Isolation of food-derived bacteria inducing interleukin-22 in B cells

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Recently, we found a novel function of the lactic acid bacterium *Tetragenococcus halophilus* derived from miso, a fermented soy paste, that induces interleukin (IL)-22 production in B cells preferentially. IL-22 plays a critical role in barrier functions in the gut and skin. We further screened other bacteria species, namely, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Weissella*, *Pediococcus*, and *Bacillus*, in addition to *Tetragenococcus* and found that some of them possessed robust IL-22-inducible function in B cells *in vitro*. This process resulted in the augmented expression of activation markers CD86 and CD69 on B and T cells, respectively. However, these observations were not correlated with IL-22 production. We isolated *Bacillus coagulans* sc-09 from miso and determined it to be the best strain to induce robust IL-22 production in B cells. Furthermore, feeding *B. coagulans* sc-09 to mice augmented the barrier function of the skin regardless of gut microbiota.

Key words: food, IL-22, bacteria, B cell, skin barrier, miso

INTRODUCTION

Miso and soy sauce, which are traditional fermented foods in Japan, contain various microorganisms. In addition to a fungus (*Aspergillus oryzae*) and yeast, *Tetragenococcus halophilus*, a salt-tolerant lactic acid bacterium; other lactic acid bacteria; and *Bacillus* strains contribute to the fermentation processes of miso and soy sauce. Recently, the beneficial effects of these microorganisms and fermented foods on human health have been reported [1–4].

Recently, we isolated a strain of lactic acid bacteria, *T. halophilus* No. 1, which has immune regulatory functions, from miso, a fermented soy paste [5]. Administration of this strain augmented serum IgA and immune responses in mice. Notably, *T. halophilus* No. 1 induced interleukin (IL)-22 cytokine production in B cells. Thus, for the first time, we found that a subpopulation of B cells produce IL-22. Furthermore, *T. halophilus* induced production of interferon (IFN)-γ in B cells. We termed IL-22-producing and IFN-γ–producing B cell subpopulations as Bi22 and Big cells, respectively.

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IL-22 is a member of the IL-10 family [6–8]. It was originally thought to be produced from T helper (Th)1 cells among CD4 T cells, and then subsequently it was found to be produced from Th17 and Th22 cells. Furthermore, γδT cells, NKT cells, and innate lymphoid cells are also known to produce IL-22. IL-22 has been identified in various tissues, such as the intestines, lung, liver, kidney, thymus, pancreas, and skin. It contributes to tissue regeneration and regulates host defense at barrier surfaces, such as the gut and skin. IL-22 is also involved in inflammatory tissue pathology. However, a comprehensive understanding of IL-22 remains elusive.

As IL-22 is a multifunctional cytokine, especially with respect to host defense functions, probiotics that induce IL-22 may be valuable to human health. Therefore, in this study, we investigated food-derived microorganisms that induce IL-22 production, identified IL-22-inducing bacteria, and assessed their *in vivo* functions.

MATERIALS AND METHODS

Ethics statement

C57BL/6 mice were maintained in our animal facility under specific pathogen free (SPF) conditions in accordance with guidelines of the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University. Germ-free (GF) mice (C57BL/6NJcl) were obtained from CLEA Japan, Inc. All experimental procedures on animals were approved by the Institutional Animal Care and Use Committee

of Tokyo Medical and Dental University (No. A2018-C3), and all experiments were carried out in accordance with the approved guidelines.

Bacteria

Bacteria were isolated from Japanese fermented foods, including miso, soy sauce, and amazake. Lactic acid bacteria were selected using MRS agar (Oxoid Ltd.) with $CaCO_3$. Salt-tolerant lactic acid bacteria, such as *T. halophilus*, were separated in 10SG10N agar (10% soy sauce, 10% NaCl, 1% glucose, 1% yeast extract, 0.5% polypeptone, 0.2% sodium acetate trihydrate, 0.02% $MgSO_4 \cdot 7H_2O$, 0.001% $MnSO_4 \cdot 4H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0025% Tween 80, and 1.5% agar; pH 6.8). Bacteria, such as *Bacillus subtilis*, were isolated in a standard methods agar (5.0 g/L pancreatic digest of casein, 2.5 g/L yeast extract, 1.0 g/L dextrose, 15.0 g/L agar; pH 7.0 ± 0.2). These bacteria were identified by microscopy and 16S rDNA analysis. Isolated bacteria were cultured, and cultures were sterilized by autoclaving at 121°C for 15 min. The bacteria were then collected by centrifugation, washed three times with water, and then lyophilized. These bacteria were directly used as a dietary supplement. Alternatively, these bacteria were suspended in PBS and used for *in vitro* immunological assay.

PCR amplification and bacterial 16S rDNA sequencing

Total bacterial DNA was extracted using a NucleoSpin Microbial DNA kit (Macherey-Nagel GmbH & Co. KG). Bacterial 16S rDNA was amplified by PCR using primers 10F (5′-GTT TGA TCC TGG CTC A-3′) and 1500R (5′-TAC CTT GTT ACG ACT T-3′). PCR products were purified using a FastGene Gel/PCR Extraction Kit (Nippon Genetics Co., Ltd). The purified PCR products were sequenced by FASMAC Co., Ltd., Japan, using an Applied Biosystems 3130 XL Genetic Analyzer (Applied Biosystems, Switzerland). To identify the bacterial species, the NCBI BLAST database was used for comparisons.

Cells and mice

The spleen cells of the C57BL/6 mice were prepared as described previously [9]. $B220^+$ B cells were isolated from the spleen cells using a BD™ IMag Cell Separation System in accordance with the manufacturer's instructions (Becton, Dickinson and Company). $B220^+$ cells were recovered with a purity of >95%.

C57BL/6 mice (8 weeks old) were fed either a standard control diet (CE2, CLEA Japan, Inc.) or a diet supplemented with 1% *Bacillus coagulans* sc-09 for 3 weeks under SPF conditions. To investigate the effect of IL-22, recombinant mouse IL-22 (Tonbo) was administered to control mice by tail vein injection. IL-22 monoclonal antibodies (mAb; Thermo Fisher Scientific) were administered by tail vein injection to the mice fed the diet supplemented with 1% *B. coagulans* sc-09. The GF mice (C57BL/6NJcl; 8 weeks old) were either fed a standard control diet (CE2, CLEA Japan, Inc.) or a diet supplemented with 1% *B. coagulans* sc-09 for 4 weeks under an aseptic environment.

In vitro immunological assays

In vitro immunological assays were performed as described previously [5]. A total of 2×10^6 spleen cells were cultured in 1 mL of RPMI 1640 medium containing 10% FCS with or without 10 μg of bacterial cells for 2 days. Activation cell surface markers CD69 and CD86 on spleen cells were evaluated by flow cytometry. Viability was defined as the ration of viable cells to total cells and was determined as described previously [5]. The viability of total spleen cells in the control was 12.0% on average.

Cytokine assays

Spleen cells were cultured for 2 days at a concentration of 2×10^6 cells/mL in RPMI 1640 medium containing 10% FCS with or without 10 μg of bacterial cells. BD GolgiStop™ (in accordance with the manufacturer's instructions; Becton, Dickinson and Company) was added to the medium at 6 hr before the end of the cultivation period. To measure intracellular cytokines, a BD Fixation/Permeabilization Solution Kit (Becton, Dickinson and Company) was used. Then, permeabilized cells were treated with phycoerythrin (PE)-labeled anti-IL-22 antibodies (clone 1H8PWSR, eBioscience). Cells were analyzed by flow cytometry. IL-22-positive cells in $B220^+$ cells cultured without bacteria served as the control, and their number was defined as 100%. Based on this finding, the relative proportion of IL-22 positive cells cultured with bacteria was calculated as the relative IL-22 expression (%).

Flow cytometry

The cells were analyzed on a MACSQuant Flow Cytometer (Miltenyi Biotec) using the following antibodies: violetFluor™ 450-labeled anti-B220 antibodies (clone RA3-6B2) and APC-labeled anti-CD86 antibodies (clone GL-1) purchased from Tonbo Biosciences and Brilliant Violet 510™ anti-mouse CD4 antibodies (clone RM4-5) and PE-labeled anti-CD69 antibodies (clone H1.2F3) purchased from BioLegend. Dead cells were excluded using propidium iodide (PI) staining. Data analysis was conducted with FlowJo (FlowJo, LLC).

Evaluation of skin barrier function

Transepidermal water loss (TEWL) in mouse skin was measured using a DermaLab Combo system (Cortex Technology). TEWL measurements were recorded once the reading had stabilized at approximately 30 sec after the probe was placed on the skin.

Statistical analysis

Regarding the experimental date in Table 1, samples that had been measured one time and found to have increased were measured 1–6 more times, and the mean value and standard error (SE) were determined. Experimental data in Figs. 2, 3 are indicated as the mean ± SE. Experimental data

Table 1. IL-22 production and CD86 expression in B cells caused by *in vitro* stimulation of bacteria

Strain		Relative IL-22 expression (%)		Relative CD86 expression (%)		n
		x^-	SE	x^-	SE	
Control		100		100		
Tetragenococcus halophilus	ta-01	100		255		1
Tetragenococcus halophilus	ta-02	98		172		1
Tetragenococcus halophilus	ta-03	97		172		1
Tetragenococcus halophilus	ta-04	119	1	183	6	3
Tetragenococcus halophilus	ta-05	99		171		1
Tetragenococcus halophilus	ta-06	100		192		1
Tetragenococcus halophilus	ta-07	98		183		1
Tetragenococcus halophilus	ta-08	109		191		1
Tetragenococcus halophilus	ta-09	98		198		1
Tetragenococcus halophilus	ta-10	96		170		1
Tetragenococcus halophilus	ta-11	99		172		1
Tetragenococcus halophilus	ta-12	102		178		1
Tetragenococcus halophilus	ta-13	129	12	198	5	3
Tetragenococcus halophilus	ta-14	100		169		1
Tetragenococcus halophilus	ta-15	95		166		1
Tetragenococcus halophilus	ta-16	114		179		1
Tetragenococcus halophilus	ta-17	99		172		1
Tetragenococcus halophilus	ta-18	97		170		1
Tetragenococcus halophilus	ta-19	112		158		1
Tetragenococcus halophilus	ta-20	94		141		1
Tetragenococcus halophilus	ta-21	138	13	162	12	3
Tetragenococcus halophilus	ta-22	95		137		1
Tetragenococcus halophilus	ta-23	92		129		1
Tetragenococcus halophilus	ta-24	94		144		1
Tetragenococcus halophilus	ta-25	105		146		1
Tetragenococcus halophilus	ta-26	100		153		1
Tetragenococcus halophilus	ta-27	101		148		1
Tetragenococcus halophilus	ta-28	100		105		1
Tetragenococcus halophilus	ta-29	97		115		1
Tetragenococcus halophilus	ta-30	91		104		1
Tetragenococcus halophilus	ta-31	100		104		1
Tetragenococcus halophilus	ta-32	97		106		1
Tetragenococcus halophilus	ta-33	100		109		1
Tetragenococcus halophilus	ta-34	111		155		1
Tetragenococcus halophilus	ta-35	115		215		1
Tetragenococcus halophilus	ta-36	106		179		1
Tetragenococcus halophilus	ta-37	112		167		1
Tetragenococcus halophilus	ta-38	123	1	168	15	3
Tetragenococcus halophilus	ta-39	107		156		1
Tetragenococcus halophilus	ta-40	97		166		1
Tetragenococcus halophilus	ta-41	99		169		1
Tetragenococcus halophilus	ta-42	100		177		1
Tetragenococcus halophilus	ta-43	97		162		1
Tetragenococcus halophilus	ta-44	102		215		1
Tetragenococcus halophilus	ta-45	97		138		1
Tetragenococcus halophilus	ta-46	97		136		1
Tetragenococcus halophilus	ta-47	100		189		1
Tetragenococcus halophilus	ta-48	99		174		1
Tetragenococcus halophilus	ta-49	137	3	235	8	3
Tetragenococcus halophilus	ta-50	118		111		1
Tetragenococcus halophilus	ta-51	169	33	262	28	5
* ***Tetragenococcus halophilus***	**ta-52**	394	27	348	15	7
Tetragenococcus halophilus	ta-53	91		256		1
Tetragenococcus halophilus	ta-54	95		187		1
Tetragenococcus halophilus	ta-55	96		158		1
Tetragenococcus halophilus	ta-56	92		148		1
Tetragenococcus halophilus	ta-57	90		221		1
Tetragenococcus halophilus	ta-58	92		211		1
Tetragenococcus halophilus	ta-59	93		169		1
Tetragenococcus halophilus	ta-60	99		173		1
Tetragenococcus halophilus	ta-61	94		165		1
Tetragenococcus halophilus	ta-62	92		156		1
Tetragenococcus halophilus	ta-63	91		187		1
Tetragenococcus halophilus	ta-64	91		154		1
Tetragenococcus halophilus	ta-65	90		171		1
Tetragenococcus halophilus	ta-66	94		160		1
Tetragenococcus halophilus	ta-67	94		142		1
Tetragenococcus halophilus	ta-68	113		169		1
Tetragenococcus halophilus	ta-69	90		143		1
Tetragenococcus halophilus	ta-70	90		162		1
Tetragenococcus halophilus	ta-71	92		156		1
Tetragenococcus halophilus	ta-72	88		215		1
Tetragenococcus halophilus	ta-73	92		149		1
Tetragenococcus halophilus	ta-74	105		161		1
Tetragenococcus halophilus	ta-75	93		186		1
Tetragenococcus halophilus	ta-76	90		146		1
Tetragenococcus halophilus	ta-77	90		167		1
Tetragenococcus halophilus	ta-78	92		149		1
Tetragenococcus halophilus	ta-79	90		143		1
Tetragenococcus halophilus	ta-80	106		177		1
Tetragenococcus halophilus	ta-81	94		147		1
Tetragenococcus halophilus	ta-82	95		166		1
Tetragenococcus halophilus	ta-83	108		199		1
Tetragenococcus halophilus	ta-84	94		159		1
Tetragenococcus halophilus	ta-85	93		240		1
Tetragenococcus halophilus	ta-86	100		165		1
Tetragenococcus halophilus	ta-87	92		160		1
Tetragenococcus halophilus	ta-88	95		185		1
Tetragenococcus halophilus	ta-89	98		148		1
Tetragenococcus halophilus	ta-90	94		160		1
Tetragenococcus halophilus	ta-91	94		162		1
Tetragenococcus halophilus	ta-92	93		152		1
Tetragenococcus halophilus	ta-93	99		166		1
Tetragenococcus halophilus	ta-94	92		177		1
Tetragenococcus halophilus	ta-95	92		178		1
Enterococcus faecalis	fa-01	104		155		1
Enterococcus faecalis	fa-02	123	3	155	7	3
Enterococcus faecalis	fa-03	110		153		1
Enterococcus faecalis	fa-04	95		124		1
Enterococcus faecalis	fa-05	100		121		1
Enterococcus faecalis	fa-06	92		120		1
Enterococcus faecalis	fa-07	102		107		1
Enterococcus faecalis	fa-08	110		126		1
Enterococcus faecalis	fa-09	113		137		1
Enterococcus faecalis	fa-10	96		129		1
Enterococcus faecalis	fa-11	112		139		1
Enterococcus faecalis	fa-12	101		181		1
Enterococcus faecium	fc-01	89		130		1
Enterococcus faecium	fc-02	92		125		1
Enterococcus faecium	fc-03	92		113		1
Enterococcus faecium	fc-04	93		111		1
Enterococcus faecium	fc-05	90		115		1
Enterococcus faecium	fc-06	94		111		1
Enterococcus faecium	fc-07	90		115		1
Enterococcus faecium	fc-08	90		115		1
Enterococcus faecium	fc-09	92		113		1
Enterococcus faecium	fc-10	89		116		1
Enterococcus faecium	fc-11	98		135		1
Enterococcus faecium	fc-12	108		143		1
Enterococcus faecium	fc-13	95		131		1
Enterococcus faecium	fc-14	94		126		1
Enterococcus faecium	fc-15	96		132		1
Enterococcus faecium	fc-16	99		134		1

Table 1. Continue

Strain		Relative IL-22 expression (%)		Relative CD86 expression (%)		n
		x⁻	SE	x⁻	SE	
Enterococcus faecium	fc-17	121	6	170	7	3
Enterococcus faecium	fc-18	99		160		1
Enterococcus faecium	fc-19	134	4	188	12	3
Enterococcus faecium	fc-20	141	3	158	6	3
Enterococcus faecium	fc-21	99		138		1
Enterococcus faecium	fc-22	98		131		1
Enterococcus faecium	fc-23	119		142		1
Enterococcus faecium	**fc-24**	215	19	197	9	7
Enterococcus faecium	fc-25	111		146		1
Enterococcus faecium	fc-26	106		155		1
Enterococcus faecium	fc-27	95		136		1
Lactobacillus acidipiscis	lb-01	218	30	343	102	3
Lactobacillus acidipiscis	lb-02	217	17	221	11	3
Lactobacillus acidipiscis	lb-03	243	48	392	54	3
Lactobacillus brevis	lb-04	190	53	218	18	3
Lactobacillus brevis	lb-05	115		225		1
Lactobacillus brevis	lb-06	90		197		1
Lactobacillus brevis	lb-07	100		218		1
Lactobacillus brevis	lb-08	96		194		1
Lactobacillus brevis	lb-09	88		169		1
Lactobacillus brevis	lb-10	118		190		1
Lactobacillus brevis	lb-11	209	39	166	17	3
Lactobacillus buchneri	lb-12	240	27	147	8	3
Lactobacillus buchneri	lb-13	89		177		1
Lactobacillus casei	lb-14	92		206		1
Lactobacillus casei	lb-15	93		316		1
Lactobacillus casei	lb-16	89		205		1
Lactobacillus casei	lb-17	97		157		1
Lactobacillus curvatus	lb-18	96		198		1
Lactobacillus fermentum	lb-19	100		215		1
Lactobacillus fermentum	lb-20	102		223		1
Lactobacillus fermentum	lb-21	94		193		1
Lactobacillus fermentum	lb-22	96		208		1
Lactobacillus fermentum	lb-23	95		193		1
Lactobacillus fermentum	lb-24	96		197		1
Lactobacillus fermentum	lb-25	156	39	228	53	2
Lactobacillus fermentum	lb-26	90		162		1
Lactobacillus fructivorans	lb-27	89		190		1
Lactobacillus fructivorans	lb-28	112		291		1
Lactobacillus fructivorans	lb-29	104		192		1
Lactobacillus fructivorans	lb-30	96		297		1
Lactobacillus fructivorans	lb-31	95		312		1
Lactobacillus fructivorans	lb-32	171	35	352	40	3
Lactobacillus helveticus	lb-33	91		122		1
Lactobacillus helveticus	lb-34	210	9	184	38	3
Lactobacillus paracasei	lb-35	91		167		1
Lactobacillus paracasei	lb-36	94		179		1
Lactobacillus pentosus	lb-37	98		195		1
Lactobacillus pentosus	lb-38	100		167		1
Lactobacillus plantarum	lb-39	108		130		1
Lactobacillus plantarum	lb-40	96		218		1
Lactobacillus plantarum	lb-41	111		186		1
Lactobacillus plantarum	lb-42	95		156		1
Lactobacillus plantarum	lb-43	124	28	197	4	3
Lactobacillus plantarum	lb-44	102		212		1
Lactobacillus plantarum	lb-45	122		218		1
Lactobacillus plantarum	lb-46	112		182		1
Lactobacillus plantarum	lb-47	96		185		1
Lactobacillus plantarum	lb-48	92		266		1
Lactobacillus plantarum	lb-49	92		175		1
Lactobacillus plantarum	lb-50	94		181		1
Lactobacillus plantarum	lb-51	114		121		1
Lactobacillus plantarum	lb-52	95		163		1
Lactobacillus plantarum	lb-53	91		174		1
Lactobacillus rhamnosus	lb-56	102		262		1
Lactobacillus sakei	lb-58	110		193		1
Lactobacillus sakei	lb-59	92		193		1
Lactobacillus sp.	lb-60	105		232		1
Lactobacillus sp.	lb-61	155	6	166	35	3
Lactococcus lactis	lc-01	118		226		1
Lactococcus lactis	lc-02	100		217		1
Lactococcus lactis	lc-03	126		202		1
Lactococcus lactis	lc-04	108		145		1
Lactococcus lactis	lc-05	97		222		1
Lactococcus lactis	lc-06	100		200		1
Lactococcus lactis	lc-07	98		187		1
Lactococcus plantarum	lc-08	138	12	282	101	3
Leuconostoc citreum	ls-01	98		231		1
Leuconostoc citreum	ls-02	102		142		1
Leuconostoc citreum	ls-03	94		205		1
Leuconostoc citreum	ls-04	94		237		1
Leuconostoc mesenteroides	ls-05	96		183		1
Leuconostoc mesenteroides	ls-06	98		201		1
Leuconostoc mesenteroides	ls-07	99		211		1
Leuconostoc mesenteroides	ls-08	97		185		1
L. pseudomesenteroides	ls-09	93		205		1
L. pseudomesenteroides	ls-10	98		188		1
Pediococcus acidilactici	pc-01	128		212		1
Pediococcus acidilactici	pc-02	142		166		1
Pediococcus acidilactici	pc-03	183		227		1
Pediococcus acidilactici	pc-04	147		186		1
Pediococcus acidilactici	pc-05	142		247		1
Pediococcus acidilactici	pc-06	165		122		1
Pediococcus acidilactici	pc-07	217	33	250	23	3
Pediococcus acidilactici	pc-08	128		242		1
Pediococcus acidilactici	pc-09	146		200		1
Pediococcus acidilactici	pc-10	122		279		1
Pediococcus acidilactici	pc-11	167		321		1
Pediococcus acidilactici	pc-12	128		296		1
Pediococcus acidilactici	pc-13	110		167		1
Pediococcus acidilactici	pc-14	146		204		1
Pediococcus acidilactici	pc-15	171		234		1
Pediococcus acidilactici	pc-16	151		208		1
Pediococcus acidilactici	pc-17	286	34	237	52	3
Pediococcus acidilactici	pc-18	147		282		1
Pediococcus acidilactici	**pc-19**	438	54	284	25	7
Pediococcus acidilactici	pc-20	193	22	308	62	3
Pediococcus acidilactici	pc-21	149		370		1
Pediococcus acidilactici	pc-22	94		230		1
Pediococcus acidilactici	pc-23	105		263		1
Pediococcus acidilactici	pc-24	104		244		1
Pediococcus acidilactici	pc-25	245	7	299	90	3
Pediococcus acidilactici	pc-26	248	16	186	16	3
Pediococcus acidilactici	pc-27	107		222		1
Pediococcus acidilactici	pc-28	97		253		1
Pediococcus acidilactici	pc-29	166		253		1
Pediococcus acidilactici	pc-30	110		276		1
Pediococcus acidilactici	pc-31	114		232		1
Pediococcus acidilactici	pc-32	95		253		1
Pediococcus acidilactici	pc-33	103		300		1
Pediococcus acidilactici	pc-34	93		201		1
Pediococcus acidilactici	pc-35	97		209		1
Pediococcus acidilactici	pc-36	99		265		1
Pediococcus acidilactici	pc-37	87		173		1

Table 1. Continue

Strain		Relative IL-22 expression (%)		Relative CD86 expression (%)		n
		x⁻	SE	x⁻	SE	
Pediococcus acidilactici	pc-38	225	28	447	99	3
Pediococcus acidilactici	pc-39	212	22	404	138	3
Pediococcus dextrinicus	pc-40	152		230		1
Pediococcus pentosaceus	pc-41	119		171		1
Pediococcus pentosaceus	pc-42	212	24	195	36	3
Pediococcus pentosaceus	pc-43	136		176		1
Pediococcus pentosaceus	pc-44	139		239		1
Pediococcus pentosaceus	pc-45	148		168		1
Pediococcus pentosaceus	pc-46	104		175		1
Pediococcus pentosaceus	pc-47	140		159		1
Pediococcus pentosaceus	pc-48	165		195		1
Pediococcus pentosaceus	pc-49	90		145		1
Pediococcus pentosaceus	pc-50	134		112		1
Pediococcus pentosaceus	pc-51	91		189		1
Pediococcus pentosaceus	pc-52	197	11	158	25	3
Pediococcus pentosaceus	pc-53	141		95		1
Pediococcus pentosaceus	pc-54	120		201		1
Pediococcus pentosaceus	pc-55	99		163		1
Pediococcus pentosaceus	pc-56	158		225		1
Pediococcus pentosaceus	pc-57	103		190		1
Pediococcus pentosaceus	pc-58	98		171		1
Pediococcus pentosaceus	pc-59	119		215		1
Pediococcus pentosaceus	pc-60	90		156		1
Pediococcus pentosaceus	pc-61	151		223		1
Pediococcus pentosaceus	pc-62	98		172		1
Pediococcus pentosaceus	pc-63	116		211		1
Pediococcus stilesii	pc-64	92		179		1
Weissella cibaria	ws-01	111		177		1
Weissella cibaria	ws-02	131		178		1
Weissella cibaria	ws-03	138		235		1
Weissella confusa	ws-04	107		241		1
Weissella confusa	ws-05	135		210		1
Weissella confusa	ws-06	161	7	322	67	3
Weissella halotolerans	ws-07	119		186		1
Weissella hellenica	ws-08	102		197		1
Weissella mesenteroides	ws-09	100		179		1
Weissella paramesenteroides	ws-10	134		140		1
Weissella paramesenteroides	ws-11	115		112		1
Weissella paramesenteroides	ws-12	86		159		1
Weissella paramesenteroides	ws-13	122		184		1
Weissella paramesenteroides	ws-14	123		171		1
Weissella paramesenteroides	ws-15	122		179		1
Weissella paramesenteroides	ws-16	115		160		1
Weissella paramesenteroides	ws-17	142		194		1
Weissella paramesenteroides	ws-18	194	11	215	32	3
Weissella soli	ws-19	124		134		1
Weissella viridescens	ws-20	134		178		1
Weissella viridescens	ws-21	138		167		1
Bacillus coagulans	sc-01	376	70	555	46	3
Bacillus coagulans	sc-02	168	20	262	58	3
Bacillus coagulans	sc-03	179	43	441	29	3
Bacillus coagulans	sc-04	243	22	688	48	3
Bacillus coagulans	sc-05	334	75	404	19	5
Bacillus coagulans	sc-06	423	54	414	17	5
Bacillus coagulans	sc-07	204	28	392	27	3
Bacillus coagulans	sc-08	444	104	385	17	5
* ***Bacillus coagulans***	**sc-09**	1,062	158	501	53	7
Bacillus coagulans	sc-10	338	75	400	8	5
Bacillus coagulans	sc-11	253	16	417	27	3
Bacillus coagulans	sc-12	419	90	371	18	5
Bacillus coagulans	sc-13	143	19	364	27	3
Bacillus coagulans	sc-14	332	54	343	31	5
Bacillus coagulans	sc-15	309	41	353	17	5
Bacillus coagulans	sc-16	249	29	330	20	5
Bacillus coagulans	sc-17	131	10	182	58	3
Bacillus coagulans	sc-18	376	35	493	25	3
Bacillus coagulans	sc-19	349	31	474	28	3
Bacillus coagulans	sc-20	480	100	415	4	5
Bacillus subtilis	bs-01	282		525		1
Bacillus subtilis	bs-02	355		474		1
Bacillus subtilis	bs-03	236		485		1
Bacillus subtilis	bs-04	176		561		1
Bacillus subtilis	bs-05	457	37	460	90	4
Bacillus subtilis	bs-06	271		434		1
Bacillus subtilis	bs-07	427	31	321	18	4
Bacillus subtilis	bs-08	353		453		1
Bacillus subtilis	bs-09	230		333		1
Bacillus subtilis	bs-10	245		215		1
Bacillus subtilis	bs-11	154		248		1
Bacillus subtilis	bs-12	332		471		1
Bacillus subtilis	bs-13	218		553		1
Bacillus subtilis	bs-14	135		206		1
Bacillus subtilis	bs-15	262		453		1
Bacillus subtilis	bs-16	252		622		1
Bacillus subtilis	bs-17	245		475		1
Bacillus subtilis	bs-18	262		526		1
Bacillus subtilis	bs-19	298		311		1
Bacillus subtilis	bs-20	193		424		1
Bacillus subtilis	bs-21	133		366		1
Bacillus subtilis	bs-22	178		535		1
Bacillus subtilis	bs-23	145		307		1
Bacillus subtilis	bs-24	223		378		1
Bacillus subtilis	bs-25	372	74	437	51	4
Bacillus subtilis	bs-26	211		282		1
Bacillus subtilis	bs-27	161		436		1
Bacillus subtilis	bs-28	159		369		1
Bacillus subtilis	bs-29	166		470		1
* ***Bacillus subtilis***	**bs-30**	766	55	430	96	4
Bacillus subtilis	bs-31	118		280		1
Bacillus subtilis	bs-32	267		325		1
Bacillus subtilis	bs-33	147		341		1
* ***Bacillus subtilis***	**bs-34**	971	53	495	38	4
Bacillus subtilis	bs-35	171		262		1
Bacillus subtilis	bs-36	494	54	655	96	4
Bacillus subtilis	bs-37	176		561		1
Bacillus subtilis	bs-38	275		449		1
Bacillus subtilis	bs-39	513	68	294	44	4
Bacillus subtilis	bs-40	249		496		1
Bacillus amyloliquefaciens	bi-01	369		499		1
Bacillus amyloliquefaciens	bi-02	213		422		1
Bacillus benzoevorans	bi-03	348		473		1
Bacillus benzoevorans	bi-04	228		304		1
Bacillus firmus	bi-05	161		189		1
Bacillus megaterium	bi-06	148		209		1
Bacillus megaterium	bi-07	176		412		1
Bacillus megaterium	bi-08	267		434		1
Bacillus megaterium	bi-09	264		270		1
Bacillus novalis	bi-10	351		507		1
Bacillus pumilus	bi-11	264		522		1
Bacillus tequilensis	bi-12	219		378		1

x⁻: mean value; SE: standard error; n: number. The strains with high values are shown in bold and marked with an asterisk.

in Figs. 4, 5 are indicated as the mean ± standard deviation (SD). Statistical significance was evaluated using a two-tailed Student's t-test for unpaired data in Figs. 2, 3, 4b, and 5. The Tukey test was used for Fig. 4a. P values <0.05 were considered to be statistically significant.

RESULTS

Screening of IL-22-inducing bacteria in B cells

We isolated 367 bacteria from Japanese fermented foods, such as miso, soy sauce, and amazake. We collected 95 *Tetragenococcus*, 39 *Enterococcus*, 58 *Lactobacillus*, 8 *Lactococcus*, 10 *Leuconostoc*, 64 *Pediococcus*, 21 *Weissella*, and 72 *Bacillus* bacterial isolates. To evaluate the ability of these bacteria in inducing IL-22 production in immune cells, we established an *in vitro* immunological assay using mouse spleen cells [5]. The ability to induce IL-22 production was distinct for each bacterial species (Table 1 and Fig. 1). Most *Tetragenococcus, Enterococcus*, *Lactococcus*, *Leuconostoc*, and *Weissella* bacterial strains did not enhance the induction of IL-22 production. *Lactobacillus* and *Pediococcus* strains possessed higher abilities to induce IL-22 production than these lactic acid bacterial strains. Additionally, most of the *Bacillus* strains had higher abilities to induce IL-22 production than the lactic acid bacteria; *B. coagulans* sc-09, which was isolated from miso, had the highest ability to induce IL-22 production. *B. subtilis* bs-30 and bs-34 also possessed high IL-22-inducing ability. High IL-22-inducing bacterial strains also augmented activation marker CD86 on B cells. However, their abilities were not always proportional, suggesting that their inducing mechanisms were different.

Activation of B and T cells by IL-22-inducing bacterial strains

As shown in Table 1, the strains with high ability to induce IL-22 production also activated B cells. We assessed if six strains (*T. halophilus* ta-52, *Enterococcus faecium* fc-24, *Pediococcus acidilactici* pc-19, *B. coagulans* sc-09, *B. subtilis* bs-30, and *B. subtilis* bs-34) played a role in survival and activation of B and T cells based on activation markers, such as CD86 on B cells and CD69 on T cells, and determined their cell viability. All the strains augmented the viability of splenocytes, including B and T cells (Fig. 2A–C), and significantly increased CD86 expression on B cells and CD69 expression on $CD4^+$ T cells (Fig. 2D and E). These results suggest that all tested strains activated B and $CD4^+$ T cells and induced IL-22 in B cells.

Next, we examined whether the effect of these strains on IL-22 induction in B cells was direct or indirect. We isolated B cells, treated them with bacteria, and measured IL-22 production. As shown in Fig. 3A and B, these strains increased CD86 expression and B cell viability. In addition, IL-22 production similarly increased (Fig. 3C) in consistency with the results presented in Table 1. Among these strains, *B. coagulans* sc-09 most efficiently induced IL-22-producing B cells. This result indicates that these bacterial strains directly induce IL-22 production in B cells.

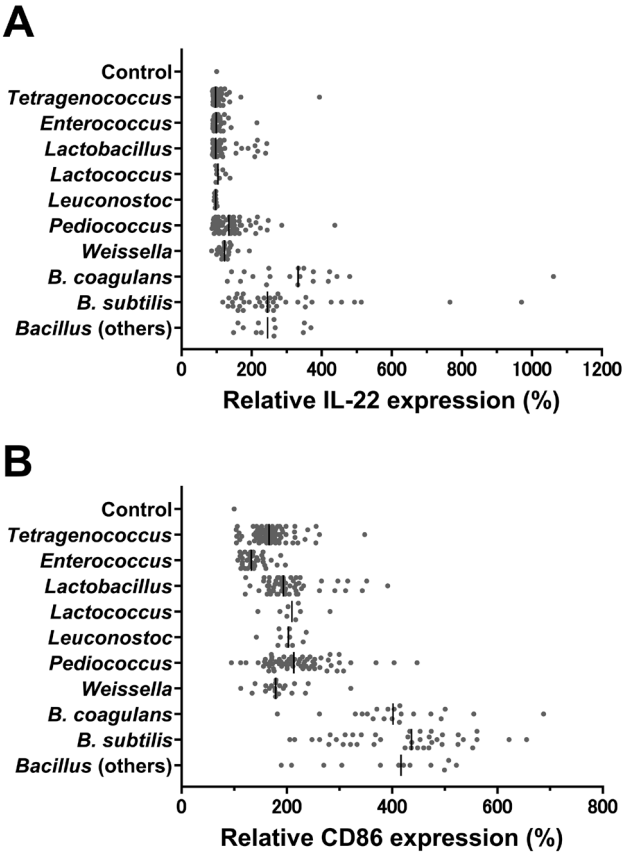

Fig. 1. Bacteria capable of inducing IL-22 and CD86.

The results in Table 1 are expressed in a column scatter plot. (A) Relative IL-22 expression in B cells. (B) Relative CD86 expression on B cells. The plots in the figure are divided according to category of bacteria, such as *Tetragenococcus* and *Lactobacillus*, and the relative value of each bacterium is plotted. The median value of each category is indicated by a bar.

B. coagulans sc-09 augments skin barrier function independent of commensal bacteria

We examined the influence on skin barrier function by feeding mice *B. coagulans* sc-09. Specifically, we fed the mice 1% *B. coagulans* sc-09 for 3 weeks and measured TEWL. TEWL was significantly reduced in the skin of *B. coagulans* sc-09–fed mice as compared with that of the control mice (Fig. 4A). When the IL-22 mAb was administered to the *B. coagulans* sc-09–fed mice, TEWL increased and became significantly higher than that in the control mice. In contrast, TEWL significantly decreased in the control mice administered IL-22 by intravenous injection.

To determine whether this function is mediated by commensal bacteria, we utilized GF mice. We fed 1% *B. coagulans* sc-09 to GF mice for 4 weeks and measured TEWL. As shown in Fig. 4B, even in experiments with GF mice,

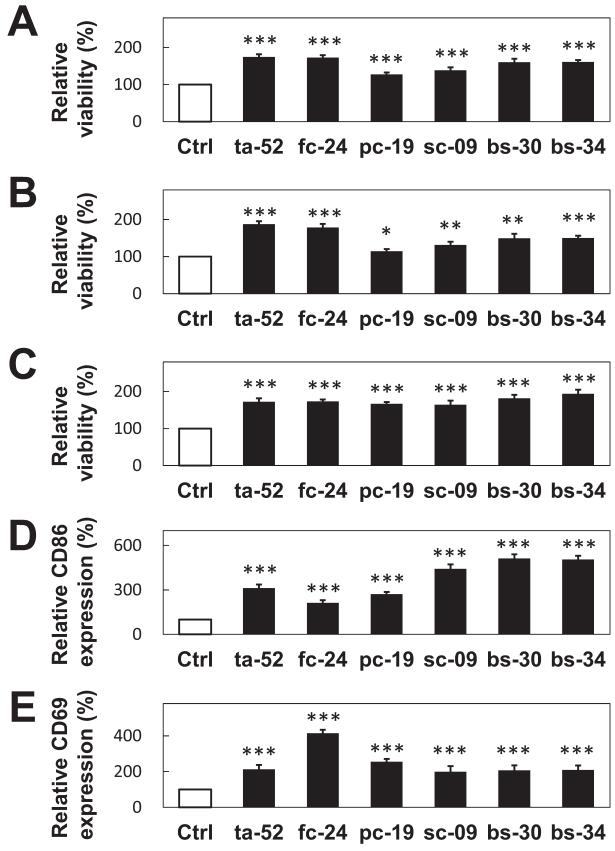

Fig. 2. CD86 expression on B cells and CD69 expression on T cells cultured with bacterial strains.

The spleen cells from C57BL/6 mice were cultured with 10 μg of bacterial cells inducing high IL-22 production in 1 mL of RPMI 1640 medium containing 10% FCS for 2 days. The cells were collected and stained with anti-B220, anti-CD4, anti-CD69, and anti-CD86 mAb. Dead cells were stained with PI. The cells were analyzed by flow cytometry. (A–C) The viabilities of total spleen cells (A), $B220^+$ cells (B), and $CD4^+$ cells (C) cultured without bacterial cells, which served as controls, were defined as 100%. Based on this parameter, the relative viabilities of cells cultured with bacteria were calculated. Bars indicate the mean ± SE (n=6). (D, E) The $CD86^+$ cells in $B220^+$ cells and $CD69^+$ cells in $CD4^+$ cells cultured without bacteria served as controls, and their numbers were defined as 100%. Accordingly, the relative proportions of $CD86^+$ cells and $CD69^+$ cells, respectively, in $B220^+$ cells (D) and $CD4^+$ cells (E) cultured with bacteria were calculated. Bars indicate the mean ± SE (n=6). *p<0.05 vs. control by t-test. **p<0.01 vs. control by t-test. ***p<0.001 vs. control by t-test.

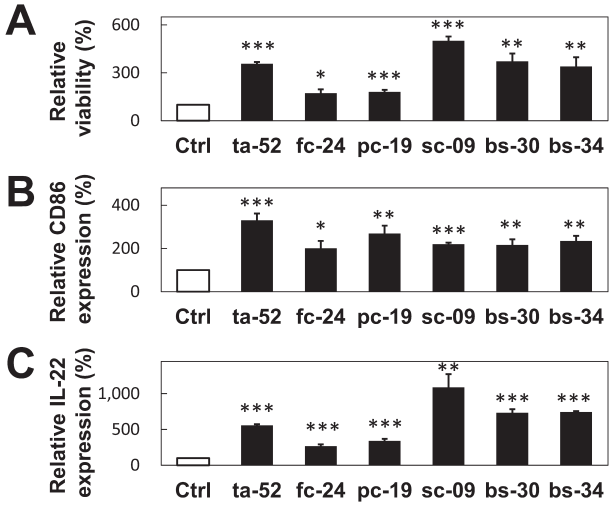

Fig. 3. CD86 expression and IL-22 production in B cells cultured with bacterial strains.

Spleen $B220^+$ cells prepared from C57BL/6 mice were cultured with 10 μg of bacterial cells that highly induced IL-22 production in 1 mL of RPMI 1640 medium containing 10% FCS for 2 days. The cells were collected and stained with anti-B220 and anti-CD86 mAb. Dead cells were stained with PI. The cells were analyzed by flow cytometry. Viability was assessed (A), and the viability of $CD86^+$ cells in $B220^+$ cells (B) cultured without bacterial cells, which served as control, was defined as 100%. On the basis of this parameter, the relative viability of cells and the relative CD86 expression of cells cultured with bacteria were calculated. Bars indicate the mean ± SE (n=4). (C) Cells cultured for 2 days were further incubated with GolgiStop and then collected and treated using a BD Fixation/Permeabilization Solution Kit. Subsequently, cells were stained and analyzed by flow cytometry. IL-22-positive cells in $B220^+$ cells cultured without bacteria served as the control (0.08%), and their number was defined as 100%. Based on this parameter, the relative IL-22 expression of cells cultured with bacteria was calculated. Bars indicate the mean ± SE (n=4). *p<0.05 vs. control by t-test. **p<0.01 vs. control by t-test. ***p<0.001 vs. control by t-test.

TEWL significantly decreased in the skin of *B. coagulans* sc-09-fed mice as compared with that of the control mice. This decrease in TEWL in the *B. coagulans* sc-09-fed GF mice shows that skin barrier function is independent of commensal bacteria. These results indicate that *B. coagulans* sc-09 is effective in enhancing skin barrier function.

We examined the effect of *B. coagulans* sc-09 on IL-22 production in SPF mice. IL-22-producing B cells (Bi22 cells) in Peyer's patches and mesenteric lymph nodes of *B. coagulans* sc-09-fed mice tended to be increased in comparison with those of control mice (Fig. 5), suggesting that *B. coagulans* sc-09-mediated IL-22 production contributed to the skin barrier function.

DISCUSSION

In this study, we screened bacteria from Japanese fermented foods for their ability to induce IL-22 production in B cells. We found that the ability to induce IL-22 production is dependent on the bacterial species, and *Bacillus* bacterial strains possessed high IL-22 induction potency. Among these strains, *B. coagulans* sc-09 was the highest IL-22 induction strain and had the ability to improve skin barrier function *in vivo*.

TEWL measurement is often used as an indicator for

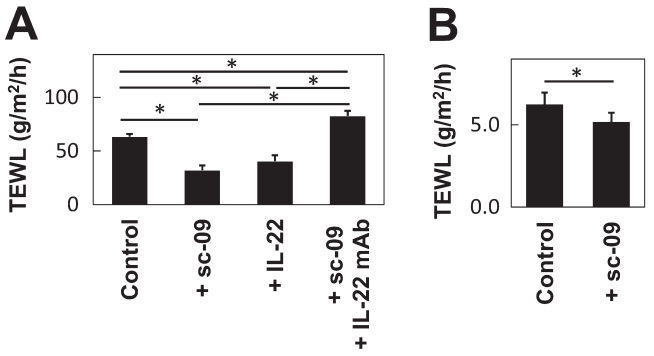

Fig. 4. Effect of *B. coagulans* sc-09 on murine skin barrier.

(A) C57BL/6 mice were divided into four groups (n=3 mice/group), with two groups fed a diet containing 1% *B. coagulans* sc-09 for 3 weeks and two groups fed a diet without *B. coagulans*. One group was specifically fed a diet containing 1% *B. coagulans* sc-09 and intravenously injected with the IL-22 antibody (20 µg/body) on the 14th and 17th days of feeding, respectively. The other group fed a diet without *B. coagulans* was intravenously injected with recombinant mouse IL-22 (2 µg/body) on the 14th and 17th days of feeding, respectively. On the 20th day of feeding, the backs of the mice were shaved, and on the 21st day, the TEWL of the skin on the back of the mice was measured (n=4). Bars indicate the mean ± SD of triplicate experiments. *p<0.05 vs. control by Tukey test. (B) Effect of *B. coagulans* sc-09 on skin barrier in GF mice. Diet containing 1% *B. coagulans* sc-09 was fed to GF mice. After feeding for 4 weeks in an aseptic environment, the TEWL of the back skin of the mice was measured. Just before the measurement, the hair on the backs of the mice was cut with clippers. Measurement of TEWL was performed four times each. Bars indicate the mean ± SD (n=5 mice). *p<0.05 vs. control by t-test.

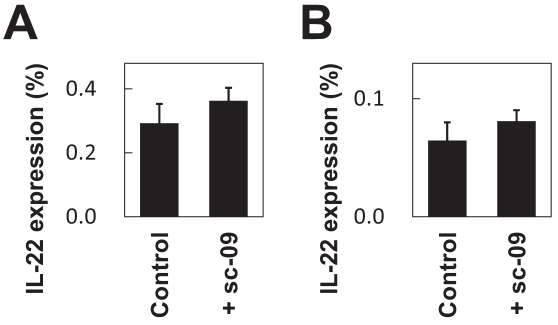

Fig. 5. Effect of *B. coagulans* sc-09 on IL-22 production in the Peyer's patches and mesenteric lymph nodes in mice.

Diet containing 1% *B. coagulans* sc-09 was fed to C57BL/6 mice for 3 weeks (n=3 mice/group). Then, cells were collected from Peyer's patches and mesenteric lymph nodes, and the percentages of IL-22-producing cells in B cells were analyzed by FACS. Mice fed without *B. coagulans* sc-09 were used as the control. Bars indicate the mean ± SD for Peyer's patches (A) and mesenteric lymph nodes (B). The p values in A and B are 0.245 and 0.265, respectively.

evaluating skin barrier function [10]. Because administration of IL-22 decreased TEWL and neutralization of IL-22 increased TEWL, the improvement of skin barrier function caused by *B. coagulans* sc-09 uptake may be attributed to IL-22. Although the significance of IL-22 produced by B cells is unknown, increased IL-22 may facilitate skin barrier function [7]. Our results suggest that IL-22-inducing bacteria have immunomodulatory abilities in addition to enhancement of skin barrier function.

Strains with high abilities to induce IL-22 production also possessed high abilities to activate B cells (Fig. 3); however, these capabilities were not directly proportional to each other (Table 1). Furthermore, only some subpopulations of activated B cells seemed to differentiate into IL-22-producing B cells (Bi22), as Bi22 cells are a minor population in $B220^+$ B cells. This finding suggests that B cell activation and IL-22 induction are distinctly regulated. In our previous report [5], we showed that *T. halophilus* No. 1 induced multiple subsets in B cells similar to Th cells exist. Thus, some of the microorganisms harboring B cell activation ability may promote differentiation into a subset of B cells producing IL-22.

IL-22 is highly expressed in the skin and digestive and respiratory organs [7]. In the skin and intestines, IL-22 induces the production of antibacterial peptides and is considered to be involved in pathogen defense. Recently, reports have shown that *Lactobacillus plantarum* stimulation of NKs can enhance IL-22 production and defend against enterotoxigenic *Escherichia coli*-induced damage of the intestinal epithelial barrier [11]. Thus, IL-22-inducing bacteria including *B. coagulans* sc-09 may act on the barrier function of the intestinal tract, although IL-22 is produced in various types of immune cells.

Here, we found that *B. coagulans* sc-09 has a strong IL-22-inducing function in B cells. *B. coagulans* is a spore-forming bacterium that produces lactic acid. *B. coagulans* spores are probiotics and have beneficial effects in humans, such as amelioration of irritable bowel syndrome [12, 13], bacterial vaginosis [14], and intestinal disorders [15–17], and absorption of amino acids from proteins [18, 19]. In addition, their use in broilers and fish yields growth-promoting and disease-preventing effects [20, 21]. *B. coagulans* sc-09 isolated from miso appears to be a probiotic that improves skin barrier function and modulates immune function. Beneficial effects of *B. coagulans* on IL-22 induction in immune cells appear to contribute to human health when it is supplied as an ingredient in foods and supplements.

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