56°C for 30 min, 100 U of penicillin/mL, 100 μ g of streptomycin/mL, and 5×10^{-5} M 2-mercaptoethanol. The J774.1 cells were seeded at 5×10^5 cells/mL into 24-well culture plates (Falcon 2046; Becton Dickinson Labware, Oxnard, CA) in 1 mL per well and were incubated for 48 h, after which the medium was changed and the J774.1 cells were incubated with or without lactococcal suspensions (1 or 10 μ g). After incubation, the collected supernatants were promptly used for analysis or stored at -80°C until use for another day. The IL-12p40 content of the culture supernatant was determined by ELISA with an Opt EIA IL-12p40 kit (Pharmingen, San Diego, CA).

Cell Lysis by Lysozyme

The assay was easily tested by the agar plate method (Veiga et al., 2009). Culture dilutions of each strain were spotted on MRS medium plates containing 50 μ g of lysozyme/mL. Plates were incubated at 30°C overnight.

Lysozyme resistance was also performed by using a modified version of the method of Šimelyte et al. (2000). One milligram (wet weight) of whole cells or cell wall was suspended in 60 mM phosphate buffer (pH 6.2) supplemented with 1% NaCl (Heine et al., 1995) and then incubated with 100 μ g/mL of lysozyme

(chicken; Sigma Chemical Co.) at 37°C. Cell lysis by lysozyme was determined as the amount of decrease in absorbance at a wavelength of 620 nm compared with untreated cells (Kimoto-Nira et al., 2008).

Autolysis of the Tested Strains

Autolysis of the tested strains was determined by the methods of Veiga et al. (2009). Cells were grown in M17 plus 0.5% lactose to an optical density at 620 nm (OD_{620}) of 0.3, washed with 0.85% NaCl, resuspended at an OD_{620} of 0.5 in 50 mM sodium phosphate (pH 7.0), and incubated at 30°C. Lysis of strains H61 and no. 13 was monitored by measuring the OD_{620} decreases in the cell suspensions.

We examined the autolysin activities of autoclaved H61 and no. 13 cells by using the methods of Buist et al. (1995) and Meyrand et al. (2007). Four milliliters of overnight cultures was centrifuged at $1,800 \times g$ for 15 min at 4°C, and the supernatant fractions were dialyzed against several changes of demineralized water, lyophilized, and dissolved in 0.8 mL of 25 mM Tris/HCl (pH 7.0) buffer. Autoclaved, lyophilized *Micrococcus lysodeikticus* ATCC 4698 cells (Sigma Chemical Co.) were diluted in 25 mM Tris/HCl buffer to an OD_{620} of 0.5. Concentrated supernatants were added to a final volume of 2 mL, and the OD_{620} of the cell suspension at 30°C was monitored with a spectrophotometer. The extent of autolysis was expressed as the percentage decrease in OD_{620} .

Cell Wall and Peptidoglycan Preparation

The bacterial cell wall fraction was prepared in accordance with the method described by Takeda et al. (1997). Briefly, an overnight culture was collected by centrifugation for 10 min at $8,000 \times g$ at 4°C. Cell pellets were washed 3 times with distilled water and then resuspended in the same solution. The cell suspensions were exposed to ultrasonication for 30 min on ice to disrupt cells and were then heated for 10 min at 100°C. Intact cells were removed by centrifugation at $1,000 \times g$ for 5 min, and the supernatants were centrifuged at $13,000 \times g$ for 20 min to collect the crude cell wall fraction. The cell wall fractions were treated with DNase and RNase (250 µg/mL each; Sigma Chemical Co.) in 50 mM Tris-HCl buffer (pH 8.2) containing 5 mM $MgSO_4$ at 37°C for 3 h. The pellet recovered by centrifugation was treated with 4% SDS for 30 min and was then digested with trypsin (0.5 mg/mL; Nacalai Tesque, Kyoto, Japan) at 37°C for 16 h. The samples were then centrifuged ($13,000 \times g$ for 20 min at 4°C) and the pellet resuspended with 1% SDS for 30 min, followed by 2 washes with 0.85% NaCl to remove the

SDS. The purified cell wall fraction was then resuspended in 0.85% NaCl (cell wall).

Teichoic acid and polysaccharides were removed from the cell wall by extraction with 3% TCA solution at 4 and 37°C, respectively, for 24 h. The residue recovered by centrifugation was re-*N*-acetylated with 30% (vol/vol) acetic anhydride in saturated $NaHCO_3$ solution at 4°C overnight. After centrifugation, the residue was washed with distilled water and then lyophilized (peptidoglycan; **PG**).

Cell Wall Modification

Cell wall samples were treated either with NaOH to deacetylate muramic acid or with TCA to remove teichoic acids. Deacetylation was carried out by incubating approximately 1 mL of wet pellets of walls with 10 mL of 0.01 M NaOH at 37°C for 60 min (Piuri et al., 2005). Trichloroacetic acid-extracted walls were prepared from the crude walls (50 mg/mL) by treatment with 10% (wt/vol) TCA for 48 h at 48°C and then washed with distilled water (de Ambrosini et al., 1996). The suspensions were then centrifuged at $13,000 \times g$ for 20 min at 4°C and washed with distilled water. Portions of these walls were used directly for incubation with lysozyme.

Cell Wall Analysis

The sugar contents of cell walls were determined by the phenol-sulfate method (Dubois et al., 1956). Milligrams of glucose per milligram of dry cell wall weight were calculated and the results were expressed as percentage glucose.

Identification of sugars in the cell wall was performed as follows: purified cell walls were hydrolyzed with 4 M trifluoroacetic acid at 100°C for 3 h, and then samples were *N*-acetylated and labeled with *p*-aminobenzoic acid ethyl ester (**ABEE**) by using an ABEE labeling kit (Seikagaku Co., Tokyo, Japan). The ABEE-converted monosaccharides were analyzed by reverse-phase HPLC using a Honepak C18 column (75 mm $\times$ 4.5 mm, i.d.; J-OIL MILLS Inc., Tokyo, Japan) and 0.2 M potassium borate buffer (pH 8.9) containing 7% acetonitrile as a solvent (Yasuno et al., 1999).

For AA analysis, a PG sample (1–2 mg of dry weight) was hydrolyzed in 1 mL of 6 N HCl at 100°C for 18 h. The hydrolysate was filtered and diluted with 0.02 N HCl. The AA composition was determined by using an automatic AA analyzer (Hitachi Ltd., Tokyo, Japan). Amino acids were separated on a lithium cation exchanger according to their isoelectric points and were detected by spectrophotometry after staining with ninhydrin.

Figure 1. Interleukin-12 production induced in cells of the murine macrophage-like line J774.1 by *Lactococcus lactis* H61 (the wild-type strain) and its derivative no. 13. The strains were incubated for 18 h at 30°C in de Man, Rogosa, and Sharpe broth and cultured with J774.1 cells for 24 h. Results are mean values, with standard deviation of 3 samples represented by vertical bars, and are representative of several separate experiments. Columns with different letters (a, b; x, y) differ significantly ($P < 0.05$, Student's *t*-test).

Statistical Analysis

Data are expressed as the mean $\pm$ standard deviation from 2 or 3 replicates and are representative of several separate experiments. Statistical analysis was performed on the differences between the parent strain and its derivative by using a *t*-test, and $P < 0.05$ was considered to be statistically significant.

RESULTS

Stimulation of IL-12 Production from J774.1 Cells by Strain H61 and Its Derivatives

Twenty-two colonies were randomly selected from cultures grown at 37°C and were subjected to the assay for IL-12 content. Among the tested strains, no

derivatives induced higher production of IL-12 by J774.1 cells than did the parent strain H61 (data not shown). Among the derivatives tested, strain no. 13 was selected for further study because it was likely that it had the lowest IL-12 inductive activity of the bacterial cells (data not shown).

Interleukin-12 production was significantly lower in response to no. 13 cells than H61 cells when 1 or 10 μ g of cells was used ($P < 0.05$; Figure 1). This derivative also halved production of IL-6 by J774.1 macrophage cells relative to the parent strain H61 (data not shown).

In the case of heat-killed cells, the addition of 10 μ g of H61 cells yielded similar amounts of IL-12 ($13,956 \pm 2,420$ pg/mL) and IL-6 ($1,724 \pm 692$ pg/mL) as when the same weight of no. 13 cells was added (IL-12: $18,321 \pm 3,489$ pg/mL, $P = 0.158$; IL-6: $1,123 \pm 329$ pg/mL, $P = 0.271$).

Growth and Biochemical Characteristics of Derivatives

We compared the growth of strain H61 and its derivative no. 13 at different temperatures (Table 1). At 30°C, the growth of strain no. 13 was significantly ($P < 0.01$) higher than that of its parent strain. At 37°C, strain no. 13 grew well, whereas strain H61 showed only slight growth. At 40°C, neither grew. In MRS broth containing 4% NaCl, they grew slightly at 30°C.

Strain H61 and its derivative no. 13 fermented lactose and did not ferment arabinose, mannitol, ribose, sorbitol, trehalose, inulin, raffinose, or starch (Table 2). The 2 strains also had the same phenotypic characteristics with regard to metabolic enzymes (Table 2).

Resistance to Lysozyme

We compared the ability of strain H61 and its derivative no. 13 to be lysed by lysozyme. Strain no. 13 was much more resistant to lysozyme than the wild-type strain H61 in MRS agar plate including lysozyme (Figure 2). In the buffer solution method, whole cells of

Table 1. Growth characteristics of *Lactococcus lactis* H61 and its derivative no. 13 in de Man, Rogosa, and Sharpe broth

Strain	Growth condition ¹			
	At 30°C	At 37°C	At 40°C	With 4% NaCl
H61	0.617 (0.01) ^a	0.017 (0.00) ^a	– ²	± ²
No. 13	0.670 (0.01) ^b	0.465 (0.02) ^b	–	±

^{a,b}Mean values within a column with unlike superscript letters were significantly different ($P < 0.01$).

¹Growth of lactococci was determined by measuring absorbance at a wavelength of 620 nm after 24-h incubation. Values are means from 3 samples. Values in parentheses are standard deviations.

²– = No growth; ± = slight growth.

Table 2. Biochemical characteristics of *Lactococcus lactis* H61 and its derivative no. 13

Characteristics	Strain ¹	
	H61	No. 13
Ammonia from arginine	–	–
Fermentation of L-arabinose	–	–
Ribose	–	–
Mannitol	–	–
Sorbitol	–	–
Lactose	+	+
Trehalose	–	–
Inulin	–	–
Raffinose	–	–
Starch	–	–
Pyrrolidonyl arylamidase	–	–
α-Galactosidase	–	–
β-Glucuronidase	–	–
β-Galactosidase	–	–
Alkaline phosphatase	–	–
Leucine arylamidase	+	+
Hydrolysis of esculin	–	–

¹+ = Positive; – = negative.

derivative no. 13 were also more resistant to lysozyme than were whole cells of strain H61 (Figure 3a).

Cell walls from strain no. 13 were also more resistant to lysozyme than were cell walls from the parent strain (Figure 3b). We treated the cell walls of the 2 strains with NaOH to de-*O*-acetylate muramic acid or with TCA to remove wall-associated polymers such as teichoic acids (Figure 3b). Sodium hydroxide treatment increased the difference in lysis sensitivity between the cell walls of the 2 strains. The degree of cell lysis after 3 h of treatment with lysozyme was expressed as a percentage of the initial value at 0 h. The lysozyme resistances of untreated and NaOH-treated cell wall from strain H61 were $55.4 \pm 5.70\%$ and $11.7 \pm 5.73\%$, re-

spectively. The values of untreated and NaOH-treated cell wall from strain no.13 were $73.7 \pm 5.69\%$ and $42.8 \pm 2.09\%$, respectively.

Trichloroacetic acid treatment made the lysozyme resistance of the cell walls of the 2 strains similar: those values at 3 h were $63.2 \pm 7.50\%$ for strain H61 and $60.3 \pm 5.44\%$ for strain no. 13. These values did not differ significantly.

In the case of heat-killed cells, the degree of cell lysis after 2 h of treatment with lysozyme was expressed as a percentage of the initial value at 0 h. The values were similar for strain H61 ($80.1 \pm 2.64\%$) and strain no. 13 ($83.3 \pm 1.62\%$). These values did not differ significantly.

Autolysis of the Tested Strains

We compared the autolysis of strain H61 and its derivative strain no. 13 after transfer of bacteria to sodium phosphate buffer solution. As shown in Figure 4, strain no. 13 autolyzed at the same rate as strain H61. The autolysin activity was tested on autoclaved cells of micrococci. The activity was measured by the decrease in optical density of the cell suspensions. The degree of cell lysis after 1 d of treatment with lysozyme was expressed as a percentage of the initial value at 0 d. The values were similar for strain H61 ($88.8 \pm 4.08\%$) and strain no. 13 ($92.4 \pm 5.29\%$). These values did not differ significantly.

Carbohydrates and AA Analyses of Cell Wall and Peptidoglycan

The sugar content of cell walls prepared from strain H61 as a proportion of dry weight was significantly higher than that of strain no. 13 cell wall ($P < 0.01$;

Figure 2. Comparison of the lysozyme (LYS) resistance level of *Lactococcus lactis* H61 and its derivative strain no. 13. Culture dilutions of each strain were spotted on de Man, Rogosa, and Sharpe (MRS) medium plates with 50 µg of lysozyme/mL or not. Plates were incubated at 30°C overnight.

Figure 3. Difference in resistance to lysozyme among whole cells and wall preparations of *Lactococcus lactis* H61 and its derivative no. 13. a) Whole cells; b) cell wall preparations. Samples were incubated with lysozyme (100 µg/mL) at 37°C. The decrease in the absorbance at a wave-length of 620 nm was determined at hourly intervals. Values were calculated by dividing the experimental data by the initial value at 0 h and expressed as a percentage of the initial value. Values shown are means (a) from 3 samples and (b) from 2 cell wall preparations tested in triplicate.

Table 3), but the cell wall of strains H61 and its derivative no. 13 had a similar sugar composition. The ratio of these carbohydrates (glucose:*N*-acetylglucosamine:galactose:rhamnose) in cell walls of strain H61

and strain no. 13 was 1.0:1.5:1.1:4.5 and 1.0:1.6:1.1:4.5, respectively.

We performed qualitative and quantitative analyses of the AA composition of purified PG from each strain. Both PG mainly included alanine, glutamic acid/glutamine, lysine, and aspartic acid/asparagine. No significant difference was observed in the AA ratio between these strains (data not shown).

DISCUSSION

To clarify the factors associated with the modification of IL-12 induction by *L. lactis* ssp. *cremoris* H61, in the present study, we obtained a derivative of strain H61 (no. 13) that had less ability than the parent strain to induce IL-12 production by macrophages. Although the 2 strains had many properties in common, it is notable that they differed in resistance to lysozyme, which de-

Figure 4. Autolysis of *Lactococcus lactis* H61 and its derivative strain no. 13 in buffer solution. Cells were grown in M17 plus 0.5% lactose medium to an optical density at 620 (OD₆₂₀) of 0.3, washed, resuspended at an OD₆₂₀ of 0.5 in 50 mM sodium phosphate buffer (pH 7.0), and incubated at 30°C. Lysis of H61 (■) and no. 13 (◇) was monitored by measuring the decreases in OD₆₂₀ in the cell suspensions. The results shown are means from 3 samples and representative of 2 separate experiments.

Table 3. Sugar content of cell walls prepared from *Lactococcus lactis* H61 and its derivative no. 13

Strain	% of cell wall as glucose
H61	10.3 (0.657) ^a
No. 13	7.25 (0.317) ^b

^{a,b}Mean values with unlike superscript letters were significantly different (*P* < 0.01).

¹Values are means of 2 cell wall samples tested in duplicate or triplicate. Values in parentheses are standard deviations.

grades the sugar backbone of PG in bacterial cell walls by hydrolyzing the bond between *N*-acetylglucosamine and *N*-acetylmuramic acid. It has been reported that the cell walls of some lactic acid bacteria can induce the production of cytokines such as IL-6 and IL-12 by macrophages (Tejada-Simon and Pestka, 1999; Kimoto-Nira et al., 2008). We, therefore, tried to determine the properties of strains H61 and no. 13 by analyzing differences in lysozyme resistance.

Lysis of viable cells is carried out by lysozyme and autolysin. We, therefore, hypothesized that differences in autolysin activity may be responsible for the difference in lysozyme resistance between strain H61 and its derivative. However, the autolytic activity of viable cells of these strains was not different, indicating that the structural or chemical properties of the cell wall of these strains must be different. Resistance to lysozyme has been related to the presence of *O*-acetyl groups, the attachment of teichoic acid to PG, or a high degree of peptide crosslinking in the cell wall. *Bacillus cereus* PG is resistant to lysozyme because of the presence of unacetylated amino groups on the majority of its glucosamine residues, but it can be converted to a lysozyme-sensitive form by acetylation with acetic anhydride (Araki et al., 1972). Conversely, the PG of lactobacilli and other organisms are resistant to lysozyme because their amino sugars are *O*-acetylated, and they can be made lysozyme sensitive by de-*O*-acetylation with a diluted base (Logardt and Neujahr, 1975). In the present study, lysozyme resistance in the cell walls of the 2 strains was decreased by deacetylation, which is consistent with a previous report in lactobacilli (Logardt and Neujahr, 1975). In addition, the effect of deacetylation by NaOH on lysozyme resistance of the cell wall of strain H61 was more drastic than the effect on strain no. 13, indicating that the cell wall of strain H61 is more highly *O*-acetylated than that of strain no. 13.

These findings raise some questions. The cell wall of strain H61 may be highly *O*-acetylated, which would be expected to lead to higher lysozyme resistance, but the lysozyme resistance of strain H61 was lower than that of strain no. 13. Furthermore, Shida et al. (2006) reported that in heat-killed strains of lactobacilli, differences in resistance to M1 enzyme, an *N*-acetylmuramidase, might explain differences in the ability of bacteria to induce IL-12 production by macrophages: strains that have rigid cell walls are highly resistant to M1 enzyme and stimulate more IL-12 production by macrophage cells than do strains with soft cell walls. Also, Shida et al. (2006) suggest that the intact remaining of cell wall structure of lactobacilli is an important element in the ability to induce IL-12. Our current results are opposite to those in their report: strain H61 cells, which are more

easily lysed, induced more IL-12 by macrophages than did strain no. 13 cells, which are rigid. However, Shida et al. (2006) used heat-killed cells, whereas our study dealt with viable cells, suggesting that dead cells have different properties than viable ones. We, therefore, also tested heat-killed cells of strains H61 and no. 13, but detected no difference in IL-12 production between viable and heat-killed cells. This indicates that induction of IL-12 and IL-6 by these strains was altered by the viability of the bacterial cells. In addition, lysozyme resistance was similar in the heat-killed cells of these strains. Thus, in this case, no relationship between lysozyme resistance and ability to induce IL-12 was apparent. Recently, it was shown that RNA released from certain lactic acid bacterial cells disrupted by autoclaving or heat can induce IL-12 by macrophages (Inoue et al., 2011; Masuda et al., 2011). Thus, in the case of heat-killed cells, a mechanism other than the degree of lysozyme resistance could induce IL-12 by macrophages. The relationship between lysozyme resistance (i.e., whether the cells have a rigid or soft cell wall) and activity for inducing IL-12 in viable cells should be carefully investigated in the near future.

The cell walls of lactic acid bacteria include PG, polysaccharide, teichoic acid, lipoteichoic acid (**LTA**), and other cell wall-associated proteins. In this study, removal of teichoic acid and LTA by TCA treatment made the cell walls of strains H61 and no. 13 similarly resistant to lysozyme. Thus, the teichoic acid and LTA contents must differ between the 2 types of cell walls. Bacterial LTA is thought to be responsible for enhancing the immune system via toll-like receptor 2, which is present on immunocompetent cells (Takeda et al., 2003). Although we did not determine the teichoic acid and LTA content in this study, the sugar content of strain H61 was significantly higher than that of strain no. 13, which has less activity for inducing IL-12. Strain H61 does not produce polysaccharide in growth medium; thus, the sugar fraction on the cell surface, including teichoic acid and LTA, may have a positive effect on the modification of IL-12 inductive activity of strain H61. However, the teichoic acid and LTA contents may be low in strains H61 and no. 13, because TCA treatment did not drastically affect the lysozyme resistance of the 2 cell walls, compared with the untreated cell walls. In addition, the similar level of IL-12 production induced by heat-killed cells of strains H61 and no.13 suggests that compounds that are sensitive to heat, such as proteins, could also be associated with the difference observed between these strains in the ability to induce IL-12 production. On the other hand, it has been reported that no relationship exists between the chemical composition of the cell wall and immune modulation in lactobacilli (De Ambrosini et al., 1996).

Careful purification of the cellular components, including the cell wall and cytoplasmic fractions, from both strain H61 and strain no. 13 is required to identify the specific molecules that induce IL-12 by macrophages.

CONCLUSIONS

Strain no. 13, a derivative of strain H61 with less activity for inducing IL-12 by macrophages, was different in the degree of *O*-acetylation and had lower sugar content in the cell wall compared with the parent strain H61. This information may lead to clarifying the factors associated with the induction of IL-12 in lactic acid bacteria, including *Lactococcus lactis* H61. The present study also showed that modification of the cell wall of strain H61 may be a useful way to regulate its ability to induce IL-12 production by macrophages. Further studies on the differences between strains H61 and no. 13 at the genetic level (for example, using a DNA microarray) would let us clarify the genes associated with the ability to induce IL-12. In near future, in vivo work including their stimulation of immunity of strains H61 and no. 13 will be needed.

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