

Analysis of gene expression profiles of *Lactobacillus paracasei* induced by direct contact with *Saccharomyces cerevisiae* through recognition of yeast mannan

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Co-culture of lactic acid bacteria (LAB) and yeast induces specific responses that are not observed in pure culture. Gene expression profiles of *Lactobacillus paracasei* ATCC 334 co-cultured with *Saccharomyces cerevisiae* IFO 0216 were analyzed by DNA microarray, and the responses induced by direct contact with the yeast cells were investigated. Coating the LAB cells with recombinant DnaK, which acts as an adhesive protein between LAB and yeast cells, enhanced the ratio of adhesion of the LAB cells to the yeast cells. The signals induced by direct contact were clarified by removal of the LAB cells unbound to the yeast cells. The genes induced by direct contact with heat-inactivated yeast cells were very similar to both those induced by the intact yeast cells and those induced by a soluble mannan. The top 20 genes upregulated by direct contact with the heat-inactivated yeast cells mainly encoded proteins related to exopolysaccharide synthesis, modification of surface proteins, and transport systems. In the case of the most upregulated gene, LSEI_0669, encoding a protein that has a region homologous to polyprenyl glycosylphosphotransferase, the expression level was upregulated 7.6-, 11.0-, and 8.8-fold by the heat-inactivated yeast cells, the intact yeast cells, and the soluble mannan, respectively, whereas it was only upregulated 1.8-fold when the non-adherent LAB cells were not removed before RNA extraction. Our results indicated that the LAB responded to direct contact with the yeast cells through recognition of mannan on the surface of the yeast.

Key words: lactic acid bacteria, yeast, co-culture, adhesion, mannan, DnaK

INTRODUCTION

Lactic acid bacteria (LAB) are useful microorganisms that play important roles in the production of fermented foods such as yoghurt, bread, and alcoholic beverages [1, 2]. In these foods, fermentation processes often proceed in the presence of with yeast, and interactions between these microorganisms affect their growth and metabolism [3–5]. LAB also exist in the intestinal microbiota as probiotics, functioning to modulate gut mucosal immune systems and maintain homeostasis [6, 7], and the intestinal microbiota is composed of a complex community of microorganism species [8]. To understand the behaviors of LAB in these environments,

it is necessary to consider the interactions of LAB with other microorganisms. However, most studies on LAB have been conducted using pure culture of a single lactic acid bacterium. This approach is effective for simplifying complicated phenomena, e.g., proliferation, substrate consumption, and production of metabolites; however, it does not allow for evaluation of the actual behaviors of these microorganisms in their native environments, such as cell adhesion and exchange of substrates and metabolites among the cohabitating microorganisms.

In co-cultures of LAB and yeast, metabolism of yeast has been reported to affect the growth and metabolism of LAB [3–5]. We also reported that the production of kefiran, an exopolysaccharide (EPS) produced by *Lactobacillus kefiranofaciens* JCM 6985, was enhanced in co-culture with *Saccharomyces cerevisiae* IFO 0216 by preventing the accumulation of lactic acid produced by the LAB [9]. In this case, inactivation of the yeast by heat treatment or disruption with glass beads promoted kefiran production, whereas the yeast encapsulated in alginate beads did not even if the yeast was active [10]. These findings indicate that the enhancement of kefiran

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production by the LAB is induced by direct and physical contact with a component(s) on the surface of yeast cells. However, most studies on co-cultures of LAB and yeast have not considered this type of interactions and have instead focused on changes in metabolism due to the co-existence of LAB with yeast [11–13].

Since the surface of yeast cells is covered with mannan, we hypothesized that LAB would respond to direct contact with yeast cells through recognition of the surface mannan. To observe of the specific signals from the LAB cells adhered to yeast in the co-culture of LAB and yeast, however, there are some difficulties relating to the fact that not all the LAB cells aggregate with the yeast cells; that is, some LAB cells bind to yeast but some do not [13, 14]. This means that the signals from the cells adhering to yeast cells are unclear when the ratio of adhesion of LAB cell to yeast is not high enough because the signals are diluted by those from the non-adherent cells. Thus, in order to evaluate the specific signals from LAB cells adhering to yeast cells, enhancement of the ratio of adhesion of LAB cells to yeast cells and removal of the non-adherent LAB cells would be required.

In this study, we aimed to evaluate the responses of LAB induced by direct contact to yeast cells using *Lactobacillus paracasei* ATCC 334 in co-culture with *S. cerevisiae* IFO 0216 as a model strains. We previously reported that DnaK of *Lactococcus lactis* IL 1403, a cytosolic heat shock protein, acts as an adhesive protein between LAB and yeast cells [15]. To clarify the specific signals from the LAB cells adhered to yeast, the ratio of adhesion of the LAB to the yeast was enhanced by coating the LAB cells with recombinant DnaK, and the non-adherent LAB cells were removed by centrifugation. Under these manipulations, changes in gene expression of the LAB induced by direct contact with the yeast cells and the addition of soluble yeast mannan were investigated by DNA microarray assay.

MATERIALS AND METHODS

Strains and culture conditions

S. cerevisiae IFO 0216, a prototroph diploid strain, was used as a model yeast, since it was reported to interact with *L. kefiranofaciens* JCM 6985 in co-culture [9]. *Lactobacillus paracasei* ATCC 334 was used as a model LAB because its complete genome sequence is available, whereas that of JCM 6985 is not.

L. paracasei ATCC 334 was cultured statically in a digested skimmed milk (DSM) medium (described below) at 30°C to an OD₆₀₀ of 1.0. For preparation of *S. cerevisiae* IFO 0216, YPL medium (1% yeast extract,

2% Bacto peptone, 1% L-lactic acid, pH 5.0) was used to adapt the yeast to lactic acid because the yeast can utilize lactic acid but cannot utilize lactose, which is the major carbon source in the DSM medium. The yeast precultured in YPD medium (1% yeast extract, 2% Bacto peptone, 2% glucose, pH 5.5) was inoculated to YPL medium and cultivated at 30°C with shaking at 150 rpm to an OD₆₀₀ of 2.0. Co-culture of *L. paracasei* ATCC 334 and *S. cerevisiae* IFO 0216 were aerobically carried out in the DSM medium at 30°C with shaking at 80 rpm.

For preparation of the DSM medium, skimmed milk powder (100 g, Meiji, Tokyo, Japan) was dissolved in 700 ml of deionized water and digested with 2.5 g of protease (Amano protease A, Amano Pharmaceutical, Tokyo, Japan) at 47°C for 4 hr. During digestion, the pH was controlled at 6.5–7.5 with NaOH. After incubation at 80°C for 10 min and centrifugation (10,000 × g, 10 min, 4°C), the pH of the supernatant was adjusted to less than pH 6.5 in a volume of 1,000 ml. The supernatant was autoclaved at 110°C for 15 min and used as the DSM medium.

Preparation of mannan

Mannan from *S. cerevisiae* (Sigma-Aldrich, Tokyo, Japan) was dissolved in deionized water and dialyzed against 1,000 volumes of deionized water using a dialysis membrane (size 8, Wako Pure Chemical Industries, Osaka, Japan). After autoclaving at 121°C for 15 min, the mannan concentration was determined by an anthrone sulfuric acid assay [16].

Preparation of DnaK

Purification of recombinant DnaK was performed as described previously [15]. *Escherichia coli* BL21 (DE3) harboring the pET21a(+) vector combined with the DnaK gene from *L. lactis* IL 1403 (gene ID: 1114585) was cultured in Luria-Bertani medium (0.5% yeast extract, 1% Bacto tryptone, 0.5% NaCl, pH 7.5) containing 100 µg/ml ampicillin at 37°C to an OD₆₀₀ of 1.5. Isopropyl-β-D-thiogalactopyranoside was added to a concentration of 0.5 mM, and cultivation was continued for 3 hr. Cells were harvested by centrifugation (4,170 × g, 15 min, 4°C) and resuspended in 50 mM sodium phosphate buffer (pH 7.8) containing 300 mM NaCl. After ultrasonication, the recombinant DnaK was purified from the supernatant using an Ni-NTA Superflow BioRobot (Qiagen, Tokyo, Japan) according to the manufacturer's instructions. The purified proteins were concentrated by ultrafiltration and then dialyzed against PBS (10 mM sodium phosphate, 137 mM NaCl, pH 7.4). The protein concentration was determined on the basis of absorbance at 280 nm as

previously described [15].

DnaK coating of LAB

The LAB cells were washed with PBS and incubated in the DnaK solution described above at 4°C for 10 min. After removal of free DnaK by centrifugation at 10,000 × *g* for 5 min, the LAB cells were resuspended in PBS.

Measurement of the ratio of adhesion of LAB cells to yeast cells

The ratio of adhesion of LAB to yeast was measured based on the difference in sedimentation velocity of non-adherent and adherent cells. *L. paracasei* ATCC 334 cultured in the DSM medium (pH 4.5) and *S. cerevisiae* IFO 0216 cultured in YPL medium were resuspended in PBS. The LAB cells and the yeast cells were mixed to final concentrations of 1.0 × 10⁹ and 2.0 × 10⁷ cells/ml, respectively, and incubated at 30°C for 30 min. After centrifugation (500 × *g*, 2 min, 20°C) to remove the yeast cells and the LAB cells adhering to the yeast cells, the optical density at 660 nm (OD₆₆₀) was measured. The ratio of adhesion of the LAB to the yeast was calculated as follows:

$$\text{Adhesion ratio (\%)} = \frac{C - B}{A} \times 100,$$

where *A* and *B* represent the OD₆₆₀ of the suspension of the LAB cells without the yeast cells before and after centrifugation, respectively, and *C* represents the OD₆₆₀ of the suspension of the LAB and the yeast cells after centrifugation.

Preparation of total RNA

A cell suspension of *L. paracasei* ATCC 334 was incubated with RNAprotect Cell Reagent (Qiagen) for 5 min at 25°C. In co-cultures with *S. cerevisiae* IFO 0216, total RNA preparation was carried out in the presence of the yeast cells. After centrifugation (5,000 × *g*, 10 min, 4°C), cells were dissolved in TE buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) containing 10 µg/ml mutanolysin (Sigma-Aldrich) and 30 mg/ml lysozyme

(Seikagaku Corporation, Tokyo, Japan) for 30 min at 30°C. Total RNA was extracted from the cell lysate by using an RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions and then concentrated by ethanol precipitation.

DNA microarray assay

To investigate the effects of the direct contact with the yeast cells on the gene expression profiles of *L. paracasei* ATCC 334 (GenBank accession no. CP000423), DNA microarray assays (4-plex; number of target, 2,766; length of probes, 60 mer; total number of probes: 65,997) were performed by Roche NimbleGen (Tokyo, Japan). Probes were designed to minimize the homology to the genome sequence of *S. cerevisiae* to avoid cross-hybridization. As a control, total RNA was extracted from the LAB cells that were incubated alone without DnaK coating. Expression ratios (non-log scaled fold change) were calculated from the normalized signal intensities of each probe for comparison between the control group and experimental groups (*n*=1). The ranks represent the descending or ascending order of expression ratios in each group.

Real-time polymerase chain reaction (PCR)

To quantify the expression levels of the genes identified by DNA microarray assay, real-time RCP was performed using SYBR Premix *Ex Taq* II (Takara Bio Inc.) and a StepOnePlus Real-Time PCR System (Life Technologies, Tokyo, Japan) according to the manufacturer's instructions. cDNA was synthesized from total RNA using random hexamers and an oligo (dT) primer using a PrimeScript RT reagent kit (Takara Bio Inc., Shiga, Japan). The primers used in this study are listed in Table 1. The PCR protocol was as follows: one cycle at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec and 60°C for 30 sec. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (LSEI_0967) was used as an internal control. The data obtained from real-time PCR were analyzed by the ΔΔCt method of relative quantification.

Table 1. Primers used for real-time PCR

Gene ID	Sense primer	Antisense primer	Amplification size (bp)
LSEI_0967	5'-acacgattctcacagcacag-3'	5'-accgtaacaacagaaacag-3'	107
LSEI_0669	5'-ccggtgtgagttttatgtcg-3'	5'-acgattgtcaccaaccagatg-3'	109
LSEI_2709	5'-cacttgcacctgtcttggatg-3'	5'-atcgaaggcgaagaacacg-3'	135

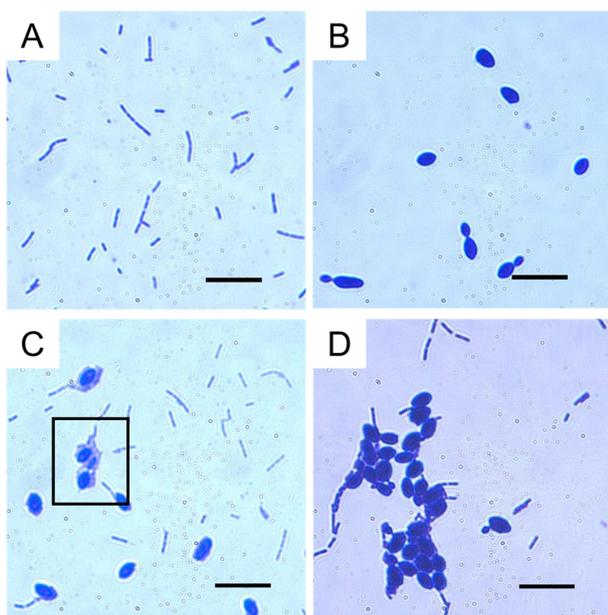


Fig. 1. Observation of *L. paracasei* ATCC 334 and *S. cerevisiae* IFO 0216 with an optical microscope.

Cells were immobilized on a slide glass and Gram's stained. (A) Pure culture of *L. paracasei* ATCC 334. (B) Pure culture of *S. cerevisiae* IFO 0216. (C) Co-culture of the LAB and the yeast. The square shows small aggregation, in which the yeast was surrounded by the LAB. (D) Large aggregation of the LAB and the yeast occurred in the co-culture. Scale bars: 10 μ m.

RESULTS

Co-aggregation of LAB and yeast in co-culture

L. paracasei ATCC 334 (1.0×10^7 cells/ml), *S. cerevisiae* IFO 0216 (1.0×10^6 cells/ml), or both microorganisms were inoculated into the DSM medium. Observations under an optical microscope revealed that the LAB cells and the yeast cells were scattered in each pure culture (Fig. 1A, B). In the co-culture of the LAB and the yeast, in contrast, a part of the LAB cells adhered to the yeast cells (Fig. 1C), and some yeast cells aggregate across the LAB cells (Fig. 1D). In the aggregates, the LAB cells were considered to mediate the adhesion of individual yeast cell.

Effects of DnaK on adhesion of LAB cells to yeast cells

To evaluate the effects of direct contact with the yeast on the behaviors of the LAB, the ratio of adhesion of the LAB cells to the yeast cells should not be low. As shown in Fig. 2, however, the ratio of adhesion of *L. paracasei* ATCC 334 cells to *S. cerevisiae* IFO 0216 cells in the DSM medium was only $9.6 \pm 0.9\%$. This culture condition is not suitable for microarray analysis because

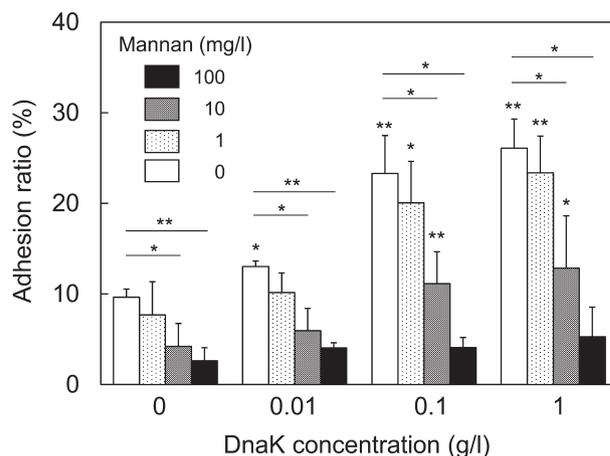


Fig. 2. Effects of mannan and DnaK concentrations on adhesion of *L. paracasei* ATCC 334 to *S. cerevisiae* IFO 0216.

The data are expressed as means \pm SDs of triplicate samples. * $p < 0.05$ for each group or vs. the group without DnaK at each mannan concentration by Student's t-test. ** $p < 0.01$ for each group or vs. the group without DnaK at each mannan concentration by Student's t-test.

the array signals from the adhered cells were diluted approximately 10-fold by those from the non-adherent cells. We previously reported that DnaK from *L. lactis* IL 1403 has affinity with both LAB cells and yeast mannan and that addition of the recombinant DnaK promotes the aggregation of *L. lactis* IL 1403 with yeast [15]. Therefore, we examined whether coating of the LAB cells with DnaK could increase the adhesion ratio. When the LAB cells were precoated with the recombinant DnaK at concentrations of 0.01, 0.1, or 1 g/l, the adhesion ratios increased to $13.0 \pm 0.6\%$, $23.3 \pm 4.2\%$, and $26.0 \pm 3.2\%$, respectively (Fig. 2). On the other hand, the adhesion was inhibited in a dose-dependent manner by addition of soluble mannan to the cell suspensions.

Change in gene expression profiles of LAB induced by adhesion to yeast

Changes in the gene expression profiles of the LAB cells induced by addition of the yeast cells or the soluble mannan were analyzed using a DNA microarray. The LAB cells were precoated with 0.1 g/l DnaK solution to enhance the adhesion and incubated in the DSM medium (pH 4.5, adjusted with lactic acid; cell density: 1×10^9 cells/ml) for 30 min at 30°C with the intact or the heat-inactivated yeast cells (2×10^7 cells/ml). The non-adherent LAB cells were removed from the supernatant after a gentle centrifugation ($500 \times g$ for 2 min) to clarify the signals from the adhered cells. Total RNA

Table 2. The top 20 genes of *L. paracasei* ATCC 334 upregulated by adhesion to *S. cerevisiae* IFO 0216

Gene ID	Predicted function	Localization	Expression ratio				
			a	b	c	d	e
LSEI_0669	Polyprenyl glycosylphosphotransferase ¹⁾	Membrane	1.9	7.6 (1)	11.0 (1)	8.8 (1)	1.8
LSEI_2659	Predicted holin-like toxin	Membrane	1.4	5.6 (2)	8.3 (3)	5.9 (8)	1.6
LSEI_1045	Hypothetical protein	Membrane	1.6	5.5 (3)	8.8 (2)	6.4 (3)	1.9
LSEI_0069	Hypothetical protein	Membrane	1.4	5.3 (4)	7.2 (5)	3.8 (63)	1.5
LSEI_2895	Peptide ABC transporter permease	Membrane	1.6	5.2 (5)	6.9 (7)	5.6 (14)	1.4
LSEI_0457	Sortase (surface protein transpeptidase)	Membrane	1.3	5.0 (6)	7.9 (4)	5.5 (15)	1.7
LSEI_0329	Kinase	Intracellular	1.6	5.0 (7)	7.0 (6)	6.4 (4)	1.6
LSEI_2740	Phosphotransferase system, fructose-specific IIC component	Membrane	1.3	4.7 (8)	6.6 (8)	4.3 (41)	1.6
LSEI_0079	Hypothetical protein	Membrane	1.6	4.5 (9)	5.5 (18)	4.9 (23)	1.2
LSEI_2700	Phosphotransferase system IIA component	Intracellular	1.6	4.3 (10)	6.5 (10)	5.7 (11)	1.6
LSEI_0059	Hypothetical protein	Membrane	1.4	4.2 (11)	6.5 (9)	4.9 (22)	1.5
LSEI_0294	ABC-type cobalt transport system, permease component	Membrane	1.5	4.2 (12)	5.7 (14)	4.6 (29)	1.5
LSEI_0049	Metal-dependent membrane protease	Membrane	1.3	4.0 (13)	6.1 (11)	3.8 (61)	1.3
LSEI_0368	Sugar metabolism regulatory protein	Intracellular	1.3	4.0 (14)	5.8 (13)	4.5 (31)	1.4
LSEI_2709	Transcriptional antiterminator	Intracellular	1.5	3.8 (15)	5.7 (16)	5.7 (13)	1.3
LSEI_2354	Hypothetical protein	Intracellular	1.4	3.7 (16)	5.8 (12)	5.9 (7)	1.5
LSEI_0298	Hypothetical protein	Membrane	1.6	3.7 (17)	4.9 (25)	3.7 (65)	1.3
LSEI_1905	LPXTG-anchored protein ¹⁾	Intracellular	1.8	3.6 (18)	5.7 (15)	6.2 (6)	1.3
LSEI_0882	Membrane-associated phospholipid phosphatase	Membrane	1.4	3.6 (19)	4.6 (30)	4.2 (45)	1.4
LSEI_2671	5-Keto-4-deoxyuronate isomerase	Intracellular	1.3	3.6 (20)	4.8 (27)	3.6 (68)	1.4

Gene expression levels relative to that in which intact LAB cells (without DnaK coating) were cultivated in skimmed milk medium are shown. Data in parentheses represent the rank of the expression ratio in descending order in each group. a, Heat-inactivated yeast cells were added to DnaK-coated LAB, and non-adherent LAB cells were not removed. b, Heat-inactivated yeast cells were added to DnaK-coated LAB cells, and non-adherent LAB cells were removed. c, Intact yeast cells were added to DnaK-coated LAB, and non-adherent LAB cells were removed. d, Mannan (10 mg/l) was added to the intact LAB. e, LAB with DnaK coating.

¹⁾ The function was predicted based on BLAST, although the function of the gene was originally annotated as hypothetical protein.

was extracted from the LAB cells sedimented with the yeast cells, and the expression levels of genes relative to the case in which the LAB were incubated in the DSM medium alone were analyzed by the DNA microarray.

Table 2 shows the top 20 genes in the LAB that were upregulated by addition of the heat-inactivated yeast cells with subsequent removal of the non-adherent LAB cells (column b). The genes upregulated by addition of the heat-inactivated yeast cells were similar to both those upregulated by the intact yeast cells (column c) and those upregulated by the soluble mannan (column d). The LAB cells were precoated in these experiments with DnaK to enhance the adhesion of the LAB cells to the yeast cells. However, no significant changes in the expression levels were observed by coating the LAB cells with DnaK (column e). When the DnaK-treated LAB cells were incubated with the heat-inactivated yeast cells but the non-adherent LAB cells were not removed, the changes in the expression levels were unclear (column a), and no genes showed expression ratios of more than 2.0. The relative standard deviations of the expression ratios of all 2,766 genes for the DnaK-precoated LAB cells incubated with the heat inactivated yeast cells

without and with removal of the non-adherent cells were 0.14 and 0.50, respectively, showing that the removal of the non-adherent LAB cells clarified the responses of the LAB to the heat-inactivated yeast. In the case of the most upregulated gene, LSEI_0669, the expression ratio for the heat-inactivated yeast cells was more than 4-fold increased by removal of the non-adherent LAB cells. The expression ratios of other genes for the heat-inactivated yeast cells were also increased approximately 3-fold by removal of the non-adherent cells. These results agree with the results showing that the ratio of adhesion of the LAB to the yeast was $23.3 \pm 4.3\%$ at the DnaK concentration of 0.1 g/l (Fig. 2).

On the other hand, Table 3 shows the top 20 genes of the LAB that were downregulated by incubation with the heat-inactivated yeast cells (column b). Without removal of the non-adherent LAB cells, since the expression ratio was 0.62 (for LSEI_2393) even in the lowest case, it was difficult to identify the downregulated genes. With removal of the non-adherent LAB cells, it was found that the genes such as LSEI_0793 encoding D-Ala-teichoic acid biosynthesis protein were downregulated. These genes were also downregulated when the LAB cells were

incubated with the intact cells or the soluble mannan.

Effects of DnaK coating and removal of non-adherent cells on the apparent increase in expression level

To confirm the effects of the DnaK coating and removal of the non-adherent LAB cells on the signal/noise ratio of microarray assays, the relative increases in the expression levels of LSEI_0669 and LSEI_2709 were quantitatively analyzed by real-time PCR (Fig. 3). The apparent relative expression levels of LSEI_0669 and LSEI_2709 were increased to 2.5 ± 1.2 and 1.9 ± 1.0 , respectively, by incubation with the heat-inactivated yeast. With removal of the non-adherent LAB cells, the apparent relative expression levels were increased to 6.6 ± 2.6 and 4.7 ± 2.0 , respectively. When the LAB cells were precoated with the recombinant DnaK that acts as an adhesive protein for yeast cells, the apparent relative expression levels were further increased to 11.6 ± 6.2 and 8.2 ± 3.0 , respectively.

Predicted functions of genes induced by adhesion to yeast cells

As shown in Table 2, half of the genes upregulated by the addition of the yeast cells or the soluble mannan were enzymes or proteins related to sugar metabolism and transport. We analyzed whether these proteins contained membrane-spanning domains and signal peptides using the SOSUI database (<http://bp.nuap.nagoya-u.ac.jp/sosui/>) [17]. Thirteen of the 20 proteins encoded by these genes contained at least one membrane-spanning domain. According to BLAST analysis (<http://www.ncbi.nlm.nih.gov/>), LSEI_0669, which showed the most dramatic change in expression ratio in the microarray analysis, encodes a protein (236 aa) that possesses a domain homologous to the highly conserved sugar transferase domain of polyprenyl glycosylphosphotransferase involving EPS biosynthesis on the C-terminal side. On the other hand, most of the genes downregulated by the addition of the yeast cells or mannan encoded intracellular proteins (Table 3), and some of them were related to the purine and pyrimidine catabolism.

DISCUSSION

Co-culture of LAB and yeast has been studied to examine changes in growth, substrate consumption, and production of metabolites [3–5]. In our study on the co-culture of *L. paracasei* ATCC 334 and *S. cerevisiae* IFO 0216, we focused on the effects of direct contact with the yeast cells on the gene expression profile of the LAB and aimed to show that the LAB would respond to direct

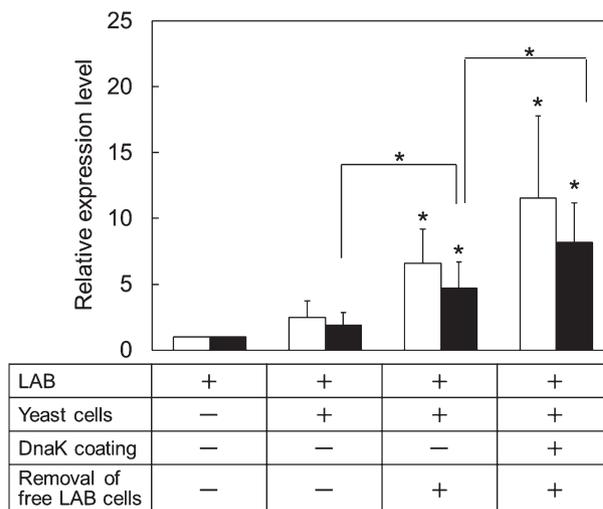


Fig. 3. Effects of removal of free LAB cells on apparent gene expression levels of LSEI_0669 (open bar) and LSEI_2709 (closed bar).

DnaK was added at 0.1 g/l. The data are expressed as means \pm SDs of triplicate samples. * $p < 0.05$ for each group or vs the control (only LAB) by Student's t-test.

contact with yeast mannan. However, it was difficult to analyze the responses of the LAB when they were cultivated with the yeast cells because a part of them adhered to the yeast cells, while the responses were clear when they cultivated with the soluble mannan because all the LAB cells were exposed to mannan molecules. Most of the LAB cells did not form aggregates with the yeast cells (Fig. 1C, D), and the original ratio of adhesion of the LAB cells to the yeast cells was under 10% (Fig. 2), which resulted in that the specific responses induced by direct contact with the yeast cells being unclear (Table 2, column a). Therefore, we examined the ability of the recombinant DnaK to enhance the adhesion of the LAB cells to the yeast cells [15]. It was found that indeed coating of the LAB cells with DnaK increased the adhesion ratio, and the adhesion was inhibited by addition of mannan (Fig. 2). These results indicated that the target site of the LAB cells is mannan on the cell surface of the yeast. By removal of the non-adherent LAB cells, it ultimately became possible to clarify the specific signals induced by direct contact with the yeast cells (Tables 2 and 3).

The DNA microarray analysis revealed that the genes highly upregulated by incubation with the heat-inactivated yeast cells were similar to both those upregulated by the intact yeast cells and those upregulated by the soluble mannan. These results indicated that direct contact with mannan would be the main trigger of these responses and

Table 3. The top 20 genes of *L. paracasei* ATCC 334 downregulated by adhesion to *S. cerevisiae* IFO 0216

Gene ID	Predicted function	Localization	Expression ratio				
			a	b	c	d	e
LSEI_0793	Putative D-Ala-teichoic acid biosynthesis protein	Membrane	0.83	0.27 (1)	0.82 (1042)	0.35 (46)	0.86
LSEI_2122	Hypothetical protein	Intracellular	0.94	0.28 (2)	0.21 (1)	0.37 (64)	0.79
LSEI_0572	Hypothetical protein	Intracellular	0.92	0.38 (3)	0.26 (2)	0.33 (35)	0.78
LSEI_0417	Hypothetical protein	Intracellular	0.82	0.38 (4)	0.29 (4)	0.22 (1)	0.98
LSEI_1400	Hypothetical protein	Intracellular	0.89	0.40 (5)	0.29 (7)	0.23 (3)	0.87
LSEI_2559	Hypothetical protein	Intracellular	0.77	0.40 (6)	0.32 (14)	0.34 (40)	0.95
LSEI_2115	3-Oxoacyl-acyl carrier protein reductase	Intracellular	1.19	0.40 (7)	0.31 (11)	0.33 (34)	0.87
LSEI_1658	Rhodanese-related sulfurtransferase	Intracellular	0.95	0.40 (8)	0.31 (10)	0.34 (37)	0.92
LSEI_0962	Transcriptional regulator	Intracellular	0.67	0.42 (9)	0.36 (31)	0.45 (155)	0.84
LSEI_1795	Acetyltransferase	Intracellular	0.72	0.42 (10)	0.34 (20)	0.40 (93)	0.86
LSEI_2349	Purine catabolism regulatory protein-like family ¹⁾	Intracellular	0.80	0.43 (11)	0.31 (13)	0.59 (446)	0.93
LSEI_1788	Hypothetical protein	Intracellular	0.95	0.43 (12)	0.38 (38)	0.45 (152)	0.81
LSEI_1669	Uridine kinase	Intracellular	0.87	0.43 (13)	0.35 (25)	0.31 (24)	0.87
LSEI_2260	Hypothetical protein	Intracellular	0.80	0.44 (14)	0.38 (39)	0.41 (103)	0.86
LSEI_2271	Peptidylprolyl isomerase	Intracellular	0.93	0.44 (15)	0.29 (8)	0.90 (1339)	0.90
LSEI_1399	Rossmann fold nucleotide-binding protein for DNA uptake	Intracellular	0.95	0.44 (16)	0.35 (24)	0.32 (26)	0.82
LSEI_1316	Hypothetical protein	Intracellular	0.92	0.44 (17)	0.33 (17)	0.29 (17)	0.84
LSEI_0570	Adenine specific DNA methylase Mod	Intracellular	0.78	0.44 (18)	0.40 (48)	0.48 (191)	0.87
LSEI_1113	Growth regulator	Intracellular	0.82	0.44 (19)	0.40 (50)	0.63 (573)	0.93
LSEI_1330	Metallo-beta-lactamase superfamily hydrolase	Intracellular	0.83	0.45 (20)	0.27 (3)	0.23 (2)	0.92

Gene expression levels relative to that in which intact LAB cells (without DnaK coating) were cultivated in skimmed milk medium are shown. Data in parentheses represent the rank of the expression ratio in ascending order in each group. a, Heat-inactivated yeast cells were added to DnaK-coated LAB, and non-adherent LAB cells were not removed. b, Heat-inactivated yeast cells were added to DnaK-coated LAB cells, and non-adherent LAB cells were removed. c, Intact yeast cells were added to DnaK-coated LAB, and non-adherent LAB cells were removed. d, Mannan (10 mg/l) was added to the intact LAB. e, LAB with DnaK coating.

¹⁾ The function was predicted based on BLAST, although the function of the gene was originally annotated as hypothetical protein.

that metabolites from the yeast cells did not influence notably the responses of the LAB. These results also suggested the possibility that LAB have mannose receptors, and the mechanisms of mannan recognition of LAB must be considered in the future.

The gene most upregulated, LSEI_0669, both by the yeast cells and mannan codes a protein homologous to polyprenyl glycosylphosphotransferase involved in EPS biosynthesis. We previously reported that EPS production by *L. kefiranofaciens* JCM 6985 was enhanced by co-culture with *S. cerevisiae* IFO 0216 cells [10]. These results suggested that the production of EPS by LAB would be stimulated by direct contact with yeast cells through yeast surface mannan. On the other hand, when LAB grow alone in viscous environments, such as in fermentation foods, in where diffusion of molecules is slow, lactic acid produced by LAB would accumulate around them and cause acid stress. In our previous report, yeast assimilated lactic acid produced by LAB in co-culture of LAB with yeast [10], and this phenomenon was also observed in this study (data not shown). These results indicated that the adhesion of LAB cells to yeast cells could relieve the lactic acid-induced

stress arising from LAB in such a viscous environment. EPS production increases the viscosity around LAB cells and leads to acid-induced stress caused by lactic acid produced by the LAB cells themselves. Therefore, it is reasonable to suggest that the adhesion of LAB cells to yeast cells triggers EPS production in the LAB cells because the adhesion leads to efficient consumption of lactic acid by the yeast cells.

Here, we focused on a protein encoded by the LSEI_0457 gene induced by yeast cells or soluble mannan, whose product is predicted to function as a sortase. In gram-positive bacteria, sortase recognizes surface proteins possessing the LPxTG motif, cleaves between threonine and glycine residues, and links covalently the carboxyl group of threonine to an amino group of the peptidoglycan [18, 19]. Proteins with the LPxTG motif, such as LspA of *Lactobacillus salivarius* UCC118 and the protein encoded by hmpref0536_10633 of *Lactobacillus reuteri*, have been known to mediate adhesion of each LAB to human epithelial cells [20, 21]. *L. paracasei* ATCC 334 possesses several proteins with the LPxTG motif that are encoded by LSEI_0445, LSEI_0465, LSEI_0564, LSEI_0607, LSEI_1314, LSEI_1905, LSEI_2320,

LSEI_2363, LSEI_2365, LSEI_2431, and LSEI_2896. The LSEI_1905 gene was highly upregulated by the yeast cells and the soluble mannan as shown in Table 2 (rank 18 in column b, rank 6 in column d), whereas the function of the gene is unknown. The protein encoded by LSEI_2320 exhibits 98% and 38% identity with internalin J proteins of *Lactobacillus casei* and *Lactobacillus rhamnosus*, respectively, according to the results of BLAST analysis. It was reported that the internalin J encoded by LGG_02337 of *L. rhamnosus* GG participates in mucus-specific adhesion of the strain [22]; however, LSEI_2320 was upregulated neither by the yeast cells nor the soluble mannan (the expression ratios for the heat-inactivated yeast cells, the intact yeast cells, and the soluble mannan were 0.97, 0.74, and 1.03, respectively). The expression level of the sortase gene, LSEI_0457, might be elevated in response to the upregulation of LSEI_1905 encoding a protein with the LPxTG motif. These results suggested that direct contact with yeast cells or soluble mannan would cause the LAB to modify the surface proteins, whereas the functions of proteins with the LPxTG motif are not fully understood.

In summary, we analyzed the effects of direct contact with *S. cerevisiae* IFO 0216 on the gene expression profile of *L. paracasei* ATCC 334 by improvement of the adhesion ratio using the adhesion protein DnaK and by removal of the non-adherent LAB cells. This revealed that direct contact of the LAB cells with the yeast cells induced the expression of genes involved in the EPS synthesis pathway, modification of the cell surface, and metabolite transfer. It is notable that most of the genes induced in the LAB by direct contact with the yeast (Table 2) possessed homologs among *Lactobacillus* species. For example, the protein encoded by LSEI_0669 has corresponding homologs in *L. casei*, *L. rhamnosus*, and *Lactobacillus zae* with 98%, 33%, and 28% identity, respectively. The hypothetical protein (379 aa) encoded by LSEI_0079 also has corresponding homologs in *L. casei*, *Lactobacillus camelliae*, *Lactobacillus plantarum*, and *Lactobacillus curvatus* with 99%, 46%, 28%, and 27% identity, respectively. These results suggested that the phenomena induced by direct contact with yeast cells were not limited to *L. paracasei* ATCC 334 but could widely occur in lactobacilli.

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