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ARTICLE

Antibiotic susceptibility of plant-derived lactic acid bacteria conferring health benefits to human

Masafumi Noda[1] · Narandalai Danshiitsoodol[1] · Yusuke Inoue[2] · Tomoko Okamoto[1] · Nasrin Sultana[1] · Masanori Sugiyama[1]

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Abstract

Lactic acid bacteria (LAB) confer health benefits to human when administered orally. We have recently isolated several species of LAB strains from plant sources, such as fruits, vegetables, flowers, and medicinal plants. Since antibiotics used to treat bacterial infection diseases induce the emergence of drug-resistant bacteria in intestinal microflora, it is important to evaluate the susceptibility of LAB strains to antibiotics to ensure the safety and security of processed foods. The aim of the present study is to determine the minimum inhibitory concentration (MIC) of antibiotics against several plant-derived LAB strains. When aminoglycoside antibiotics, such as streptomycin (SM), kanamycin (KM), and gentamicin (GM), were evaluated using LAB susceptibility test medium (LSM), the MIC was higher than when using Mueller–Hinton (MH) medium. Etest, which is an antibiotic susceptibility assay method consisting of a predefined gradient of antibiotic concentrations on a plastic strip, is used to determine the MIC of antibiotics world-wide. In the present study, we demonstrated that Etest was particularly valuable while testing LAB strains. We also show that the low susceptibility of the plant-derived LAB strains against each antibiotic tested is due to intrinsic resistance and not acquired resistance. This finding is based on the whole-genome sequence information reflecting the horizontal spread of the drug-resistance genes in the LAB strains.

Introduction

Lactic acid bacteria (LAB), which consist of over 10 genera including *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Streptococcus*, *Pediococcus*, and *Weissella* [1–3], contribute to promote human health [4, 5]. "Probiotics" are the living microorganisms that contribute health benefits to the host when administered orally in appropriate amounts [6]. LAB, which are typical probiotics, have been used to produce fermented foods, beverages, and supplements that claim some health benefits [7–10].

✉ Masanori Sugiyama
sugi@hiroshima-u.ac.jp

[1] Department of Probiotic Science for Preventive Medicine, Graduate School of Biomedical and Health Sciences, Hiroshima University, Kasumi 1-2-3, Minami-ku, Hiroshima 734-8551, Japan

[2] Mitsui Sugar Co., Ltd., Hakozaki-cho 36-2, Nihonbashi, Chuo-ku, Tokyo 103-0015, Japan

The LAB strains are roughly classified into two groups by the isolated environment: one is isolated from animal sources (designated as animal-derived LAB), such as skin and intestine. Another is isolated from plant sources (designated as plant-derived LAB), such as fruits, vegetables, and medicinal plants. The animal-derived LAB strains have been widely used to manufacture yogurt and cheese preparation, whereas the plant-derived ones have been useful to produce fermented dishes, mainly in East- and Southeast-Asians. We have isolated many kinds of plant-derived LAB strains from vegetables, flowers, fruits, and medicinal herbs. In a plant-derived LAB library composed of over 700 strains, we have found that several LAB strains are very useful for immune modulation, the improvement of liver function, and the reduction of obesity [11–14]. The LAB strains we characterize in our academic laboratories could be the source of health promoting organisms for the health food industry.

Since LAB strains are often administrated orally as living organisms contained in fermented foods, and considering that the emergence of antibiotic-resistant bacteria is caused by the clinical use of antibiotics (antibacterial agents),

evaluation of the susceptibility of LAB strains to antibiotics is very important for food safety and security. In other words, if certain LAB strains that live in the intestinal tract are exposed to antibiotics, they may acquire resistance to antibiotics by the horizontal transfer of the drug-resistance gene. The risk of horizontal gene transfer is higher than when the intrinsic resistance gene is encoded on the chromosome if the resistance gene is mediated by a plasmid or transposon [15, 16].

In this study, we evaluated the antibiotic susceptibilities of plant-derived LAB isolates that have been previously confirmed to exhibit the health-promoting effects. Further, the risks of horizontal spread were evaluated through whole-genome sequence analysis of the strains.

Materials and methods

Bacterial strains, growth media, and culture conditions

The antibiotic susceptibility of six strains of the plant-derived LAB was investigated. Table 1 shows the LAB strains isolated previously by our research group [11–14, 17–21]. To culture LAB strains, MRS medium (Becton, Dickinson, and Company, Franklin Lakes, NJ, USA) was used. To evaluate the MIC, the LAB strains were cultured in MH medium (Becton, Dickinson, and Company) or LSM medium [22] composed of 90% Iso-Sensitest broth [23] (Oxoid, Cheshire, UK) and 10% (w/v) MRS medium. If necessary, 1.5 (w/v) % agar was added to the medium.

Evaluation of susceptibility to antibiotics using the broth microdilution method

Broth microdilution test method was performed using a U-bottom 96-well microtiter plate. Antibiotics evaluated in the study were tetracycline (TC), erythromycin (EM), chloramphenicol (CL), ampicillin (AM), vancomycin (VA), gentamicin (GM), clindamycin (CM), kanamycin (KM), streptomycin (SM), and tylosin (TS), which were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Each antibiotic was diluted into LSM or MH medium under the appropriate concentration. A 200 µl portion of the LSM or MH medium was transferred into each well to contain given concentrations (serially diluted) of antibiotics. After inoculation of LAB cells at 5×10^5 colony-forming unit (CFU) ml^{-1}, the plate was incubated at 28 or 37 °C until cell growth was observed in the control well containing medium without antibiotics. After cultivation, the bacterial growth measured as turbidity or cell density in the well bottom. The MIC is defined as the lowest concentration of the added antibiotic at which growth of the

Table 1 Summary of whole genome features of LAB strains used in this study

Species	Strain	Genome size (bp)	GC content (%)	CDSs	tRNA genes	rRNA genes	Number of plasmids	Isolation origin	Accession No.	References
Enterococcus avium	G-15	3,623,727	39.7	3630	67	15	0	Carrot leaf	AP019814	[11, 17]
Enterococcus mundtii	15-1A	3,112,343	38.5	2987	65	18	4	Mibuna leaf	AP019810	[11, 18]
Lactobacillus paracasei	IJH-SONE68	3,084,917	46.6	2963	59	15	2	Fig leaf	AP018392	[19]
Lactobacillus plantarum	SN13T	3,612,790	46.4	3398	75	16	1	Banana leaf	AP019815	[11, 12]
Lactobacillus plantarum	SN35N	3,267,626	44.5	3146	75	16	4	Pear	AP018405	[12, 20]
Pediococcus pentosaceus	LP28	1,774,865	37.1	1683	51	6	1	Longan	DF970691	[11, 13, 14, 21]

Table 2 MIC values against tested antibacterial agents measured by broth microdilution method using LSM (μg ml^{-1})

Agents	Strain	G-15	15-1A	IJH-SONE68	SN13T	SN35N	LP28
TC	MIC	≤0.063	0.17 ± 0.04	0.33 ± 0.08	8	8	8
	efsa cut-off value	4	4	4	32	32	8
EM	MIC	0.0625	0.5	0.08 ± 0.02	0.125	0.125	0.125
	efsa cut-off value	4	4	1	1	1	1
CL	MIC	2	4	2.7 ± 0.7	2.7 ± 0.7	2	2
	efsa cut-off value	16	16	4	8	8	4
AM	MIC	≤8[a]	0.5	2	8	4	4
	efsa cut-off value	2	2	4	2	2	4
VA	MIC	0.5	0.5	1 024	2 048	1 024	1 024
	efsa cut-off value	4	4	NR	NR	NR	NR
GM	MIC	16	≤32	8	2	11 ± 3	13 ± 3
	efsa cut-off value	32	32	32	16	16	16
CM	MIC	0.05 ± 0.01	≤16	0.026 ± 0.005	0.125	≤0.016	≤0.016
	efsa cut-off value	4	4	1	2	2	1
KM	MIC	90 ± 20	128	64	50 ± 10	256	256
	efsa cut-off value	1 024	1 024	64	64	64	64
SM	MIC	128	≤128	32	32	128	128
	efsa cut-off value	128	128	64	NR	NR	64
TS	MIC	1	4	0.5	0.5	1	1
	efsa cut-off value	4	4	NR	NR	NR	NR

The data are expressed as mean ± SE ($n = 3$). Where the data have no SE, only mean value is written

[a]The MIC values that are greater than efsa cut-off value are indicated as underlined bold texts

NR Not required

plant-derived LAB strain is completely inhibited or at which small button of bacteria (no more than 2 mm in diameter) was only observed [24, 25]. This examination was repeated at least three times, and the average value was recorded.

Evaluation of MIC using Etest strips

MIC measurement using the Etest strip method (bioMérieux, Lyon, France) was performed according to the manufacturer's instructions: LAB cells grown until the stationary phase were suspended in a brain heart infusion (BHI) medium. The cell suspension (5×10^8 CFU ml^{-1}) soaked in a sterile cotton swab was streaked with a plate rotation 60 degrees three times on the LSM agar medium. After drying the surface of plate, Etest strips were applied to the agar medium, followed by incubation at 28, 37, or 45 °C until cell growth was observed on the control agar plate. The experiment was repeated at least three times, and the average value was recorded.

Whole-genome sequencing and annotation

The chromosomal DNA from each LAB strain was isolated, as described previously [20]. The whole-genome sequencing of G-15, 15-1A, and SN13T strains was done on a next-generation sequencing platform, PacBio RS II (Pacific Biosciences, Menlo Park, CA, U.S.A.), as performed in previous studies [19, 20]. After de novo assembling, the obtained genomic contig sequence data was annotated using the Microbial Genome Annotation Pipeline (MiGAP) [26]. The genome data were analyzed using the In Silico Molecular Cloning Genomic Edition (In Silico Biology, Inc., Yokohama, Japan).

Phylogenetic analysis

The predicted proteins that have the motifs of serine hydrolase and metal-dependent hydrolase coded on G-15 chromosomal DNA (G15_0480, G15_0661, G15_0663, G15_0941, G15_0720, G15_0818, G15_0918, G15_1504, G15_1771, and G15_2962) were phylogenetically analyzed with typical class A–D β-lactamases as follows: class A enzymes from *Escherichia* (*E.*) *coli* (CU928163 and NC_019081), *Klebsiella* (*K.*) *pneumoniae* (AY850171), and *Proteus vulgaris* (D29982); Class B ones from *K. pneumoniae* (NC_014312) and *Pseudomonas* (*P.*) *aeruginosa* (NC_022345); Class C ones from *E. coli* (HQ185697), *K. pneumoniae* (AF259520), and *P. aeruginosa* (AF490770); and Class D ones from *Acinetobacter baumannii* (NC_025109), *E. coli* (NC_022374), and *K. pneumoniae* (NC_019160).

The eighteen predicted proteins of the 15-1A strain annotated to efflux pump (EM151A_0036, EM151A_0081,

Table 3 MIC values against tested antibacterial agents measured by broth microdilution method using MH broth (μg ml^{-1})

Agents	Strain	G-15	15-1A	IJH-SONE68	SN13T	SN35N	LP28
AM	MIC	4[a]	0.25	2	2	1	1
	efsa cut-off value	2	2	4	2	2	4
GM	MIC	8	32	4	0.5	2	2
	efsa cut-off value	32	32	32	16	16	16
KM	MIC	64	64	32	16	64	32
	efsa cut-off value	1 024	1 024	64	64	64	64
SM	MIC	64	64	16	8	32	16
	efsa cut-off value	128	128	64	NR	NR	64

The data are expressed as mean ± SE ($n = 3$). Where the data have no SE, only mean value is written

[a]The MIC values that are greater than efsa cut-off value are indicated as underlined bold texts

NR Not required

Table 4 MIC values against tested antibacterial agents measured by E-test strip (μg ml^{-1})

Agents	Strain	G-15	15-1A	IJH-SONE68	SN13T	SN35N	LP28
TC	MIC	0.037 ± 0.005	0.10 ± 0.01	0.25	3 ± 2	4 ± 1	3 ± 1
	efsa cut-off value	4	4	4	32	32	8
EM	MIC	0.094	0.5 ± 0.2	0.15 ± 0.05	0.4 ± 0.3	0.12 ± 0.07	0.2 ± 0.1
	efsa cut-off value	4	4	1	1	1	1
CL	MIC	1.5 ± 0.3	2.7 ± 0.3	2	1.2 ± 0.4	1.1 ± 0.2	1.3 ± 0.4
	efsa cut-off value	16	16	4	8	8	4
AM	MIC	6 ± 1[a]	0.27 ± 0.06	0.6 ± 0.1	5 ± 2	1.8 ± 0.2	2 ± 0.5
	efsa cut-off value	2	2	4	2	2	4
VA	MIC	0.34 ± 0.04	0.29 ± 0.04	≤256	≤256	≤256	≤256
	efsa cut-off value	4	4	NR	NR	NR	NR
GM	MIC	4 ± 1	8 ± 4	3 ± 2	1.3 ± 0.4	5 ± 3	5 ± 2
	efsa cut-off value	32	32	32	16	16	16
CM	MIC	0.06 ± 0.02	1	0.13 ± 0.06	0.8 ± 0.6	≤0.016	0.03 ± 0.02
	efsa cut-off value	4	4	1	2	2	1
KM	MIC	19 ± 3	30 ± 10	50 ± 20	20 ± 10	48	28 ± 3
	efsa cut-off value	1 024	1 024	64	64	64	64
SM	MIC	110 ± 10	120 ± 10	27 ± 3	43 ± 5	50 ± 20	150 ± 20
	efsa cut-off value	128	128	64	NR	NR	64

The data are expressed as mean ± SE ($n = 3$). Where the data have no SE, only mean value is written

[a]The MIC values that are greater than efsa cut-off value are indicated as underlined bold texts

NR Not required

EM151A_0695, EM151A_1022, EM151A_1101, EM151A_1104, EM151A_1120, EM151A_1681, EM151A_1744, EM151A_1998, EM151A_2046, EM151A_2214, EM151A_2250, EM151A_2303, EM151A_2455, EM151A_2653, EM151A_2698, and EM151A_3018) were also analyzed with MsrA proteins from *S. dysgalactiae* subsp. *equisimilis* (AP011114) and *Streptococcus* sp. (DQ131177) and MefA proteins from *S. pyogenes* (KJ809088) and *S. viridans* (EF042094).

The phylogenetic trees were drawn with the ClustalW program in Molecular Evolutionary Genetics Analysis (MEGA) software ver. 6.0 [27] using the unweighted pair group method with arithmetic (UPGMA) [28]. The bootstrap values [29] were determined from 5 000 replications.

Acute oral toxicity and mutagenicity tests

The acute oral toxicity using rats of each LAB strain, 15-1A, SN13T and LP28, was evaluated by the New Drug Development Research Center, Inc., and the Japan Food Research Laboratories (for the IJH-SONE68 strain), as described in our previous studies [17, 19]. The test was performed according to the OECD Guidelines for the Testing of Chemicals, Guideline 420 (2001).

The mutagenicity test (*umu* test) of the culture supernatant of the LAB strain was performed using a Umulac AT test kit (Protein Purify Ltd., Maebashi, Japan) according to the manufacturer's protocol. The induction of the *umu* gene is responsible for DNA damage that was calculated by the

umuC-lacZ fusion gene expression in *Salmonella enterica* serovar Typhimurium NM2009. If the sample added into the reaction mixture enhances the activity of β-galactosidase to more than twice that of background reaction, it is speculated that the sample exhibits the mutagenic property at the given concentration.

Results

Susceptibility to antibiotics as measured by the broth microdilution method

The MICs of antibiotics measured by the broth microdilution method with the LSM are shown in Table 2. The MIC ($16 \leq \mu g\ ml^{-1}$) of clindamycin (CM) on *Enterococcus* (*E.*) *mundtii* 15-1A isolated from *Brassica rapa* L. subsp. *nipposinica* (L.H. Bailey) Hanelt var. *linearifolia* [18] was higher than the cutoff value (4-$\mu g\ ml^{-1}$). However, the MIC of CM on other LAB isolates was lower than the cutoff value. In addition, among the MICs of ampicillin (AM), kanamycin (KM), streptomycin (SM), and gentamicin (GM) on the LAB strains, some strains had higher MICs than the cutoff values defined by the European Food Safety Authority (EFSA). The EFSA provides independent scientific advice on risks in the food chain. The MICs of AM, KM, SM, and GM were also evaluated using MH medium, designed to test the sensitivity of pathogens to antibiotics [30], and not for LSM. As shown in Table 3, when using MH medium, the MIC of each antibiotic was eight times lower than with the LSM. The MICs of other antibiotics except AM against *Enterococcus* (*E.*) *avium* G-15 were less than or equal to the cutoff values.

Antibiotic MIC determination using Etest strips

The MICs of tested antibiotics were also measured using Etest strips located on the LSM agar. As shown in Table 4, almost all MICs were lower or higher than those determined by the broth microdilution method using LSM or MH medium, respectively. With the Etest strip, the MIC of AM against *E. avium* G-15 and *Lactobacillus* (*Lb.*) *plantarum* SN13T were higher than the cutoff values. Further, the MIC of SM on *Pediococcus* (*P.*) *pentosaceus* LP28 was also higher than the cutoff value.

Whole-genome sequence analyses

Each whole-genome sequence of *E. avium* G-15, *E. mundtii* 15-1A, and *Lb. plantarum* SN13T was determined. These strains have been isolated from a carrot, *Citras Iyo*, and a pork sausage fermented by wrapping it with a banana leaf, called "nem chua," respectively. The whole-DNA sequences are summarized in Table 1, together with those of the fig leaf–derived *Lb. paracasei* IJH-SONE68, *Lb. plantarum* SN35N, and *P. pentosaceus* LP28 determined previously [19–21]. The whole-genome sequences of the G-15, 15-1A, and SN13T strains reveal that the three whole genomes are circular DNA, and the sizes are 3,623,727, 3,112,343, and 3,612,790 bp, with GC contents of 39.7%, 38.5%, and 46.4%, respectively. To obtain the GenBank/EMBL/DDBJ accession numbers, the nucleotide sequence data of the G-15, 15-1A, and SN13T strains have been deposited in the International Nucleotide Sequence Database (AP019814, AP019810, and AP019815, respectively). The average genome sizes of other *Lb. plantarum* and *E. mundtii* strains, which were calculated from the completely sequenced genomic DNA data registered in the NCBI genome database, were 3318 (from 2952 to 3697; $n = 90$) and 3 174 (from 2827 to 3505; $n = 4$) kb, respectively. However, at this time, the complete genomic sequence of *E. avium* has not been previously registered in the NCBI database.

From the whole-genome sequence data, it was found that *E. mundtii* 15-1A harbors three kinds of plasmids, which were designated pEM15-1A-1, 2, and 3 (AP019811, AP019812, and AP019813, respectively). The sizes of pEM15-1A-1, pEM15-1A-2, and pEM15-1A-3 are 67,440, 59,372, and 56,627 bp, respectively. The GC content of each plasmid is 36.1%, 33.9%, and 35.7%, respectively. *Lb. plantarum* SN13T harbors a plasmid designated pSN13T-1 (AP019816), with a size of 72,292 bp and a GC content of 40.4%. Significantly, *E. avium* G-15 did not have any plasmids.

Safety evaluations of the plant-derived LAB strains

We evaluated the acute toxicity of the *E. mundtii* 15-1A, *Lb. paracasei* IJH-SONE68, *Lb. plantarum* SN13T, and *P. pentosaceus* LP28 cells using rats, confirming that the ingestion of each strain did not induce illness or death. Additionally, no differences were noted in the health of the animals and no signs of inflammation in the organs were observed by histology. Furthermore, the *umu* test demonstrates that no culture supernatant of any strain induces mutagenesis. We have previously determined that the G-15 and SN35N cells and the culture supernatant have no acute toxicity or mutagenic activity, respectively [17, 20].

Discussion

In general, LAB require a nutrient-rich medium containing carbohydrates, vitamins, amino acids, and minerals for growth. In order to satisfy the nutrition requirements, de Man, Rogosa, and Sharpe (MRS) medium has been

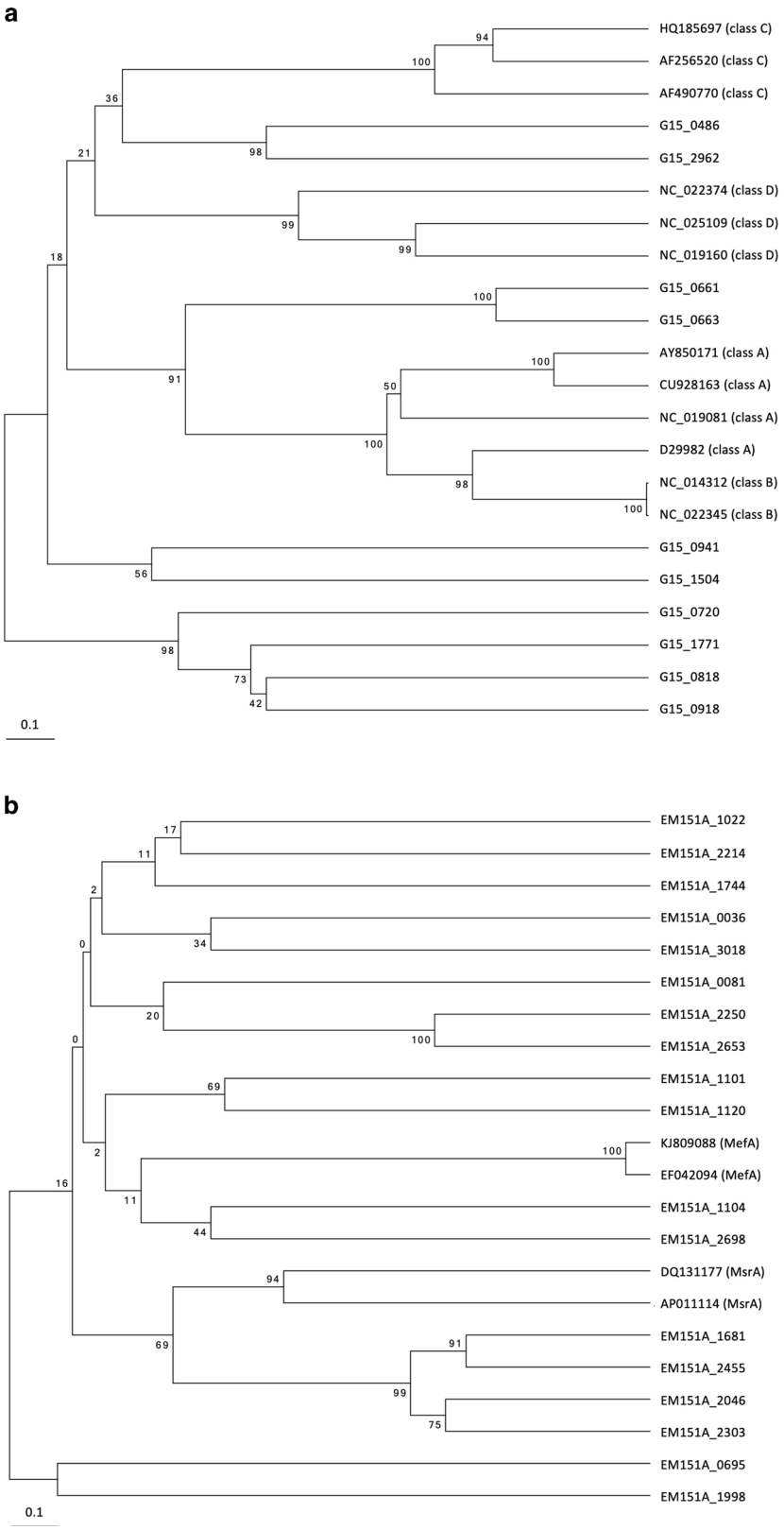

Fig. 1 Phylogenetic trees on the β-lactamases **a** and multi-drug efflux pumps **b**. The phylogenetic trees were drawn with the ClustalW program in Molecular Evolutionary Genetics Analysis (MEGA) software ver. 6.0 [27] using the unweighted pair group method with arithmetic (UPGMA) [28]. The bootstrap values [29] were determined from 5 000 replications. The bottom horizontal bars show a distance of 0.1 substitutions per site

developed [31]. Although some nutrients contained in the MRS medium could be antagonistic to antibiotic, but the mechanism is not fully understood [22, 32, 33]. To avoid antibiotic-inhibitory activity by different medium constituents, MH medium was used to determine the MIC values. However, the MH medium appears to be inadequate

for cultivating LAB. In fact, our isolates of *Lb. reuteri*, *Lb. sakei*, and *Lb. amylovorous* grew very poorly in MH medium (data not shown). In the present study, we evaluated the susceptibility of health-promoting LAB strains against several antibiotics using LSM, which was designed as an alternative to the MH medium for LAB [22].

The MICs of AM and aminoglycosides (SM, KM, GM), which were determined using the broth microdilution method with LSM, were higher than those with MH medium, as shown in Tables 2 and 3, respectively. The MICs of GM on *Staphylococcus aureus*, *E. faecalis*, and *E. coli*, which have been determined using the broth microdilution method with the LSM, were also higher than those with the MH one [22]. Judging from the report by another research group, some compounds in LSM are likely to interfere with the antimicrobial activity of some antibiotics.

Interestingly, when Etest strip was employed, almost all of the MICs were lower than those determined using the broth microdilution method, especially for aminoglycosides (Table 4). Furthermore, the low sensitivity of *E. avium* G-15 to AM observed in the assay using the MH medium was also observed in the Etest assay. The Etest strip method has been reported to be an applicable technique for evaluating the MICs of Gram-positive non-spore-forming anaerobes, including bifidobacteria and some LAB strains [34–36]. However, when the MH agar medium was used instead of the LSM agar one for the Etest assay, the inhibitory zone was very unclear, suggesting that the MH medium is not suitable (data not shown). Therefore, the Etest assay with the LSM may be useful to evaluate the MICs on LAB.

β-Lactamases (EC3.5.2.6), which are produced by bacteria and confer resistance to β-lactam antibiotics, are classified into four groups (Classes A, B, C, and D): penicillinase and cephalosporinase belong to Class A and C, respectively. Oxacillinase (Class D) belongs to the serine hydrolase family enzyme. The Class B enzyme carbapenemase is a member of metal-dependent hydrolase [37]. The whole-genome sequence of *E. avium* G-15 suggests that there are six (*G15_0480*, *G15_0661*, *G15_0663*, *G15_0941*, *G15_1504*, and *G15_2962*) and four (*G15_0720*, *G15_0818*, *G15_0918*, and *G15_1771*) genes encoding proteins that have the motifs of serine hydrolase and metal-dependent hydrolase on the chromosomal DNA, respectively. The 10 predicted proteins were compared with 12 typical enzymes of Class A–D β-lactamases by phylogenic tree analysis, showing that not all of the predicted hydrolases are classified as β-lactamases (Fig. 1a). It has been reported that the low sensitivity or resistance of *Enterococcus* species to β-lactams is a genus-specific feature caused by low affinity of their penicillin-binding proteins [38, 39]. In addition, Murray reported in her review that the MICs of AM and penicillin for *E. faecalis* strains are 1–4 and 2–8 μg ml^{-1}, respectively, and those values are 10–100 times higher than those of most streptococci [38]. Therefore, the MIC of AM on *E. avium* G-15, which has been shown to be two times higher than the cutoff value in the MH medium, is thought to be due to intrinsic resistance rather than acquired resistance.

The MIC of CM on *E. mundtii* 15-1A was 16 times higher than the cutoff value when using broth microdilution method, although the resistance was not observed when using the Etest method. Mutations in ribosomal proteins L4 and L22 (K63E and Δ82–84, respectively, *E. coli* numbering) [40, 41] and 23S rRNA (A2058 and A2059, *E. coli* numbering) [42] are known factors that decrease the sensitivities of bacteria to the macrolide-lincosamide-streptogramin (MLS) group of antibiotics, including EM and CM. Based on the whole-genome sequence, *E. mundtii* 15-1A has no mutations on corresponding residues or nucleotides in deduced ribosomal proteins (encoded by *EM151A_1637* and *EM151A_1641*) or 23S rRNA sequences, respectively.

Furthermore, multi-drug efflux pumps have been reported to contribute to antibiotic resistance in bacteria. Some pathogenic *Streptococcus* spp. have efflux pumps named MsrA and MefA that give the bacteria an acquired resistance against MLS antibiotics [43]. Among the predicted CDSs (coding sequences) in *E. mundtii* 15-1A, there are 18 gene products annotated to efflux pump. However, all of the predicted pumps were found to be clearly different from MsrA and MefA *via* phylogenic tree analysis of those proteins (Fig. 1b). Therefore, those predicted genes do not seem to play a role in acquired MLS antibiotic resistance. It has also been reported that some *E. mundtii* isolates have low sensitivities against lincosamides without known resistance genes, such as *ermA*, *ermB*, *ermC*, *mefA*, and *msrA* [44]. In fact, the PCR analyses performed on *E. mundtii* 15-1A using primer sets designed for amplifying those five genes [45–47] indicate that the 15-1A strain does not have those resistance genes (data not shown). Thus, the observed higher MICs of MLSs on *E. mundtii* 15-1A are thought to be not acquired but intrinsic characteristics. The accumulation of genomic and strain characteristics of minor enterococci, including *E. avium* and *E. mundtii*, will provide detailed information on the differences in susceptibility against antibiotics within the genus.

Although *Streptococcus* (*S.*) *thermophilus* has been widely and traditionally used to manufacture yogurt [48], the genus *Streptococcus* includes some harmful species, such as *S. agalactiae*, *S. pneumoniae*, and *S. pyogenes*. Bolotin *et al*. reported that all streptococci lack the gene encoding RecQ helicase [49], which is present in bacteria from mammals and increases genome stability [47]. Furthermore, the pathogenic streptococci, but not *S. thermophilus*, lack the *sbcC* and *sbcD* genes that contribute to genome stabilization by repairing the recombinogenic

double-strand DNA brakes, suggesting that genomic instability might allow pathogenic streptococci to acquire virulence-related genes [49, 50]. Whole-genome sequencing revealed that the six strains analyzed in the present study have all three of those genes (*recQ*, *sbcC*, and *sbcD*). The result indicates that our isolates are genomically stable and resistant to undesirable genes that bring antibiotic resistance and pathogenicity *via* horizontal gene transfer. In addition, it has been shown that the isolates do not cause disease or mutagenesis after administration, suggesting that those isolates are useful for the production of fermented foods and healthcare materials.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

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