

Effect of Formic Acid on Exopolysaccharide Production in Skim Milk Fermentation by *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL1073R-1

Junko NISHIMURA^{1*}, Yasushi KAWAI¹, Ryota ARITOMO¹, Yoshiyuki ITO²,
Seiya MAKINO², Shuji IKEGAMI², Emiko ISOGAI³ and Tadao SAITO¹

¹Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, 1–1 Tsutsumidori-Amamiyamachi, Aobaku, Sendai 981-8555, Japan

²Food Science Institute, Meiji Co., Ltd., 540 Naruda, Odawara, Kanagawa 250-0862, Japan

³Laboratory of Animal Microbiology, Graduate School of Agricultural Science, Tohoku University, 1–1 Tsutsumidori-Amamiyamachi, Aobaku, Sendai 981-8555, Japan

Received March 30, 2012; Accepted July 3, 2012

In yogurt, the formation of formate by *Streptococcus thermophilus* stimulates the activity of *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*). However, there have been no reports how formic acid acts on the exopolysaccharide (EPS) production of *L. bulgaricus*. Here, the effect of formate on the EPS production in skim milk by *L. bulgaricus* OLL1073R-1 was investigated. After incubation for 24 hr with 100 mg/l formate, cell proliferation and lactic acid production were accelerated. The viable and total cell numbers were increased about ten- and four-fold, respectively. The amount of EPS in culture with formate (~116 µg/ml) was also four-fold greater than that of the control (~27 µg/ml). Although elongation of cells was observed at 6 hr of cultivation in both cultures, cells cultivated with formate returned to a normal shape after incubation for 24 hr. The sensitivity to cell wall hydrolase and composition of surface layer proteins, as well as the cell membrane fatty acid composition of *L. bulgaricus* OLL1073R-1, were not influenced by formate. However, differences were observed in intracellular fatty acid compositions and sensitivity to antibiotics. Cell length and surface damage returned to normal in cultures with formate. These observations suggest that formic acid is necessary for normal cell growth of *L. bulgaricus* OLL1073R-1 and higher EPS production.

Key words: cell division, exopolysaccharide, formic acid, *Lactobacillus delbrueckii* subsp. *bulgaricus*

INTRODUCTION

The physiological functions of dairy lactic acid bacteria are known, and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) is one of the most important yogurt starters. Yogurt is generally made from milk using a culture of *L. bulgaricus* and *Streptococcus thermophilus* (*S. thermophilus*). In the fermentation process, *L. bulgaricus* and *S. thermophilus* exhibit a symbiotic relationship: *S. thermophilus* provides formic acid, pyruvic acid, folic acid [1] and carbon dioxide [2, 3] to *L. bulgaricus*, and then *L. bulgaricus* leads to *S. thermophilus* growth by peptides or amino acids production. Suzuki et al. reported that formic acid produced by *S. thermophilus* is taken into the cells of *L.*

bulgaricus and utilized as a precursor in purine synthesis [4, 5]. *S. thermophilus* has pyruvate formate lyase, an enzyme that *L. bulgaricus* lacks [6, 7]; thus, provision of formic acid from *S. thermophilus* is necessary for the normal proliferation of *L. bulgaricus*.

Recently, exopolysaccharides (EPS) have been introduced as food emulsifiers/thickeners and functional food ingredients. The bacterial polysaccharides also confer health benefits in humans and livestock. Previously, we characterized EPS produced by *L. bulgaricus* OLL1073R-1 [8]. For yogurt fermented with this strain, acidic polysaccharides (APS: phosphopolysaccharide) exerted beneficial immunological effects such as the enhancement of lymphocyte mitogenicity, macrophage function [9–11], and NK cell activity [12], and the reduction of cold prevalence [13] and reduced influenza virus infections [14]. It is considered that these beneficial activities are attributable to the EPS produced by this strain. At present, yogurt fermented with this strain is marketed in Japan, but its EPS production is lower than that of other EPS-producing strains [15, 16].

In this study, we investigated the effects of several

*Corresponding Author. Mailing address: Junko Nishimura, Laboratory of Animal Products Chemistry, Graduate School of Agricultural Science, Tohoku University, 1-1 Tsutsumidori-Amamiyamachi, Aobaku, Sendai 981-8555, Japan. Tel: +81-22-717-8712, Fax: +81-22-717-8715. E-mail: juemura@bios.tohoku.ac.jp

environmental conditions, including differing formic acid concentration in skim milk culture, on EPS production in *L. bulgaricus* OLL1073R-1.

MATERIALS AND METHODS

Bacterial strain and media

L. bulgaricus OLL1073R-1 was obtained from the cell culture collection of Meiji Co., Ltd. (Tokyo, Japan). Skim milk (10%) with or without formate was prepared at a concentration of 100 mg/l [17, 18] by addition sodium formate (10 mg/100 mL, 1.47 mM) based on formic acid production from *S. thermophilus* of 40 to 600 mg/l [19, 20]. The media were sterilized by low-temperature long-time (LTLT) pasteurization (63°C for 30 min) to avoid the formation of formic acid [21].

Culture conditions

The strain was propagated twice in 10% (w/v) skim milk (115°C, 15 min). The *L. bulgaricus* culture was inoculated into 10% skim milk with or without formate and incubated at 37°C for 24 hr.

After cultivation of OLL1073R-1 in skim milk without formate for 24 hr, formate (100 mg/l) was added to the medium and then incubated for a further 24 hr. A part of the same culture was inoculated into another batch of skim milk with formate (100 mg/l) and cultivated for 24 hr.

Fermentation conditions with formate

Total cell number, cell viability and pH of cultures were examined every three hours during incubation. Total cell numbers were determined according to the method of Sieuwerts et al. [17]. Briefly, turbidity ($OD_{600\text{ nm}}$) was determined after mixing one volume of culture with nine volumes of 0.2% (w/v) NaOH and 0.2% sodium EDTA acid solution. Optical density was monitored using a UVmini-1240 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Cell viability was determined by pour plating serial dilutions on de Man, Rogosa, and Sharpe (MRS) agar plates (Difco & BBL, Franklin Lakes, NJ, USA; 2% agar), incubating at 37°C for 48 hr, and enumerating the colony-forming units (CFU)/ml. For each culture, 6 CFU counts were performed. The pH was measured with a pH/ION meter (F-24, HORIBA, Tokyo, Japan) in duplicate.

Preparation and quantification of EPS

Cultures were sampled (1 mL) for EPS determination. Proteins were removed by adding 300 μL of 5 M trichloroacetic acid and centrifuging at $750 \times g$ for 10 min at room temperature. The supernatant (500 μL)

was collected, adjusted to neutral pH using 2.5 M NaOH (125 μL), and then filtered through an ultrafiltration kit (MW: 10,000 cut off; USY-1, Advantec Toyo Kaisha, Ltd., Tokyo, Japan). After washing five times with distilled water, retentates (EPS) were recovered in 500 μL distilled water. The EPS content as neutral sugars was determined using the phenol- H_2SO_4 method [22].

Microscopic analysis

After cultures were diluted 1:10 with 0.2% NaOH/EDTA solution, bacterial cells were collected by centrifugation ($750 \times g$, 10 min, 4°C) and washed with sterilized distilled water (SDW). The resuspended cells were dried on a cover glass and fixed with cold acetone. Cells were coated with platinum palladium using a magnetron sputter coater (MSP-1S, Vacuum Device Inc., Mito, Japan) and observed using scanning electron microscopy (SEM; SU8000, Hitachi, Tokyo, Japan).

Sensitivity to cell wall hydrolase

Bacterial sensitivity to cell wall hydrolase was spectrophotometrically determined using a 96-well microplate. After 24 hr of culture, cells were collected by adding nine volumes of 0.2% NaOH/EDTA solution, sterilization in boiling water, washing with SDW, lyophilization and resuspension at 1 mg/ml in SDW. The cell suspensions (95 μL), N-acetylmuramidase SG (from *Streptomyces globisporus* 1829, Seikagaku Biobusiness, Tokyo, Japan; 5 μL , 1 mg/ml SDW or 5 mg/ml SDW) and 10 mM Tris-maleate-NaOH buffer (containing 4 mM MgCl_2 , pH 7.0; 100 μL) were mixed in each well and incubated at 37°C for 3 hr. The optical density at 600 nm was monitored with an absorbance microplate reader system (Viento VS, Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan) and the Gen5 software (Biotek, Winooski, VT, USA).

SDS-PAGE of surface layer protein

Cells after cultivation for 24 hr were collected with 0.2% NaOH/EDTA solution. Surface layer proteins (SLPs) from the cells were suspended in 4 M guanidine hydrochloride and stirred at 37°C for 2 hr. Following precipitation with cold acetone, SLPs were examined by SDS-PAGE according to the method of Laemmli [23] using a 15% (wt/vol) polyacrylamide separation gel and stained with a Rapid CBB kit (Kanto Chemical Co., Inc., Tokyo, Japan).

Fatty acid analysis of the cell membrane using gas chromatography

Cells prepared in 0.2% NaOH/EDTA solution were

washed three times with PBS and then lyophilized. The lipids were extracted in 1:2:0.8 (v:v:v) chloroform:methanol:water following the method of Bligh and Dyer [24]. Neutral and polar lipids were separated using a Sep-Pak Plus NH₂ Cartridge (Waters Corporation, Milford, MA, USA). Samples were converted to fatty acid methyl esters and analyzed using a Hitachi G-6000 gas chromatograph equipped with flame ionization detectors and a TC-70 column (0.25 mm × 60 m, GL Sciences Inc., Tokyo, Japan) in He carrier gas. The injection and detection port temperature were both 260°C, and the column temperature was held at 140°C for 5 min, increased at 4°C/min to 180°C, held at 180°C for 10 min, increased at 20°C/min to 250°C and then held at 250°C for 10 min. Peaks were identified by comparing the retention times to a FAME standard mix containing 37 components.

Resistance activity against antibiotics

Resistance against two antibiotics was evaluated using a plate assay. Vancomycin hydrochloride (1,050 IU/mg) and bacitracin (40 units/mg) were prepared at 5.12 mg/ml, serially diluted with sterilized distilled water and used at the following concentrations: 32, 64 and 128 µg/ml for vancomycin and 32, 64, 128 and 256 µg/ml for bacitracin.

Minimum inhibitory concentration (MIC) was determined using the plate assay method. Cultures in 10% skim milk with or without formate were serially diluted with saline solution, 100 µL of the 10² dilutions were lawn plated on BCP plate count agar (Eiken Chemical Co., Ltd., Tokyo, Japan) into which 6-mm diameter wells were cut and 20 µL of antibiotic solutions were added. The plates were incubated at 37°C for 24 hr. Each sample was assayed in five independent determinations, and the diameter of the clear zone around the well was measured using a caliper.

Statistics

Statistical significance of differences was determined using the Student's t-test.

RESULTS

*Effect of environmental conditions on growth and EPS production of *L. bulgaricus* OLL1073R-1*

The addition of formic acid in 10% skim milk improved the cell number and viability of *L. bulgaricus* OLL1073R-1 and lowered the pH (Fig. 1). The differences were especially large from the exponential to stationary phase, and the differences were significant for incubation

at 6 to 24 hr ($p < 0.001$). The addition of formate increased the amount of EPS more than fourfold compared with the control (116.0 vs. 26.6 µg/ml) incubation for 24 hr (Fig. 2).

Effect of formate on morphological alteration of cells

Morphological changes in *L. bulgaricus* OLL1073R-1 after 6 and 24 hr of incubation are shown in SEM images (Fig. 3). In both cultures, cells were abnormally elongated to over 50 µm in length after 6 hr of incubation (Fig. 3-A, B). After incubation for 24 hr, cells grown with formate changed and became shorter than those grown without formate (Fig. 3-C1, D1). In the culture without formate, cells were easily damaged by the electron irradiation under reduced pressure in the pretreatment for SEM, but very few cells were damaged in the culture with formate (Fig. 3-C2, D2).

After the *L. bulgaricus* OLL1073R-1 culture without formate (shown in Fig. 3-C1) was inoculated into another batch of skim milk with formate (100 mg/l) and incubated for another 24 hr, the cell shape became almost normal (Fig. 4-B1). The shape of the cells in the culture grown in the presence of formate for two cycles was not changed (Fig. 4-A1). Comparison of the cell surface structure showed that the improvement was observed in both cells (Fig. 4-A2, B2). Inoculation into the medium with formate resulted in the normalization of cell length and reduced damage to the cell surface.

Chemistry of the cell surface

Changes were detected in the peptide glycan and cell membrane of the cell surface after formate addition (Fig. 5–7). Although significant differences were noted in cell lysis between with or without formate (Fig. 5(A), 1 hr and 2 hr: $p < 0.001$), the effect of formic acid addition could not be determined. Surface layer proteins from *L. bulgaricus* OLL1073R-1 cells cultivated with or without formate showed similar band patterns of surface layer proteins; however, there were some differences in the low molecular weight proteins (MW: 5–20 kDa). The N-terminal amino acid sequences of five bands corresponding to low molecular weight proteins (<20kDa) were analyzed using FASTA. The proteins were considered to have originated from the medium and cytoplasm. Further, determination of the fatty acid composition of the cell membrane and cytoplasm of *L. bulgaricus* OLL1073R-1 cultivated with or without formate showed the following distribution: 6 polar lipids, 4 of which were identified, and 18 to 20 neutral lipids, 12 of which were identified. The abundance ratio of fatty acids in the polar lipid did not differ between with or

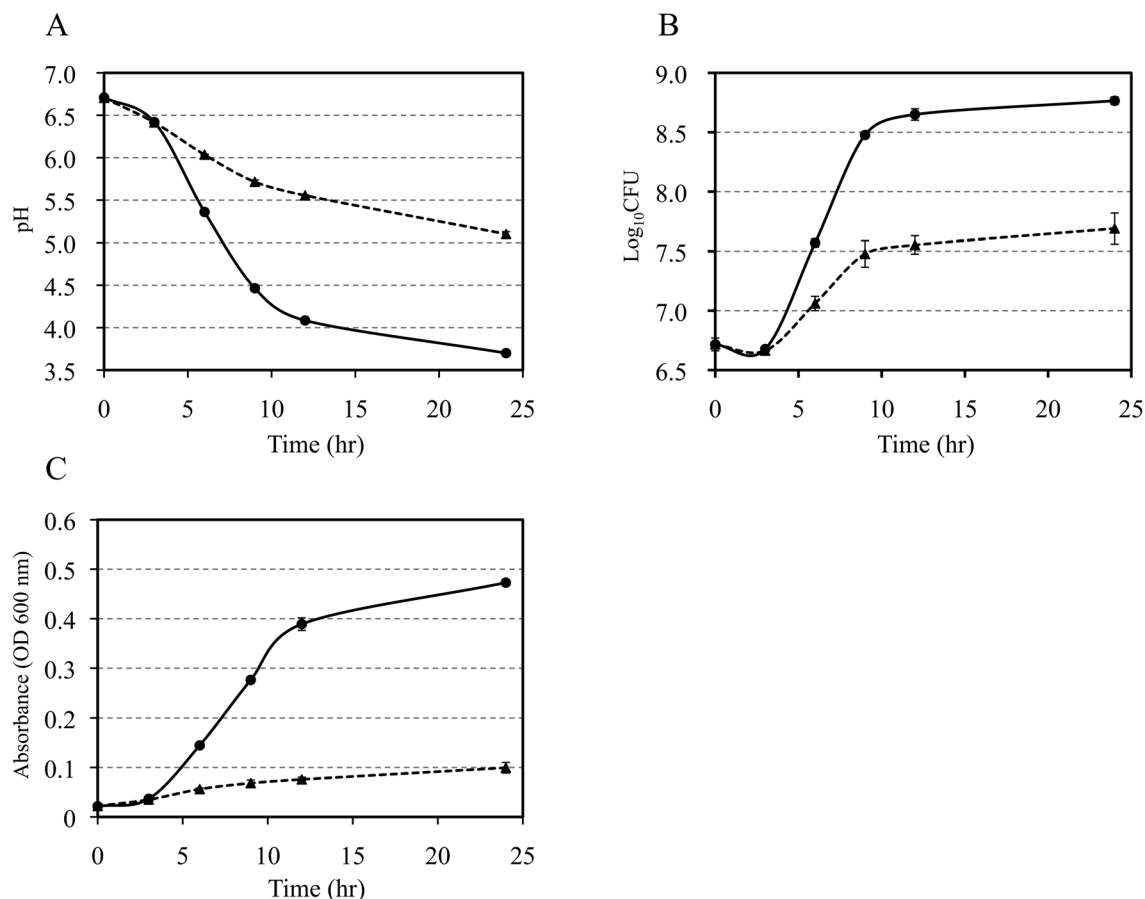


Fig. 1. Culture kinetics of *L. bulgaricus* OLL1073R-1 in skim milk medium incubated at 37°C for (A) culture pH, (B) cell viability and (C) total cell number of the *L. bulgaricus* OLL1073R-1 strain in skim milk medium (● and solid line) with or (▲ and dotted line) without formate addition. The average of two pH readings and the mean and standard deviation of six different samples for viability and optical density are presented.

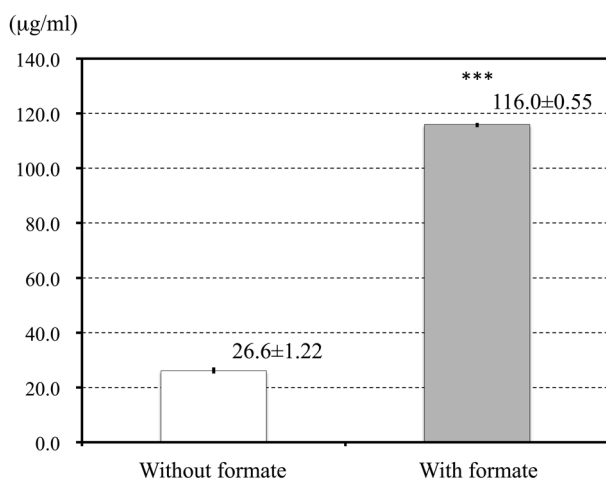


Fig. 2. EPS concentration after incubation for 24 hr in the (□) absence or (■) presence of formate. Each bar represents the mean and the standard deviation of four different samples. (***) $p < 0.001$.

without formate. For the neutral lipids, the concentrations of two peaks (C18 and C18:1n9c) were lower in cells cultured with formate than without formate, and three peaks (C18:3n3, C20:1 and C20:2/C22) were higher.

Antibiotic sensitivity

The diameters of the inhibition zones for different concentrations of antibiotics against *L. bulgaricus* OLL1073R-1 on BCP plate count agar are shown in Table 1. Applying a regression formula to the data for the two antibiotics in cultures with or without formate put the MICs of vancomycin and bacitracin in cultures with formate at 0.14 and 1.21 µg/ml, respectively, and at 0.47 and 1.91 µg/ml, respectively, for cultures without formate. Thus, the addition of formate statistically increased ($p < 0.05$) antibiotic resistance in *L. bulgaricus* OLL1073R-1.

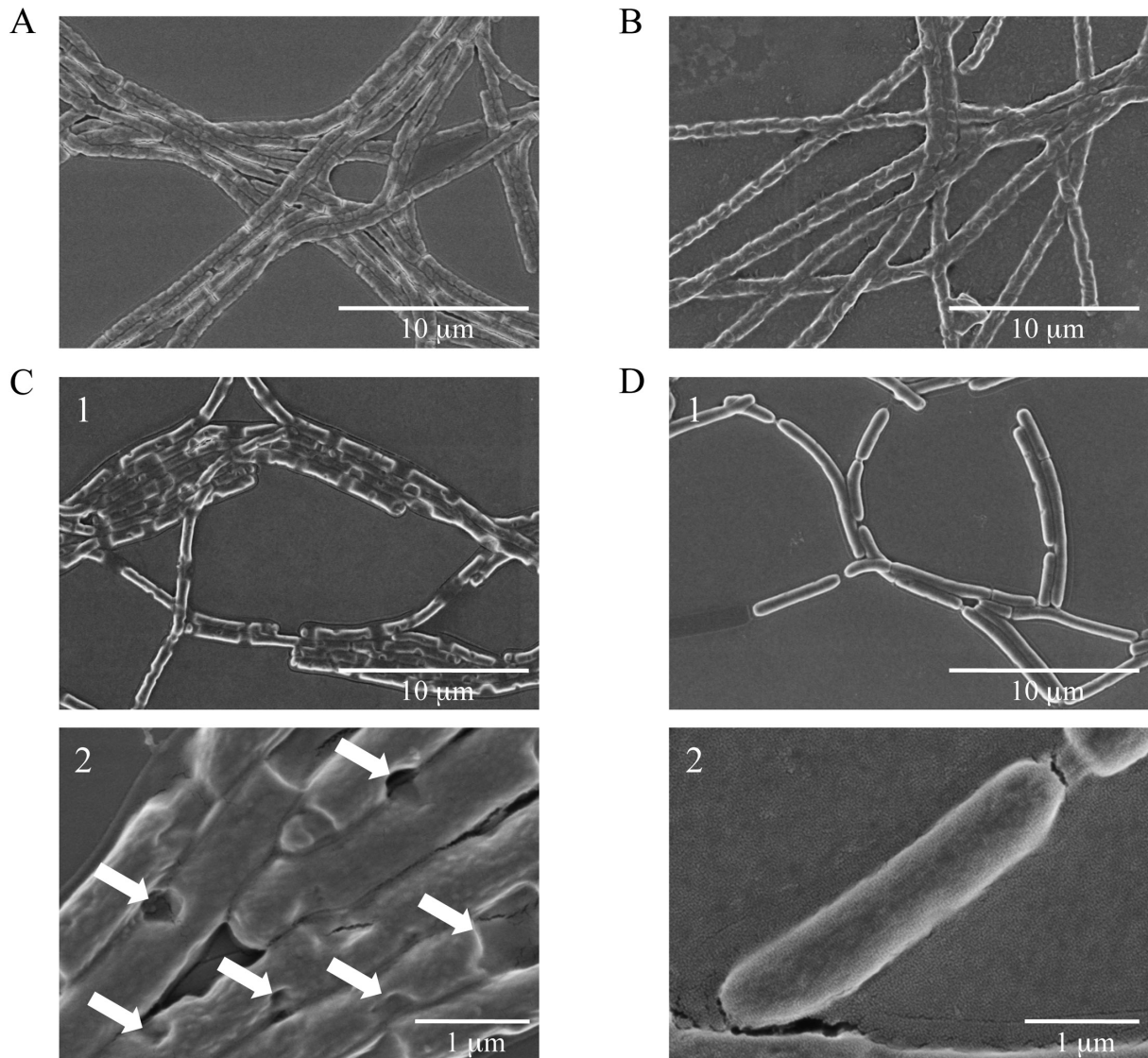


Fig. 3. Scanning electron micrographs of *L. bulgaricus* OLL1073R-1 grown in skim milk medium. *L. bulgaricus* OLL 1073R-1 cells were cultured in the (A) absence or (B) presence of formate for 6 hr and (C) absence or (D) presence of formate for 24 hr and are shown at two magnifications: 1, $\times 5,000$; 2, $\times 30,000$. Arrows indicate the part of remarkable cell damage.

DISCUSSION

In yogurt fermentation, *L. bulgaricus* utilizes formic acid supplied from *S. thermophilus*. Here, we made a detailed investigation of the contribution of formate to EPS production of *L. bulgaricus* OLL1073R-1. Increased cell number, decreased pH and increased EPS production were observed with the addition (100 mg/l) of sodium formate (Fig. 1), and the amount of EPS production increased more than fourfold compared with the control (Fig. 2). These data show that the increase of EPS production is closely related to cell numbers,

indicating that EPS production is dependent on cell proliferation rate. Therefore, increasing cell number is important for the higher EPS production in skim milk in yogurt production. Recently, Mende et al. reported that the addition of a mixture of salts including nucleobases, vitamins, and oleic acid and containing sodium formate to a semi-defined medium increased EPS production in *L. bulgaricus* [25] without affecting cell growth [26]. They suggested that the metabolic pathway of EPS biosynthesis may be activated by a combination of several chemical components, including formic acid.

The cell surface structures (Figs. 3 and 4) and antibiotic

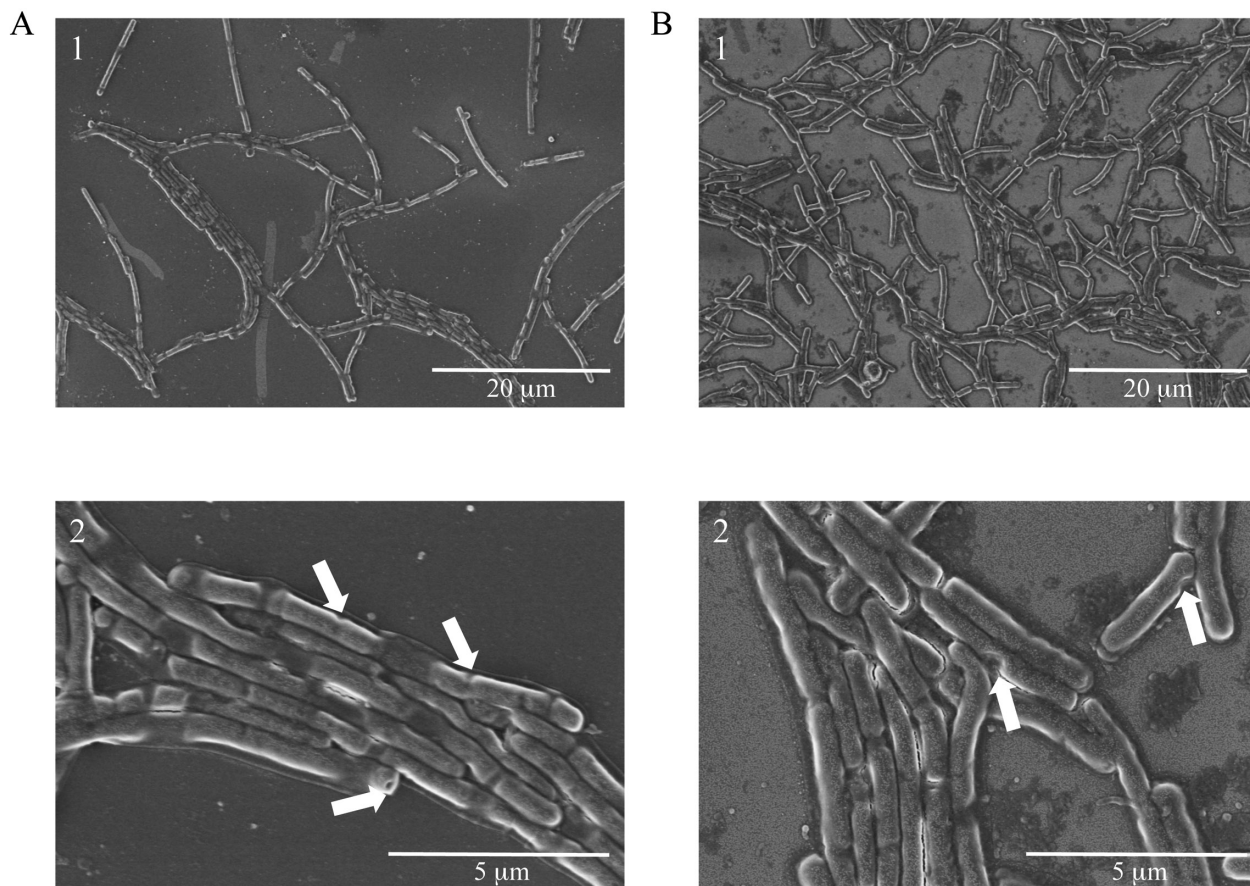


Fig. 4. Scanning electron micrographs of *L. bulgaricus* OLL1073R-1 strains grown (A) with successive addition of formate after incubation for 24 hr or (B) without formate for 24 hr and then inoculated into skim milk medium with formate and incubated for 24 hr. Two magnifications are shown: 1, $\times 2,000$; 2, $\times 10,000$. Arrows indicate the part of remarkable cell damage.

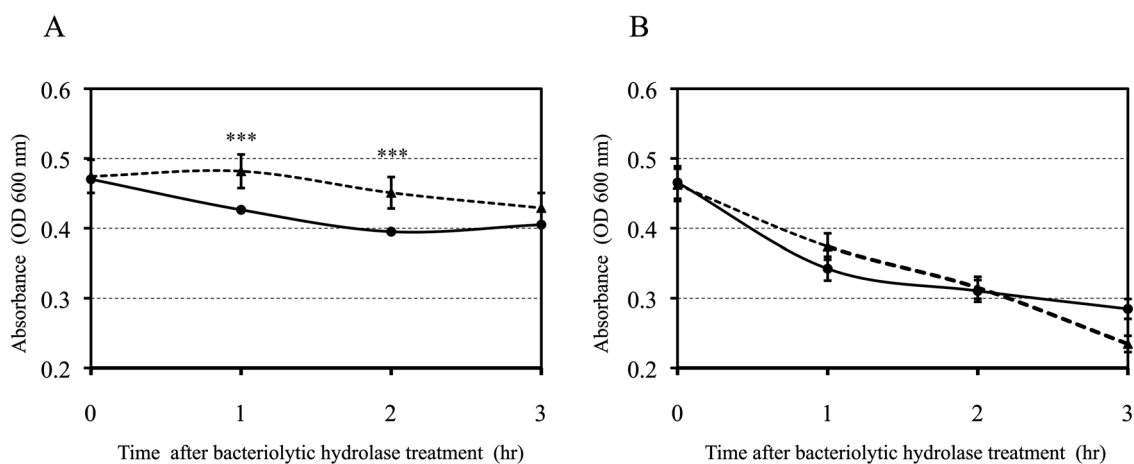


Fig. 5. Hydrolysis patterns of N-acetylmuramidase SG in *L. bulgaricus* OLL1073R-1. The cell numbers in the presence of (A) 25 and (B) 125 $\mu\text{g/ml}$ N-acetylmuramidase SG are shown. Solid (\bullet) and dotted (\blacktriangle) lines indicate cultures with or without N-acetylmuramidase SG, respectively. Optical density was monitored at 600 nm. Data are presented as the mean and standard deviation of six experiments (***) $p < 0.001$.

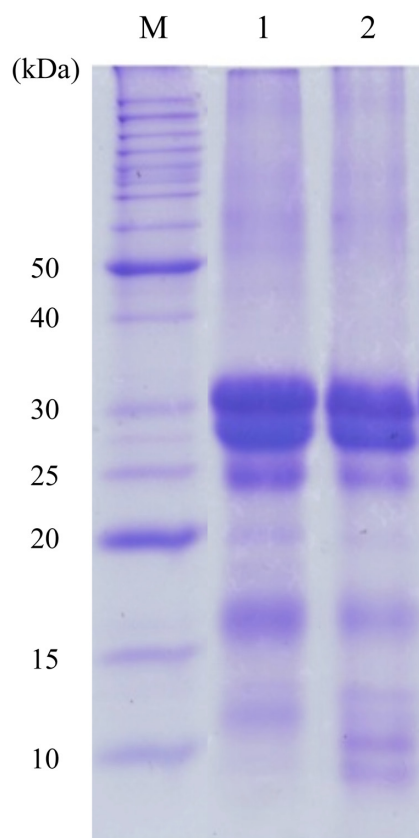


Fig. 6. SDS-PAGE (15%) of cell SLPs from *L. bulgaricus* OLL1073R-1 stained with Coomassie blue. Lane M, molecular weight standard marker (BenchMark™ protein ladder, Invitrogen); lane 1, SLPs in culture without formate; lane 2, SLPs in culture with formate.

sensitivity (Table 1) of *L. bulgaricus* OLL1073R-1 cells were affected by the addition of formate in this study. These findings suggest that formic acid is related to the normal construction of peptidoglycan, including cross-linkage formation in cell wall synthesis. In intermediate lipid biosynthesis during cell wall construction, vancomycin and bacitracin interfere with the reaction of the lipid carrier in the process of peptidoglycan cross-linkage formation. Vancomycin is a kind of glycopeptide and lipoglycopeptide antibiotic and inhibits bacterial cell wall peptidoglycan synthesis [27]. Vancomycin attaches to the terminal D-alanyl-D-alanine group of the disaccharide pentapeptide and blocks the synthesis of peptidoglycans by producing steric hindrance (inhibits transfer of GlcNAc-MurNAc-pentapeptide-(Gly)₅ by a lipid carrier (undecaprenyl-PP) toward the cell wall). On the other hand, bacitracin interferes with the dephosphorylation of the lipid carrier during the process of peptidoglycan cross-linkage formation. Namely, tight binding of bacitracin

with undecaprenyl pyrophosphate prevents recycling of the sugar carrier, thereby inhibiting cell wall synthesis and forming a tertiary complex in the presence of bivalent ions, which prevents pyrophosphatase from interacting with C-55 isoprenyl pyrophosphate, thus reducing the amount of C-55 isoprenyl phosphate that is available for carrying sugar-peptide units [27]. It is considered that formic acid might bind to the D-Ala-D-Ala terminus of the pentapeptide peptidoglycan precursor, resulting in normalization of cell shape.

A lipid carrier is also necessary for EPS production [28, 29]. The lipid carrier does not compete with cell division and EPS synthesis because cell wall construction and cell division mainly occur in the exponential phase, while EPS synthesis occurs in the stationary phase [19, 29]. Based on these findings, formate is suggested to promote both cell division and EPS synthesis via a lipid carrier.

In this study, some changes were observed in intracellular fatty acid composition. Lipid composition of the cell membrane in *L. bulgaricus* was reported to be different following exposure to low pH, high or low temperatures or oxidative or osmotic stress conditions [30]. The change of lipid composition reflects adaptation to environment. Thus, the intracellular fatty acid composition may be related to antibiotic sensitivity. In addition, the C20:5n3 peak, which is eicosapentaenoic acid (EPA), was detected in intracellular fatty acids prepared from the culture without formate. Recently, EPA was found to be related to cell division in *Shewanella livingstonensis* [31]. Although *L. bulgaricus* and *S. livingstonensis* are far taxonomically, it seems that the processes of EPA metabolism, formic acid utilization and cell elongation may be related between these two strains.

In cocultivation with *L. bulgaricus* and *S. thermophilus*, formic acid is produced by *S. thermophilus* and is supplied to *L. bulgaricus* from the early exponential phase [19]. In this experiment, an unusual morphological shape was observed in the exponential phase (after 6 hr of cultivation) of the culture with formate, but the cell length returned to normal when the cultivation continued for 24 hr to the stationary phase. Thus, intake of formic acid is necessary for both cell growth and normal cell division. Suzuki et al. showed that formic acid is a necessary precursor of purine synthesis in DNA reproduction [4, 5] and that cell elongation is induced by having RNA and/or protein that is insufficient for separation [4]. Although it is not found the mechanism that lack of formic acid causes abnormal cell division, formic acid may play a critical role in the biosynthesis of cell division proteins, such as filamentation temperature-sensitive protein Z (FtsZ) [32], or in the other proteins involved in the process of peptide

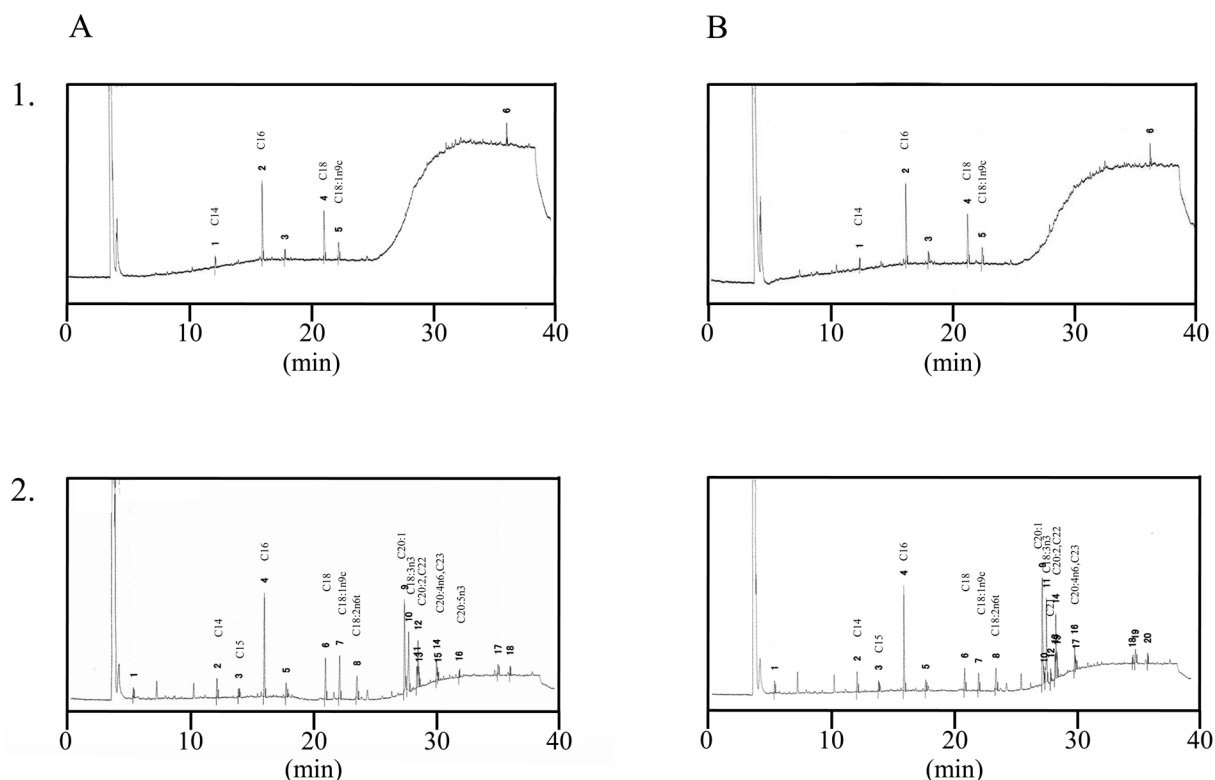


Fig. 7. Gas chromatograms of fatty acid analysis of the cell membrane and cytoplasm from *L. bulgaricus* OLL1073R-1 strains grown at 37°C in skim milk medium (A) without formate or (B) with formate. 1, polar lipids; 2, neutral lipids.

Table 1. Diameters of inhibition zones around antibiotics at varying concentrations with or without formate

	Without formate Diameter (cm)	With formate Diameter (cm)
Vancomycin		
32 µg/ml	2.330 ± 0.054	2.140 ± 0.043***
64 µg/ml	2.578 ± 0.099	2.386 ± 0.088*
128 µg/ml	2.894 ± 0.024	2.538 ± 0.036***
Bacitracin		
32 µg/ml	1.882 ± 0.037	1.746 ± 0.055**
64 µg/ml	2.203 ± 0.043	2.052 ± 0.067**
128 µg/ml	2.515 ± 0.044	2.235 ± 0.061***
256 µg/ml	2.830 ± 0.082	2.507 ± 0.085***

The data are shown as means ± standard deviations of five different plates (*p<0.05; **p<0.01; ***p<0.001).

glycan formation.

In this study, we suggest that the control of formic acid levels is important to the regulation of EPS production of *L. bulgaricus* OLL1073R-1 in yogurt fermentation. The selection and application of strains of *S. thermophilus* with high formic acid production would be useful for the development of EPS-rich yogurt cultures in the future.

ACKNOWLEDGMENTS

We are grateful to Dr. Toshiki Morichi for valuable comments about unusual cell division when formic acid is absent. We also thank Ms. Kumiko Ito for operating the electron microscope and Ms. Yumiko Makino for amino acid sequencing. This work was supported by a grant to Prof. Saito.

REFERENCES

1. Sybesma W, Starrenburg M, Tijsseling L, Hoefnagel MH, Hugenholtz J. 2003. Effects of cultivation conditions on folate production by lactic acid bacteria. *Appl Environ Microbiol* 69: 4542–4548. [[Medline](#)] [[CrossRef](#)]
2. Driessen FM, Kingma F, Stadhouders J. 1982. Evidence that *Lactobacillus bulgaricus* in yoghurt is stimulated by carbon dioxide produced by *Streptococcus thermophilus*. *Neth Milk Dairy J* 36: 135–144.
3. Sieuwerts S, de Bok FA, Hugenholtz J, van Hylckama Vlieg JE. 2008. Unraveling microbial interactions in food fermentations: from classical to genomics approaches. *Appl Environ Microbiol* 74: 4997–5007. [[Medline](#)] [[CrossRef](#)]
4. Suzuki I, Kato S, Kitada T, Yano N, Morichi T. 1986. Growth of *Lactobacillus bulgaricus* in milk. 1. Cell

- elongation and the role of formic acid in boiled milk. *J Dairy Sci* 69: 311–320. [CrossRef]
5. Suzuki I, Kato S, Kitada T, Yano N, Morichi T. 1986. Growth of *Lactobacillus bulgaricus* in milk. 2. Characteristics of purine nucleotides, pyrimidine nucleotides, and nucleic acid synthesis. *J Dairy Sci* 69: 971–978. [Medline] [CrossRef]
 6. van de Guchte M, Penaud S, Grimaldi C, Barbe V, Bryson K, Nicolas P, Robert C, Oztas S, Mangenot S, Couloux A, Loux V, Dervyn R, Bossy R, Bolotin A, Batto JM, Walunas T, Gibrat JF, Bessières P, Weissenbach J, Ehrlich SD, Maguin E. 2006. The complete genome sequence of *Lactobacillus bulgaricus* reveals extensive and ongoing reductive evolution. *Proc Natl Acad Sci USA* 103: 9274–9279. [Medline] [CrossRef]
 7. van de Guchte M, Serror P, Chervaux C, Smokvina T, Ehrlich SD, Maguin E. 2002. Stress responses in lactic acid bacteria. *Antonie van Leeuwenhoek* 82: 187–216. [Medline] [CrossRef]
 8. Uemura (Nishimura) J, Itoh T, Kaneko T, Noda K. 1998. Chemical characterization of exocellular polysaccharide from *Lactobacillus delbrueckii* subsp. *bulgaricus* OLL 1073R-1. *Milchwissenschaft* 53: 443–446.
 9. Kitazawa H, Harata T, Uemura (Nishimura) J, Saito T, Kaneko T, Itoh T. 1998. Phosphate group requirement for mitogenic activation of lymphocytes by an extracellular phosphopolysaccharide from *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Int J Food Microbiol* 40: 169–175. [Medline] [CrossRef]
 10. Kitazawa H, Ishii Y, Uemura (Nishimura) J, Kawai Y, Saito T, Kaneko T, Noda K, Itoh T. 2000. Augmentation of macrophage functions by an extracellular phosphopolysaccharide from *Lactobacillus delbrueckii* ssp. *bulgaricus*. *Food Microbiol* 17: 109–118. [CrossRef]
 11. Nishimura-Uemura J, Kitazawa H, Kawai Y, Itoh T, Oda M, Saito T. 2003. Functional alteration of murine macrophages stimulated with extracellular polysaccharides from *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. *Food Microbiol* 20: 267–273. [CrossRef]
 12. Makino S, Ikegami S, Kano H, Sashihara T, Sugano H, Horiuchi H, Saito T, Oda M. 2006. Immunomodulatory effects of polysaccharides produced by *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. *J Dairy Sci* 89: 2873–2881. [Medline] [CrossRef]
 13. Makino S, Ikegami S, Kume A, Horiuchi H, Sasaki H, Orii N. 2010. Reducing the risk of infection in the elderly by dietary intake of yoghurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1. *Br J Nutr* 104: 998–1006. [Medline] [CrossRef]
 14. Nagai T, Makino S, Ikegami S, Itoh H, Yamada H. 2011. Effects of oral administration of yogurt fermented with *Lactobacillus delbrueckii* ssp. *bulgaricus* OLL1073R-1 and its exopolysaccharides against influenza virus infection in mice. *Int Immunopharmacol* 11: 2246–2250. [Medline] [CrossRef]
 15. van den Berg D, Robijn GW, Janssen AC, Giuseppin M, Vreeker R, Kamerling JP, Vliegthart J, Ledebor AM, Verrips CT. 1995. Production of a novel extracellular polysaccharide by *Lactobacillus sake* 0-1 and characterization of the polysaccharide. *Appl Environ Microbiol* 61: 2840–2844. [Medline]
 16. Goh KK, Haisman DR, Singh H. 2005. Development of an improved procedure for isolation and purification of exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* NCFB 2483. *Appl Microbiol Biotechnol* 67: 202–208. [Medline] [CrossRef]
 17. Sieuwerts S, Molenaar D, van Hijum SA, Beerthuyzen M, Stevens MJ, Janssen PW, Ingham CJ, de Bok FA, de Vos WM, van Hylckama Vlieg JE. 2010. Mixed-culture transcriptome analysis reveals the molecular basis of mixed-culture growth in *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. *Appl Environ Microbiol* 76: 7775–7784. [Medline] [CrossRef]
 18. Audet P, Paquin C, Lacroix C. 1989. Sugar utilization and acid production by free and entrapped cells of *Streptococcus salivarius* subsp. *thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus*, and *Lactococcus lactis* subsp. *lactis* in a whey permeate medium. *Appl Environ Microbiol* 55: 185–189. [Medline]
 19. El-Abbassy MZ, Sitohy M. 1993. Metabolic interaction between *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in single and mixed starter yoghurts. *Food/Nahrung* 37: 53–58.
 20. Moreira M, Storani EAB, de Antoni G. 2001. Sodium formate as an alternative for the replacement of streptococci in the elaboration of Quartirolo cheese. *J Food Compost Anal* 14: 533–540. [CrossRef]
 21. Kern J, Weiser HH, Harper WJ, Gould IA. 1954. Letter to the editor: Observations on organic acids formed during the heat sterilization of milk. *J Dairy Sci*, 37: 904.
 22. Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. *Anal Chem* 28: 350–356. [CrossRef]
 23. Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685. [Medline] [CrossRef]
 24. Bligh EG, Dyer WJ. 1959. A rapid method of total lipid extraction and purification. *Can J Biochem* 37: 911–917. [Medline] [CrossRef]
 25. Petry S, Furlan S, Crepeau MJ, Cerning J, Desmazeaud M. 2000. Factors affecting exocellular polysaccharide production by *Lactobacillus delbrueckii* subsp. *bulgaricus* grown in a chemically defined medium. *Appl Environ Microbiol* 66: 3427–3431. [Medline]

- [CrossRef]
26. Mende S, Krzyzanowski L, Weber J, Jaros D, Rohm H. 2012. Growth and exopolysaccharide yield of *Lactobacillus delbrueckii* ssp. *bulgaricus* DSM 20081 in batch and continuous bioreactor experiments at constant pH. *J Biosci Bioeng* 113: 185–191. [Medline] [CrossRef]
 27. Bryskier A., editor. 2005. Antimicrobial agents: Antibacterials and antifungals, ASM Press, Washington DC.
 28. Stingle F, Newell JW, Neeser JR. 1999. Unraveling the function of glycosyltransferases in *Streptococcus thermophilus* Sfi6. *J Bacteriol* 181: 6354–6360. [Medline]
 29. Broadbent JR, McMahon DJ, Welker DL, Oberg CJ, Moineau S. 2003. Biochemistry, genetics, and applications of exopolysaccharide production in *Streptococcus thermophilus*: A Review. *J Dairy Sci* 86: 407–423. [Medline] [CrossRef]
 30. Tymczyszyn EE, Gómez-Zavaglia A, Disalvo EA. 2005. Influence of the growth at high osmolality on the lipid composition, water permeability and osmotic response of *Lactobacillus bulgaricus*. *Arch Biochem Biophys* 443: 66–73. [Medline] [CrossRef]
 31. Kawamoto J, Kurihara T, Yamamoto K, Nagayasu M, Tani Y, Mihara H, Hosokawa M, Baba T, Sato SB, Esaki N. 2009. Eicosapentaenoic acid plays a beneficial role in membrane organization and cell division of a cold-adapted bacterium, *Shewanella livingstonensis* Ac10. *J Bacteriol* 191: 632–640. [Medline] [CrossRef]
 32. Bi EF, Lutkenhaus J. 1991. FtsZ ring structure associated with division in *Escherichia coli*. *Nature* 354: 161–164. [Medline] [CrossRef]