



Communication

Establishment of a Novel Method of Screening Anti-Allergic Lactic Acid Bacteria

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In this study, we attempted to establish a novel method of screening anti-allergic lactic acid bacteria (LAB). We cloned the human histidine decarboxylase (HDC) promoter into the promoter-less pPhi-Yellow-RPL-dest1 vector and established KU812F cells transduced with this vector (pHDCp-Phi-Yellow/KU812F). After adding LAB to these cells, the change in fluorescence intensity was monitored by flow cytometry. After screening, we identified several LAB strains that downregulated HDC promoter activity. Functional analysis of these LAB strains indicated that two LAB strains inhibited histamine release from KU812F cells, indicating that this assay system can be used to screen for anti-allergic LAB in a high-throughput manner.

Key words: lactic acid bacteria; histidine decarboxylase; allergy; KU812F

Lactic acid bacteria (LAB) are known to have various beneficial effects including i) improving intestinal tract health, ii) enhancing the immune system, iii) synthesizing and enhancing the bioavailability of nutrients, iv) reducing the symptoms of lactose intolerance, v) decreasing the prevalence of allergies, and vi) reducing the risk of certain cancers.¹⁾ Among these functions, the ability of LAB to alleviate some of the symptoms of atopy and to reduce the development of allergies has been extensively studied. These anti-allergic effects of LAB have been shown to be elicited by enhancement of the production of type I and type II interferon, reducing the production of IL-4 and IL-5, or induction of the production of IL-12 and IL-18.²⁾ To date, various aspects of the anti-allergic activity of particular LAB strains have been investigated, but there exist only a limited number of assay systems to screen for anti-allergic LAB in a high-throughput manner. In this study, we attempted to establish a novel system to screen for anti-allergic LAB.

We focused on histidine decarboxylase (HDC),³⁾ which catalyzes the reaction that produces histamine from histidine. We cloned the human HDC promoter by PCR using primers 5'-CTCGAGATGGGCAATGC-

CACCAAAGC-3' and 5'-AAGCTTCTCCCTTGGGCT-CTGGCTCTT-3' and human genome DNA prepared from KU812F cells. After checking of the sequence, the HDC promoter was inserted into the promoter-less pPhi-Yellow-RPL-dest1 vector (pHDCp-Phi-Yellow; Evrogen, Moscow, Russia). We transfected this vector into KU812F cells, an immature prebasophilic cell line (HSRRB, Osaka, Japan), and established a stable cell line (pHDCp-Phi-Yellow/KU812F cells). These cells were cultured in RPMI-1640 medium (Nissui, Tokyo) supplemented with 10% heat-inactivated fetal bovine serum (Trace Scientific, Victoria, Australia) and antibiotics (100 U·mL⁻¹ of penicillin G and 100 µg·mL⁻¹ of streptomycin). Next, we seeded this cell line in 24-well plates (Becton Dickinson, Franklin Lakes, NJ) at a density of 1×10^5 cells/mL, and added 1–10 µg/mL of LAB. The LAB strains used are listed in Table 1. LAB were freeze-dried after heating at 100°C for 30 min and suspended in 2.24×10^{-2} M phosphate buffer containing 1.37×10^{-1} M NaCl (PBS).⁴⁾ After 7 d of culture, the change in the yellow fluorescence derived from pHDCp-Phi-Yellow was monitored by flow cytometry (EPICS XL, Beckman Coulter, Miami, FL). As shown in Fig. 1, although the LAB strains of a particular species do not necessarily have similar activity, several LAB strains were found to downregulate HDC promoter activity in KU812F cells strongly. Most LAB strains belonging to *L. gasseri* and *L. sakei* downregulated HDC promoter activity. LAB strains T1802 (*L. paracasei*), NHK1 (*L. gasseri*), NHK2 (*L. salivaris*), H0709, H1202, and NHMN1 (*P. acidilactici*) also showed strong down-regulation of HDC promoter activity.

Next, we evaluated the abilities of the LAB strains to downregulate transcription of the endogenous HDC gene in KU812F cells. For this experiment, we selected four LAB strains (T2102, NHK2, H0709, and NHMN1) that downregulate HDC promoter activity, and one LAB strain (C0201) that upregulates HDC promoter activity, and evaluated the effects of these strains on transcription of the HDC gene by quantitative RT-PCR (Fig. 2A). Based on the preliminary experiments, we treated KU812F cells with LAB strains for 7 d to obtain

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Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HDC, histidine decarboxylase; LAB, lactic acid bacteria; PBS, phosphate-buffered saline

reproducible results. Quantitative RT-PCR was performed using SYBR Premix Ex Taq (Takara, Shiga, Japan) and a Thermal Cycler Real Time System TP-800 instrument (Takara) using the following primers: HDC forward, 5'-GCTCCACATCGATGCTGCTTA-3' and HDC reverse, 5'-GATGGGATTCACACTGAAGGTCTG-3'; glyceraldehyde-3-phosphate dehydrogenase (GAPDH) forward, 5'-GCACCGTCAAGGCTGAGAAC-3' and GAPDH reverse, 5'-ATGGTGGTGAAGACGCCAGT-3'. GAPDH was used as internal control.

The results clearly indicated that LAB strains that downregulate HDC promoter significantly repressed transcription of the HDC gene in KU812F cells. However, C0201 also repressed transcription of the HDC gene. We assume that the repression induced by the LAB strains that downregulate HDC promoter and by C0201 were caused by different mechanisms.

To test this hypothesis, we further evaluated the functionalities of the LAB strains. We tested the effects of these strains on histamine release from KU812F cells induced by ionomycin treatment (Fig. 2B). To assess the histamine release inhibitory activity of LAB, we conducted a β -hexosaminidase release assay using KU812F cells.^{5,6} KU812F cells were treated with 10 μ g/mL of LAB and cultured for 3 d. Cells were

treated with 10 μ M ionomycin at 37 °C for 1 h, and then 40 μ L of supernatant was incubated with 100 μ L of substrate solution (2 mM *p*-nitrophenyl-*N*-acetyl- β -D-glucosaminide in 0.4 M citric acid) and 100 μ L of 0.2 M phosphate buffer, pH 4.5, at 37 °C for 30 min. Glycine (0.2 M) was added to stop the reaction, and the amount of β -hexosaminidase released, which corresponds to the level of histamine released, was calculated based on the absorbance at 405 nm. The results indicated that H0709 and especially NHMN1 inhibited histamine release from the KU812F cells, suggesting that H0709 and NHMN1 might be anti-allergic LAB strains, and we further indicated that our system (Fig. 1) is useful in screening for anti-allergic LAB strains in a high-throughput manner. The other LAB strains that downregulate HDC promoter did not always inhibit histamine release from the ionomycin-treated KU812F cells. We intend to clarify the reasons for this ineffectiveness of HDC promoter-downregulating LAB strains in a future study, and to test further the functionalities of these LAB strains using other systems, such as antigen-triggered histamine release assay. In addition, we intend to confirm and validate the false-negative rate of this system using HDC promoter-upregulating LAB strains in histamine release assay.

C0201 also repressed histamine release from KU812F cells, which is consistent with the results shown in Fig. 2A, but during the course of the experiment we observed more extensive accumulation of C0201 around KU812F cells than H0709 (Fig. 2C). KU812F cells and LAB were stained independently with PKH26 (Sigma, St. Louis, MO) and PKH67 (Sigma) respectively. These stained cells were incubated and observed under a confocal laser scanning microscope (FV1000, Olympus, Tokyo). We observed that the pHDCp-Phi-Yellow/KU812F cells emitted bright yellow fluorescence just before death (unpublished results). All of these results suggest that C0201 had a cytotoxic effect on KU812F cells and killed them. Thus the increased yellow fluorescence, the decreased HDC transcription, and the decreased histamine release observed in KU812F cells treated with C0201 might have been caused by a cytotoxic effect of C0201 on KU812F cells. Further, we intend to determine whether C0201 induces necrosis or apoptosis in KU812F cells, and whether the LAB strains

Table 1. List of LAB

LAB species	LAB strain
<i>Lactobacillus plantarum</i>	L32, SK0201, SK0605, C0101, C1102, T2002, T2101, NHMT1
<i>Lactobacillus paracasei</i>	L31, C0102, C0301, T1802, T2003
<i>Lactobacillus gasseri</i>	L33, NHT3, NHK1, NHMF2
<i>Lactobacillus brevis</i>	C1104, T2102, NHH8
<i>Lactobacillus sakei</i>	H1001, H1201
<i>Lactobacillus curvatus</i>	H1101, T1801
<i>Lactobacillus delbrueckii</i> subsp. <i>lactis</i>	C0201
<i>Lactobacillus rhamnosus</i>	C1103
<i>Lactobacillus salivarius</i>	NHK2
<i>Lactobacillus fermentum</i>	T2103
<i>Lactococcus lactis</i>	C0402, C0504, SK0303
<i>Pediococcus acidilactici</i>	C0701, H0709, H1202, NHMN1
<i>Leuconostoc mesenteroides</i>	SK0302
<i>Lactococcus garvieae</i>	SK0603
<i>Tetragenococcus halophilus</i>	H0701

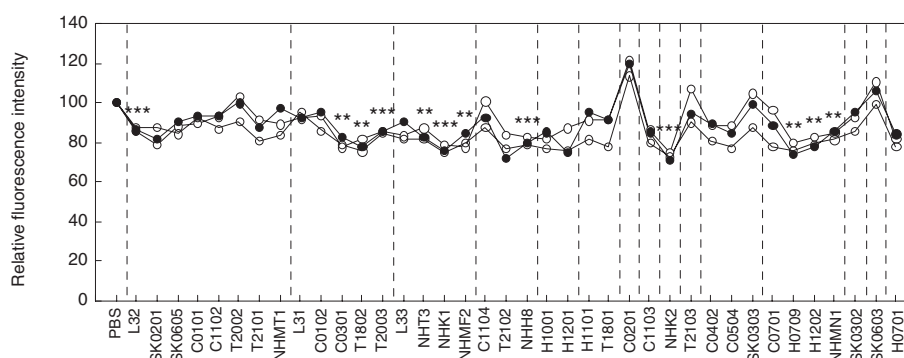


Fig. 1. Screening for LAB Strains That Downregulate HDC Promoter Activity.

pHDCp-Phi-Yellow/KU812F cells were treated with LAB strains for 7 d, and yellow fluorescence was monitored by flow cytometry. Relative fluorescence intensity was determined by dividing the peak fluorescence intensity of pHDCp-Phi-Yellow/KU812F cells treated with LAB by that of non-treated pHDCp-Phi-Yellow/KU812F cells. All experiments were repeated three times, and are indicated as different symbols in the figure. Statistical significance of differences was determined by a two-sided Student's *t*-test. Differences at $p < 0.005$ were considered significant (**, $p < 0.0005$; ***, $p < 0.0001$).

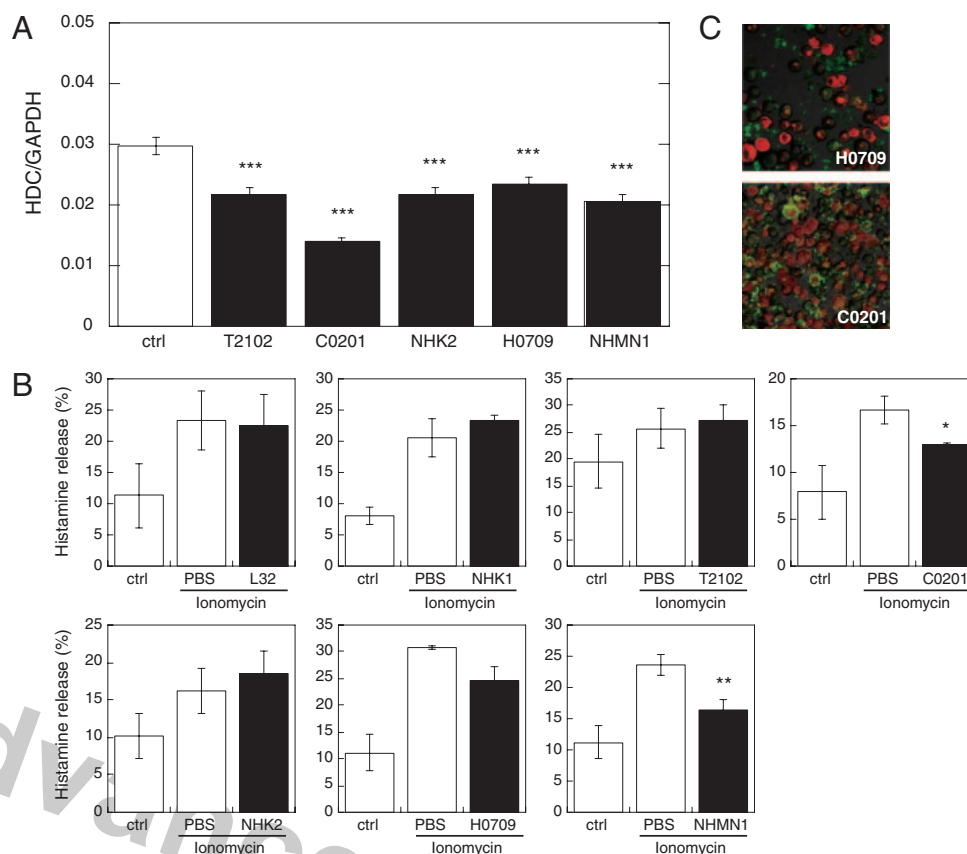


Fig. 2. Functionalities of the LAB Strains.

A, Effects of the LAB strains on the expression of the endogenous HDC gene in KU812F cells. KU812F cells were treated with the various LAB strains for 7 d, and expression of the HDC gene in the KU812F cells was evaluated by quantitative RT-PCR. Statistical significance of the differences was determined by two-sided Student's *t*-test. Differences $p < 0.001$ were considered significant (***, $p < 0.001$). B, Effects of LAB strains on histamine release from KU812F cells triggered by ionomycin. After KU812F cells were treated with the LAB strains for 3 d, the cells were treated with $10 \mu\text{M}$ ionomycin for 1 h. The amount of histamine was evaluated based on β -hexosaminidase activity. Statistical significance of differences was determined by two-sided Student *t*-test. Differences $p < 0.01$ were considered significant (*, $p < 0.01$; **, $p < 0.005$). C, Interaction between LAB and KU812F cells. KU812F cells stained with PKH26 were incubated with LAB strains stained with PKH67 for 24 h, and were observed under a confocal laser scanning microscope.

that induce necrosis or apoptosis produce a false-positive result in future study.

Considering all of our results, our system using pHDCp-Phi-Yellow/KU812F cells and flow cytometry can be used in primary screening for anti-allergic LAB strains in a high-throughput manner.

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