<table>
<thead>
<tr>
<th><strong>Title</strong></th>
<th>Diversity and mechanisms of alkali tolerance in lactobacilli</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Author(s)</strong></td>
<td>Sawatari, Yuki; Yokota, Atsushi</td>
</tr>
<tr>
<td><strong>Citation</strong></td>
<td>Applied and Environmental Microbiology, 73(12), 3909-3915</td>
</tr>
<tr>
<td><strong>Issue Date</strong></td>
<td>2007-06</td>
</tr>
<tr>
<td><strong>Doc URL</strong></td>
<td><a href="http://hdl.handle.net/2115/28128">http://hdl.handle.net/2115/28128</a></td>
</tr>
<tr>
<td><strong>Rights</strong></td>
<td>Copyright © American Society for Microbiology.</td>
</tr>
<tr>
<td><strong>Type</strong></td>
<td>article</td>
</tr>
<tr>
<td><strong>File Information</strong></td>
<td>AEM73-12.pdf</td>
</tr>
</tbody>
</table>

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

---

*Footnotes or additional information*
Diversity and Mechanisms of Alkali Tolerance in Lactobacilli

Yuki Sawatari and Atsushi Yokota*

Laboratory of Microbial Physiology, Research Faculty of Agriculture, Hokkaido University, Kita 9 Nishi 9, Kita-ku, Sapporo, Hokkaido 060-8589, Japan

Received 6 December 2006/Accepted 8 April 2007

We determined the maximum pH that allows growth (pHmax) for 34 strains of lactobacilli. High alkali tolerance was exhibited by strains of Lactobacillus casei, L. paracasei subsp. tolerans, L. paracasei subsp. paracasei, L. curvatus, L. pentosus, and L. plantarum that originated from plant material, with pHmax values between 8.5 and 8.9. Among these, L. casei NRIC 1917 and L. paracasei subsp. tolerans NRIC 1940 showed the highest pHmax, at 8.9. Digestive tract isolates of L. gasseri, L. johnsonii, L. reuteri, L. salivarius subsp. salivarius, and L. salivarius exhibited moderate alkali tolerance, with pHmax values between 8.1 and 8.5. Dairy isolates of L. delbrueckii subsp. bulgaricus, L. delbrueckii subsp. lactis, and L. helveticus exhibited no alkali tolerance, with pHmax values between 6.7 and 7.1. Measurement of the internal pH of representative strains revealed the formation of transmembrane proton gradients (ΔpH) in a reversed direction (i.e., acidic interior) at alkaline external-pH ranges, regardless of their degrees of alkali tolerance. Thus, the reversed ΔpH did not determine alkali tolerance diversity. However, the ΔpH contributed to alkali tolerance, as the pHmax values of several strains decreased with the addition of nigericin, which dissipates ΔpH. Although neutral external-pH values resulted in the highest glycolysis activity in the presence of nigericin regardless of alkali tolerance, substantial glucose utilization was still detected in the alkali-tolerant strains, even in a pH range of between 8.0 and 8.5, at which the remaining strains lost most activity. Therefore, the alkali tolerance of glycolysis reactions contributes greatly to the determination of alkali tolerance diversity.

Lactic acid bacteria (LAB) produce lactic acid as a main product of sugar fermentation. Many studies have examined the response of LAB to acid stress, including the mechanisms underlying acid tolerance (3, 4, 28). In contrast, very few studies have examined the stress response of LAB to alkaline environments. Several alkali–tolerant/alkaliphilic LAB, which are capable of growing at pH values of >9.5 as well as producing large amounts of lactic acid from glucose, have been reported to date: Enterococcus hirae (formerly Streptococcus faecalis) (8), Pediococcus urinaeequi (23), Alkalibacterium olivapolviliticus (12, 24), Marinilactibacillus psychrotolerans (12), M. piezotolerans (27), Halolactibacillus halophilus, and H. miurensis (13).

The mechanism underlying the alkali tolerance of LAB has been examined in E. hirae ATCC 9790T. Biochemical experiments and mutant analyses suggest that E. hirae ATCC 9790T maintains an internal pH lower than the external pH by the operation of an ATP-driven K⁺/H⁺ antiporter during growth at an external pH of around 9.5 (14–17). However, E. hirae ATCC 9790T has no such system to maintain a near-neutral internal pH in an alkaline medium (22). Thus, the contribution of internal-pH maintenance to alkali tolerance in this bacterium is controversial. Adaptation to alkaline stress has been reported for E. faecalis ATCC 19433T, in which the induction of two stress proteins, DnaK and GroEL, was detected (9).

Lactobacilli, although generally not considered alkali tolerant, have been used as a starter for the fermentation of alkaline materials to prepare fermented foods. For example, Lactobacillus pentosus CECT 5138 has been studied as a starter to ferment lye-treated green olives (pH, >9.0) for the preparation of Spanish-style green olives (25). In our previous study (26), in which noodle dough with a pH of around 8.5 was fermented, L. plantarum NRIC 0380 was screened as a starter strain for fermented instant Chinese noodles.

Because there has been little examination of alkali tolerance and sensitivity in Lactobacillus strains, we investigated the effects of alkaline stress on the growth, physiology, and metabolism of lactobacilli from various sources for more effective use of this group of bacteria in food industries.

MATERIALS AND METHODS

Bacterial strains and chemicals. The bacterial strains used (Table 1) were obtained from the Japan Collection of Microorganisms (JCM, Wako, Japan), the Culture Collection Center, Tokyo University of Agriculture (NRIC, Tokyo, Japan); the Laboratory of Applied Microbiology, Research Faculty of Agriculture, Hokkaido University (AHU, Sapporo, Japan); and the Wageningen Center for Food Sciences (Wageningen, The Netherlands). [2-(Hydroxy-1,1-bis(2-hydroxyethyl)amino)-1-propanesulfonic acid: N-Tris(hydroxymethyl)methyl-3-amino-2-propanesulfonic acid (TAPS); N-Tris(hydroxymethyl)methyl-4-amino-2-propanesulfonic acid (TABS); 2-(cyclohexylamino)ethanesulfonic acid (CHES); and nigericin were obtained from Sigma-Aldrich (St. Louis, MO). 2-(Morpholino)-ethanesulfonic acid monohydrate (MES) and 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) were obtained from Dojindo Laboratories (Mashikimachi, Kumamoto, Japan). 3-(N-Morpholino)propanesulfonic acid (MOPS) was purchased from Nacalai Tesque (Kyoto, Japan). The 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDASE) was obtained from Molecular Probes (Eugene, OR). Other chemicals used were of the highest quality available commercially.

Determination of maximum alkaline pH allowing growth of lactobacilli. For the preparation of seed cultures, frozen stocks of Lactobacillus strains and E. hirae JCM 8720T kept under −80°C were transferred to 3 ml of half-strength MRS (1/2 MRS) medium (6) and incubated overnight at 30°C or 37°C, as indicated in the legend to Table 1. All cultures were grown in screw-cap test tubes tightly sealed with rubber septums. No other anaerobic treatment was necessary.

* Corresponding author. Mailing address: Laboratory of Microbial Physiology, Division of Applied Bioscience, Research Faculty of Agriculture, Hokkaido University, Kita 9 Nishi 9, Kita-ku, Sapporo, Hokkaido 060-8589, Japan. Phone: 81-11-706-2501. Fax: 81-11-706-4961. E-mail: yokota@chem.agr.hokudai.ac.jp.

Published ahead of print on 20 April 2007.
The 1/2 MRS medium contained 10 g/liter glucose, 5 g/liter Bacto Proteose Peptone No. 3 (Becton Dickinson, Franklin Lakes, NJ), 5 g/liter Lab-Lemco powder (Oxoid, Basingstoke, Hampshire, UK), 2.5 g/liter Bacto yeast extract (Becton Dickinson), 0.5 g/liter Tween 80, 1 g/liter K2HPO4, 2.5 g/liter NaCH3CO2·H18528·3H2O, 1 g/liter triammonium citrate, 0.1 g/liter MgSO4·H18528·7H2O, and 0.025 g/liter MnSO4·H18528·4H2O; the pH was adjusted to 6.5, using HCl. The seed culture was inoculated into 3 ml of buffered 1/2 MRS medium at an optical density at 660 nm (OD 660) of around 0.03. The buffered 1/2 MRS medium was prepared by supplementing 1/2 MRS medium with an appropriate buffer at 100 mM to prevent acidification of the culture medium, using the following buffers: MOPS (pH 6.5 to 7.9), HEPES (pH 7.1 to 8.1), TAPS (pH 8.1 to 9.1), and CHES (pH 8.7 to 9.7). The pH of these media was adjusted using NaOH or HCl.

Growth experiments using the lactobacilli strains were conducted at a pH range of 6.5 to 9.1; experiments using *E. hirae* JCM 8729T were conducted at a pH range of 6.5 to 9.7. Incubation was conducted at the same temperatures and same anaerobic conditions as used for the seed cultures for up to 24 h, and growth was monitored by the OD660, using a spectrophotometer (Spectronic 20D; Milton Roy Company). Strains that reached an OD 660 of >0.15 within 24 h of being cultured were considered to exhibit positive growth. At this point, acidification of the media appeared to be negligible (around 0.05 pH unit). Additionally, specific growth rates ($\mu$; expressed in h$^{-1}$) were calculated during the exponential growth phase, using the following equation: $\mu = (\ln x_t - \ln x_0)/(t_2 - t_1)$, where $x_t$ and $x_0$ are the OD660 at times $t_2$ and $t_1$, respectively. Normally, calculation was conducted at the early exponential phase (OD$_{max}$ ~0.3), at which point acidification of the medium (around 0.15 pH unit) did not seem to affect $\mu$ values significantly.

**Intracellular pH measurement.** The internal pH of five representative strains of lactobacilli (see Fig. 2) and *E. hirae* JCM 8729T was measured at external-pH range of 6.5 to 9.7. Incubation was conducted at the same temperatures and same anaerobic conditions as used for the seed cultures for up to 24 h, and growth was monitored by the OD$_{max}$ using a spectrophotometer (Spectronic 20D; Milton Roy Company). Strains that reached an OD$_{max}$ of >0.15 within 24 h of being cultured were considered to exhibit positive growth. At this point, acidification of the media appeared to be negligible (around 0.05 pH unit). Additionally, specific growth rates ($\mu$; expressed in h$^{-1}$) were calculated during the exponential growth phase, using the following equation: $\mu = (\ln x_t - \ln x_0)/(t_2 - t_1)$, where $x_t$ and $x_0$ are the OD$_{max}$ at times $t_2$ and $t_1$, respectively. Normally, calculation was conducted at the early exponential phase (OD$_{max}$ ~0.3), at which point acidification of the medium (around 0.15 pH unit) did not seem to affect $\mu$ values significantly.

**Intracellular pH measurement.** The internal pH of five representative strains of lactobacilli (see Fig. 2) and *E. hirae* JCM 8729T was measured at external-pH

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. casei</td>
<td>JCM 1134T</td>
<td>Cheese</td>
</tr>
<tr>
<td></td>
<td>NRIC 1917</td>
<td>Sugar cane wine</td>
</tr>
<tr>
<td>L. paracasei subsp. tolerans*</td>
<td>JCM 1171T</td>
<td>Pasteurized milk</td>
</tr>
<tr>
<td></td>
<td>NRIC 1940</td>
<td>Coconut juice</td>
</tr>
<tr>
<td>L. paracasei subsp. paracasei*</td>
<td>JCM 8130T</td>
<td>Milk products</td>
</tr>
<tr>
<td></td>
<td>NRIC 1946</td>
<td>Coconut juice</td>
</tr>
<tr>
<td>L. curvatus</td>
<td>JCM 1096T</td>
<td>Milk</td>
</tr>
<tr>
<td></td>
<td>AHU GD12</td>
<td>“Ragi tape” (starter for sake)</td>
</tr>
<tr>
<td>L. pentosus</td>
<td>JCM 1558T</td>
<td>Corn silage</td>
</tr>
<tr>
<td></td>
<td>NRIC 0413</td>
<td>Fermented tea leaves</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>JCM 1149T</td>
<td>Pickled cabbage</td>
</tr>
<tr>
<td></td>
<td>WCFS1</td>
<td>Human saliva</td>
</tr>
<tr>
<td></td>
<td>NRIC 0380</td>
<td>Pickles</td>
</tr>
<tr>
<td>L. salivarius subsp. salicinatus</td>
<td>JCM 1150T</td>
<td>Saliva</td>
</tr>
<tr>
<td>L. salivarius subsp. salivarius</td>
<td>JCM 1040</td>
<td>Human intestine</td>
</tr>
<tr>
<td>JCM 1044</td>
<td>Human intestine</td>
<td></td>
</tr>
<tr>
<td>JCM 1231T</td>
<td>Human saliva</td>
<td></td>
</tr>
<tr>
<td>L. gasseri</td>
<td>JCM 1131T</td>
<td>Human intestine</td>
</tr>
<tr>
<td>JCM 5813</td>
<td>Infant feces</td>
<td></td>
</tr>
<tr>
<td>L. johnsonii</td>
<td>JCM 1022</td>
<td>Human intestine</td>
</tr>
<tr>
<td>JCM 2012T</td>
<td>Human blood</td>
<td></td>
</tr>
<tr>
<td>L. sakei subsp. sakei*</td>
<td>JCM 1157T</td>
<td>“Moto” (starter for sake)</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>JCM 1112T</td>
<td>Adult intestine</td>
</tr>
<tr>
<td>L. brevis*</td>
<td>NRIC 0137</td>
<td>Fermented bamboo shoots</td>
</tr>
<tr>
<td>L. delbrueckii subsp. delbrueckii</td>
<td>JCM 1012T</td>
<td>Sour grain mash</td>
</tr>
<tr>
<td>L. fermentum</td>
<td>JCM 1173T</td>
<td>Fermented beets</td>
</tr>
<tr>
<td></td>
<td>NRIC 0131</td>
<td>Pickled mustard</td>
</tr>
<tr>
<td>L. mali</td>
<td>JCM 1116T</td>
<td>Apple juice from cider press</td>
</tr>
<tr>
<td>L. delbrueckii subsp. bulgaricus</td>
<td>JCM 1001</td>
<td>Not described</td>
</tr>
<tr>
<td>JCM 1002T</td>
<td>Bulgarian yogurt</td>
<td></td>
</tr>
<tr>
<td>L. delbrueckii subsp. lactis</td>
<td>JCM 1248T</td>
<td>Emmental (Swiss) cheese</td>
</tr>
<tr>
<td>L. helveticus</td>
<td>JCM 1062</td>
<td>Dairy products</td>
</tr>
<tr>
<td>JCM 1120T</td>
<td>Emmental (Swiss) cheese</td>
<td></td>
</tr>
<tr>
<td>L. acidophilus</td>
<td>JCM 1132T</td>
<td>Human feces</td>
</tr>
<tr>
<td>E. hirae</td>
<td>JCM 8729T</td>
<td>Not described</td>
</tr>
</tbody>
</table>

* Gray bars indicate the pH range allowing growth for each strain. Cultures were grown in buffered 1/2 MRS media at 37°C, except for species denoted with asterisks, which were cultured at 30°C. Buffers used in the buffered 1/2 MRS medium included MOPS (#), HEPES (%), and CHES (&); TAPS buffer was used for the rest of the cultures. The data are derived from at least two independent experiments that gave similar results. When different pH$_{max}$ values were obtained for a given strain, the lower value was adopted.
values of 6.5 to 9.0, using the pH-sensitive fluorescence probe 5 (and 6)-carboxyfluorescein succinimidyl ester (cFSE), based on the method originally described by Breeuwer et al. (2), with some modification. The fluorescence intensity of this probe is increased at alkaline pH and reduced at acidic pH. Seed culture was inoculated into an appropriate volume of 1/2 MRS medium in a screw-cap bottle at an OD660 of about 0.05. The culture was conducted to an OD660 of around 0.5 and incubated for 30 min at the temperatures described in the footnote to Table 1. After being centrifuged and washed with buffer A (150 mM potassium phosphate buffer with 1 mM MgSO4 · 7H2O [pH 6.5]), the cells were resuspended in buffer A containing 4 μM cFDASE, a precursor molecule of cFSE, at an OD660 of around 0.5 and incubated for 30 min at the temperatures described in the footnote to Table 1. During this incubation, the membrane-permeating cFDASE was cleaved by intracellular esterases, and the resultant cFSE molecules were conjugated to the aliphatic amines of intracellular proteins. After being centrifuged and washed with buffer A, the cells were resuspended in the same volume of buffer A. The nonconjugated probe was eliminated by the addition of glucose at a final concentration of 10 mM and subsequent incubation for 1 h at the temperatures described in the footnote to Table 1. After being centrifuged and washed with buffer A, the cells were resuspended in buffer A, and their energy was depleted through incubation for 1 h at the temperatures described in the footnote to Table 1. After being centrifuged and washed with buffer A, the cells were resuspended in buffer A and their energy was depleted through incubation for 1 h at the temperatures described in the footnote to Table 1. Subsequently, the energy-depleted cells were added at an OD660 of about 4, and then nigericin in ethanol solution was added at a final concentration of 7 μM. Control cultures received 0.1% (vol/vol) ethanol. Specific growth rates were calculated during the exponential growth phase as described above. Acidification of the culture medium during the calculation appeared to be at negligible levels owing to the added buffer system.

Measurement of glycolysis activity in the presence of nigericin. To evaluate the pH dependency of lactobacilli glycolysis activity, the activities of the four strains used in the preceding section together with that of E. hirae JCM 8729T were measured in the presence of nigericin at various pHs between 4.0 and 9.5. The cells were cultured until the exponential phase (OD660 = 0.6) in 1/2 MRS medium, harvested, and washed with buffer A, as described under “Intracellular pH measurement.” The washed cell pellet was resuspended in the same buffer and incubated for 1 h at the same temperatures in which the cells were cultured to deplete their metabolic energy. The cells were washed once with buffer A and resuspended in the same buffer at an OD660 of about 80. The basal composition of the reaction mixture for the glycolysis reaction contained 150 mM potassium phosphate buffer, 1 mM MgSO4 · 7H2O, and one of the following buffers at 100 mM with the pH adjusted to the desired values, using KOH: MES (pH 6.5), MOPS (pH 7.5), TAPS (pH 8.0 to 9.0), or TABS (pH 9.5). Subsequently, the energy-depleted cells were added at an OD660 of about 4, and then nigericin in ethanol solution was added at a final concentration of 7 μM. The control series received 0.1% (vol/vol) ethanol. The reaction was started by the addition of glucose at a final concentration of 10 mM. The mixtures were incubated at the temperature in which they were cultured, for appropriate time periods (see Fig. 3). After being incubated, the mixtures were centrifuged at 18,120 × g for 5 min at 4°C, and the supernatant was filtered through a 0.2-μm-pore-size membrane. The glucose concentrations of the filtered supernatants were measured, using Glucose C2 (Wako Pure Chemical Industries, Osaka, Japan).

RESULTS

Maximum pH allowing the growth of lactobacilli. We determined the maximum pH that allows growth (pHmax) for 34 strains of lactobacilli from various sources. We used E. hirae JCM 8729T, a well-known alkali-tolerant strain (8), as a reference. As expected, E. hirae JCM 8729T showed growth up to a pH of 9.7 (Table 1). Relatively high-alkali-tolerant strains in the lactobacilli included the plant isolates of L. casei, L. paracasei subsp. tolerans, L. paracasei subsp. paracasei, L. curvatus,
L. pentosus, and L. plantarum, with pHmax values between 8.5 and 8.9. Of these, L. casei NRIC 1917 and L. paracasei subsp. tolerans NRIC 1940 exhibited the highest alkali tolerance, with a pHmax of 8.9. Within the same species of lactobacilli, i.e., L. casei, L. paracasei subsp. tolerans, and L. paracasei subsp. paracasei, isolates from plant materials showed higher alkali tolerance than those from other sources. In contrast, strains of L. brevis, L. delbrueckii subsp. delbrueckii, L. fermentum, and L. mali that were isolated from plant materials showed lower pHmax values of between 7.5 and 7.7. Strains of L. gasseri, L. johnsonii, L. reuteri, L. salivarius subsp. salicinus, and L. salivarius subsp. salivarius, which were isolated from digestive tracts, exhibited moderate alkali tolerance, with pHmax values between 8.1 and 8.5. The dairy isolates of L. delbrueckii subsp. bulgaricus, L. delbrueckii subsp. lactis, and L. helveticus showed no alkali tolerance, with pHmax values between 6.7 and 7.1. The least-alkali-tolerant strains among those tested were L. helveticus JCM 1120T and L. acidophilus JCM 1132T, which had pHmax values as low as 6.7.

Among six representative strains of lactobacilli and E. hirae JCM 8729T, E. hirae JCM 8729T showed the highest μ as a function of the initial pH of the culture medium under the tested pH range of between 6.5 and 9.7 (Fig. 1). However, in all strains, μ decreased with the increasing initial pH of the medium.

**Intracellular pH measurements.** Next, we clarified the mechanism(s) determining the diversity of alkali tolerance. Previous studies suggested that antiporters acidify cytoplasmic pH under alkaline conditions in several alkali-tolerant species, including E. hirae ATCC 9790T (15–17), and some alkaliphiles (20, 21). To verify the contribution of this mechanism in alkali tolerance, internal-pH measurements were performed, using five strains of lactobacilli with different pHmax values and E. hirae JCM 8729T under a range of external-pH values from 6.5 to 9.0 (Fig. 2). An ATP-driven K⁺/H⁺ antiporter has been suggested for E. hirae ATCC 9790T (JCM 8729T) (15–17), whereas Na⁺/H⁺ antiporter activity has been implied for the alkaliphilic bacteria Bacillus firmus RAB (20) and Exiguobacterium aurantiacum NCIB 11798 (21). Thus, both NaOH and KOH were used to adjust the assay mixtures for pH measurements. At an external pH of 6.5, an ΔpH (i.e., an alkaline interior) was generated in all tested strains by energization with glucose. When the external pH was increased using KOH, the internal pH also rose, but at a slower rate, thereby decreasing the ΔpH. Above a specific external pH, ΔpH was reversed (i.e., the interior became acidic), and under highly alkaline external pH, the internal pH increased while maintaining the magnitude of the reversed ΔpH at about 0.5 in all tested strains, including E. hirae JCM 8729T. We did not observe the remarkable cytoplasmic pH regulation previously reported at alkaline external pH in E. hirae ATCC 9790T (14). The formation of the reversed ΔpH was observed not only in the highly alkali-tolerant strains E. hirae JCM 8729T and L. acidophilus JCM 1132T, but also in the least-alkali-tolerant strain, L. acidophilus JCM 1132T (Fig. 2). Similar results were obtained when NaOH, rather than KOH, was used to adjust the pH, except that a significantly larger reversed ΔpH was observed in L. acidophilus JCM 1132T than that observed when KOH was used for pH adjustment. Internal-pH regulation does not influence the diversity of lactobacilli alkali tolerance.

**Growth in the presence of nigericin.** The reversed ΔpH observed in the preceding experiments was expected to contribute to growth in alkaline conditions. To verify this, we examined growth in the presence or absence of nigericin, which dissipates ΔpH, using four strains of lactobacilli and E. hirae JCM 8729T in buffered K-1/2 MRS. We observed dramatic decreases in the μ values of all the Lactobacillus strains and E.
hiae JCM 8729\textsuperscript{T} in the presence of nigericin under a range of acidic pH from 6.5 to 7.0 (Fig. 3A). This was expected from previous studies showing that maintenance of intracellular pH by H\textsuperscript{+}-ATPase is important for growth and viability in acidic conditions (1, 18, 29). Under a range of alkaline pH, the \( \mu \) values of \textit{L. johnsonii} JCM 1022, \textit{L. plantarum} NRIC 0380, and \textit{L. paracasei} subsp. \textit{tolerans} NRIC 1940 were decreased in the presence of nigericin and the pHmax of each strain was decreased by 0.5. Thus, the reversed \( \Delta \text{pH} \) formed in alkaline conditions contributes to the alkali tolerance of the tested \textit{Lactobacillus} strains. Furthermore, we observed that the pHmax of a given strain in the presence of nigericin was positively dependent on the pHmax in the absence of nigericin (Fig. 3A), suggesting that the degree of resistance of the cellular metabolism, including catabolism and anabolism to an alkaline environment, may determine the alkali tolerance of a given strain.

**Glycolysis activity in the presence of nigericin.** To evaluate the preceding hypothesis, we measured the glycolysis activity of the strains in the presence or absence of nigericin at various external pH values. As expected, we observed dramatic decreases in glycolysis activity at a range of acidic pH values for all strains tested (Fig. 3B). The glycolysis activities of the highly alkali-tolerant strains NRIC 1940 and \textit{E. hirae} JCM 8729\textsuperscript{T} were also greatly decreased at a range of alkaline pH above 9.0 in the presence of nigericin (Fig. 3B). However, the optimum pH for glycolysis activity in the presence of nigericin of all strains tested was neutral, regardless of the strain’s alkali tolerance. Interestingly, substantial glucose utilization in the highly alkali-tolerant strains NRIC 1940 and \textit{E. hirae} JCM 8729\textsuperscript{T} was still detected up to a pH range of between 8.0 and 8.5 in the presence of nigericin, whereas the remaining strains lost most glycolysis activity. Therefore, the diversity of alkali tolerance is strongly dependent on the alkali tolerance of cellular metabolism, especially glycolysis reactions involved in the generation of energy.

**DISCUSSION**

We determined the pHmax of many lactobacilli for the first time. The pHmax ranged between 6.7 and 8.9, demonstrating the diversity of alkali tolerance in lactobacilli (Table 1). Strains of \textit{L. casei}, \textit{L. paracasei} subsp. \textit{tolerans}, \textit{L. paracasei} subsp. \textit{paracasei}, \textit{L. curvatus}, \textit{L. pentosus}, and \textit{L. plantarum} originating from plant material exhibited high alkali tolerance, with pHmax values of \( \geq 8.5 \). It is not clear if there is a correlation between the plant environment and the strain’s alkali tolerance. Most of these strains belong to two phylogenetic groups of lactobacilli: the \textit{L. casei} group (strains tested, \textit{L. casei}, \textit{L. paracasei} subsp. \textit{paracasei}, and \textit{L. paracasei} subsp. \textit{tolerans}) and the \textit{L. plantarum} group (strains tested, \textit{L. plantarum} and \textit{L. pentosus}) (5). Therefore, we speculated that strains belonging to these groups may have a tendency for alkali tolerance from an evolutionary viewpoint. The next-highest alkali-tolerant...
growth (Fig. 3A), especially on the glycolysis reactions of a
including catabolism and anabolism, as observed in terms of
strongly on the alkali tolerance of the cellular metabolism,
environment and, therefore, have not evolved high tolerance.
dairy strains may have little chance of encountering an alkaline
ues between 6.7 and 7.1. Dairy starter fermentations generally
larger reversed
this strain possibly has certain system(s) to create significantly
as 6.7. However, as discussed later in the Discussion section,
activity profile of
pH optima to be around 7.5 (Fig. 3A). The pH/glycolysis ac-
higher than those of the other tested strains, which showed the
registration in alkaline
environment than catabolic reactions (glycolysis reactions).
We investigated the physiological response of many Lacto-
strains to an alkaline environment. The most impor-
tant factor determining the diversity of alkali tolerance in lac-
tobacilli was the alkali tolerance of the strains’ glycolysis
activity profile was also observed to a lesser extent in
subsp. tolerans NRIC 1940, which showed the highest alkali
tolerance to the corresponding stress in various LAB strains (3,
pH under alkaline conditions than any other
strains tested (Fig. 2), suggesting survival under an alkaline
environment (without growth). We found extremely low alkali
tolerance in the dairy strains of
subsp. bulgaricus, 
subsp. lactis, and
helveticus, with pHmax values between 6.7 and 7.1. Dairy starter fermentations generally
under severe acidic conditions of around pH 4. Thus,
dairy strains may have little chance of encountering an alkaline
environment and, therefore, have not evolved high tolerance.
We found that the diversity of alkali tolerance depended
strongly on the alkali tolerance of the cellular metabolism,
including catabolism and anabolism, as observed in terms of
growth (Fig. 3A), especially on the glycolysis reactions of a
given strain (Fig. 3B). In fact, the alkali-tolerant reference
strain,
JCM 8729T, exhibited the optimum pH for
growth of 8.5 in the presence of nigericin, which is significantly
higher than those of the other tested strains, which showed the
pH optima to be around 7.5 (Fig. 3A). The pH/glycolysis ac-
tivity profile of
JCM 8729T also shifted to the alkaline
side compared to those of the other tested strains in the pres-
ence of nigericin (Fig. 3B). A dramatic decrease in glycolysis
activity, which was not observed in the other strains, occurred
at pH 6.0 (Fig. 3B). This shift in the pH/glycolysis activity
profile was also observed to a lesser extent in
subsp. tolerans NRIC 1940, which showed the highest alkali
tolerance of the tested lactobacilli (Fig. 3B). Nevertheless, the
optimal pH for glycolysis activity was neutral in all strains,
regardless of their alkali tolerance (Fig. 3B), which is consist-
tent with a previous finding of neutral optimal pH in many
enzymes of LAB involved in carbohydrate and amino acid
metabolism (11). It is interesting that in
JCM 1132T, substantial glycolysis activity in the absence of nigericin
was still observed at pH 7.5 or even at pH 8.5 (Fig. 3B), while
the pHmax was 6.7 (Table 1). These results suggest that in this
strain anabolic reactions may be more sensitive to an alkaline
environment than catabolic reactions (glycolysis reactions).
Reversed ΔpH formation was observed at alkaline external
pH in all of the tested lactobacilli, as well as in
JCM 8729T, with a magnitude of around 0.5 (Fig. 2). The dissipation
of this ΔpH by the addition of nigericin lowered the pHmax of
each tested strain by 0.5 (Fig. 3A). These consistent results
clearly indicate the contribution of the reversed ΔpH to alkali
tolerance. The formation of the reversed ΔpH under alkaline
external pH has already been reported for
ATCC 9790T (14, 18, 19), and such ATP-dependent K+/H+ antiporter
and/or physicochemical forces as the Donnan potential
have been suggested to contribute to these phe-
nomena. In the case of
JCM 1132T,
and
subsp. tolerans NRIC 0380, and
subsp. tolerans NRIC 1940, glu-
cose utilization was still observed under the alkaline pH range
in which the reversed ΔpH was observed (Fig. 2 and 3B),
thereby ensuring an energy supply under these conditions.
Therefore, the observed reversed ΔpH formation in these Lacto-
strains (Fig. 2) may possibly be mediated not only by
some physicochemical force but also by certain energy-depen-
dent Na+/K+ antiporter(s). The larger reversed ΔpH ob-
served in
JCM 1132T when NaOH was used for
pH adjustment than that observed when KOH was used (Fig. 2)
suggested the possible operation of an Na+/H+ antiporter.
In this bacterium, since the pHmax is 6.7 (Table 1), the ob-
served reversed ΔpH (Fig. 2) may not contribute to the growth
but may contribute to the survival of this bacterium under
alkaline stress conditions.
We did not detect the remarkable regulation of cytoplasmic
pH in
E. hirae JCM 8729T (ATCC 9790T) in external alkaline
pH (Fig. 2) by such transporters as the ATP-driven K+/H+
antiporter reported previously for this bacterium (14–17).
Similar results have been reported by Mugikura et al. (22).
These discrepancies may have been caused by differences in
the methods used for intracellular-pH measurements. It has been
argued that the amines used for the measurement of internal
pH by Kakinuma (14) tend to overestimate ΔpH because of the
binding of amines to intracellular materials such as RNA as
well as to the cell surface (22). In contrast, Mugikura et al. (22)
used weak acid for internal pH measurements, and its binding
to intracellular materials is negligible. We used a pH-sensitive
fluorescent probe, cFSE, which allows the direct monitoring of
intracellular pH.
We investigated the physiological response of many Lacto-
strains to an alkaline environment. The most impor-
tant factor determining the diversity of alkali tolerance in lac-
tobacilli was the alkali tolerance of the strains’ glycolysis
reactions. In addition, the reversed ΔpH formed in alkaline
environments, probably because of the Donnan potential
and/or certain energy-dependent Na+/K+ antiporter(s),
contributed to the alkali tolerance. Although a variety of mech-
isms aid LAB survival in acidic environments, lactobacilli
seem to have poor evolutionary preparation for growth in
alkaline environments. It has been reported that prior expo-
sure to weak environmental stresses dramatically increases the
tolerance to the corresponding stress in various LAB strains (3,
4, 28). Potential alkaline stress adaptation, as has been re-
ported for
ATCC 19433T (9), needs to be checked in
lactobacilli for a more-comprehensive understanding of the
mechanism of alkali tolerance. We have illustrated previously
unexplored aspects of lactobacilli in alkaline conditions. We
believe that these findings will help to understand the basic
biology of lactobacilli and expand the application range of this
group of bacteria.
ACKNOWLEDGMENTS
NRIC cultures were kindly supplied by Sanae Okada, Culture Col-
lection Center, Tokyo University of Agriculture, Tokyo, Japan. 
Wageningen Center for Food Sciences, Wageningen, The Nether-
lands.
A portion of this work was conducted under the framework of the
Research and Development Program for New Bio-industry Initiatives,
sponsored by the Bio-oriented Technology Research Advance-
Institution (BRAIN). Y.S. received a scholarship from Kagome, Nagoya, Japan.

REFERENCES


