NADH Oxidase of *Streptococcus thermophilus* 1131 is Required for the Effective Yogurt Fermentation with *Lactobacillus delbrueckii* subsp. *bulgaricus* 2038

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We previously reported that dissolved oxygen (DO) suppresses yogurt fermentation with an industrial starter culture composed of *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) 2038 and *Streptococcus thermophilus* 1131, and also found that reducing the DO in the medium prior to fermentation (deoxygenated fermentation) shortens the fermentation time. In this study, we found that deoxygenated fermentation primarily increased the cell number of *S. thermophilus* 1131 rather than that of *L. bulgaricus* 2038, resulting in earlier L-lactate and formate accumulation. Measurement of the DO concentration and hydrogen peroxide generation in the milk medium suggested that DO is mainly removed by *S. thermophilus* 1131. The results using an H₂O-forming NADH oxidase (Nox)-defective mutant of *S. thermophilus* 1131 revealed that Nox is the major oxygen-consuming enzyme of the bacterium. Yogurt fermentation with the *S. thermophilus* *Anox* mutant and *L. bulgaricus* 2038 was significantly slower than with *S. thermophilus* 1131 and *L. bulgaricus* 2038, and the DO concentrations of the mixed culture did not decrease to less than 2 mg/kg within 3 hr. These observations suggest that Nox of *S. thermophilus* 1131 contributes greatly to yogurt fermentation, presumably by removing the DO in milk.

Key words: yogurt fermentation, NADH oxidase, *Lactobacillus delbrueckii* subsp. *bulgaricus*, *Streptococcus thermophilus*

INTRODUCTION

Yogurt, a very important dairy product, is fermented by a mixed culture of two thermophilic lactic acid bacteria: *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*). Protocooperation (or symbiosis) exists between these two bacteria via an exchange of metabolites that are necessary for the growth of each bacterium in milk. For example, *L. bulgaricus* provides amino acids and peptides for *S. thermophilus*, and *S. thermophilus* provides formate and carbon dioxide for *L. bulgaricus* [1–3]. Several reviews on protocooperation have been published [4–7] and the mutual benefits often result in higher acidification rates, which are important for yogurt fermentation.

Many new aspects of protocooperation in yogurt fermentation have been revealed using the current post-

genomic techniques. The complete genomes of five *S. thermophilus* strains [8–12] and four *L. bulgaricus* strains have been characterized [13–16] and post-genomic studies to analyze the protocooperation have recently been reported. Microarray and proteome analyses of *S. thermophilus* LMG18311 and a qRT-PCR analysis of *L. bulgaricus* ATCC 11842 have shown that the coculture results in not only nutritional exchanges but also dramatic physiological changes in these two bacteria. Transcriptional changes for genes related to nitrogen, nucleotide bases and iron metabolism were observed in *S. thermophilus* LMG18311 [17]. Another study [18] showed, using microarray of *S. thermophilus* CNRZ1066 and *L. bulgaricus* ATCC BAA-365, that the interactions between the purine, amino acid, exopolysaccharide and long-chain fatty acid metabolisms are affected by coculture. The genome of *S. thermophilus* LMD-9 encodes eight two-component systems, and qRT-PCR analysis data indicated that coculture with *L. bulgaricus* ATCC11842 induced expression of two response regulators among them [19].

Recently, we reported that dissolved oxygen (DO) greatly affects yogurt fermentation with an industrial

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starter culture composed of *L. bulgaricus* 2038 and *S. thermophilus* 1131 [20]. The starter began to produce acid actively only after the DO concentration in the yogurt mix was reduced to almost 0 mg/kg. Fermentation was suppressed in the presence of more than 1 mg/kg of DO, and this suppression was compensated partially by the addition of formate to the medium [21]. We have also found that the fermentation time was shortened by 30 min if DO in the yogurt mix was removed in advance (deoxygenated fermentation). These observations clearly demonstrated that DO in milk would greatly affect yogurt fermentation, and at the same time, they indicated that these bacteria have a DO-consuming enzyme(s) presumably required for fermentation.

Like other lactic acid bacteria, *S. thermophilus* and *L. bulgaricus* do not produce cytochrome oxidases required for energy-linked respiratory metabolism due to the inability to synthesize heme, an essential cofactor for cytochrome oxidase. Instead of heme cofactor oxidases, lactic acid bacteria consume molecular oxygen through the action of flavoprotein oxidases, including NADH oxidase, pyruvate oxidase, α -glycerophosphate oxidase, L-amino acid oxidase, and lactate oxidase. Although these oxidases have not been directly linked to energy metabolism, many functions of these oxidases in the physiology of lactic acid bacteria have been reported [22–30]. Teraguchi et al. characterized the NADH oxidase activity of *S. thermophilus* and suggested that high NADH oxidase activity might shift metabolism from homo lactic acid to mixed acid fermentation by affecting the cellular NAD^+/NADH ratio [31]. NADH oxidase activity of *L. bulgaricus* has been reported to be a source of hydrogen peroxide (H_2O_2) when the bacterium was cultured under aerobic conditions [32]. However, the role of these oxidases in yogurt fermentation has not been elucidated.

In the present study, the beneficial effects of deoxygenated fermentation on yogurt fermentation were investigated from the viewpoint of protocoooperation. We also analyzed the contributions of these bacterial oxidases in yogurt fermentation, and identified the NADH oxidase of *S. thermophilus* 1131 as the major enzyme required for the DO reduction and fermentation of milk.

MATERIALS AND METHODS

Strains and culture conditions

Yogurt starter strains of *L. bulgaricus* 2038 and *S. thermophilus* 1131 were obtained from the stock cultures of the Food Science Institute of Meiji Co., Ltd., which are used in the commercial production of Meiji Bulgaria

Yogurt LB81 in Japan. For the coculture medium, 10% (wt/wt) skim milk was prepared by heating it to 95°C for 2 min and immediately cooling it to 43°C. For monoculture experiments of *L. bulgaricus* 2038 or *S. thermophilus* 1131, either 1 mM sodium formate (Wako Pure Chemical Industries, Ltd., Japan) or 0.1% (wt/wt) casein peptides (CE90GMM, Nippon Shinyaku Co., Ltd., Japan) was added, respectively, to the skim milk medium prepared above. Yogurt fermentation with or without deoxygenated fermentation was performed as described previously [20, 21]. Since the *Anox* mutant (described below) did not grow well on skim milk medium, the preculture of this strain was prepared on M17L medium [M17 (Becton, Dickinson and Company) supplemented with 0.5% lactose] in monoculture. The precultures of *S. thermophilus* 1131 and the *Anox* mutant were grown for 16.5 hours at 37°C on M17L using an Anaero Pack system (anaerobic cultivation system; Mitsubishi Gas Chemical Company Inc.) and then inoculated to 10% skim milk supplemented with 0.1% casein peptides to give the final OD_{660} of 0.05. The growths of *S. thermophilus* 1131 and the *Anox* mutant were monitored at 43°C under static conditions. For coculture experiments, the preculture obtained above containing *S. thermophilus* 1131 or *Anox* was inoculated to 10% skim milk together with a culture of *L. bulgaricus* 2038 grown on MRS (Becton, Dickinson and Company) at 37°C. *S. thermophilus* 1131 or the *Anox* mutant and *L. bulgaricus* 2038 were inoculated to 10% skim milk medium at a 1:1 ratio, corresponding to a final OD_{660} of 0.025.

Measurements of CFU, DO, lactate and formate

In coculture experiments, a portion of skim milk medium was withdrawn at predetermined time intervals and spread on M17G agar plates and on Rogosa agar plates. The plates were incubated at 43°C for 48 hours under anaerobic conditions using an Anaero Pack system. The numbers of colony-forming units (CFU) for *S. thermophilus* and *L. bulgaricus* were determined by counting colony numbers on M17G plates and on Rogosa plates, respectively.

Concentration of DO in the skim milk medium was measured using optical oxygen sensors (VisiFerm DO optical sensors, Hamilton Company, Reno, NV, USA).

L-lactate, D-lactate and formate in the skim milk medium was measured using an F-kit (Roche Diagnostics K.K.) suitable for each material as described previously [20, 21].

Measurement of hydrogen peroxide in skim milk medium

In order to measure small quantities of H_2O_2 in

the milk medium, the following improved method was used in this study. An aliquot of culture medium (0.5 g) was harvested at predetermined time intervals and diluted with 0.5 mL of distilled water, and 30 μ L of Carrez I and Carrez II solutions [33] were added. After centrifugation at 12,000 rpm for 10 min at 4°C, the supernatant was diluted by three-fold with 100 mM PIPES (pH 6.5). One hundred microliters of this diluted sample received 100 μ L of 1 mM DA64 (Wako Pure Chemical Industries Ltd., Osaka, Japan), a sensitive chromophore N-(carboxymethylaminocarbonyl)-4,4'-bis(dimethylamino)-diphenylamine sodium salt [34], and 2 μ L of peroxidase (Wako Pure Chemical Industries Ltd., Osaka, Japan). After 10 min of incubation at 16°C, the absorbance at 727 nm was determined.

Construction of a nox knockout mutant of S. thermophilus 1131

Plasmids containing the *nox* gene, inactivated by insertion of a spectinomycin resistance cassette, were constructed as follows. A 1369-bp internal fragment of the *nox* gene was obtained by PCR using the primer pairs PYY0034 (5'-CTCGAGTCAAAAATCGTAGTTGTCGG-3') and PYY0037 (5'-CTCGAGTATTCAGCGGAGATAGCTGC-3'), genomic DNA from *S. thermophilus* 1131, and Takara Ex Taq DNA polymerase (Takara Bio Inc., Otsu, Japan). The amplified fragment was cloned into pGEM-T (Promega K.K., Tokyo, Japan) to obtain pGEM-T-*nox*. The primer pairs PYY0035 (5'-GCACCAACGACTGCTACAC-3') and PYY0036 (5'-TGCGTACGATGTAGATATGG-3') were used to introduce blunt ends in the middle of the *nox* gene of pGEM-T-*nox* by PCR. pSPC1 [25] was digested with *Bam*HI to obtain a DNA fragment containing a terminator-less spectinomycin resistance cassette. Then the *Bam*HI fragment was ligated with the blunt ends of the PCR product of pGEM-T-*nox* after generating blunt ends by treatment with the Klenow fragment of *E. coli* DNA polymerase I (Takara Bio Inc., Otsu, Japan). pGEM-T-*nox* carrying the spectinomycin resistance cassette in the middle of the *nox* gene in the same direction was selected and designed as pGEM-T-*nox*::*spc*^r. The *nox* gene carrying the spectinomycin resistance cassette was then transferred into the *Xho*I site of pG⁺host6 (Appligene, Pleasanton, CA, USA), a temperature-sensitive cloning vector for gram-positive bacteria. The resulting plasmid was designated as pG⁺host6-*nox*::*spc*^r and introduced into *S. thermophilus* 1131 by electroporation. Double-cross-over events leading to the expected gene replacements were obtained as previously

described [35]. Correct insertion of the spectinomycin resistance cassette into genomic DNA was confirmed by PCR analysis using primer pairs PYY0034 and PYY0037.

Determination of NADH oxidase and whole-cell oxygen consumption activities of S. thermophilus

To determine NADH oxidase activity, 1 mL of overnight culture of each strain was transferred into a 300 mL flask containing 50 mL of fresh M17G medium, and incubated at 37°C with vigorous shaking (180 cycles/min) under aerobic conditions until the mid-log phase ($OD_{600} = \sim 1.0$). Then the bacterial cells were collected by centrifugation at 6,300 g for 5 min at 4°C, and washed with 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA, and suspended in 0.8 mL of the same buffer. The cell suspensions were transferred into a screw cap tube containing 0.3 g zirconium beads and disrupted by a Mini-Beadbeater (BioSpec Products, Inc., Bartlesville, OK, USA) at 4,600 rpm for 120 sec. The cell debris was removed by centrifugation at 16,000 g for 15 min at 4°C, and the resulting supernatant was used for NADH oxidase activity measurement as described previously [25]. One unit of enzyme was defined as the amount that catalyzed oxidation of 1 μ mol of NADH to NAD per min at 25°C. Protein concentrations were determined according to the Bradford method [36] using bovine serum albumin as a standard.

To monitor oxygen consumption activity of *S. thermophilus* cells, each strain was grown up to an early log phase ($OD_{600} = \sim 0.5$) in M17G medium with vigorous shaking (180 cycles/min) as described above. The bacterial cells were collected by centrifugation at 6,300 g for 5 min at 4°C, and washed with phosphate buffered saline (PBS) and suspended in 0.8 mL of the same buffer. Then an aliquot of the suspension was injected into a rubber-capped vial filled with 3.5 mL of oxygen-saturated PBS to give an OD_{600} of 2.0. After the vial containing the bacterial suspension was incubated for 5 min at 37°C with stirring, 20% glucose solution was injected into the vial at a final concentration of 0.1%, and the rate of oxygen consumption of the bacterial suspension was monitored using an by the oxygen meter (Fibox 3, PreSens, Regensburg, Germany) through a sensor tip fixed to the bottom of the vial.

RESULTS

Growth acceleration by deoxygenated fermentation was more prominent in S. thermophilus 1131 than L. bulgaricus 2038

The viable cell numbers and lactate productions of *S. thermophilus* 1131 and *L. bulgaricus* 2038 in yogurt fermentation were compared under deoxygenated and normal fermentation conditions [20, 21]. Deoxygenated fermentation in coculture significantly increased the number of colony-forming units of *S. thermophilus* 1131 and production of both L-lactate and formate at 90 min (Fig. 1). However, the number of colony-forming units and L-lactate concentrations of the cultures with or without deoxygenated fermentation were almost the same at the end of fermentation. Although the number of colony-forming units of *L. bulgaricus* 2038 and D-lactate production were also slightly affected by deoxygenated fermentation, the changes were small compared with those of *S. thermophilus* 1131. These results indicate that L-lactate, produced by *S. thermophilus* 1131, mainly contributed to the acceleration of acidification in coculture, especially between 60 and 120 min of fermentation in the deoxygenated milk medium.

DO consumption and H₂O₂ generation in monoculture of S. thermophilus 1131 and L. bulgaricus 2038

DO concentrations and pH changes in the skim milk medium in monoculture of each lactic acid bacterium were examined in relation to H₂O₂ generation. The milk medium received either 0.1% of casein peptides or 1 mM sodium formate to promote growth of *S. thermophilus* 1131 or *L. bulgaricus* 2038, respectively. H₂O₂ concentrations in the culture medium were determined because *L. bulgaricus* produces H₂O₂ in the presence of oxygen [32, 37, 38] and *S. thermophilus* changes the expression of genes involved in iron metabolism during the coculture with *L. bulgaricus* presumably to avoid the harmful effects of H₂O₂ [17, 18].

Although it is reportedly difficult to measure H₂O₂ concentrations in milk due to the presence of several milk components including casein [17], we successfully determined H₂O₂ concentrations using a sensitive chromophore, DA-64. Linear standard curves of H₂O₂ in the milk were obtained repeatedly in a range between 18 and 300 μ M (data not shown). This new method was used to measure H₂O₂ concentrations in the skim milk medium.

As shown in Fig. 2, both lactic acid bacteria in monoculture reduced DO in the medium to less than 1 mg/kg within 120 min. Although *L. bulgaricus* 2038 produced up to 120 μ M of H₂O₂ in accordance with

Fig. 1. Influence of deoxygenated fermentation on viable cell numbers and lactate and formate concentrations in coculture of *S. thermophilus* 1131 and *L. bulgaricus* 2038.

For deoxygenated fermentation (DF), skim milk medium was deoxygenated with nitrogen gas before fermentation. Normal fermentation (NF) was performed without this treatment. Incubation was at 43°C. In (A), CFU of *S. thermophilus* 1131 in DF (■) and NF (□) and CFU of *L. bulgaricus* 2038 in DF (◆) and NF (◇) are shown. In (B), L-Lactate concentrations in DF (■) and NF (□) and D-Lactate concentrations in DF (◆) and NF (◇) are shown. In (C), formate concentrations in DF (◆) and NF (◇) are shown. The error bars represent the standard deviations of three independent experiments.

the decrease of DO, no H₂O₂ (less than 18 μ M, the quantitative determination limit) was detected in the monoculture of *S. thermophilus* 1131. These results indicate that both lactic acid bacteria are able to consume DO in the skim milk medium and that *L. bulgaricus* 2038 does not completely reduce molecular oxygen to H₂O, resulting in production of H₂O₂.

DO consumption and H₂O₂ generation in coculture

The concentrations of DO and H₂O₂ in the culture medium together with the pH values were measured in coculture of *S. thermophilus* 1131 and *L. bulgaricus* 2038. As shown in Fig. 3, DO in the medium decreased to less than 1 mg/kg within 90 min, and H₂O₂ was not detected until 60 min of fermentation. The maximum concentration of H₂O₂ was around 40 μ M, which was approximately 3 times lower than that of the *L. bulgaricus* 2038 monoculture (Fig. 2B).

Fig. 2. DO consumption and H_2O_2 generation in monoculture of *S. thermophilus* 1131 and *L. bulgaricus* 2038.

DO ($\square$) and H_2O_2 ($\blacksquare$) concentrations and pH ($\circ$) in monoculture of *S. thermophilus* 1131 (A) and *L. bulgaricus* 2038 (B) at 43°C are shown. The error bars represent the standard deviations of three independent experiments.

Fig. 3. DO consumption and H_2O_2 generation in coculture. DO ($\blacksquare$) and H_2O_2 ($\square$) concentrations and pH ($\circ$) in coculture of *S. thermophilus* 1131 and *L. bulgaricus* 2038 at 43°C are shown. The error bars represent the standard deviations of three independent experiments.

The result showing that H_2O_2 was not detected in the first 60 min of fermentation, when the rapid decrease of DO occurred, strongly suggests that the DO decrease in coculture can be attributed to *S. thermophilus* 1131. This result together with the increased cell number and production of L-lactate in yogurt fermentation (Fig. 1A and 1B) indicates that the metabolic activity of *S. thermophilus* 1131 is the main contributor to DO reduction during yogurt fermentation.

Fig. 4. NADH oxidase and whole-cell oxygen consumption activities of *S. thermophilus* 1131 and the Δnox mutant. Log-phase cultures of *S. thermophilus* 1131 and Δnox were used for NADH oxidase activity (A) and whole-cell oxygen consumption determinations (B). The error bars represent the standard deviations of three independent experiments.

Construction of an NADH oxidase knockout mutant of *S. thermophilus* 1131

The results obtained above strongly suggest the importance of the DO reduction activity of *S. thermophilus* 1131 during yogurt fermentation. To identify the oxidase(s) that contributes to DO reduction during yogurt fermentation, we focused on an H_2O -forming NADH oxidase (Nox) homologue of *S. thermophilus* 1131.

Fig. 5. Growth, DO concentration, and formate production in monoculture of *S. thermophilus* 1131 and *Anox* mutant. Growth of *S. thermophilus* 1131 and *Anox* mutant were monitored at 43°C in skim milk medium supplemented with 0.1% casein peptides. In (A), pH (■) and DO (●) of *S. thermophilus* 1131, and pH (□) and DO (○) of the *Anox* mutant are shown. In (B), formate concentrations in monoculture of *S. thermophilus* 1131 (■) and the *Anox* mutant (□) are shown. The error bars represent the standard deviations of three independent experiments.

An *S. thermophilus* 1131 *nox*-inactivated strain was constructed by insertion into *nox* of a terminator-less spectinomycin resistance gene as described in Materials and Methods. Insertion of the spectinomycin resistance gene into the chromosomal *nox* gene was confirmed by PCR analysis (data not shown). Log phase cultures of the resulting *Anox* mutant and *S. thermophilus* 1131 were prepared under aerobic conditions and used for NADH oxidase and rate of whole-cell oxygen consumption determinations. As shown in Fig. 4, NADH oxidase activity was diminished in the *Anox* mutant to less than 4% of that in wild-type strain. Inactivation of the *nox* gene also reduced the rate of whole-cell oxygen consumption by 65%. These results indicate that NADH oxidase encoded by the *nox* gene is the major oxygen-consuming enzyme in *S. thermophilus* 1131.

Comparison of growth and DO consumption in monoculture of *S. thermophilus* 1131 and the *Anox* mutant

Growth properties of *S. thermophilus* 1131 and the *Anox* mutant were compared in a monoculture on skim milk medium supplemented with 0.1% casein peptides. As shown in Fig. 5, *S. thermophilus* 1131 effectively reduced the DO concentrations of the medium, and fermentation seemed to be accelerated after the DO concentrations were below 1 mg/kg. By contrast, the *Anox* mutant did

Fig. 6. Growth and DO concentration in coculture with *L. bulgaricus* 2038. pH (■) and DO (●) in coculture of *S. thermophilus* 1131 and *L. bulgaricus* 2038, and pH (□) and DO (○) in coculture of the *S. thermophilus* *Anox* mutant and *L. bulgaricus* 2038 at 43°C are shown. The error bars represent the standard deviations of three independent experiments.

not rapidly reduce the DO concentrations, and the pH value of the medium was kept high even after 4 hours of fermentation. We also compared the growth of both strains on M17L medium under the same static conditions and found no significant differences between the two strains (data not shown). These results demonstrated that NADH oxidase of *S. thermophilus* 1131 was required for effective fermentation in the skim milk medium but not in M17L medium.

Formate concentrations of the skim milk medium were determined (Fig. 5B), because NADH oxidase has a possible role in activating formate production in *S. thermophilus*. Under anaerobic conditions, formate is generally produced by pyruvate formate lyase (Pfl), an oxygen-sensitive enzyme whose activity is easily inactivated by molecular oxygen [40]. NADH oxidase may be able to activate Pfl by removing DO in the medium. As shown in Fig. 5B, formate was detected in the culture medium of *S. thermophilus* 1131 after 2 hours of fermentation, but not in that of the *Anox* mutant, supporting this hypothesis.

Growth and DO removal of *S. thermophilus* 1131 and the *Anox* mutant in coculture with *L. bulgaricus* 2038

The behavior of the *S. thermophilus* *Anox* mutant in coculture with *L. bulgaricus* was examined. The

coculture conditions were essentially the same as standard coculture conditions except that M17L was used as a preculture of *S. thermophilus* 1131 and the *Anox* mutant. The consumption of DO and decrease in pH caused by coculture of the *Anox* mutant were by far slower than those caused by coculture of the wild type (WT) strain, resulting in a strong retardation of yogurt fermentation (Fig. 6). The H_2O_2 concentrations in the coculture medium of the *Anox* mutant were in the range of 40 to 70 μ M, a little higher than the concentration observed in Fig. 3, indicating that NADH oxidase activity might contribute to the reduction of H_2O_2 generation during the coculture.

DISCUSSION

We have previously reported that deoxygenated fermentation shortened the yogurt fermentation time by 30 min, and the details of this preferable effect were investigated in the present study. Monitoring the growth of *S. thermophilus* 1131 and *L. bulgaricus* 2038 in skim milk demonstrated that DO removal mainly accelerates the growth of *S. thermophilus* 1131. Formate production was also increased by pre-fermentation removal of DO from the medium. Accumulation of formate would favor to the growth of *L. bulgaricus* 2038, because formate is a well-known growth factor for *L. bulgaricus* in milk [41]. However, DO removal stimulated the growth of *S. thermophilus* 1131. This result implies that formate accumulation may also facilitate the growth of *S. thermophilus* in milk. Using proteome analysis, Derzelle et al. identified Pfl of *S. thermophilus* IMG18311 as a protein strongly induced during growth in skim milk medium [42]. They also demonstrated that the addition of formate or purine bases diminishes the overexpression of Pfl and increases the growth yield of *S. thermophilus* in skim milk medium [42]. Their observations suggest that formate produced by Pfl may be utilized in an essential biosynthetic pathway such as purine biosynthesis of *S. thermophilus* in milk. It would therefore not be surprising if the deoxygenated milk activated the Pfl, thereby promoting the growth of *S. thermophilus* 1131 by the addition of formate.

In this study, we identified Nox of *S. thermophilus* 1131 as the major oxygen-consuming enzyme that promotes the fermentation of milk. The *S. thermophilus Anox* mutant could not effectively reduce the DO concentrations and pH values of the skim milk medium in either monoculture or coculture. Nox is a flavoprotein conserved in most Streptococcaceae and has been reported to be a major part of the oxidase machinery in several lactic acid bacteria

[25, 28, 39]. Although most flavoprotein oxidases of lactic acid bacteria generate H_2O_2 as an end product of the reaction, Nox is able to reduce molecular oxygen to H_2O without producing H_2O_2 .

As mentioned above, a possible role for Nox in milk fermentation is activation of Pfl, an oxygen-sensitive enzyme, by the removal of DO from the medium. Consistent with this hypothesis, formate was detected in the culture medium of *S. thermophilus* 1131 but not that of the *Anox* mutant. However, we need to also pay attention to other possibilities concerning the function of Nox in *S. thermophilus* 1131 because various roles have been proposed as physiological functions of Nox in streptococci and lactococci. One major role of Nox is regulation of the intercellular ratios of NAD^+ /NADH by oxidizing NADH with molecular oxygen. Overexpression or a defect of Nox has been shown to affect the intercellular NAD^+ /NADH ratio and change the fate of the metabolic end products in *Lactococcus lactis*, *Streptococcus mutans*, and *Streptococcus agalactiae* [25, 28, 43]. In *Streptococcus thermophilus*, the difference in NADH oxidase activities between strains has been proposed to affect the extent of mixed acid fermentation [31]. Regeneration of NAD^+ by Nox has been shown to be necessary for the assimilation of sugar alcohols in *S. mutans* and for fatty acid synthesis in *S. agalactiae*, under aerobic conditions [25, 28]. Although the mechanism is not clearly understood, Nox activity is involved in the development of natural competence in *Streptococcus pneumoniae* [43, 44]. Nox has also been shown to contribute to the oxidative stress resistance of *S. pyogenes*, *S. mutans*, and *S. pneumoniae* [45–47]. In these bacteria, Nox has been proposed to reduce the generation of reactive oxygen species (ROS) by affecting cellular metabolism or removing DO, a precursor of ROS, from the medium [45–47]. Although we propose the possibility that Nox can promote formate production by establishing anaerobic conditions, further study is necessary to understand the precise role(s) of Nox in milk fermentation and protocoooperation with *L. bulgaricus*.

Recently, Techon et al. characterized the *noxE* (or *nox*, *nox2*) deficient mutant of *L. lactis* and demonstrated that NoxE was required for the reduction of DO and redox potential of milk [39]. However, in the case of *L. lactis*, NoxE deficiency only slightly affected the growth of the bacterium in milk. They also found that the *L. lactis* strains isolated from cheese did not exhibit NADH oxidase activity [32], indicating that Nox is not important for the growth of *L. lactis* in milk. Although the knowledge about NADH oxidase activities of *S. thermophilus* isolated from dairy products is limited,

it is useful to assess whether the results obtained in the present study for *S. thermophilus* and *L. bulgaricus* are also relevant to other strains and strain combinations.

In this study, we demonstrated the H₂O₂-producing activity of *L. bulgaricus* 2038 in milk, while *S. thermophilus* 1131 did not produce detectable H₂O₂ under the same conditions (Fig. 2). During the coculture of these bacteria in milk, H₂O₂ was transitionally detected at 90 and 120 min of fermentation (Fig. 3). This suggested that *L. bulgaricus* 2038 might also contribute to DO reduction and that either *S. thermophilus* 1131 or the skim milk medium may scavenge H₂O₂. To assess the fate of H₂O₂ during coculture, we measured the whole-cell H₂O₂-scavenging activity of *S. thermophilus* 1131. H₂O₂ degradation was not promoted by the addition of *S. thermophilus* 1131 cells to the PBS or skim milk medium to a final concentration of 10⁹ CFU/ml (data not shown). Instead, this experiment indicated that the medium itself has the ability to decrease H₂O₂, i.e., 200 µM H₂O₂ added to the skim milk medium at 43°C decreased to about 90 µM in one hour. The presence of H₂O₂ during milk fermentation has been suggested in several studies [17, 18]. Further research will clarify the effects of H₂O₂ on yogurt fermentation.

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