J. Microbiol. Biotechnol. (2007), **17**(2), 218–225

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

© The Korean Society for Microbiology and Biotechnology

Metabolic Characterization of Lactic Acid Bacterium *Lactococcus garvieae* sk11, Capable of Reducing Ferric Iron, Nitrate, and Fumarate

YUN, SU HEE, TAE SIK HWANG, AND DOO HYUN PARK*

Department of Biological Engineering, Seokyeong University, Seoul 136-704, Korea

Received: July 30, 2006
Accepted: September 28, 2006

Abstract A lactic acid bacterium capable of anaerobic respiration was isolated from soil with ferric iron-containing glucose basal medium and identified as *L. garvieae* by using 16S rDNA sequence homology. The isolate reduced ferric iron, nitrate, and fumarate to ferrous iron, nitrite, and succinate, respectively, under anaerobic N_2 atmosphere. Growth of the isolate was increased about 30–39% in glucose basal medium containing nitrate and fumarate, but not in the medium containing ferric iron. Specifically, metabolic reduction of nitrate and fumarate is thought to be controlled by the specific genes *fnr*, encoding FNR-like protein, and *nir*, regulating fumarate-nitrate reductase. Reduction activity of ferric iron by the isolate was estimated physiologically, enzymologically, and electrochemically. The results obtained led us to propose that the isolate metabolized nitrate and fumarate as an electron acceptor and has specific enzymes capable of reducing ferric iron in coupling with anaerobic respiration.

Key words: *Lactococcus garvieae*, anaerobic respiration, ferric iron reduction, fumarate-nitrate reductase, *fnr*, FNR-like protein

L. garvieae is known as a major pathogen of fish, which is primarily isolated from blood of ill fishes and animals [8, 43]. The fish diseases attributed to *L. garvieae* have been reported in different parts of the world, including Japan, Korea, Italy, Spain, France, Australia, Israel, and the United States [7]. Typically, *L. garvieae* has been isolated from animal blood or skin, which has character different from other *Lactococcus* sp. Little is known about the ecological distribution and biochemical characteristics of *L. garvieae* [8]. Generally, pathogenic bacteria grow or can grow in media containing animal tissue infusion or animal blood [1, 9, 10, 40]. However, *L. garvieae* sk11 is different from the pathogenic *L. garvieae* and other lactic acid bacteria, which metabolically reduce ferric iron to ferrous iron, nitrate to nitrite, and fumarate to succinate, coupled with oxidation of glucose. The dissimilatory ferric iron reduction can be defined as the use of ferric iron as an external electron acceptor in bacterial respiration [21]. Ferric iron is known to be reduced by anaerobic respiration bacteria such as *Geobacter metallireducens* [4, 34], *Shewanella putrefaciens* [2], *Thiobacillus ferrooxidans* [39], and *Anaeromxyobacter dehalogenans* [16]. A distinction of dissimilatory ferric iron reduction from assimilatory reduction is that significant quantities of ferrous iron are accumulated outside the cell during growth under normal physiological conditions [5]. Metabolic reduction of nitrate to nitrite and fumarate to succinate is known to be controlled by the *fnr* gene [17, 19, 25, 33, 38]. The *fnr* product FNR, which is a pleiotropic activator of the gene involved in anaerobic respiration, regulates the nitrate and fumarate reduction coupled to oxidation of different reducing power such as NADH, $FADH_2$, quinone, and cytochromes [41].

In this study, we characterized the metabolic and enzymatic reduction of ferric iron to ferrous iron, nitrate to nitrite, and fumarate to succinate by growing of *L. garvieae* sk11 cells. The metabolic ferric iron reduction was electrochemically analyzed by using the resting cells, because the ferric iron might be chemically reduced by metabolites of growing cells. We also confirmed that the isolate possessed *fnr* and *nir* genes that were amplified by PCR, using a primer designed with a gene encoding the FNR-like protein of *L. lactis* and the *fnr* gene of *E. coli*.

MATERIALS AND METHODS

Isolation of *L. garvieae* sk11

Strain sk11 was isolated from the riverside of Han River located in Daesung-ri Chungpyung-myun Gapyung-eup Kyunggi-do Korea in the middle of April. About 50 g (wet

*Corresponding author
Phone: 82-2-940-7190; Fax: 82-2-919-0345;
E-mail: baakdoo@skuniv.ac.kr

weight) of soil samples was taken from a 5-cm depth of wet land after removal of surface soil. Each sample obtained from 30 places were suspended in 100 ml of sterile saline, and the suspension was spread on agar medium in 6 h after sampling. The agar medium contained glucose basal medium and 30 mM ferric iron [$Fe(OH)_3$]. The glucose basal medium was composed of 50 mM glucose, 2.0 g/l yeast extract, 50 mM phosphate buffer (pH 7.0), and 0.5 ml of trace mineral stock solution. The trace mineral stock solution contained 0.01 g/l $MnSO_4$, 0.01 g/l $MgSO_4$, 0.01 g/l $CaCl_2$, 0.002 g/l $NiCl_2$, 0.002 g/l $CoCl_2$, 0.002 g/l $SeSO_4$, 0.002 g/l WSO_4, 0.002 g/l $ZnSO_4$, 0.002 g/l $Al_2(SO_4)_3$, 0.0001 g/l $TiCl_3$, 0.002 g/l $MoSO_4$, and 10 mM EDTA. The agar plates were incubated under anoxic nitrogen atmosphere at 30°C for 72 h. The black colonies grown on agar plate were selectively transferred into broth medium and incubated for 48 h. In our previous experiment, we found that the colonies of ferric iron-reducing bacteria growing on agar plate changed to black or dark brown, and one strain was finally selected on the basis of ferric iron-reducing activity and identified by 16S rDNA sequence homology.

Bacterial Cultivation

Strain sk11 was cultivated in glucose basal medium. Bacterial growth was measured by optical density at 660 nm, and dry cell mass was determined with bacterial filtrate dried at 110°C for 3 to 5 h until the weight of filtrate was constant. Bacterial filtrate was obtained from 1 l of bacterial culture (OD_{660}=1.0) by filtration with membrane filter (diameter 47 mm, pore 0.22 mm). The dry cell mass of sk11 was (0.195±0.007 g) OD per liter.

Identification of Isolate

Strain sk11 was identified by 16S rDNA sequencing. 16S ribosomal DNA was amplified by direct PCR using the universal primers of forward 5'-GAGTTGGATCCTG-GCTCAG-3' and reverse 5'-AAGGAGGGGATCCAGCC-3'. The reaction mixture consisted of 300 mM Tris-HCl (pH 8.8), 100 mM $(NH_4)_2SO_4$, 100 mM KCl, 20 mM $MgSO_4$, 20 pM each of primers, 20 mM each of dNTP, 2U of Taq polymerase (Genenmed, U.S.A.), and 20 ng of template. Amplification was conducted for 30 cycles of 1 min at 95°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C using a PCR machine (T Gradient model, Biometera, Germany). The PCR products were directly sequenced with an ABI Prism 3700 Genetic analyzer by a professional company (Macrogen Inc., Korea). The 16S rDNA sequences were analyzed using the GenBank database and identified on the basis of 16S rDNA sequence homology [3]. To examine substrate utilization, the isolate was applied to an API50CHL strip (bioMérieux Korea) according to the user manual of the API kit. The API50CHL strip is a test kit for biochemical identification of *Lactobacillus* or *Lactococcus* sp.

Metabolic Reduction of Electron Acceptors

Ferric iron, nitrate, or fumarate was used as an electron acceptor to test the respiratory metabolism of sk11. Thirty mM each of electron acceptors was added to the glucose basal medium: 80 ml of medium was prepared in an anaerobic serum vial (total volume of 165 ml) of which the head space was filled with 99.999% nitrogen. Five % of the pre-cultivated bacterial cells or resting cells were inoculated into serum vials, which were incubated at 30°C without shaking. The resting cells were prepared from 24-h-old culture by aseptic centrifugation at 5,000 ×*g* and 4°C for 30 min. The resting cells were useful to obtain desired results in a short time. Each experiment was repeated five times. Biomass, substrate, metabolites, and electron acceptors of bacterial culture isolated from the bioreactor were quantitatively analyzed.

Analysis

Ferrous iron was analyzed by the Ferrozine method [21, 32]. Nitrate and nitrite were analyzed by ion chromatography (IC, Dionex DX-500, U.S.A.) equipped with an anion exchange column (IonPac, Dionex AS14A, 4×250 mm). A solution of 8.0 mM sodium carbonate and 1.0 mM sodium bicarbonate was used as the mobile phase at a flow rate of 2.0 ml/min. The column temperature was adjusted to 30°C. Samples were prepared by centrifugation of bacterial cultures at 12,000 ×*g* and 4°C for 30 min and then filtration by membrane filters of pore size 0.22 µm. The filtrate was diluted appropriately to near detection limit and then injected into the injector, and the injection volume was adjusted to 5 µl with the sample loop. Glucose and metabolites were analyzed by HPLC (YoungLin, Korea) equipped with an Aminex HPX-87H ion-exchange column (Bio-Rad, CA, U.S.A.) and refractive index detector. The column and detector temperature was adjusted to 35°C. Sulfuric acid (0.008 N) was used as a mobile phase at a flow rate of 0.6 ml/min. Samples were prepared by centrifugation at 12,000 ×*g* at 4°C for 30 min and then filtration by membrane filters with a pore size of 0.22 µm. The filtrate was as a sample injected into the injector of HPLC, and the injection volume was automatically controlled with a 20-µl loop. The concentration was calculated by using the peak area obtained with standard materials. For determination of the enzymatic reduction of ferrous ion, nitrate, and fumarate, the cell extract was used as a biocatalyst instead of bacterial cells. All chemicals used in this research were purchased from Sigma-Aldrich (U.S.A.).

Enzymatic Reduction of Electron Acceptors

Cell extract was obtained from a 24-h-old culture of bacterial cells, which was used as a biocatalyst for ferric iron, fumarate, and nitrate reduction tests. Thus, bacterial cells were harvested and washed three times with 50 mM Tris-Cl buffer (pH 7.5) by centrifugation at 4°C and 5,000 ×*g*

Table 1. Primers prepared on the basis of *Lactococcus lactis fnr*-like gene [10] and *E. coli fnr* gene [34].

Source of primer	Primer number	Sequence (F, forward; R, reverse)	Location	Annealing temp. °C
Lactococcus lactis	I	F, 5'-acaatgattattgcaagtat-3' R, 5'-cctgtcctttaacaacgtcc-3'	1–1,000	50.6
	II	F, 5'-gaatgtagcgtctgttggat-3' R, 5'-gctatcaaagcttttaaata-3'	1,001–2,000	No product*
	III	F, 5'-tatctcaatataactttttt-3' R, 5'-cttgtgatactttaaaaatt-3'	2,001–3,000	45.9
	IV	F, 5'-ttcattaccgaggatccaaa-3' R, 5'-atctagtattttgattatct-3'	3,001–4,000	45.2
	V	F, 5'-attgatagccttgaagatga-3' R, 5'-aaaaatcattccaactttac-3'	4,001–5,000	No product*
	VI	F, 5'-caaaatccactcactgcgct-3' R, 5'-gttctaaaagttgaaagatt-3'	5,001–5,124	45.9
Escherichia coli	VII	F, 5'-tccggtaaaatgccgaaatt-3' R, 5'-cggctgatggtttcaaccgt-3'	524–1,457	40.8

*Annealing temperature used for amplification was a gradient from 46.5°C to 60°C at intervals of 1.5°C in the consideration of the optimal annealing temperature (55.8°C) offered by the primer producer (Bioneer, Korea). However, no amplification product was produced at any annealing temperature specified.

for 30 min. The washed cells were disrupted by ultrasonic treatment (400 W) at 4°C for 20 min. Protein concentration was determined with Bradford reagent (BioRad) as a coloring reagent and bovine serum albumin as a protein standard. The reaction mixture was composed of 5.0 mM NADH, 10 mM ferric iron, and 50 mM Tris-HCl buffer (pH 7.5), and was prepared in anaerobic serum vials whose head space was filled with 99.999% nitrogen after completely removing oxygen from the serum vials. Enzymatic reaction was started by addition of the cell extract to the reaction mixture, and the final protein concentration of the cell extract was adjusted to 10 mg/ml.

fnr Amplification and Sequence Analysis

The *fnr* of sk11 was amplified by direct PCR using seven pairs of primers, of which six pairs were designed using the full sequence (5,124 bp) of gene encoding *Lactococcus lactis* FNR-like protein (GenBank Accession No. AJ006750) [12] and one pair was designed with the full sequence (1,641 bp) of *E. coli fnr* gene (GenBank Accession No. J01608) [35], as shown in Table 1. Amplification was conducted by the same method and procedures used for the 16S rDNA amplification, except for the annealing temperature. The PCR products were sequenced directly with an ABI Prism 3700 Genetic analyzer by a professional company (Macrogen Inc., Korea). The product sequences were analyzed using the GenBank database.

Electrochemical Measurement of Ferric Iron Reduction

A two-compartment microbial fuel cell (MFC) system [31, 36], equipped with graphite anode modified with ferric iron (ferric anode) [29], native graphite cathode (cathode), and native graphite anode (anode) [18], was used to electrochemically measure bacterial ferric iron reduction. The ferric anode functions as an electron acceptor and the cathode functions as a catalyst for the oxidation reaction of proton and electron transferred from the anode compartment by H_2O_2 (H_2O_2+$2H^+$+$2e^-$→$2H_2O$) that functions as an electron acceptor. The MFC and ferric anode were prepared according to the method developed by Park *et al.* [29] and Park and Zeikus [27, 28]: 200 mM phosphate buffer (pH 7.0) containing 200 mM NaCl and 1.0% hydrogen peroxide (oxidant) was used as the catholyte, and 50 mM phosphate buffer (pH 7.0) containing 50 mM glucose was used as the anolyte. By addition of resting sk11 and *W. kimchii* sk10 [30, 31] cells to the anode compartment, current production was measured. *W. kimchii* sk10 was used as a control, which was isolated from kimchi and identified with the 16S rDNA sequence (GenBank Accession No. DQ846737), because it does not have a metabolic function to reduce ferric iron. Initial bacterial density was adjusted to OD 5.0 at 660 nm. Resting cells were obtained by centrifugation (at 5,000 ×*g* and 4°C for 30 min) of a 24-h-old culture of sk11 grown in ferric iron containing glucose basal medium and *W. kimchii* sk10 grown in MRS medium.

Results and Discussion

Identification of the Isolate

The isolate was identified as *L. garvieae* by 16S rDNA sequence analysis and registered at the GenBank database system (http://www.ncbi.nlm.nih.gov/BankIt/) under accession number DQ485325. The 16S rDNA of the isolate was 99% identical with that of all other *L. garvieae* registered in the GenBank database system. On the basis of metabolite (lactate) analysis, *L. garvieae* sk11 was confirmed to be a homofermentative lactic acid bacterium. The strain sk11 grown in anaerobic condition produced lactate as a single

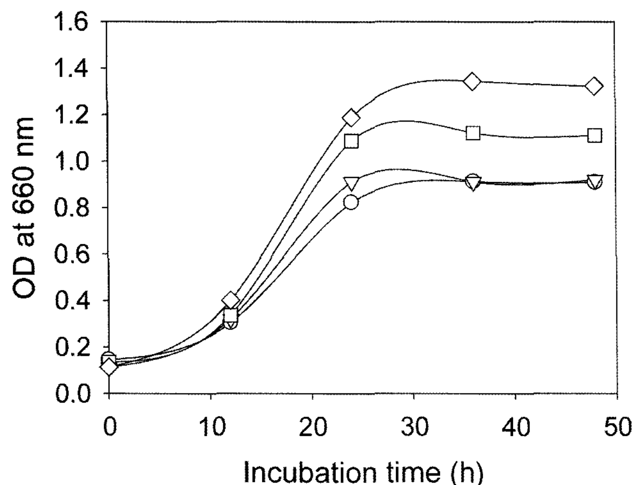

Fig. 1. Effect of ferric ion, nitrate, and fumarate on growth of *L. garvieae* sk11.
The bacterial cells were cultivated in glucose basal medium (○) containing ferric ion (▽), nitrate (□), or fumarate (◇).

metabolite when analyzed by HPLC. Theoretically, lactic acid bacteria can produce only lactic acid as the major metabolite, but other fermentative bacteria produce lactic acid as a minor metabolite or mixed acids. The strain sk11 was 76.3% identical with *Lactococcus lactis* ssp *lactis* 1 and 19.6% identical with *Lactobacillus brevis* 1 in the biochemical test with the API kit. It should be mentioned that the biochemical test with the API kit was performed for biochemical classification of sk11, but not for its identification. Both genetic and biochemical classification tests revealed that sk11 belongs to the genus *Lactococcus*.

Effect of Electron Acceptors on Bacterial Growth

In anaerobic environment, ferric ion, nitrate, or furmarate can function as an electron acceptor for the bacteria capable of producing free energy by respiration [23, 41]. Lactic acid bacteria are typical hetetotrophic organisms, which are dependent on fermentative metabolism and produce fixed quantity of free energy (ATP), reducing power (NADH), and metabolite (lactic acid) [13]. Generally, the growth of lactic acid bacteria is proportional to the free energy produced from glycolysis [23]. However, as shown in Fig. 1, the growth of *L. garvieae* sk11 was increased in the presence of nitrate or fumarate than without them. This indicated that *L. garvieae* sk11 may produce additional free energy coupled to reduction of nitrate or fumarate. As shown in Fig. 2, the resting cells of *L. garvieae* sk11 reduced relatively similar concentrations of nitrate and fumarate in coupling with similar concentration of glucose consumption, but much lower concentration of ferric iron than nitrate and fumarate, indicating that the metabolic system for fumarate and nitrate reduction may be different from that for ferric iron reduction. Based on this result, the

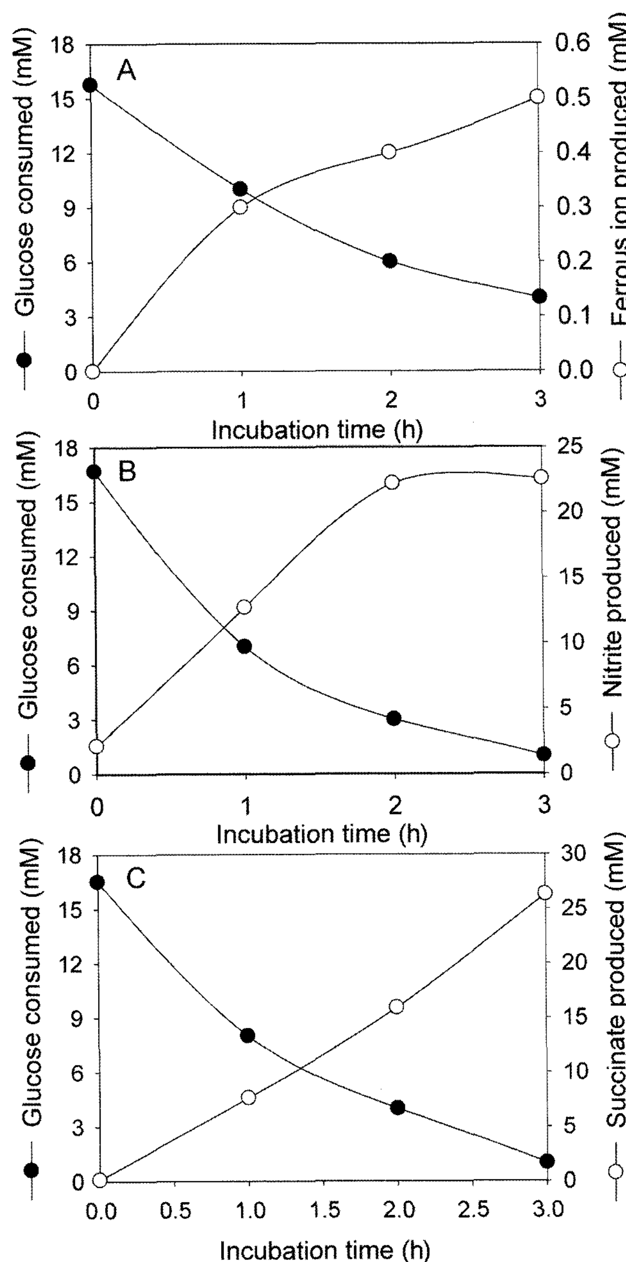

Fig. 2. Metabolic reduction of ferric ion, nitrate, and fumarate by resting cells of *L. garvieae* sk11.
Resting cells were prepared from 24-h-old culture by centrifugation. Biomass was adjusted to an OD_{660} of 5.0 for increase of reaction efficiency.

growth yield, the reduction of electron acceptors, and the ratio of lactate production to glucose consumption (lactate/glucose ratio) were measured, and the result is shown in Table 2. In lactic acid fermentation metabolism, the reducing power (NADH) is reoxidized to NAD^+ coupled to reduction of pyruvate to lactate, in which the redox balance between NADH/pyruvate and NAD^+/lactate has to be theoretically 1.0, and 2.0 moles of lactate has to be produced from 1.0 mole of glucose [30]. However, the lactate/glucose

Table 2. Growth and metabolism of *L. garvieae* sk11 cultivated on glucose basal medium containing ferric ion, nitrate, or fumarate. The bacterial cells were cultivated under strict anaerobic condition for 48 h. Initial concentration of glucose, ferric iron, nitrate, and fumarate was adjusted to 50, 30, 30, and 30 mM, respectively.

Combination of electron donor and acceptors	Cell mass OD_{660} (dry weight, g)	Substrate consumption and metabolite production (mM)			
		Glucose (growth yield)*	Lactate	e^--Acceptors reduced	Lactate/Glucose
Glucose	0.861±0.11 (0.1679±0.02)	22.46±0.21 (7.476)	40.82±1.94	–	1.817±0.087
Glucose+Fe(III)	0.889±0.07 (0.1733±0.04)	23.08±0.13 (7.509)	34.39±1.26	Fe(II), 1.66±0.06	1.491±0.055
Glucose+NO_3^-	1.120±0.16 (0.2184±0.03)	24.42±0.30 (8.943)	25.84±1.04	NO_2^-, 5.53±0.23	1.058±0.043
Glucose+Fumarate	1.210±0.19 (0.2360±0.04)	26.10±0.24 (9.042)	28.98±1.22	Succinate, 14.37±0.62	1.110±0.047

*Growth yield: g cell produced/mol glucose consumed.

ratio of *L. garvieae* sk11 was 1.817±0.087, which is lower than the theoretical value of 2.0. This is most likely due to the effect of bacterial culture pH: At pH higher than 5.0, lactic acid production may be inhibited [23].Therefore, the lactate/glucose ratio may be less than the theoretical value in the glucose basal medium because 50 mM phosphate buffer (pH 7.0) was present in the medium. The lactate/glucose ratio was 1.491±0.055, 1.058±0.043, and 1.110±0.047 in the presence of ferric iron, nitrate, and fumarate, respectively. It appears that a part of NADH reduced in glycolysis was reoxidized coupled to reduction of electron acceptors, by which additional free energy may be produced and growth yield may be increased. However, the growth yield in the presence of ferric iron was not significantly increased. This it is not an unusual phenomenon, as similar phenomena were also found in many other ferric iron-reducing bacteria. Metabolic ferric iron reduction can be defined as the use of ferric iron as an external electron acceptor in anaerobic bacterial respiration. However, there has been no evidence to show that the growth of the metal-reducing bacteria was stimulated or that ATP production was increased when ferric iron was presented to the growth medium as an electron acceptor [22, 24]. Park and Kim [26] suggested that ferric iron functions as an electron sink, but not as an electron acceptor, in which NADH may be reoxidized coupled to reduction of ferric iron outside the bacterial cell without free energy production. This could be an explanation of why the growth yield of *L. garvieae* sk11 in the presence of ferric iron was not increased. Another possibility about ferric iron reduction is the chemical reaction by bacterial metabolites produced by growing cells. The chemical reduction of ferric iron is difficult to measure by biochemical methods. The lactate/glucose ratio in the growth condition with electron acceptors can be balanced by using ferrous iron (1.491±0.055), succinate (14.37±0.62 mM), and nitrite (5.53±0.23 mM) metabolically reduced. Theoretically, 1.0 mol, 2.0 mol, and 2.0 mol of NADH are oxidized, coupled to reduction of 1.0 mol fumarate, ferric iron, and nitrate, respectively. On the basis of the NADH/electron acceptors balances, the theoretical lactate production can be calculated as follows: 28.98±1.22 mM (lactate) plus 14.37±0.62 mM (succinate) equals 43.35±1.84 mM in the presence of fumarate, 34.39±1.26 (lactate) plus double 1.66±0.06 (ferrous iron) equals 37.71±1.32 mM in the presence of ferric iron, and 25.84±1.04 mM (lactate) plus double 5.53±0.23 mM (nitrite) equals 36.9±1.27 mM in the condition with nitrate, which is close to the lactate (40.82±1.94 mM) produced in glucose basal medium without electron acceptor. However, the concentration of ferrous iron and nitrite metabolically produced was slightly lower in comparison with the lactate/glucose ratio. This is most likely due to a lower stability of ferrous iron and nitrite than succinate, specially as ferrous iron can be very easily oxidized by molecular oxygen. The respiration of lactic acid bacteria has been studied by molecular and proteomic approaches by several researchers. Vido *et al.* [42] reported the proteomic evidence on heme-dependent respiration of *L. lactis*, and Duwat *et al.* [6] suggested that *L. lactis* may grow and survive by respiratory metabolism in the presence of heme and oxygen, but oxygen has negative effects on both growth and survival in the absence of heme. Furthermore, Gaudu *et al.* [11] reported that CcpA (glycolytic gene expression regulator) may regulate aerobic and respiratory growth of *L. lactis*. Our present result obtained by physiological approaches supports these above-mentioned studies, and may provide an additional support to the respiratory metabolism of lactic acid bacteria.

Genetic Evidence of Respiratory Metabolism of *L. garvieae*

Growth yield and fumarate-nitrate reduction activity of *L. garvieae* sk11 are physiological evidences to demonstrate that it has specific enzymes capable of catalyzing the respiratory metabolism. Specifically, fumarate-nitrate reductase is known to be regulated by the *fnr* gene, which is expressed under anaerobic condition [12, 19]. To prove the presence of a gene encoding fumarate-nitrate reductase, we designed

seven primers on the basis of the *L. lactis* gene encoding FNR-like protein and the *E. coli fnr* gene whose full sequence has been reported [11, 35]. During the amplification of DNA with the seven *fnr* primers (Table 1), 5 products were obtained, and their sequence homology was analyzed using the GenBank database system. The DNA fragment amplified with the forward primer I was 94% identical with the 65th to 816th base of *L. lactis fnr* gene (AJ006750.1/LLA 006750) and that with the reverse primer I was 92% identical with the 2197th to 2938th base of *L. lactis fnr* gene, whereas the DNA fragment amplified with forward primer VII was 92% identical with the 975th to 1134th base of *E. coli nir* gene (J01608.1/ECONIRR) and that with reverse primer VII was 91% identical with the 916th to 1083rd base of *E. coli nir* gene. The *fnr* and *nir* sequences of *L. garvieae* sk11 were registered at GenBank with accession numbers of DQ485326 for *fnr* and DQ485327 for *nir*. These results provide simple genetic evidence to support the physiological results that *L. garvieae* sk11 can respire with nitrate and fumarate, which may be genetically related at least with other bacterial *fnr* [14, 15, 24, 32]. Based on the above physiological evidence of fumarate-nitrate reduction and genetic evidence of *fnr*, we suggest that *L. garvieae* living in soil may control respiratory and fermentative metabolism according to the environmental condition. It could be an advantageous function for energy production and competition with other bacteria.

Enzymatic Reduction of Ferric Iron to Ferrous Iron

Ferric iron can chemically be reduced to ferrous iron by organic acids such as oxalate, citrate, and ethylenediamine tetra acetic acid (EDTA) [21]. The chemical reduction of ferric to ferrous iron, therefore, cannot be excluded, when *L. garvieae* sk11 was growing under anaerobic condition. With no genetic evidence, the best solution to explain how ferric iron can be reduced in coupling to bacterial metabolism is to use an enzyme reaction. In the test with cell extract, 10.3 mM ferrous iron was produced per min and mg protein of cell extract in coupling to oxidation of 0.25 mM NADH (data not shown), indicating that *L. garvieae* sk11 may have a specific enzyme catalyzing the reduction of ferric iron to ferrous iron. However, it is not enough to definitely prove that ferric iron can metabolically be reduced, because the ferrous iron concentration is too low to prove biochemical reduction of ferric iron. Theoretically, a low concentration of ferric iron may be reduced by various coenzymes of cell extract under anaerobic condition: NADH added to the reaction mixture may induce the reduction reaction of various coenzymes. Under the same reaction condition, 0.25 mM nitrate and 0.16 mM fumarate were reduced per min and mg protein of cell extract, which had a tendency very similar to the reduction reaction of ferric iron, nitrate, and fumarate by the resting cells (Fig. 2).

Electrochemical Analysis of Metabolic Ferric Ion Reduction

A possibility for both the chemical and metabolic reductions of ferric iron cannot be excluded; however, it is difficult to prove by biochemical reaction with crude enzyme presentation. The electrochemical method may be a useful tool to measure the metabolic reduction of ferric iron, because ferric iron can be immobilized to the electrode and can mediate a redox reaction between the electrode and the bacterial cells [27, 28]. The ferrous iron of anode (redox potential=+0.1 volt) reduced in coupling to bacterial metabolism is reoxidized in coupling to reduction of H_2O_2 (redox potential=+0.9 volt) to H_2O on the cathode surface. Electrons can be driven from anode to cathode by the redox potential difference through an electric circuit line. The quantity of electrons driven from anode to cathode is the current value. As shown in Fig. 3, meaningful current production

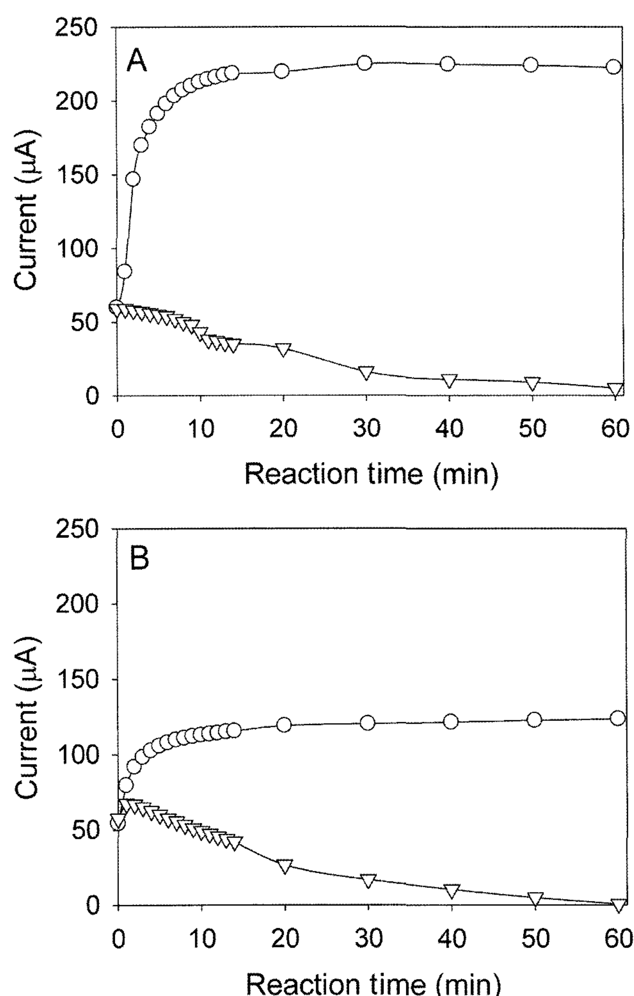

Fig. 3. Current production by resting cells of *L. garvieae* sk11 (**A**) and *W. kimchii* sk10 (**B**) in a two-compartment microbial fuel cell (MFC) composed of graphite anode modified with ferric ion (○) or native graphite anode (▽).

Commonly, native graphite electrode was used as the cathode.

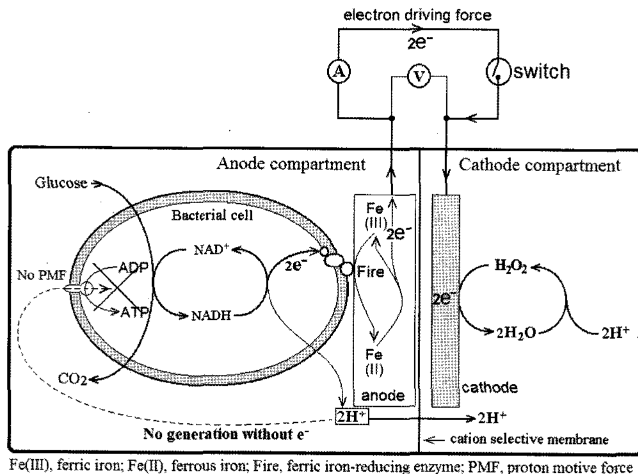

Fig. 4. Schematic structure of microbial fuel cell operated with *L. garvieae* sk11 or *W. kimchii* sk10 resting cells.
Ferric iron contained in the anode can be reduced to ferrous iron by enzyme catalysis and ferrous iron has to be reoxidized coupled to reduction of hydrogen peroxide. Ferrous iron (E=+0.1 volt) and hydrogen peroxide (E=+0.9 volt) function like electron donor and electron acceptor, respectively, by the redox potential (E) differences. A proton motive force cannot be generated because electrons are transferred from bacterial cells to ferric iron, in which proton has to be transferred into the cathode compartment.

was observed only in the MFC system with the combination of ferric anode and *L. garvieae* sk11, but not in the MFC system with the combination of anode and *L. garvieae* sk11, or anode and *W. kimchii* sk10. It is reasonable to assume that *L. garvieae* sk11 has a specific enzyme for metabolic reduction of ferric iron outside bacterial cells, and that electrons may be sunk outside bacterial cells through the ferric iron-reducing enzymes without free energy (ATP) production, as shown in Fig. 4. The current generated by *W. kimchii* was significantly lower than that by *L. garvieae* sk11 in MFC with ferric anode; however it was a little higher than that by *L. garvieae* sk11 or *W. kimchii* in MFC with anode. This shows a possibility that ferric ions immobilized to the graphite electrode may be chemically reduced by the metabolites produced by *W. kimchii*. The ferric iron reduction activity of *L. garvieae* sk11 was metabolically, enzymologically, and electrochemically tested and proven, but not proven genetically. The relationship between *fnr* and ferric iron-reducing enzyme of *L. garvieae* sk11 needs to be studied. Lascelles and Burke [20] reported that the electron carriers acting in the nitrate reductase complex function as a reductant for ferric iron reduction, and Sørensen [37] reported that bacterial ferric iron reduction is activated by addition of nitrate to anaerobic sediment.

In summary, *L. garvieae* sk11 can produce free energy by fermentation and anaerobic respiration with nitrate and fumarate as an electron acceptor, which may be controlled by *fnr* and *nir*. No evidence that *L. garvieae* can produce free energy coupled to ferric iron reduction was found. At present, we are in a process to purify the cytoplasmic membrane from cell extract to test its reduction activity of ferric iron. In future, the mechanism of ferric iron reduction will be studied enzymologically by using purified enzyme and with genetic basis.

References

1. Aguirre, M. and M. D. Collins. 1993. Lactic acid bacteria and human clinical infection. *J. Appl. Bacteriol.* **75:** 95–107.
2. Arnold, R. G., M. R. Hoffmann, T. J. DIChristina, and F. W. Picardal. 1990. Regulation of dissimilatory Fe(III) reduction activity in *Shewanella putrefaciens. Appl. Environ. Microbiol.* **56:** 2811–2817.
3. Chin, H. S., F. Breidt, H. P. Fleming, W. C. Shin, and S. S. Yoon. 2006. Identification of predominant bacterial isolates from the fermenting *kimchi* using ITS-PCR and partial 16S rDNA sequence analyses. *J. Microbiol. Biotechnol.* **16:** 68–76.
4. Coppi, M. V., R. A. O'Neil, and D. R. Lovley. 2004. Identification of an uptake hydrogenase required for hydrogen-dependent reduction of Fe(III) and other electron acceptors by *Geobacter sulfurreducens J. Bacteriol.* **186:** 3022–3028.
5. Dobbin, P. S., J. P. Carter, C. G. D. San Juna, M. von Hobe, A. K. Powell, and D. J. Richardson. 1999. Dissimilatory Fe(III) reduction by *Clostridium beijerincki* isolated from freshwater sediment using Fe(III) maltol enrichment. *FEMS Microbiol.* **176:** 131–138.
6. Duwat, P., S. Sourice, B. Cesselin, G. Lamberet, K. Vido, P. Gaud, Y. L. Loir, F. Violet, P. Loubiére, and A. Gruss. 2001. Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival. *J. Bacteriol.* **183:** 4509–4516.
7. Eldar, A., M. Goria, C. Ghittino, A. Zlotkin, and H. Bercovier. 1999. Biodiversity of *Lactococcus garvieae* strains isolated from fish in Europe, Asia and Australia. *Appl. Environ. Microbiol.* **65:** 1005–1008.
8. Elliot, J. A., M. D. Collins, N. E. Pigott, and R. R. Facklam. 1991. Differenctiation of *Lactococcus lactis* and *Lactococcus garvieae* from humans by comparison of whole-cell protein patterns. *J. Clin. Microbiol.* **29:** 2731–2734.
9. Facklam, R. R. and J. A. Elliot. 1995. Identification, classification and clinical relevance of catalase-negative, Gram-positive cocci, excluding the streptococci and entercoocci, *Clin. Microbiol. Rev.* **8:** 479–495.
10. Fefer, J. J., K. R. Ratzan, S. E. Sharp, and E. Saiz. 1998. *Lactococcus garvieae* endocarditis: Report of a case and review of the literature. *Diagn. Microbiol. Infect. Dis.* **32:** 127–130.
11. Gaudu, P., G. Lunberet, S. Ponet, and A. Grus. 2003. CcpA regulation of aerobic and respiration growth of *L. lactis. Mol. Microbiol.* **50:** 183–192.
12. Gostic, D. O., H. G. Griffin, C. A. Shearman, C. Scott, J. Green, M. J. Gasson, and J. R. Guest. 1999. Two operons that encode FNR-like proteins in *Lactococcus lactis. Mol. Microbiol.* **31:** 1523–1535.
13. Gottschalk, G. 1986. *Bacterial Metabolism*, 2nd Ed. pp. 208–224. Springer-Verlag, New York.

14. Grabbe, R., A. Kuhh, and R. A. Schmitz. 2001. Cloning, sequencing and characterization of Fnr from *Klebsiella pneumoniae*. *Antonie Van Leeuwenhoek* **79:** 319–326.
15. Hattori, T., K. Takahashi, T. Nakanishi, H. Ohta, K. Fukui, S. Taniguchi, and M. Takigawa. 1996. Novel FNR homologues identified in four representative oral facultative anaerobes: *Capnocytophaga ochracea*, *Capnocytophaga sputigena*, *Haemophilus aphrophilus*, and *Actinobacillus actinomycetemcomitans*. *FEMS Microbiol. Lett.* **137:** 213–220.
16. He, Q. and R. A. Sanford. 2003. Characterization of Fe(III) reduction by chlororespiring *Anaeromxyobacter dehalogenans*. *Appl. Envir. Microbiol.* **69:** 2712–2718.
17. Iuchi, S. and E. C. Lin. 1987. Molybdenum effector of fumarate reductase repression and nitrate reductase induction in *Escherichia coli*. *J. Bacteriol.* **169:** 3720–3725.
18. Jeon, S. J., I. H. Shin, B. I. Sang, and D. H. Park. 2005. Electrochemical regeneration of FAD by catalytic electrode without electron mediator and biochemical reducing power. *J. Microbiol. Biotechnol.* **15:** 281–286.
19. Jones, H. M. and R. P. Gunsalus. 1985. Transcription of the *Escherichia coli* fumarate reductase genes (frdABCD) and their coordinate regulation by oxygen, nitrate, and fumarate. *J. Bacteriol.* **164:** 1100–1109.
20. Lascelles, J. and K. A. Burke. 1978. Reduction of ferric iron by L-lactate and DL-glycerol-3-phosphate in membrane preparations from *Staphylococcus aureus* and interactions with the nitrate reductase system. *J. Bacteriol.* **134:** 585–589.
21. Lovely, D. R. and E. J. P. Phillips. 1986. Availability of ferric iron for microbial reduction in bottom sediments of the freshwater tidal Potomac River. *Appl. Envir. Microbiol.* **52:** 751–757.
22. McInerney, M. J. and P. S. Beaty. 1988. Novel mode of microbial energy metabolism: Organic carbon oxidation coupled to disimmilatory reduction of iron or manganese. *Appl. Environ. Microbiol.* **54:** 1472–1480.
23. Moat, A. G., J. W. Foster, and M. P. Spector. 2002. *Microbial Physiology*, 4th Ed. pp. 368–393. Wiley-Less. New York.
24. Ohmura, N., N. Matsumoto, K. Sasaki, and H. Saiki. 2002. Electrochemical regeneration of Fe(III) to support growth on anaerobic iron respiration. *Appl. Envir. Microbiol.* **68:** 405–407.
25. Overton, T., E. G. F. Reid, R. Foxall, H. Smith, S. J. W. Busby, and J. A. Cole. 2003. Transcription activation at *Escherichia coli* FNR-dependent promoters by the gonococcal FNR protein: Effects of a novel s18f substitution and comparisons with the corresponding substitution in *E. coli* FNR. *J. Bacteriol.* **185:** 4734–4747.
26. Park, D. H. and B. H. Kim. 2001. Growth properties of the iron-reducing bacteria, *Shewanella putrefaciens* IR-1 and MR-1, coupling to reduction of Fe(III) to Fe(II). *J. Microbiol.* **39:** 273–278.
27. Park, D. H. and J. G. Zeikus. 2002. Impact of electrode composition on electricity generation in a single-compartment fuel cell using *Shewanella putrefaciens*. *Appl. Microbiol. Biotechnol.* **59:** 58–61.
28. Park, D. H. and J. G. Zeikus. 2003. Improved fuel cell and electrode designs for producing electricity from microbial degradation. *Biotechnol. Bioeng.* **81:** 348–355.
29. Park, D. H., Y. K. Park, and E. S. Choi. 2004. Application of single-compartment bacterial fuel cell (SCBFC) using modified electrodes with metal ions to wastewater treatment reactor. *J. Microbiol. Biotechnol.* **14:** 1120–1128.
30. Park, S. M., H. S. Kang, and D. H. Park. 2004. Metabolic flux shift of *Weissella kimchii* sk10 grown under aerobic conditions. *J. Microbiol. Biotechnol.* **14:** 919–923.
31. Park, S. M., H. S. Kang, D. W. Park, and D. H. Park. 2005. Electrochemical control of metabolic flux of *Weissella kimchii* sk10: Neutral red immobilized in cytoplasmic membrane as electron channel. *J. Microbiol. Biotechnol.* **15:** 80–83.
32. Phillips, E. J. P. and D. R. Lovely. 1987. Determination of Fe(III) and Fe(II) in oxalate extracts of sediment. *Soil Sci. Soc. Am. J.* **51:** 938–941.
33. Reents, H., R. Münch, T. Dammeyer, D. Jahn, and E. Härtig. 2006. The *fnr* regulon of *Bacillus subtilis*. *J. Bacteriol.* **188:** 1103–1112.
34. Senko, J. M. and J. F. Stolz. 2001. Evidence for iron-dependent nitrate respiration in the dissimilatory iron-reducing bacterium *Geobacter metallireducens*. *Appl. Envir. Microbiol.* **67:** 3750–3752.
35. Shaw, D. J. and J. R. Guest. 1982. Nucleotide sequence of the *fnr* gene and primary structure of the *fnr* gene protein of *Escherichia coli*. *Nucleic Acids Res.* **10:** 6119–6130.
36. Shin, I. H., S. J. Jeon, H. S. Park, and D. H. Park. 2004. Catalytic oxidoreduction of pyruvate/lactate and acetaldehyde/ethanol coupled to electrochemical oxidoreduction of NAD^+/NADH. *J. Microbiol. Biotechnol.* **14:** 540–546.
37. Sørensen, J. 1982. Reduction of ferric iron in anaerobic, marine sediment and interaction with reduction of nitrate and sulfate. *Appl. Envir. Microbiol.* **43:** 319–324.
38. Spiro, S. and J. R. Guest. 1987. *E. coli fnr* gene encoding a transcriptional activator (FNR), 5'-flanking region. *J. Gen. Microbiol.* **133:** 3279–3288.
39. Sugio, T., C. Domatsu, O. Munakata, T. Tano, and K. Imai. 1985. Role of a ferric ion-reducing system in sulfur oxidation of *Thiobacillus ferrooxidans*. *Appl. Envir. Microbiol.* **49:** 1401–1406.
40. Teixeira, L. M., V. L. C. Mergquior, C. E. Vianni, M. G. S. Carvalho, S. E. L. Fracalanzza, A. G. Steigerwalt, D. J. Brenner, and R. R. Facklam. 1996. Phenotypic and genotypic characterization of atypical *Lactococcus garvieae* strains isolated from water buffalo with subclinical mastitis and confirmation of *L. garvieae* as a senior subjective synonym of *Enterocuoccus seriolicida*. *Int. J. Syst. Bacteriol.* **46:** 664–668.
41. Thaurer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **41:** 100–180.
42. Vido, K., D. I. Bars, M.-Y. Mistou, P. Anglade, A. Gruss, and P. Gaudu. 2004. Proteome analyses of heme-dependent respiration in *Lactococcus lactis*: Involvement of the proteolytic system. *J. Bacteriol.* **186:** 1648–1657.
43. Zlotskin, A., A. Eldar, C. Ghittino, and H. Bercovier. 1998. Identification of *Lactococcus garvieae* by PCR. *J. Clin. Microbiol.* **36:** 983–985.