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Metabolic Flux Shift of *Weissella kimchii* sk10 Grown Under Aerobic Conditions

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Abstract The sk10 isolated from kimchi was identified as *W. kimchii* on the basis of 16s-rDNA sequencing. Studies were made to analyze the metabolic flux shift of the sk10 on glucose under aerobic growth conditions. The sk10 produced 38.2 mM acetate, 16.3 mM ethanol, and 33.2 mM lactate under aerobic conditions, but 2.4 mM acetate, 48.0 mM ethanol, and 44.1 mM lactate under anaerobic conditions. The NADH peroxidase (NADH-dependent hydrogen peroxidase) activity of sk10 grown under aerobic conditions was 11 times higher than that under anaerobic conditions. Under the low ratio of NADH/NAD^+, the metabolic flux toward lactate and ethanol was shifted to the flux through acetate kinase without NADH oxidation. The kinds of enzymes and metabolites of sk10 were close to those in the pathway of *Leuconostoc* sp., but the metabolites produced under aerobic growth conditions were different from those of *Leuconostoc* sp. The stoichiometric balance calculated using the concentrations of metabolites and substrate was about 97%, coincident with the theoretical values under both aerobic and anaerobic conditions. From these results, it was concluded that the metabolic flux of *W. kimchii* sk10 was partially shifted from lactate and ethanol to acetate under aerobic conditions only.

Key words: *Weissella kimchii*, metabolic flux, NADH peroxidase, phosphoketolase pathway, heterofermentation

The genus *Weissella* was first proposed by Collins *et al.* [5] on the basis of the results of a 16s rRNA phylogenetic analysis and confirmed to be Gram-positive, non-spore-forming heterofermentative, and nonmotile, but the fermentative pathway was not known. Based on 16s rRNA sequencing studies, the *Weissella* species were separated from the genus *Leuconostoc* within the lactic acid bacteria [19, 20, 30]. *W. kimchii* was first reported by Choi *et al.* [2], who isolated the bacterium from kimchi, which is a Korean vegetable food fermented by various lactic acid bacteria.

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The metabolic pathway of *Weissella* sp. has been known to be similar to that of *Leuconostoc* sp., which has a phosphoketolase (PK) pathway. In the PK pathway, 3NADH and 2ATP are produced and 3NADH and 1ATP are consumed. Finally, the ATP yield is one per mol of glucose, which is half of that of the homofermentative pathway [8]. The major metabolites of heterofermentative lactic acid bacteria are lactic acid, ethanol, and CO_2, and those of genus *Bifidobacterium* are one lactic acid and two acetic acids without CO_2 production, but metabolic shifts leading to end products other than lactate (the so-called mixed-acid fermentation) have been observed under certain fermentation conditions, such as utilization of galactose as the sole carbon and energy source [26], carbohydrate limitation [27], or aerobic conditions [1, 6]. It has recently been pointed out that diminished rates of sugar metabolism lead to shifts from homolactic to mixed-acid fermentation, while rapid flux through the central pathways results in homolactic fermentation [4, 7, 9]. We discovered that the *W. kimchii* produces four metabolites, including those with CO_2 from glucose under both aerobic and anaerobic growth conditions, and also that the metabolite concentrations were different according to the growth conditions. In the present study, we estimated the growth and metabolites of *W. kimchii* sk10 grown under aerobic and anaerobic conditions, and try to analyze the metabolic flux using the stoichiometric calculation of metabolite production and substrate consumption [29].

Materials and Methods

Organism

The strain sk10 was isolated from kimchi and the organism was identified as *W. kimchii* by the homogeneity of its 16s-rDNA sequencing and sugar fermentation profile [3].

Cultivation and Growth

sk10 was cultivated on the modified MRS medium (Proteose peptone 10 g/l, beef extract 10 g/l, yeast extract 5 g/l, sorbitan monooleate 1 g/l, ammonium citrate 2 g/l, magnesium sulfate

0.1 g/l, manganese sulfate 0.05 g/l, disodium phosphate 2 g/l). The glucose was added to the medium, and the final concentration was adjusted to 80 mM after autoclaving. The pH of the medium was adjusted to 8.0 before autoclaving, and the bacterium was cultivated at 28–30°C. The bacterial growth was measured with a spectrophotometer (Jasco Mode V-550, Tokyo, Japan) using optical density at 660 nm. Two-and-a-half l/min of air and O_2-free N_2 was supplied to the bacterial culture and sparged in the growth medium for the maintenance of complete aerobic and anaerobic conditions, respectively.

Sugar Fermentation Test

API 50CHL strips (bioMérieux, France) were used to determine the sugar-fermentation patterns of sk10. The API test was performed in accordance with the manufacturer's directions.

Preparation of Crude Enzymes

The bacterium was cultivated for 20 h and then harvested by centrifugation at 5,000 ×*g* for 30 min at 4°C. The harvested cell was washed three times with 50 mM Tris-Cl buffer (pH 7.5) to which about 0.01 μg/g cell of lysozyme was added and frozen at −85°C for 24 h. The frozen cell was thawed at 4°C and the cell was frozen twice more and thawed out to damage peptidoglycan and cytoplasmic membrane. The cell damaged by freezing and lysozyme treatment was disrupted with ultrasonicator (model Vibra Cell Sonics & Materials Inc., Danbury, CT, U.S.A.) at 400w for 10 min at 4°C. The cell debris were removed by centrifugation at 10,000 ×*g* for 40 min at 4°C and supernatant was used as crude enzyme whose protein concentration was spectrophotometrically determined with Bradford reagent. The concentration was calculated using a standard calibration curve that was made previously with bovine serum albumin.

Enzyme Assay

The activities of the key enzymes working in the phosphoketolase pathway, glucose-6-phosphate dehydrogenase (G6PiDH) and 6-phosphogluconate dehydrogenase (6piGDH), were spectrophotometically measured at 340 nm using rates of NAD^+ reduction to NADH coupled to oxidation of G6Pi and 6piG. On the other hand, the activities of alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH), and NADH peroxidase were spectrophotometrically measured at 340 nm using rates of NADH oxidation to NAD^+ coupled to enzymatic reduction of pyruvate to lactate, acetaldehyde to ethanol, and H_2O_2 to H_2O, respectively [28]. The concentration of glucose-6-phosphate, 6-phosphogluconate, pyruvate, acetaldehyde, and NADH peroxide was adjusted to 10 mM and the concentrations of NAD^+ and NADH were adjusted to 1 mM and 400 μM, respectively. The specific activity was calculated using the difference of specific absorbance coefficient (ε=6.23 $mM^{-1}cm^{-1}$) of NADH between the starting point and the ending point of the linear-slopped graph obtained in the time-coursed reaction. The specific activity corresponds to the concentration of NADH in mg $protein^{-1}min^{-1}$ produced or consumed during enzyme reaction.

Analysis

Pyruvate, lactate, acetate, and ethanol were analyzed using an HPLC system (YoungLin system M925 pump, Seoul Korea) equipped with an RI detector (RI750F model) and Aminex HPX-87H ion-exchange column (Bio-Rad, Burlington, U.S.A.). The bacterial culture that was periodically separated from bacterial growing medium was centrifuged at 10,000 ×*g* for 30 min and filtrated with a membrane filter (pore size, 0.22 μM), which was used as a sample for analysis. The concentration was calculated using a standard calibration curve that was made previously. Gas (CO_2) production from glucose fermentation was only qualitatively determined using Durham tubes.

RESULTS AND DISCUSSION

Recent research on kimchi has been focused on the identification of the microflora autogenetically grown in kimchi during fermentation, and investigation of various factors that influence kimchi fermentation [10, 11, 12, 14, 16, 21, 25]. However, the physiological and metabolic properties of lactic acid bacteria isolated from kimchi have not been investigated. In particular, the metabolic pathway of *Weissella* sp. has been known to be similar to that of *Leuconostoc* sp., which is a heterofermentation bacterium and produces lactic acid, ethanol, and CO_2. We compared the sugar fermentation pattern and 16s-rDNA sequence of sk10 with other *Weissella* sp. The sugar fermentation pattern was completely coincident and the 16s-rDNA sequence 99.8% coincident with *W. kimchii* CHJ3, respectively [3], but its metabolic and physiological properties could not be compared with other *Weissella* sp. because the relative data have not been reported. The CO_2 production was qualitatively determined using the Durham tubes but not quantitatively measured. Figure 1 shows the growth and metabolites of sk10 grown under anaerobic and aerobic conditions. As shown in Fig. 1A, the bacterial growth was not influenced by O_2 but the metabolite concentration (Figs. 1B and 1C) was greatly shifted under aerobic growth conditions. Lactate and ethanol production was relatively increased by the time-coursed growing cells under anaerobic N_2 atmosphere but decreased under aerobic atmosphere, and a very small amount of acetate was produced under anaerobic conditions, which was about one-sixteenth of that produced under aerobic conditions, as shown in Table 1. The stoichiometric parameter in Table 1 was obtained from

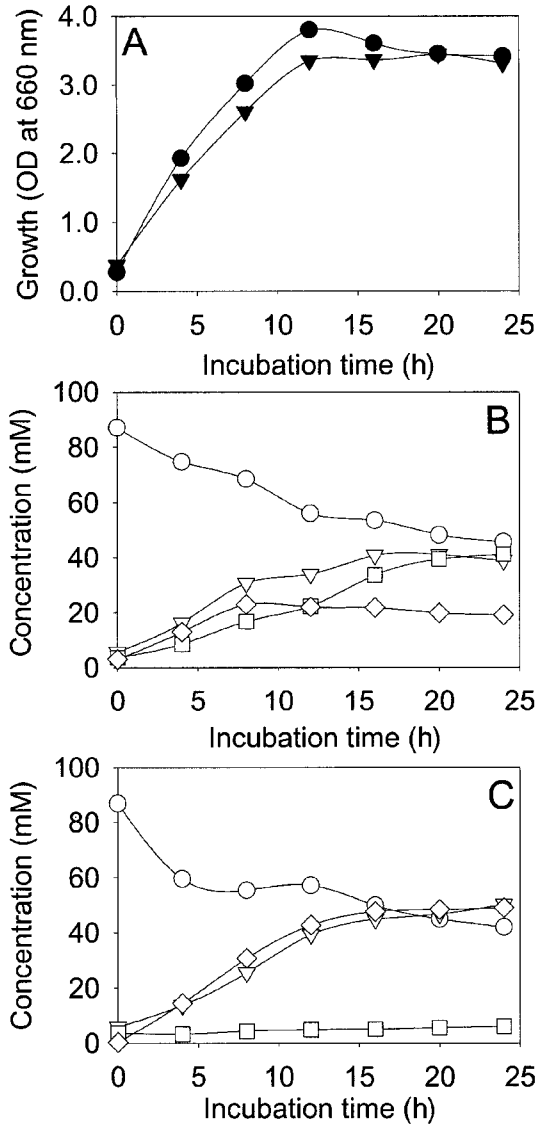

Fig. 1. Growth of *Weissella kimchii* sk10 (A) on MRS under aerobic (●) and anaerobic (▼) condition; lactate (▽), acetate (□), and ethanol (◇) production from glucose (○) fermentation of sk10 under aerobic (B) and anaerobic (C) condition.

calculation of the metabolites produced and substrate consumed by growing cells of sk10 cultivated for 24 h. On the basis of the parameters in Table 1 and the pathway of *Leuconostoc* sp., we were able to propose the pathway of *Weissella* sp., as shown in Fig. 2, and the hypothesis that O_2 may promote the metabolic flux from acetyl phosphate to acetate coupled to ATP production without NADH oxidation, by which the NADH/NAD^+ ratio and Y_{ATP} has to be increased. The metabolic flux in the higher ratio of the NADH/NAD^+ has to shift from acetate production to ethanol and lactate production coupled to NADH oxidation, but acetate production was increased under aerobic conditions, as shown in Table 1. Theoretically, the Y_{sub} of a bacterium

Table 1. Fermentation parameters of the growing cell of *Weissella kimchii* on MRS under aerobic and anaerobic conditions. The bacterial cells were cultivated for 24 h.

Fermentation parameters	Growth condition	
	Aerobic grown	Anaerobic grown
Glucose consumption (mM)	42.8	45.9
Lactate production (mM)	33.2	44.1
Acetate production (mM)	38.2	2.4
Ethanol production (mM)	16.3	48.0
Total growth (g cell/l)	1.246	1.130
Carbon recovery (%)[a]	102.5	102.9
Y_{ATP} (mol ATP/mol substrate)[b]	1.44	0.97
Y_{sub} (g dcw/mol substrate)[c]	29.1	24.6
Ratio of Y_{sub}/Y_{ATP}	20.2	25.36
NADH/NAD^+[d]	2.63	0.95

[a]Carbon recovery: (sum of metabolites)÷(glucose consumption×2)×100.
[b]1 ATP/1 acetate production, 2 ATP/1 lactate production, 1 ATP/1 glucose consumption.
[c]g cell of dry cell weight (dcw)/mol substrate consumption.
[d]2 NADH/1 glucose consumption, 2 NADH/1 ethanol production.

has to be proportional to the Y_{ATP}, but the ratio of Y_{sub} (29.1 g cell/mol glucose)/Y_{ATP} (1.44 mol/mol glucose) under aerobic conditions was relatively lower than that of Y_{sub} (24.6 g cell/mol glucose)/Y_{ATP} (0.97 mol /mol glucose) under anaerobic conditions, as shown in Table 1. The carbon recovery was 102.5% and 102.9 % under aerobic and anaerobic conditions, respectively, which is very close to the theoretical value of 100%. This difference of the ratio of Y_{sub}/Y_{ATP} between aerobic and anaerobic growth conditions is a clue that a part of ATP may be wasted due to the removal of inhibitory factors such as hydrogen peroxide produced under only aerobic conditions. Lopez *et al.* [17] have reported that shifts of homolactic fermentation toward mixed-acid fermentation have been observed at high imposed glycolytic fluxes during metabolism under aerobic conditions. We compared the specific activities of crude enzymes G6piDH, 6piGDH, LDH, ADH, and NADH peroxidase extracted from sk10 grown under anaerobic and aerobic conditions. As shown in Table 2, the specific activity of NADH peroxidase under aerobic conditions was 11 times higher than under anaerobic conditions, whereas the activities of the other enzymes were no more than twice as high or even similar to each other regardless of the growth condition. From these results, we suggest that crude enzymes other than NADH peroxidase were not influenced, or only slightly influenced, by O_2, that at least 11 times as much H_2O_2 was produced under aerobic conditions, and that the dissipated H_2O_2 was coupled to both NADH oxidation and ATP consumption. Direct NADH oxidation coupled to H_2O_2 reduction causes a reduction in the metabolic flux toward lactate via LDH, and ethanol via ADH and ALDH, as shown in Fig. 2. The ratio of NADH/NAD^+, used as

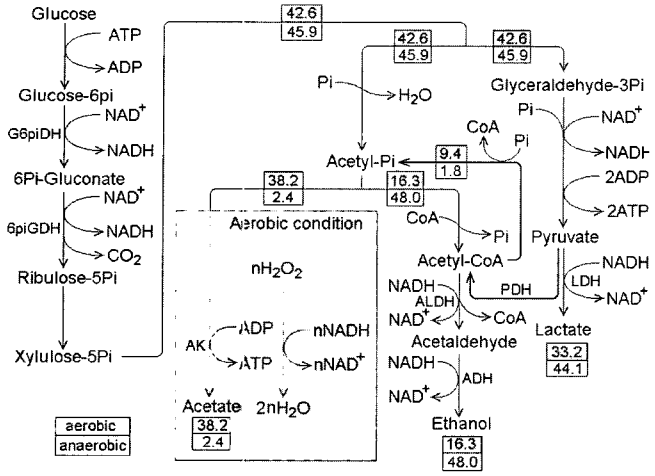

Fig. 2. Proposed glucose fermentation pathway using the flux balance of *Weissella kimchii* based on the kinds and concentration of metabolites produced from glucose under aerobic and anaerobic growth conditions.

The carbon balances for aerobic and anaerobic growth of *Weissella kimchii* sk10 are 102.5% and 102.9%, respectively. The calculation for metabolic flux balancing is performed by the following equation under aerobic condition: [glucose (42.6×2=85.2) / metabolites (38.2+16.3+33.2=87.7)], [acetate+ethanol (38.2+16.3=54.5) / (42.6+9.4=52.0)], and [lactate (33.2)= 42.6- 9.4]; under anaerobic condition: [glucose (45.9×2=91.8) / metabolites (2.4+48.0+44.1=94.5)], [acetate+ethanol (2.4+48.0=50.4) / (45.9+1.8= 47.7)], and [lactate (44.1)=45.9- 1.8]. G6piDH: Glucose-6pi dehydrogenase; 6piGDH: 6pi-gluconate dehydrogenase; ALDH: aldehyde dehydrogenase; ADH: alcohol dehydrogenase; PDH; pyruvate dehydrogenase; AK: acetate kinase.

an indicator of the redox state of the cells, was directly affected, at the expense of oxygen, by the NADH peroxidase activity, which mainly determined the observed shift of metabolic flux [7, 15]. The ratio of NADH/NAD$^+$ was 2.63 and 0.95, calculated from the metabolic balance of sk10 grown aerobically and anaerobically, respectively, as shown in Table 1. The main effect of overproducing the NADH peroxidase was an observed decrease in the NADH/NAD$^+$ ratio under aerobic conditions [13, 18]. This decrease could lead to high acetate production as a result of pyruvate dehydrogenase activity which catalyzes pyruvate oxidation to acetyl-CoA (thick line of pathway in Fig. 2), since this enzyme complex has been reported to be very sensitive to a high NADH/NAD$^+$ ratio [23, 24]. As shown in Fig. 2, for dissipation of H_2O_2 under aerobic conditions, the bacterium has to consume NADH produced in the glycolytic pathway (from glucose-6pi to ribulose-5pi), by which the NADH/NAD$^+$ ratio has to be decreased and additional ATP produced via the pathway from acetyl phosphate to acetate without oxidoreduction of NAD$^+$, which cannot be observed in growing cells under anaerobic conditions. Judging by the enzyme activity isolated from sk10, the *Weissella* sp. is thought to ferment sugar by the PK pathway, like *Leuconostoc* sp., as shown in Fig. 2. Under aerobic conditions, however, the metabolic flux of pyruvate toward lactate may shift to one toward acetate, and the decrease of NADH/NAD$^+$ ratio may cause metabolic flux shift from ethanol to acetate fermentation. Theoretically, the lactate and ethanol concentration has to be the same or very similar in the PK pathway, and lactate and ethanol production by *W. kimchii* sk10 was not greatly different under anaerobic conditions. Under aerobic condition, however, both lactate and ethanol productivities were decreased and the shift in the metabolic flux from ethanol toward acetate became more pronounced, as shown in Fig. 2. This discovery demonstrated that metabolic engineering on the level of oxidation of the key cofactor NADH can change *W. kimchii* from *Leuconostoc*-like metabolism to the higher acetic acid-producing bacterium. The observed rerouting of its metabolism toward acetate production by aeration is clearly more ineffective than the previously reported electrochemical control system [22]. Induction of activity of the overproduced NADH peroxidase by aeration changed pyruvate reduction metabolism to oxidized metabolism. Metabolic flux shift aimed at modulating key cofactors such as NADH could be a more effective way to understand bacterial fermentative metabolism, and lead to improvements in the methods used for bacterial metabolite production.

Table 2. Specific activities of key enzymes functioning in fermentative metabolism of *Weissella kimchii*, which were measured coupled to NADH oxidation or NAD$^+$ reduction.

Enzymes	Specific activity (mM mg protein^{-1} min^{-1})	
	Aerobic grown	Anaerobic grown
Glucose-6-pi dehydrogenase	0.64	1.147
6-pi-gluconate dehydrogenase	0.603	0.69
Lactate dehydrogenase	7.85	7.58
Alcohol dehydrogenase	1.78	2.78
NADH peroxidase	0.55	0.05

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