

Nucleotide Sequence Identities of *horA* Homologues and Adjacent DNA Regions Identified in Three Species of Beer-Spoilage Lactic Acid Bacteria

K. Suzuki^{1,3}, M. Sami², K. Ozaki¹ and H. Yamashita¹

ABSTRACT

J. Inst. Brew. 110(4), 276–283, 2004

The *horA* homologues and adjacent DNA regions identified in beer-spoilage *Lactobacillus lindneri* DSM 20690^T and *L. paracollinoides* DSM15502^T were examined and compared with the corresponding DNA region of beer-spoilage *L. brevis* ABBC45, a strain in which the hop-resistance gene *horA* was originally identified. The PCR analysis suggests ORFB1-B5 regions surrounding *horA* are conserved in all of the strains. The nucleotide sequence comparison of the conserved DNA regions revealed extremely high levels of identities among the three beer-spoilage strains that are not typical for distinct species. The PCR methods using primers specific to the adjacent ORFs were found to be able to differentiate beer-spoilage *Lactobacillus* strains from non-spoilers, indicating these ORFs are also useful genetic markers for determining the beer-spoilage ability of lactobacilli. The presence or absence of the adjacent ORFs in 92 bacterial strains was completely identical with that of *horA* homologues, indicating the ORFB1-B5 regions are generally conserved in beer-spoilage *Lactobacillus* strains. Taken together, these results suggest the ORFB1-B5 regions have been acquired by beer-spoilage lactobacilli through horizontal gene transfer and provide a theoretical basis for applying a trans-species genetic marker such as *horA* to deal with unencountered species of beer-spoilage lactobacilli.

Key words: Beer-spoilage ability, lactobacilli, *horA*, horizontal gene transfer, trans-species genetic marker.

INTRODUCTION

Beer has been recognized as a beverage with high microbiological stability. Only a small number of bacterial species, predominantly lactic acid bacteria, represent the majority of beer-spoilage bacteria^{1–3}. For this reason, species-specific identification methods have been prevalent in the brewing industry to determine the beer-spoilage ability of bacteria. As one of the rapid means of species-

specific identification, the PCR-based method has been widely evaluated for potential applications in microbiological quality control^{4,10,26–28}. Although species-specific PCR tests are rapid and reasonably accurate, there are two problems in applying this approach in quality control in breweries.

The first problem is that the species-specific method is unable to distinguish intra-species differences between beer-spoilage strains and non-spoilage strains. For instance, some strains of *L. brevis*, the leading cause of spoilage incidents in the brewing industry^{1–3}, are capable of vigorously growing in beer, whereas some others belonging to the same species have no beer-spoilage ability^{5,17,25}. The presence of non-spoilage strains within a beer-spoilage species inevitably leads to false-positive results as long as the brewers rely on the species-specific approaches.

Hop compounds added to confer a bitter flavor in beers were reported to dissipate the transmembrane pH gradient of bacterial cells by acting as ionophores^{15,16,18} and the differences in hop-resistance ability have been considered to be responsible for intra-species variations in beer-spoilage ability^{5,17,19,23,25}. Therefore it is difficult for species-specific approaches to deal with the strain-specific nature of hop-resistance ability. To overcome this difficulty, the genetic markers specific to beer-spoilage *L. brevis* strains were identified to assess the intra-species differences in beer-spoilage ability of this species^{7,8}. Although these genetic markers are useful for differentiating beer-spoilage ability of *L. brevis*, it requires considerable time to identify similar genetic markers for each species of beer-spoilage bacteria.

The second problem concerns unencountered species of beer-spoilage bacteria. For instance, *L. paracollinoides* was recently proposed as a new species that exhibits strong beer-spoilage ability^{6,20,21}. The presence of other novel beer-spoilage *Lactobacillus* species was also suggested⁹. This problem is in fact a serious threat to brewers since one microbiological incident significantly damages the corporate brand, resulting in the significant loss of sales. What has long been desired is a new concept of genetic markers for differentiating beer-spoilage ability beyond the species status.

The concept of trans-species genetic markers was pioneered by Sami *et al.*¹⁴. The *horA* gene, the first trans-

¹Analytical Technology Laboratory and ²Fundamental Research Laboratory, Asahi Breweries Ltd., 1-21 Midori 1-chome, Moriyashi, Ibaraki, 302-0106, Japan.

³Corresponding author. E-mail: koji.suzuki@asahibeer.co.jp

species genetic marker ever found in brewing microbiology, was originally identified in beer-spoilage *L. brevis* ABBC45¹⁴. This gene was harbored by a 15.1 kb plasmid designated as pRH45, the copy number of which multiplied with the increase in hop-resistance ability of this strain¹³. A subsequent study showed that HorA, a product of the *horA* gene, acts as a multidrug transporter and confers hop resistance on lactic acid bacteria by extruding toxic hop compounds out of the cells¹¹. Interestingly a PCR method based on the nucleotide sequence of *horA* was demonstrated to differentiate beer-spoilage ability of a wide variety of beer-spoilage *Lactobacillus* species¹⁴. The versatility of *horA* also suggests this genetic marker is applicable to unencountered species of beer-spoilage lactic acid bacteria, although no theoretical or experimental basis for this has been provided.

In this study, we analyzed the nucleotide sequences of the *horA* homologues and adjacent DNA region belonging to two species of beer-spoilage lactic acid bacteria other than *L. brevis*. On the basis of the nucleotide sequence similarity of three species, we will discuss the possibility of horizontal gene transfer to be involved in the origin of beer-spoilage lactic acid bacteria. In addition, the theoretical basis for using trans-species genetic markers to deal with unencountered beer-spoilage species will be presented.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Lactobacillus strains were grown anaerobically at 25°C in MRS broth (Merck, Darmstadt, Germany, pH adjusted to 5.5 with HCl). Anaerobic conditions were generated by the AnaeroPack (Mitsubishi Gas Chemicals, Tokyo, Japan). Cells were stored in MRS broth containing 20% glycerol at -80°C.

DNA sequencing of *horA* homologues

The nucleic acid was extracted from *Lactobacillus* strains, as described previously¹⁴. The *horA* homologues were amplified by PCR using primers based on the sequences of the adjoining regions of *horA*. The primer sequences are as follows.

Forward primer

5'-TTTGGTAAAGACCACGTCATCATC-3'

Reverse primer

5'-ACACGGTTTCGGTCAAGCCTTGGG-3'

Taq DNA polymerase and reaction mixture were supplied with the TaKaRa EX *Taq* kit (Takara Bio, Shiga, Japan). The PCR mixtures were placed in a thermal cycler (GeneAmp PCR System 9700, Applied Biosystems, Foster City, CA, USA). The cycling profile consisted of an initial heating at 94°C for 2.5 min, followed by amplification of 30 cycles for denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 3 min. The final extension was set at 72°C for 6 min. The TaKaRa EX *Taq* kit was used for subsequent PCR analysis unless otherwise described.

The PCR product was sequenced by the primer walking method, using RISA-384 (Shimadzu, Kyoto, Japan). The nucleotide sequence identity was determined using the DNASIS Pro software package (Hitachi Software Engineering, Tokyo, Japan).

Analysis of the conserved DNA regions

The ORFs of pRH45, a 15.1 kb *horA*-harboring plasmid identified in *L. brevis* ABBC45, was listed in Table I¹³. The conserved DNA regions of *L. lindneri* DSM20690^T and *L. paracollinoides* DSM15502^T were examined by PCR analysis using a strategy based on the amplification of separate portions of DNA regions upstream and downstream of *horA*.

For the analysis of the conserved DNA regions upstream of *horA*, forward primers designated as ORFB11F, ORFB12F and ORFB1F were designed on the basis of the nucleotide sequences of ORFB11, ORFB12 and ORFB1 respectively. To design a reverse primer, the consensus *horA*-specific sequence obtained from the DNA sequences of *horA* homologues was used. The primer sequences for the analysis of the conserved DNA region upstream of *horA* are as follows.

ORFB11F 5'-CCAGGGCGCTCATGGATCGAG-3'

ORFB12F 5'-ATGATGATTAATCAACGTAATCGC-3'

ORFB1F 5'-GAATTGACTGTTTGGCGTGCCAAGC-3'

horA-ER 5'-TCACCCGTTGCTCGTCGCGCTCTG-3'

Table I. Putative ORFs in pRH45 encoding proteins with the expected size exceeding 100 a.a.¹

ORF no.	Strand	Range (bp)	Size (a.a.)	Homologous proteins (accession number)	Identity ²
B1	-	70-933	287	<i>Lactobacillus delbrueckii</i> phospho-β-glycosidase (CAB06820.1)	35% (98/278)
B2	-	930-1880	316	<i>Lactobacillus gasseri</i> LPS biosynthesis protein (ZP_00046794.1)	38% (120/315)
B3	-	1898-2686	262	<i>Lactobacillus gasseri</i> hypothetical protein (ZP_00046797.1)	40% (102/252)
B4 ³	+	2898-4649	583	<i>Lactobacillus brevis</i> HorA (BAA21552.1)	100% (583/583)
B5	-	4921-5595	224	<i>Lactobacillus gasseri</i> LPS biosynthesis protein (ZP_00046796.1)	47% (66/138)
B6	-	6014-7006	330	<i>Tetragenococcus halophilus</i> replication protein A (NP_862285.1)	79% (247/311)
B7	-	8598-9071	157	<i>Pediococcus acidilactici</i> tyrosine recombinase (NP_857608.1)	94% (138/146)
B8	+	10420-10782	120	<i>Tetragenococcus halophilus</i> replication protein B (S38640)	29% (34/114)
B9	-	11315-12454	379	<i>Enterococcus faecium</i> transposase (ZP_00036235.1)	40% (156/381)
B10	-	12461-12922	153	<i>Clostridium perfringens</i> putative transposase (NP_562804.1)	45% (50/110)
B11	-	13768-14340	190	<i>Lactobacillus casei</i> transposase (CAA05973.1)	96% (168/175)
B12	+	14604-15101	165	<i>Lactobacillus plantarum</i> transposase TraISLp11 (AA038817.1)	98% (162/165)

¹ The nucleotide sequence of pRH45 has been deposited in DDBJ under accession number AB167897.

² Identity is expressed as the percentage of the number of matched amino acids in the aligned amino acid sequences, as indicated in the parenthesis.

³ ORFB4 corresponds to *horA*.

For the analysis of the conserved DNA regions downstream of *horA*, reverse primers designated as ORFB5R, ORFB6R and ORFB7R were designed on the basis of the nucleotide sequences of ORFB5, ORFB6 and ORFB7. To design a forward primer, the consensus *horA*-specific sequence was used. The primer sequences for the analysis of the conserved DNA region downstream of *horA* are as follows.

ORFB5R 5'-CGATAGTGGCGTTACCAAAATTGA-3'
 ORFB6R 5'-AGCACTTGCTGAACTCCGAAAGGG-3'
 ORFB7R 5'-AAAACGGGTAAGCCTAATACCTTG-3'
horAEF 5'-ATGCAAGCTCAGTCCAAGAACAAT-3'

The cycling profile consisted of an initial heating at 94°C for 2.5 min, followed by amplification of 30 cycles for denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 7 min. The final extension was set at 72°C for 15 min.

DNA sequencing of the conserved DNA regions

The DNA sequencing of the conserved DNA region of *L. lindneri* DSM 20690^T was carried out, using inverse PCR. The primers were designed based on the sequences of the adjoining regions of *horA* homologue. The primer sequences are as follows.

Forward primer (*horA* For1)
 5'-TGAATTGCCCTTTTAAACGTCCC-3'

Reverse primer (*horA* Rev1)
 5'-TTGATTGAAGTCCCTGGGTTTGGG-3'

Taq DNA polymerase and reaction mixture were supplied with Herculase Hotstart DNA Polymerase (Stratagene, La Jolla, CA, USA). The cycling profile consisted of an initial heating at 92°C for 2 min, followed by amplification of 30 cycles for denaturation at 92°C for 30 s, annealing at 60°C for 30 s and extension at 68°C for 20 min. The final extension was set at 68°C for 40 min.

The PCR products were subsequently fragmented, using Hydroshear (GeneMachines, San Carlos, CA, USA). The fragments were cloned into pUC118 (Takara Bio, Shiga, Japan) and the nucleotide sequences of these fragments were determined by RISA-384 (Shimadzu, Kyoto, Japan). The DNA sequence was assembled using Phred/Phrap/consed System (University of Washington, Seattle, WA, USA).

As for the conserved DNA region of *L. paracollinoides* DSM 15502^T, the ORFB1-B5 region was amplified using the following primers designed based on the adjoining regions of ORFB1 and ORFB5 respectively.

Forward primer (ORFB1SeqF)
 5'-GAAAGATCATTGTTTCACGGTC-3'

Reverse primer (ORFB5SeqR)
 5'-CTGACTGACGATCACGCAGCC-3'

The cycling profile consisted of an initial heating at 94°C for 2.5 min, followed by amplification of 30 cycles for denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 6 min. The final extension

was set at 72°C for 12 min. The amplified ORFB1-B5 region of *L. paracollinoides* DSM15502^T was sequenced by the primer walking method as described earlier.

ORF analysis and nucleotide sequence comparison of the conserved DNA regions

Putative ORFs encoding proteins exceeding 100 amino acids (a.a.) in size were identified on the basis of the nucleotide sequences, using ORF finder (National Center for Biotechnology Information (NCBI), Bethesda, MD, USA). Identification of homologous proteins was based on BLASTP analysis, the program of which was provided by NCBI. Nucleotide sequence comparisons of the conserved DNA regions were performed, using DNASIS Pro software package (Hitachi Software Engineering, Tokyo, Japan).

Evaluation of the adjacent ORFs as genetic markers for differentiating beer-spoilage ability

A total of 78 *Lactobacillus* strains and 14 species frequently isolated from brewery environments were examined for the presence of *horA* homologues and the adjacent ORFs. The primers specific to each ORF are listed in Table II. The cycling profile for ORFB1, ORFB2 and ORFB5 consisted of an initial heating at 94°C for 2.5 min, followed by amplification of 30 cycles for denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The final extension was set at 72°C for 3 min. The PCR conditions for *horA* were described previously¹⁴.

For positive PCR reactions, all the amplicons were subjected to Southern blot analysis, using the probes specific to each ORF. The specific probes were obtained from *L. brevis* ABBC45 using the identical primers listed in Table II. Southern blot analysis was performed in accordance with the manufacturer's protocol (DIG High Prime Labeling and Detection Starter Kit I, Roche Diagnostics, Mannheim, Germany).

Evaluation of beer-spoilage ability

Degassed commercial lager beers (bitterness unit 20 BU and alcohol content 5% v/v) were adjusted to pH 4.6 with 5N NaOH. The beers with these specifications represent a Japanese pilsner-type beer with relatively weak

Table II. Specific primers for PCR analysis and probes for Southern blot analysis.

Probes ¹	Specific primers	Nucleotide sequences
ORFB1	GM-ORFB1F GM-ORFB1R	5'-GGGCAATAAACCCCTTGC-3' 5'-TTGCTGAAAACGTCGTC-3'
ORFB2	GM-ORFB2F GM-ORFB2R	5'-TTCGATTTCTCGCGATGG-3' 5'-TATTGCTGAATTGTTCCC-3'
<i>horA</i> ²	LbHC-1 LbHC-2	5'-ATCCGGCGGTGGCAAATCA-3' 5'-AATCGCCAATCGTTGGCG-3'
ORFB5	GM-ORFB5F GM-ORFB5R	5'-CTTTATCAACTCCCAAGG-3' 5'-CATCAACACGTTATTACAG-3'

¹ Specific probes for Southern blot analysis were obtained from *L. brevis* ABBC45, using the specific primers to each ORF.

² The previously reported *horA*-specific primers and PCR conditions were used¹⁴.

Table III. Identities of *horA* homologues and the deduced proteins.

	<i>horA</i> homologue ¹	Deduced protein ²
<i>L. lindneri</i> DSM20690 ^T	99.7%	99.5%
<i>L. paracollinoides</i> DSM15502 ^T	99.4%	99.0%

¹ The entire region of *horA* homologue (1752 bp) was compared with that of *L. brevis* ABBC45.

² The entire region of deduced protein (583 a.a.) was compared with that of *L. brevis* ABBC45.

microbiological stability. Ten mL of beer was dispensed into 15 mL sterile polypropylene tubes and inoculated with approximately 3×10^3 cells/mL of *Lactobacillus* strains and environmental isolates. The inoculated beers were incubated anaerobically at 25°C and examined regularly for visible growth for up to 60 days.

RESULTS

DNA sequencing of *horA* homologues

The *horA* homologues were amplified from beer-spoilage *L. lindneri* DSM20690^T and *L. paracollinoides* DSM15502^T by PCR and fully sequenced. As shown in Table III, the *horA* homologues obtained from *L. lindneri* DSM20690^T and *L. paracollinoides* DSM15502^T displayed 99.7% and 99.4% identity, respectively, with that of *L. brevis* ABBC45 in nucleotide sequence. The amino acid sequences of the deduced proteins were found to be 99.5% and 99.0% identical.

Analysis of the conserved DNA regions

The extremely high similarity of *horA* and its homologues among the three beer-spoilage strains belonging to distinct species prompted us to investigate the conserved

DNA regions surrounding *horA*. To examine the upstream region of *horA*, we designed forward primers, ORFB11F, ORFB12F and ORFB1F on the basis of the nucleotide sequences of ORFB11, ORFB12 and ORFB1. As a reverse primer, a consensus *horA*-specific primer, *horAER*, was designed to amplify regions containing ORFB11-*horA*, ORFB12-*horA* and ORFB1-*horA*. For the downstream region of *horA*, we adopted a similar approach and designed reverse primers, ORFB5R, ORFB6R and ORFB7R, based on the nucleotide sequences of ORFB5, ORFB6 and ORFB7. The forward primer, *horAEF*, was also designed based on the consensus sequence of *horA* homologues.

As a result of PCR analysis, the positive reactions were obtained from *L. lindneri* DSM20690^T with all the three primer sets designed to amplify the upstream region of *horA*, suggesting the ORFB11-*horA* region is conserved in *L. lindneri* DSM20690^T (Table IV). The downstream region of *horA* was also successfully amplified with all the primer combinations, suggesting that the extensive DNA region surrounding *horA* homologue is conserved in *L. lindneri* DSM20690^T.

In contrast, only the primer pair, ORFB1F and *horAER*, yielded a positive reaction from *L. paracollinoides* DSM15502^T, whereas no positive reactions were obtained from the other combinations of primers designed to amplify the upstream region of *horA* (Table IV). For the downstream region, only one primer combination, *horAEF* and ORFB5R, yielded positive reactions. The different primers designed based on ORFB11 and ORFB12, as well as ORFB6 and ORFB7, did not yield positive reactions from *L. paracollinoides* DSM15502^T (data not shown). From these results, the ORFB1-B5 region appears to be conserved in *L. paracollinoides* DSM15502^T, although the presence of the DNA regions upstream of ORFB1 and downstream of ORFB5 has not been ruled out in this study.

Table IV. PCR analysis for the conserved DNA regions.

Strains	Upstream region of <i>horA</i> homologue			Downstream region of <i>horA</i> homologue		
	ORFB11- <i>horA</i>	ORFB12- <i>horA</i>	ORFB1- <i>horA</i>	<i>horA</i> -ORFB5	<i>horA</i> -ORFB6	<i>horA</i> -ORFB7
<i>L. lindneri</i> DSM20690 ^T	+	+	+	+	+	+
<i>L. paracollinoides</i> DSM15502 ^T	-	-	+	+	-	-
<i>L. brevis</i> ABBC45	+	+	+	+	+	+

Table V. Comparison of putative ORFs encoding proteins with the expected size exceeding 100 a.a. identified in the conserved DNA sequences determined in the current study¹.

ORF no.	Strand	<i>L. brevis</i> ABBC45		<i>L. lindneri</i> DSM20690 ^T			<i>L. paracollinoides</i> DSM15502 ^T		
		Range (bp) ²	Size (a.a.)	Range (bp)	Size (a.a.)	Identities (%) ³	Range (bp)	Size (a.a.)	Identities (%)
B1	-	1-864	287	1-867	288	99.0 (855/864)	1-864	287	100.0 (864/864)
B2	-	861-1811	316	864-1814	316	98.5 (937/951)	861-1811	316	99.6 (947/951)
B3	-	1829-2617	262	1832-2620	262	99.5 (785/789)	1829-2617	262	99.9 (788/789)
B4 ⁴	+	2829-4580	583	2832-4583	583	99.7 (1746/1752)	2829-4580	583	99.4 (1742/1752)
B5	-	4852-5526	224	4855-5751	298	99.4 (671/675)	4852-5412	186	96.4 (541/561)

¹ The nucleotide sequences of ABBC45, DSM20690^T and DSM15502^T have been deposited in DDBJ under accession number AB167897, AB167898 and AB178589, respectively. Putative ORFs were identified, using ORF finder (National Center for Biotechnology Information (NCBI), Bethesda, USA).

² The first nucleotide of ORFB1 was repositioned as 1 for easier comparison.

³ The identities with the corresponding regions of *L. brevis* ABBC45 are expressed as the percentage of the number of matched nucleotides in the aligned sequences shown in the parenthesis.

⁴ ORFB4 corresponds to *horA* and *horA* homologues.

Table VI. Evaluation of the adjacent ORFs as genetic markers for differentiating beer-spoilage ability¹.

Strain no. ²	Species	ORFB1	ORFB3	ORFB5	<i>horA</i>	Beer-spoilage ability ³
ABBC3	<i>L. brevis</i>	+	+	+	+	+
ABBC4	<i>L. brevis</i>	+	+	+	+	-
ABBC12	<i>L. brevis</i>	-	-	-	-	-
ABBC34	<i>L. brevis</i>	+	+	+	+	+
ABBC36	<i>L. brevis</i>	+	+	+	+	+
ABBC37	<i>L. brevis</i>	+	+	+	+	+
ABBC42	<i>L. brevis</i>	+	+	+	+	+
ABBC43	<i>L. brevis</i>	+	+	+	+	+
ABBC44	<i>L. brevis</i>	+	+	+	+	+
ABBC44 ^{NB}	<i>L. brevis</i>	-	-	-	-	-
ABBC45	<i>L. brevis</i>	+	+	+	+	+
ABBC45 ^C	<i>L. brevis</i>	-	-	-	-	+
ABBC45 ^{CC}	<i>L. brevis</i>	-	-	-	-	-
ABBC46	<i>L. brevis</i>	+	+	+	+	+
ABBC46 ^{NB}	<i>L. brevis</i>	-	-	-	-	-
ABBC56	<i>L. brevis</i>	+	+	+	+	+
ABBC64	<i>L. brevis</i>	+	+	+	+	+
ABBC64 ^{NB}	<i>L. brevis</i>	-	-	-	-	-
ABBC65	<i>L. brevis</i>	+	+	+	+	+
ABBC67	<i>L. brevis</i>	+	+	+	+	+
ABBC69	<i>L. brevis</i>	+	+	+	+	+
ABBC70	<i>L. brevis</i>	+	+	+	+	+
ABBC76	<i>L. brevis</i>	+	+	+	+	+
ABBC77	<i>L. brevis</i>	+	+	+	+	+
ABBC78	<i>L. brevis</i>	+	+	+	+	+
ABBC79	<i>L. brevis</i>	+	+	+	+	+
ABBC84	<i>L. brevis</i>	+	+	+	+	+
ABBC85	<i>L. brevis</i>	+	+	+	+	+
ABBC86	<i>L. brevis</i>	+	+	+	+	+
ABBC99	<i>L. brevis</i>	+	+	+	+	+
ABBC100	<i>L. brevis</i>	+	+	+	+	+
ABBC104	<i>L. brevis</i>	+	+	+	+	+
ABBC104 ^{NB}	<i>L. brevis</i>	-	-	-	-	-
ABBC400	<i>L. brevis</i>	+	+	+	+	+
ABBC400 ^{NB}	<i>L. brevis</i>	-	-	-	-	-
ABBC402	<i>L. brevis</i>	+	+	+	+	+
ABBC403	<i>L. brevis</i>	+	+	+	+	+
ABBC404	<i>L. brevis</i>	+	+	+	+	+
ABBC405	<i>L. brevis</i>	+	+	+	+	+
ABBC406	<i>L. brevis</i>	+	+	+	+	+
ABBC407	<i>L. brevis</i>	+	+	+	+	+
ABBC408	<i>L. brevis</i>	+	+	+	+	+

(continued on next page)

¹The primer pairs specific to each ORF were used. All of the positive amplicons were subjected to Southern blot analysis to determine whether the PCR products were homologues of respective ORFs. All the PCR results were found to be consistent with those of the Southern blot analysis. The superscripts, NB and CC, indicate the hop-sensitive variants obtained from beer-spoilage wild-type strains with the same strain number^{22,23}.

²ABBC and HC: our culture collections principally consisting of brewery isolates; ATCC: American Type Culture Collection; DSM: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH; JCM: Japan Collection of Microorganisms.

³Degassed commercial lager beers (bitterness unit: 20 B.U.; alcohol content: 5% v/v) were adjusted to pH 4.6 and inoculated with approximately 3×10^3 cells/ml of test strains. The inoculated beers were incubated anaerobically at 25°C and examined regularly for visible growth for up to 60 days.

DNA sequencing and comparison of the conserved DNA regions

To make a comparison among the three strains, we sequenced the conserved DNA regions containing ORFB1-B5. For *L. lindneri* DSM20690^T, the conserved region was amplified by inverse PCR, since the presence of a *horA*-harboring plasmid was detected in this strain by Southern blot analysis (unpublished data). The similar approach was not successful for *L. paracollinoides* DSM15502^T. We therefore amplified the ORFB1-B5 region by PCR using the primers designed based on the adjoining regions of ORFB1 and ORFB5.

As shown in Table V, the internal organizations of the conserved ORFB1-B5 region were found to be strikingly similar among the three beer-spoilage strains. The corresponding DNA regions of *L. lindneri* DSM20690^T and *L. paracollinoides* DSM15502^T were 99.3% and 99.4% identical with that of *L. brevis* ABBC45 over the approximately 5.5 kb DNA region. The four ORFs surrounding *horA* homologue were also remarkably similar to those of *L. brevis* ABBC45, exhibiting approximately 99% identities except for ORFB5 homologue identified in *L. paracollinoides* DSM15502^T. Relatively low similarity of the ORFB5 homologue is partly due to 18 bp DNA gap found in this strain.

Table VI (continued)¹.

Strain no. ²	Species	ORFB1	ORFB3	ORFB5	horA	Beer-spoilage ability ³
JCM1059 ^T	<i>L. brevis</i>	-	-	-	-	-
DSM1267	<i>L. brevis</i>	-	-	-	-	-
DSM2647	<i>L. brevis</i>	-	-	-	-	-
DSM20556	<i>L. brevis</i>	-	-	-	-	-
DSM15502 ^T	<i>L. paracollinoides</i>	+	+	+	+	+
LA3	<i>L. paracollinoides</i>	+	+	+	+	+
LA4	<i>L. paracollinoides</i>	+	+	+	+	+
LA7	<i>L. paracollinoides</i>	-	-	-	-	+
LA8	<i>L. paracollinoides</i>	-	-	-	-	+
LA9	<i>L. paracollinoides</i>	+	+	+	+	+
LA10	<i>L. paracollinoides</i>	+	+	+	+	+
LA11	<i>L. paracollinoides</i>	+	+	+	+	+
LA12	<i>L. paracollinoides</i>	+	+	+	+	+
LA13	<i>L. paracollinoides</i>	+	+	+	+	+
LA14	<i>L. paracollinoides</i>	+	+	+	+	+
LA15	<i>L. paracollinoides</i>	+	+	+	+	+
ABBC216	<i>L. paracollinoides</i>	-	-	-	-	-
DSM20690 ^T	<i>L. lindneri</i>	+	+	+	+	+
DSM20692	<i>L. lindneri</i>	+	+	+	+	+
HC92	<i>L. lindneri</i>	+	+	+	+	+
HC95	<i>L. lindneri</i>	+	+	+	+	+
HC98	<i>L. lindneri</i>	+	+	+	+	+
JCM1123 ^T	<i>L. collinoides</i>	-	-	-	-	-
ATCC27610	<i>L. collinoides</i>	-	-	-	-	-
ATCC27611	<i>L. collinoides</i>	-	-	-	-	-
ABBC213	<i>L. plantarum</i>	-	-	-	-	-
ABBC219	<i>L. buchneri</i>	+	+	+	+	-
ABBC222	<i>L. paracasei</i>	-	-	-	-	-
ABBC228	<i>L. fermentum</i>	-	-	-	-	-
ABBC251	<i>L. coryniformis</i>	-	-	-	-	-
ABBC257	<i>L. fructivorans</i>	-	-	-	-	-
ABBC275	<i>L. rhamnosus</i>	-	-	-	-	-
ABBC279	<i>L. casei</i>	-	-	-	-	-
ABBC281	<i>L. delbrueckii</i>	-	-	-	-	-
JCM8573 ^T	<i>L. parakefiri</i>	-	-	-	-	-
DSM5707	<i>L. parabuchneri</i>	-	-	-	-	-
HC311	<i>Lactococcus lactis</i>	-	-	-	-	-
HC367	<i>Serratia marcescens</i>	-	-	-	-	-
HC417	<i>Citrobacter freundii</i>	-	-	-	-	-
HC432	<i>Enterobacter cloacae</i>	-	-	-	-	-
HC437	<i>Staphylococcus warneri</i>	-	-	-	-	-
HC440	<i>Propionibacterium acnes</i>	-	-	-	-	-
HC442	<i>Bacillus thuringiensis</i>	-	-	-	-	-
HC453	<i>Pantoea agglomerans</i>	-	-	-	-	-
HC459	<i>Paenibacillus amylolyticus</i>	-	-	-	-	-
HC466	<i>Paenibacillus jamilae</i>	-	-	-	-	-
HC472	<i>Clostridium beijerinckii</i>	-	-	-	-	-
HC475	<i>Staphylococcus epidermidis</i>	-	-	-	-	-
HC523	<i>Sporolactobacillus racemicus</i>	-	-	-	-	-
HC534	<i>Klebsiella oxytoca</i>	-	-	-	-	-

Evaluation of the adjacent ORFs as genetic markers for differentiating beer-spoilage ability

To further determine whether the ORFs adjacent to *horA* serve as genetic markers suited to differentiating the beer-spoilage ability of detected bacteria, three sets of primers were designed on the basis of the nucleotide sequences of ORFB1, ORFB2 and ORFB5. Among the 51 beer-spoilage *Lactobacillus* strains, 48 strains yielded positive reactions simultaneously with all the primer pairs as shown in Table VI. These strains also reacted with the *horA*-specific primers previously reported¹⁴. The Southern blot analysis with the probes specific to ORFB1, ORFB2,

horA and ORFB5 showed the simultaneous presence of homologues for each ORF in all of the positively reacted strains (Table VI). These results suggest the ORFB1-B5 regions are conserved among the 48 beer-spoilage *Lactobacillus* strains examined in this study. Nevertheless three beer-spoilage strains, *L. brevis* ABBC45^C and *L. paracollinoides* LA7 and LA8, did not react positively with any of the primer pairs, consistent with the absence of positive reactions with the *horA*-specific primers in these strains.

As for 27 non-spoilage *Lactobacillus* strains and 14 strains isolated from brewery environments, no positive reactions were observed with any of the primer pairs ex-

cept for *L. brevis* ABBC4 and *L. buchneri* JCM1115^T which reacted with all the primer combinations. Taken collectively, these results indicate that ORFB1, ORFB2 and ORFB5 potentially serve as genetic markers well suited to the determination of beer-spoilage ability of detected bacteria, a feature comparable with that of *horA*.

DISCUSSION

In this study, we analyzed the *horA* homologues and the adjacent DNA regions identified in beer-spoilage *L. lindneri* DSM 20690^T and *L. paracollinoides* DSM15502^T. As a result of comparison with beer-spoilage *L. brevis* ABBC45, approximately 5.5 kb DNA regions were conserved in all of the three strains. The conserved DNA region was found to contain 5 ORFs designated as ORFB1–B5. Notably the internal organizations of these ORFs were strikingly similar among the three beer-spoilage strains. The nucleotide sequence identity of the ca 5.5 kb DNA regions found in *L. lindneri* DSM 20690^T and *L. paracollinoides* DSM15502^T was 99.3% and 99.4% respectively, as compared with that of *L. brevis* ABBC45. As for the respective ORFs in the conserved DNA regions, the *horA* homologues identified in *L. lindneri* DSM 20690^T and *L. paracollinoides* DSM15502^T exhibited 99.7% and 99.4% identity with that of *L. brevis* ABBC45 in nucleotide sequence. The amino acid sequences of the deduced proteins were also found to be remarkably similar to that of *L. brevis* ABBC45 (Table III). This level of similarity suggests the *horA* homologues also confer hop-resistance ability on *L. lindneri* DSM 20690^T and *L. paracollinoides* DSM15502^T. The other ORFs were also found to be highly similar (Table V). This level of nucleotide identities observed among the three strains belonging to distinct species suggests that the conserved DNA regions were acquired by these beer-spoilage strains through horizontal gene transfer rather than evolved independently with the speciation process.

It is also noteworthy that six strains shown in Table VI are hop-sensitive variants obtained from beer-spoilage wild-type strains^{22,23}. The loss of reactivity with all of the primer sets in these hop-sensitive variants suggests that the conserved DNA regions are unstable in beer-spoilage lactobacilli and therefore of foreign origin. The Southern blot analysis with *EcoRI*-digested genomic DNA of these variants indicated that *horA* homologues were indeed lost from all the six variants (unpublished data), further supporting this hypothesis. The *horA* regions were found to be carried by plasmids in all the six wild-type strains, which may explain the instability of the *horA* region (unpublished data). We are currently working on this aspect of the conserved DNA region identified in this study.

An interesting feature of *horA* is that this gene serves as a genetic marker well suited to differentiating beer-spoilage ability of *Lactobacillus* strains that belong to various species¹⁴. Therefore we examined whether other ORFs adjacent to *horA* also serve as a genetic marker for determining the beer-spoilage ability of detected bacteria. Our results indicate the presence or absence of ORFB1, ORFB2 and ORFB5 are perfectly identical with that of *horA* in all the strains tested in this study and these ORFs

are capable of differentiating the beer-spoilage ability of detected bacteria (Table VI). This result also suggests that the DNA regions containing ORFB1–B5 are generally conserved in beer-spoilage *Lactobacillus* strains and possibly transfers simultaneously. Although the reliability of the ORFs adjacent to *horA* in differentiating the beer-spoilage ability of lactobacilli should be reinforced by the functional characterization of these ORFs, the use of ORFB1, ORFB2 and ORFB5 will have potential applications to verifying the results yielded by the *horA*-PCR method.

One concern for the *horA* PCR method is the occurrence of false-positive results with *L. brevis* ABBC4 and *L. buchneri* ABBC219. The reason for this has not yet been identified. But the mere presence or absence of a given genetic marker has a certain limit in differentiating beer-spoilage ability because the hop-resistance gene must be functionally expressed in the host lactic acid bacteria, which may not be the case for *L. brevis* ABBC4 or *L. buchneri* ABBC219. This aspect of *horA* gene should be studied in further details.

Another concern for the *horA* PCR method is false negative results found in this study. *L. brevis* ABBC45^C, one of the strains that yielded negative reactions, is a variant obtained from the wild-type strain ABBC45. Our previous study showed that *L. brevis* ABBC45^C lost pRH45¹², a *horA*-harboring plasmid, but still exhibited beer-spoilage ability that is weaker than that of ABBC45²⁵. These findings indicate that *L. brevis* ABBC45 possesses at least two hop-resistance mechanisms. The secondary hop-resistance mechanism of ABBC45^C appears to be mediated by a proton motive force (PMF) dependent multidrug transporter²⁵, a mechanism that is different from that of *horA*, which has been demonstrated to encode an ATP-dependent multidrug transporter¹¹. The investigation into the gene that encodes a putative PMF-dependent multidrug transporter led to the identification of another trans-species genetic marker, ORF5 and its adjacent region, for determining beer-spoilage ability of detected bacteria^{22,24}. Interestingly this genetic marker was found in *L. paracollinoides* LA7 and LA8²⁴, as well as *L. brevis* ABBC45^C, which did not react with primers specific to *horA* and the adjacent ORFs. In fact ORF5 was found to be the genetic marker, the presence or absence of which is highly correlated with beer-spoilage ability of *L. brevis* and *L. paracollinoides* strains^{22,24}, suggesting ORF5 and its adjacent DNA region mediates the *horA*-independent hop-resistance mechanism. These findings also indicate that multiple trans-species genetic markers are required to develop a comprehensive microbiological control system in breweries.

Our hypothesis that hop-resistance ability of lactobacilli has been conferred by horizontal gene transfer potentially indicate that trans-species genetic markers, such as *horA*, are useful for determining the beer-spoilage ability of unencountered *Lactobacillus* species. This hypothesis also provides a theoretical basis for applying trans-species genetic markers to the quality control of breweries. The concept of trans-species genetic markers is relatively new in brewing microbiology. But if our hypothesis is correct, brewing microbiologists will have another useful means of detecting beer-spoilage bacteria. We are currently conducting research to demonstrate that the hop-resistance ability is conferred by horizontal gene transfer.

ACKNOWLEDGEMENTS

We are grateful to Hiroe Kusama of Pasona Inc. for providing technical assistance for this study.

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(Manuscript accepted for publication July 2004)