J. Microbiol. Biotechnol. (2004), **14**(5), 914–918

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Biocatalytic Oxidation-Reduction of Pyruvate and Ethanol by *Weissella kimchii* sk10 Under Aerobic and Anaerobic Conditions

KANG, HYE SUN, SUN MI PARK, AND DOO HYUN PARK*

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

Received: September 8, 2003
Accepted: December 13, 2003

Abstract This study was carried out to analyze the metabolic flux of *W. kimchii* sk10 on pyruvate and ethanol as a carbon source. The sk10 grown on ethanol produced acetate under aerobic conditions rather than under anaerobic conditions. The lactate and acetate were produced on ethanol plus pyruvate by the sk10 grown under aerobic and anaerobic conditions, respectively. The resting cell of sk10 produced 99.1 mM acetate and 17.3 mM lactate under aerobic conditions and 51.1 mM acetate and 62.4 mM lactate under anaerobic conditions from ethanol plus pyruvate, respectively. This result is thought to be due to the difference in the NADH/NAD^+ ratio depending on the growth conditions. The 11-fold overproduction of NADH peroxidase results in a low NADH/NAD^+ ratio under aerobic growth conditions. At the low NADH/NAD^+ ratio, the metabolic flux of pyruvate toward lactate has to be shifted to a flux toward acetate without NADH oxidation to NAD^+, and ethanol oxidation to acetate coupled to NAD^+ reduction to NADH has to be activated.

Key words: *Weissella kimchii*, NADH/NAD^+ ratio, metabolic flux, heterofermentation, NADH peroxidase

The genus *Weissella* has been isolated from various sources such as silage fermentation reactor [1], human feces [2], fermented vegetable food [3, 8, 10], and fermented sausages [4]. The genus *Weissella* was first proposed by Collins *et al.* [4] on the basis of 16s rRNA phylogenetic analysis by which the *Weissella* sp. was classificationally separated from the genus *Leuconostoc* within the lactic acid bacteria [4, 13, 14, 21, 22]. Kang *et al.* [7] reported that *W. kimchii* sk10 produced more acetate than lactate from glucose under aerobic conditions, but more lactate than acetate from glucose under anaerobic conditions. Recently, it was reported that sugar metabolism in *L. lactis* may be manipulated by controlling NADH oxidase activity at the level of the central intermediate pyruvate [6, 16]. We expect that a different level of pyruvate flux redistribution may be obtained as a result of the shift of the NADH/NAD^+ ratio in the metabolism of *W. kimchii* sk10. In this paper, we describe a new strategy for modifying the metabolic flux of *W. kimchii* by changing its growth conditions and carbon source. This modification was performed by overproduction of NADH peroxidase activity. We cultivated the *W. kimchii* sk 10 on modified MRS with pyruvate, ethanol, or ethanol plus pyruvate as a carbon source under aerobic and anaerobic conditions, respectively. We measured carbon source consumption, metabolite production, growth yield, and NADH/NAD^+ ratio for the calculation of the metabolic flux balancing.

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

Materials and Methods

Chemicals

All the chemicals used in this experiment were of reagent grade, and all the individual experiments were repeated two to three times with identical results.

Organism

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

Cultivation and Growth

sk10 was cultivated on 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 initial concentration of pyruvate and ethanol was adjusted to about 80 and 150 mM, respectively, and added to the medium after autoclaving. The pH of the medium was adjusted to 8.0 before autoclaving and the bacterium was cultivated at 28°C. The bacterial growth was measured

using optical density at 660 nm with a spectrophotometer (Jasco Mode V-550, Tokyo, Japan). Two-and-a-half l/min of air and O_2-free N_2 was supplied to the bacterial culture and sparged in growth medium for the maintenance of complete aerobic and anaerobic conditions, respectively. The resting cell was aseptically prepared by centrifugation of 16 h-old culture of sk10 at 5,000 ×*g* and 4°C for 30 min and resuspending in fresh medium with pyruvate or ethanol. The optical density of the resting cell was adjusted to 10 at 660 nm.

Preparation of Crude Enzymes

The bacteria were cultivated for 20 h and then harvested by centrifugation at 5,000 ×*g* for 30 min at 4°C. The harvested cells were 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 cells were thawed at 4°C and the cells were frozen twice more and thawed to damage the peptidoglycan and cytoplasmic membrane. The cells damaged by freezing and lysozyme treatment were disrupted with an 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 the 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 previously made with Bovine serum albumin.

Enzyme Assay

The alcohol dehydrogenase (ADH), lactate dehydrogenase (LDH) and NADH peroxidase activities were spectrophotometrically measured at 340 nm using rates of NADH oxidation to NAD^+ coupled to enzymatic reductions of pyruvate to lactate, acetaldehyde to ethanol, and H_2O_2 to H_2O, respectively [16]. The concentration of all the substrates (pyruvate, acetaldehyde, and hydrogen peroxide) was adjusted to 10 mM and the concentration of NADH was adjusted to 400 μM. The specific activity was calculated by the difference of specific absorbance coefficient (ε= 6.23 $mM^{-1}cm^{-1}$) of NADH between the starting and ending points of the linear-slopped graph obtained in a time-coursed reaction. The specific activity corresponds to the concentration of NADH in mg $protein^{-1}min^{-1}$ produced or consumed during the 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.). Bacterial culture was periodically separated from the bacterial growing medium, and centrifuged at 10,000 ×*g* for 30 min, filtrated with a membrane filter (pore size, 0.22 μM), and used as a sample for analysis. The concentration was calculated using a standard calibration curve that had been made previously. Gas (CO_2) production was determined using Durham tubes.

Results and Discussion

Generally, homolactic fermentation bacteria produce only lactate from sugar, but heterolactic fermentation bacteria produce two or three metabolites such as lactate, ethanol, acetate, or CO_2.

We proposed the metabolic pathway of *W. kimchii* sk10 on the basis of the phosphoketolase (PK) pathway, which was found in *Leuconostoc* sp. The major metabolites of the PK pathway are ethanol, lactate, and CO_2, but *W. kimchii* sk10 was reported to produce lactate, ethanol, acetate, and CO_2 by Kang *et al.* [7]. In homolactic and heterolactic fermentation, pyruvate is a central intermediate from sugar to metabolites. It has been known that the pyruvate may be oxidized to acetate or reduced to lactate under some specific growth conditions such as carbohydrate limitation [18], aerobic conditions [1, 5], and utilization of a sugar other than glucose as a carbon source [19]. We cultivated the *W. kimchii* sk10 on pyruvate and ethanol instead of glucose under anaerobic or aerobic conditions. We expected that sk10 would metabolically oxidize pyruvate to acetate coupled to pyruvate reduction to lactate under anaerobic conditions; however, sk10 was not grown on pyruvate under both anaerobic and aerobic conditions (data not shown) [9, 11]. When ethanol was used as the sole carbon source, sk10 did not grow and did not produce metabolite under anaerobic conditions, as shown in Figs. 1A and 1B, but it grew minutely and produced 5 mM acetate under aerobic conditions as shown in Figs. 1A and 1C. However, the growth of sk10 on ethanol plus pyruvate increased about three-fold compared to the growth on ethanol under both anaerobic and aerobic conditions, as shown in Fig. 1D. The sk10 grown on ethanol plus pyruvate produced 13.7 mM lactate and 8.9 mM acetate under anaerobic conditions as shown Fig. 1E, and produced 3.9 mM lactate and 20.8 mM acetate under aerobic conditions as shown in Fig. 1F. After 6 h incubation, sk10 did not grow anymore (Figs. 1A and 1D) but continuously produced metabolites. It is thought that the *W. kimchii* without gluconeogenesis pathway cannot synthesize the building blocks from ethanol or pyruvate, but can maintain energy metabolism for the production of ATP and NAD(P)H coupled to oxidation of ethanol to acetate. Table 1 shows the concentrations of the carbon sources consumed and the metabolites produced by the growing cells of sk10 at 24 h incubation time. As shown in Table 1, about 15% and 85% of the metabolites produced by sk10

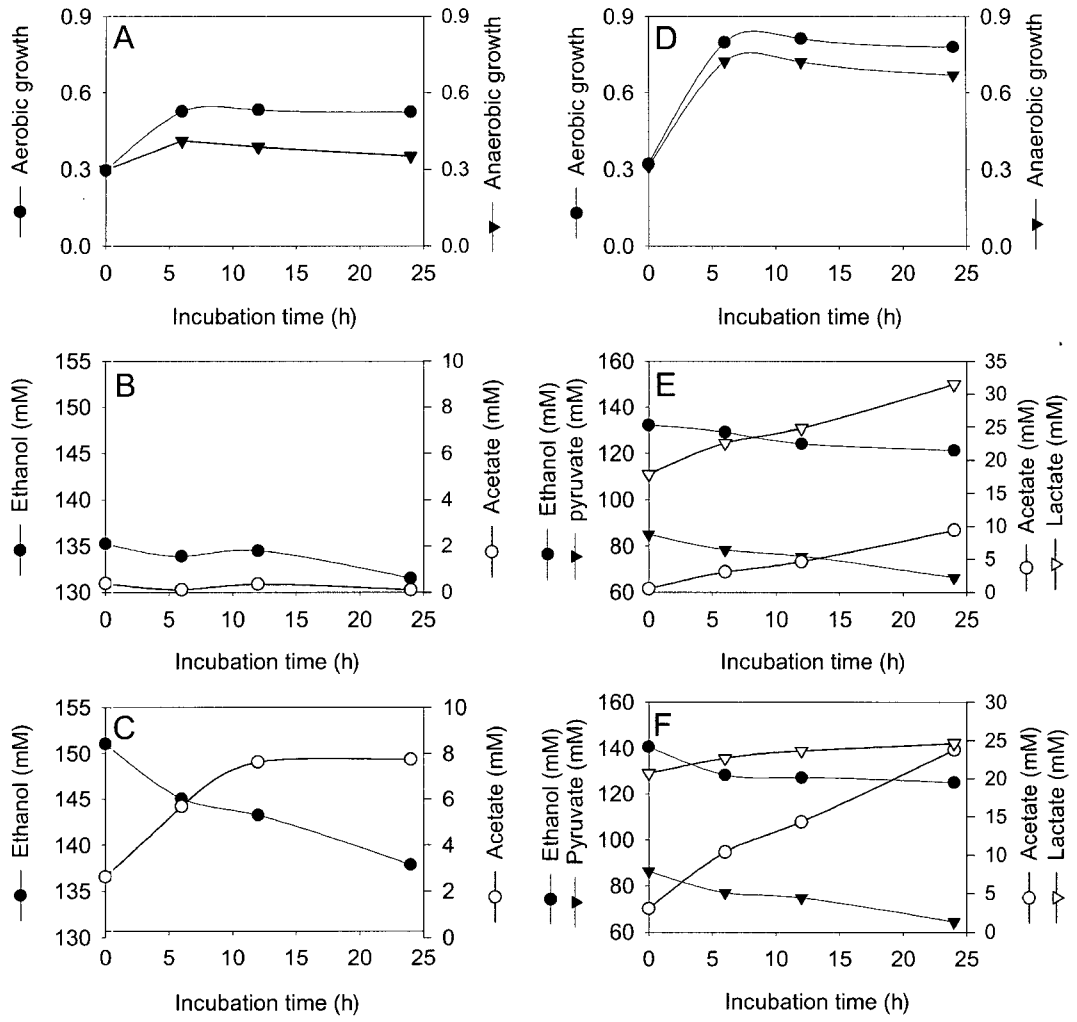

Fig. 1. Growth of *Weissella kimchii* on modified MRS medium with ethanol (A) and ethanol plus pyruvate (D); acetate production on the modified medium with ethanol under anaerobic (B) and aerobic (C) conditions, and acetate and lactate production on the modified medium with ethanol plus pyruvate under anaerobic (E) and aerobic (F) conditions.

were lactate and acetate, respectively, under aerobic conditions, and about 65% and 35% of the metabolites were lactate and acetate, respectively, under anaerobic conditions. The carbon recovery calculated with carbon source consumption and metabolites production was 65.5% and 76.1% under aerobic and anaerobic conditions, respectively. These results are appropriate for the analysis of the metabolic flux shift and the calculation of the stoichiometric balance of *W. kimchii* sk10 grown on ethanol plus pyruvate, because the carbon recovery was greatly different from the theoretical value (100%), but provided a clue that whereas the growing cell of sk10 can metabolically oxidize ethanol to acetate coupled to reduction of pyruvate to lactate, it cannot oxidize pyruvate to acetate coupled to reduction of pyruvate itself to lactate. This is thought to be due to the insufficient biomass of the growing cell, which makes it incapable of consuming most of, or all of, the carbon sources during growth. One solution for taking sufficient biomass is to use the resting cell aseptically harvested from the culture of sk10. We used as a biocatalyst the high-density resting cell (OD 10 at 660 nm), which is difficult to grow further on medium without glucose, but can produce ATP and NADH by the metabolic oxidation-reduction of pyruvate and ethanol. The pyruvate added to the medium was completely consumed by the resting cells of sk10 in 24 h incubation, and the carbon recovery was very close to 100%. As shown in Table 2, about 80% pyruvate and 100% ethanol were oxidized to acetate coupled to reduction of 20% pyruvate to lactate, but about 20% pyruvate and 100% ethanol were oxidized to acetate coupled to reduction of 80% pyruvate to lactate under anaerobic conditions. Under both anaerobic and aerobic conditions, the ethanol added to the medium was completely oxidized. The redox balance under aerobic and anaerobic conditions was 120.9 and 20.7, respectively. The key enzymes LDH and ADH for lactate and ethanol fermentations, respectively, were not influenced by O_2, but

Table 1. Fermentation parameters of the growing cell of *Weissella kimchii* on modified MRS, with ethanol plus pyruvate as the sole carbon source instead of glucose, under aerobic and anaerobic conditions.

Fermentation parameters	Growth condition	
	Aerobic grown	Anaerobic grown
Pyruvate consumption (mM)	22.1	18.7
Ethanol consumption (mM)	15.6	11.0
Lactate production (mM)	3.9	13.7
Acetate production (mM)	20.8	8.9
Total cell mass (g cell/l)	0.162	0.078
Carbon recovery (%)[a]	65.5	76.1

[a] (production of acetate and lactate) ÷ (consumption of pyruvate and ethanol)×100.

the NADH peroxidase activity of sk10 grew under aerobic conditions and it was about 11 times higher than under anaerobic conditions, as shown in Table 3. From these results, we calculated the balance of the NADH/NAD^+ ratio on the basis of the metabolite concentration and it can be described as follows: about 87% (120.9/138.2) of NADH produced from the metabolic oxidation of ethanol and pyruvate may be reoxidized to NAD^+ for dissipation of H_2O_2 or biosynthesis. In addition, about 13% (17.3/183.2) of NADH may be reoxidized for pyruvate reduction to lactate under aerobic conditions, but about 25% (20.7/83.1) of that may be reoxidized to NAD^+ for dissipation of inhibitor or biosynthesis, and 75% (62.4/83.1) of NADH may be reoxidized to NAD^+ for pyruvate reduction to lactate under anaerobic conditions. The reoxidation of NADH coupled to reduction of H_2O_2 to H_2O results in the lower NADH/NAD^+ ratio by which the metabolic flux of pyruvate towards lactate via LDH has to be diminished. The NADH/NAD^+ ratio used as an indicator of the redox

Table 2. Fermentation parameters of the resting cell of *Weissella kimchii* on modified MRS, with ethanol and pyruvate as a carbon source instead of glucose, under aerobic and anaerobic conditions.

Fermentation parameters	Growth condition	
	Aerobic grown	Anaerobic grown
Pyruvate consumption (mM)	79.1	79.3
Ethanol consumption (mM)	38.2	31.3
Lactate production (mM)	17.3	62.4
Acetate production (mM)	99.1	51.1
Redox balance[a]	138.2-17.3=120.9	83.1-62.4=20.7
Resting cell mass (g cell/l)	3.28	3.28
Carbon recovery[b] (%)	99.1 %	102.6%

[a] (NADH produced coupled to oxidation of carbon sources)-(NADH consumed coupled to reduction of pyruvate).

[b] (production of acetate and lactate) ÷ (consumption of pyruvate and ethanol)×100.

Table 3. Specific activities of key enzymes functioning in fermentative metabolism of *Weissella kimchii*, which were measured coupled to NADH oxidation.

Enzymes	Specific activity (mM mg protein^{-1} min^{-1})	
	Aerobic grown	Anaerobic grown
Lactate dehydrogenase	7.85	7.58
Alcohol dehydrogenase	1.78	2.78
Hydrogen peroxidase	0.55	0.05

balance in the metabolic pathway is directly affected by the NADH peroxidase activity at the expense of oxygen, which mainly determines the metabolic flux shift, as shown in Fig. 2 [12]. This result could only be caused by the NADH oxidation catalyzed by the overproduced NADH peroxidase under aerobic conditions, as seen by the decrease in lactate production. The NADH peroxidase (Table 3) had an effect on the acetate production by which the flux through ADH and ALDH is activated coupled to NADH reduction. The higher NAD^+/NADH ratio could lead to high acetate production by inhibition of pyruvate dehydrogenase (PDH) activity, since this enzyme complex has been reported to be very sensitive to a high NAD^+/

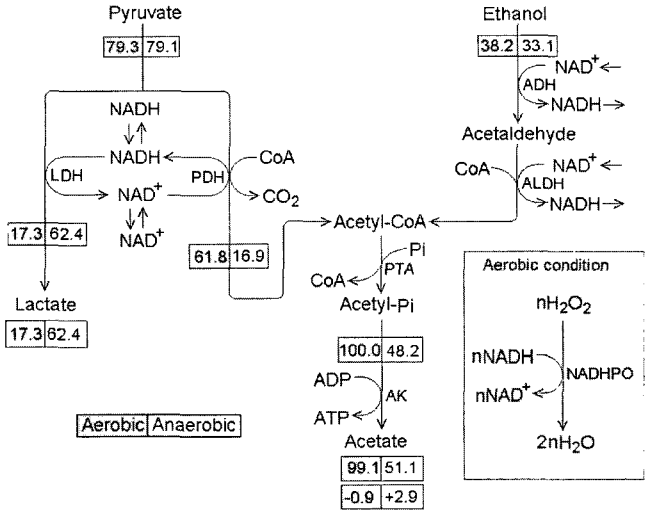

Fig. 2. Proposed pyruvate and ethanol metabolism of resting cell of *Weissella kimchii* based on the species and concentration of metabolites produced from pyruvate and ethanol under aerobic and anaerobic conditions.

The resting cell was used as a biocatalyst, which was harvested from cultivated cell for 16 h and cell density adjusted to 10 at OD_{660}. The theoretical values 100 and 48.2 are the sum of 61.8 and 38.2 and 16.9 and 31.3, respectively, which are slightly different from experimental values 99.1 and 51.1, and the final balance difference is -0.9 and +2.9 under aerobic and anaerobic conditions, respectively. The difference between theoretical and experimental values are within standard deviation obtained from 5 time-repeated tests. LDH: lactate dehydrogenase; PDH: pyruvate dehydrogenase; PTA: phosphotransacetylase; AK: acetate kinase; ADH: alcohol dehydrogenase; ALDH: aldehyde dehydrogenase; NADHPO: NADH peroxidase.

NADH ratio [16, 17]. It is possible that, with the exception of O_2 or H_2O_2, any oxidant influence on the NADH/NAD^+ ratio, such as the bioelctrochemical engineering, may be a cause of metabolic flux shift. Other new methods for control of metabolic flux are under development in our laboratory using the bioelectrochemical oxidation-reduction of electron mediators such as neutral red [15]. This is thought to be a useful tool for controlling the sugar metabolism of heterofermentation bacteria by control of the NADH/NAD^+ ratio without the application of bacterial mutation, genetic recombination, chemical oxidant, or chemical reductant [20].

Acknowledgment

This research was supported by a grant from the research program of e-Biotech (Co. Ltd.).

REFERENCES

1. Anders, R. F., D. M. Hogg, and G. R. Jago. 1970. Formation of hydrogen peroxide by group N streptococci and its effect on their growth and metabolism. *Appl. Microbiol.* **19:** 608-612.
2. Cai, Y., Y. Benno, M. Ogawa, S. Ohmomo, S. Kimura, and T. Nakase. 1988. Influence of *Lactobacillus* spp. from an inoculant and of *Weissella* and *Leuconostoc* spp. from forage crops on silage fermentation. *Appl. Environ. Microbiol.* **64:** 2982-2987.
3. Choi, H. J., C. I. Cheigh, S. B. Kim, J. C. Lee, D. W. Lee, S. W. Choi, J. M. Park, and Y. R. Pyun. 2002. *Weissella kimchii* sp. nov., a novel lactic acid bacterium from kimchi. *Int. J. Syst. Evol. Microbiol.* **52:** 507-511.
4. Collins, M. D., J. Samelis, J. Metaxopoulos, and S. Wallbanks. 1993. Taxonomic studies on some *Leuconostoc*-like organisms from fermented sausages: Description of a new genus *Weissella* for the *Leuconostoc paramesenteroides* group of species. *J. Appl. Bacteriol.* **75:** 595-603.
5. Condon, S. 1987. Responses of lactic acid bacteria to oxygen. *FEMS Microbiol. Rev.* **46:** 269-280.
6. de Vos, W. M. 1996. Metabolic engineering of sugar catabolism in lactic acid bacteria. *Antonie van Leeuwenhoek* **70:** 223-242.
7. Kang, H. S., S. M. Park, and D. H. Park. 2003. Growth and metabolic properties of *Weissella kimchii* sk10 under aerobic and anaerobic condition. *J. Microbiol. Biotechnol.* (in press).
8. Kim, T. W., S. H. Jung, J. Y. Lee, S. K. Choi, S. H. Park, J. S. Jo, and H. Y. Kim. 2003. Identification of lactic acid bacteria in kimchi using SDS-PAGE profiles of whole cell proteins. *J. Microbiol. Biotechnol.* **13:** 119-124.
9. Lee, J. H., J. H. Kim, M. R. Kim, S. M. Lim, S. W. Nam, J. W. Lee, and S. K. Kim. 2002. Effect of dissolved oxygen concentration and pH on the mass production of high molecular weight pullulan by *Aureobasidium pullulans*. *J. Microbiol. Biotechnol.* **13:** 1-7.
10. Lee, J. S., K. C. Lee, J. S. Ahn, T. I. Mheen, Y. R. Pyun, and Y. H. Park. 2002. *Weissella koreensis* sp. nov., isolated from kimchi. *Int. J. Syst. Evol. Microbiol.* **52:** 1257-1261.
11. Lee, J. W., A. Goel, M. M. Ataai, and M. M. Domach. 2002. Flux regulation patterns and energy audit of *E. coli* B/r and K-12. *J. Microbiol. Biotechnol.* **12:** 268-272.
12. Lopez deFelip, F., M. Llerebezem, W. M. de Vos, and J. Hugenholtz. 1998. Cofactor engineering: A novel approach to metabolic engineering in *Lactococcus lactis* by controlled expression of NADH oxidase. *J. Bacteriol.* **180:** 3804-3808.
13. Martinez-Murcia, A. J. and M. D. Collins. 1990. A phylogenetic analysis of the genus *Leuconostoc* based on reverse transcriptase sequencing of 16s rRNA. *FEMS Microbiol. Lett.* **70:** 73-83.
14. Martinez-Murcia, A. J. and M. D. Collins. 1991. A phylogenetic analysis of an atypical leuconostic: Description of *Leuconostoc fallax* sp. nov. *FEMS Microbiol. Lett.* **82:** 55-59.
15. Park, D. H. and J. G. Zeikus. 1999. Utilization of electrically reduced neutral red by *Actinobacillus succinogenes*: Physiological function of neutral red in membrane-driven fumarate reduction and energy conservation. *J. Bacteriol.* **181:** 2403-2410.
16. Snoep, J. L., M. J. Teixeira de Mattos, and O. M. Neijsse. 1991. Effect of the energy source on the NADH/NAD ratio and on pyruvate catabolism in anaerobic chemostat cultures of *Enterococcus faecalis* NCTC 775. *FEMS Microbiol. Lett.* **81:** 63-66.
17. Snoep, J. L., M. J. Teixeira de Mattos, M. J. C. Starrenburg, and J. Hugenholtz. 1992. Isolation, characterization and physiological role of the pyruvate dehydrogenase complex and α-acetolactate synthase of *Lactococcus lactis* subsp. *lactis* var. deiacetylactis. *J. Bacteriol.* **174:** 4838-4841.
18. Thomas, T. D., D. C. Ellwood, and V. M. C. Longyear. 1979. Change from homo- to heterolactic fermentation by *Streptococcus lactis* resulting from glucose limitation in anaerobic chemostat cultures. *J. Bacteriol.* **138:** 109-117.
19. Thomas, T. D., K. W. Turner, and V. L. Crow. 1980. Galactose fermentation by *Streptococcus lactis* and *Streptococcus cremoris*: Pathways, products and regulation. *J. Bacteriol.* **144:** 642-682.
20. Van der Werf, M. J., M. V. Guettler, M. K. Jain, and J. G. Zeikus. 1997. Environmental and physiological factors affecting the succinate product ratio during carbohydrate fermentations by *Actinobacillus* sp. 130Z. *Arch. Microbiol.* **167:** 332-342.
21. Walter, J., C. Hertel, G. W. Tannock, C. M. Lis, K. Munro, and W. P. Hammes. 2001. Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc* and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **67:** 2578-2585.
22. Yang, D. and C. R. Woese. 1989. Phylogenetic structure of the "leuconostoc": An interesting case of a rapidly evolving organism. *Syst. Appl. Microbiol.* **12:** 145-149.