

Isolation and Identification of Potential Beer-Spoilage *Pediococcus inopinatus* and Beer-Spoilage *Lactobacillus backi* Strains Carrying the *horA* and *horC* Gene Clusters

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ABSTRACT

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Four beer-spoilage strains, LA20, LA21, LA22 and LA23, were isolated from brewery environments. Based on the 16S rRNA gene sequence, LA20 was identified as *Pediococcus inopinatus* and the remaining three were identified as *Lactobacillus backi*. The homologs of *horA* and *horC*, the hop resistance genes originally identified in *L. brevis* ABBC45, were detected simultaneously in LA22 and LA23, while only a *horA* homolog and a *horC* homolog were found in LA20 and LA21 respectively. The 5.6 kb DNA regions containing the *horA* homolog in LA20 and LA22 were almost 99% identical with the corresponding region of ABBC45. Similarly the 8.2 kb regions containing the *horC* homolog in LA21 and LA22 were more than 99% identical with that of ABBC45. Interestingly the *horA*-containing 5.6 kb regions in LA20 and LA22 were found to be completely identical despite the distinct genus status. Coupled with the fact that LA20 and LA22 were isolated from the same sampling site, these results, taken collectively, reinforce our hypothesis that *horA* and *horC* genes were acquired by beer-spoilage species through horizontal gene transfer and confirm the usefulness of *horA* and *horC* as genetic markers for the species-independent determination of beer-spoilage ability in lactic acid bacteria.

Key words: Beer-spoilage ability, hop resistance gene, *horA*, *horC*, horizontal gene transfer, *Lactobacillus backi*, *Pediococcus inopinatus*

INTRODUCTION

Only a limited number of bacteria can grow in beer due to its alcohol content (0.5–10% w/w), relatively low pH (3.8–4.7), anaerobic conditions, lack of nutrition and the antibacterial effects of hop compounds^{13,18}. However, microbiological incidents cause not only vast economic dam-

age in terms of product recalls, but also irreversible loss of consumer confidence, leading to destruction of corporate brands. Therefore, beer-spoilage bacteria are one of the major problems in the brewing industry worldwide.

It has been reported that approximately 70% of microbiological incidents in beers are caused by lactic acid bacteria (LAB)². Therefore the rapid detection and identification of beer-spoilage LAB is extremely important for investigating and improving brewery environments. The well-established beer-spoilage LAB species consist of several species, including *L. brevis*, *L. lindneri*, *L. paracollinoides* and *P. damnosus*^{2–4,18}. Therefore, in the brewing industry, species-specific detection methods for these beer-spoilage species have been traditionally developed¹⁸. On the other hand, the presence of previously uncharacterized beer-spoilage species has been reported in several studies. These trends make it difficult to develop comprehensive species-specific detection methods for all of the beer-spoilage species that can be encountered in breweries.

In the course of our studies, we identified two hop resistance genes, *horA* and *horC*, in *L. brevis* ABBC45^{8,12,14,15,17,18,21}. The *horA*- and *horC*-carrying regions found in *L. lindneri*, *L. paracollinoides* and *P. damnosus* are approximately 99% identical with each corresponding region of *L. brevis* ABBC45 despite their distinct species status, indicating the emergence of beer-spoilage LAB by horizontal transfer of the hop resistance genes^{17,18,22–24}. Moreover, the wide and exclusive distributions of *horA* and *horC* genes in various beer-spoilage LAB species indicate the possibility of comprehensive species-independent detection of beer-spoilage LAB with these genetic markers¹⁷. However, the applicability of the *horA*- and *horC*-specific methods to unencountered beer-spoilage bacteria remains to be more rigorously tested, since our previous studies only evaluated four beer-spoilage species, consisting of *L. brevis*, *L. lindneri*, *L. paracollinoides* and *P. damnosus*.

In this study, we have attempted to isolate beer-spoilage LAB that have not been characterized in our previous reports, and we have reevaluated the usefulness of *horA* and *horC* genes as genetic markers for evaluating the beer-spoilage ability of LAB.

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MATERIALS AND METHODS

Isolation of bacterial strains and growth conditions

A total of 783 samples were collected from brewery environments and recycled containers. For screening beer spoilage lactic acid bacteria, the samples were inoculated into degassed commercial pilsner-type beers (pH 5.0) containing approximately 20 ppm of iso- α -acids and incubated anaerobically at 25°C for 14 days. For the samples in which growth of bacteria was observed, portions of turbid beers were spread onto MRS agar (Merck, Darmstadt, Germany) for isolation.

Identification of the isolated strains

The rod-shaped bacteria examined by microscopic observation were identified by species-specific polymerase chain reaction (PCR) for *L. brevis*, *L. lindneri* and *L. paracollinoides* using primers listed in Table I. The *Taq* DNA polymerase and reaction mixtures were supplied with *TaKaRa Ex Taq* (Takara Bio, Shiga, Japan). 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 55°C for 30 s and extension at 72°C for 1 min. The final extension was set at 72°C for 3 min. The unidentifiable strains by species-specific PCR were analyzed by sequencing the whole 16S rRNA gene region with the method described by Funahashi et al.⁷. The determined sequences were queried with Basic Local Alignment Search Tool (BLAST) analysis, using the DNA database provided by the National Center for Biotechnology Information (NCBI) Web site (<http://www.ncbi.nlm.nih.gov/>). Bacterial species of the isolated strains were determined as the species with the highest identity in the BLAST analysis. All coccal strains were similarly identified based on the sequencing analysis of the 16S rRNA gene.

Evaluation of beer spoilage ability

Approximately 10³ cells ml⁻¹ of the strains were inoculated into degassed beers with three pH levels (pH 4.2, pH 4.6, pH 5.0). The inoculated beers were incubated anaerobically at 25°C and examined regularly for visible growth for up to 60 days. The beer-spoilage ability of the tested strains was defined as strong, weak and potential when

the growth was observed in beers at pH 4.2, pH 4.6 and pH 5.0, respectively.

Detection of the *horA* and *horC* genes

The strains were subjected to the *horA*- or *horC*-specific PCR as described previously^{15,17}. The primers for PCR are listed in Table I.

Analysis of the DNA regions containing the *horA* and *horC* gene

Each DNA region containing the *horA* or *horC* gene was amplified by PCR using the primers listed in Table I. DNA polymerase and reaction mixtures were supplied with Herculase Hotstart DNA Polymerase (Stratagene, La Jolla, CA). The cycling profile for the *horA*-containing regions 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 cycling profile for the *horC*-containing regions 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 10 min. The final extension was set at 72°C for 20 min.

Each 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).

Nucleotide sequence accession numbers

The DNA Data Bank of Japan (DDBJ) accession numbers of the *horA*-containing regions determined in this study are AB279601 (*P. inopinatus* LA20) and AB279602 (*L. backi* LA22). The *horC*-containing regions are AB279603 (*L. backi* LA21) and AB279604 (*L. backi* LA22).

RESULTS AND DISCUSSION

Isolation of beer-spoilage bacteria

The screening using degassed beer adjusted to pH 5.0 was carried out to isolate the beer-spoilage bacteria from brewery environments and recycled containers. Portions

Table I. List of specific primers used in this study.

Target genes	Specific primers	Nucleotide sequences (5'→3')	References
<i>L. brevis</i> 16S rRNA gene	Forward	CTGATTTCAACAATGAAGC	11
	Reverse	CCGTCAATTCCTTTGAGTTT	
<i>L. lindneri</i> 23S rRNA gene and ITS region	Forward	AACTTACACCGATCAAAAATC	1
	Reverse	CTTAACCTTGCATGCAACT	
<i>L. paracollinoides</i> 16S rRNA gene and ITS region	Forward	CACCCAAAGTCGGTTCGG	20
	Reverse	GTTCTCGGCTTAATTACTG	
<i>horA</i> homolog	Forward	ATCCGGCGGTGGCAAATCA	15
	Reverse	AATCGCCAATCGTTGGCG	
<i>horC</i> homolog	Forward	GTCAACGAAGACAAAGGAGCTCTC	17
	Reverse	GGGCGAACCGTGAACAAATAG	
<i>horA</i> and flanking regions	Forward	GAAAGATCATTTTGTTCACGGTC	24
	Reverse	CTGACTGACGATCACGCAGCC	
<i>horC</i> and flanking regions	Forward	ATCCCTCTCTCGATTGATGGTTG	22
	Reverse	GATGGCGACAACAAGGTCAAAC	

of the beers, in which the growth of microorganisms was observed, were spread on MRS agar for isolation. Subsequently the bacterial isolates were grouped based on the characteristics of colonies on agar plates and the morphological features of strains by microscopic observation. The rod-shaped strains were identified by the species-specific PCR using primers for three beer-spoilage species, *L. brevis*, *L. lindneri* and *L. paracollinoides* (Table II). Most rod-shaped isolates were identified as one of these beer-spoilage species. Rather surprisingly, *L. paracollinoides* was the most frequent species isolated in this study^{16,20}. Although the frequent findings of *L. paracollinoides* cannot be ruled out due to the possibilities of sampling bias, the strains identified as this species may be more widely distributed in beer-related environments than many brewing microbiologists previously assumed. Since *L. paracollinoides* has been recently proposed as a new species, the unexpected abundance of this species suggests a potential threat when a previously uncharacterized beer-spoilage species abruptly becomes a dominant bacterial flora in a particular brewery environment¹⁶.

The three rod-shaped isolates could not be identified by the species-specific PCR and therefore the identification of these isolates, designated LA21, LA22 and LA23, was implemented by the whole 16S rRNA gene sequencing. The 16S rRNA gene sequence of each strain showed more than 99% identity to that of *Lactobacillus backi* DSM 18080^T (GenBank accession no. DQ406862)⁵. Therefore LA21, LA22 and LA23 were identified as *L. backi*.

In regard to the cocci, five strains were identified as *P. damnosus* on the basis of 16S rRNA gene sequencing analysis. On the other hand, the 16S rRNA gene sequence of LA20 showed 100% identity to that of *P. inopinatus* DSM 20285^T (EMBL accession no. AJ271383) and LA20 was identified as *P. inopinatus*.

Beer-spoilage ability of LA20, LA21, LA22 and LA23

Although *L. brevis*, *L. lindneri*, *L. paracollinoides* and *P. damnosus* have been well characterized as strong beer-

spoilage LAB capable of growing in beers adjusted to pH 4.2, the information regarding the beer-spoilage ability of *P. inopinatus* and *L. backi* was relatively scarce¹⁸. Therefore, the beer-spoilage ability of LA20, LA21, LA22 and LA23 was investigated using degassed beers adjusted to pH 5.0, pH 4.6 and pH 4.2. *P. inopinatus* LA20 was capable of growing in beer of pH 5.0 within eight days, but did not grow at lower pH values (Table III). On the other hand, *L. backi* LA21, LA22 and LA23 were able to grow in beers of all pH levels tested (Table III). Based on these results, *P. inopinatus* LA20 was considered as potential spoiler, while *L. backi* LA21, LA22 and LA23 were judged as strong beer-spoilage strains.

Detection of the *horA* and *horC* genes

In the brewing industry, species-specific methods have been developed for determining beer-spoilage ability of the detected strains¹⁸. Although this approach can rapidly determine whether detected isolates belong to the well-known beer-spoilage species, the species-specific methods are not only unable to determine the intra-species differences in beer-spoilage ability but are also incapable of detecting previously uncharacterized beer-spoilage species. Based on our previous results, that all of the investigated beer-spoilage strains belonging to four beer-spoilage species have either or both of the *horA* and *horC* homolog, we have recently suggested that the combined use of *horA* and *horC* is useful for evaluating the beer-spoilage ability of LAB in a species-independent manner^{15,17}. Therefore *P. inopinatus* LA20 and the three *L. backi* strains isolated in this study were examined for the presence or absence of the *horA* and *horC* genes. Consequently, the *horA* gene was detected in LA20, LA22 and LA23. On the other hand, the *horC* gene was detected in LA21, LA22 and LA23 (Table IV). Since all of the strains possess at least one of the genetic markers, these results once again confirmed the usefulness of the species-independent *horA*- and *horC*-specific methods for detecting previously unencountered beer-spoilage LAB. In addition, further investigations concerning the remaining 34 beer-spoilage iso-

Table II. Identification of isolates from brewery and other beer-related environments.

Presumptive identification	Number of isolates
<i>L. brevis</i>	6
<i>L. lindneri</i>	5
<i>L. paracollinoides</i>	18
<i>L. backi</i>	3
<i>P. damnosus</i>	5
<i>P. inopinatus</i>	1

Table III. Evaluation of beer-spoilage ability and detection of genetic markers.

Strains	pH of beers ¹		
	pH 4.2	pH 4.6	pH 5.0
<i>P. inopinatus</i> LA20	–	–	+ (8)
<i>L. backi</i> LA21	+ (50)	+ (30)	+ (5)
<i>L. backi</i> LA22	+ (35)	+ (12)	+ (5)
<i>L. backi</i> LA23	+ (38)	+ (12)	+ (5)

¹+: Positive growth, –: Negative growth. The time required for growth in beer is indicated in parenthesis (days).

Table IV. Identification of isolates and detection of genetic markers.

Strains	Detection of genetic markers ¹	
	<i>horA</i>	<i>horC</i>
<i>P. inopinatus</i> LA20	+	–
<i>L. backi</i> LA21	–	+
<i>L. backi</i> LA22	+	+
<i>L. backi</i> LA23	+	+

¹+: The presence of genetic markers is detected. –: The presence of genetic markers is undetectable.

Table V. Evaluation of *horA*- and *horC*-specific methods for detecting strains belonging to the established beer spoilage species.

Species	Total number of strains	Number of <i>horA</i> -positive strains	Number of <i>horC</i> -positive strains
<i>L. brevis</i>	6	6	6
<i>L. lindneri</i>	5	3	5
<i>L. paracollinoides</i>	18	18	18
<i>P. damnosus</i>	5	5	5

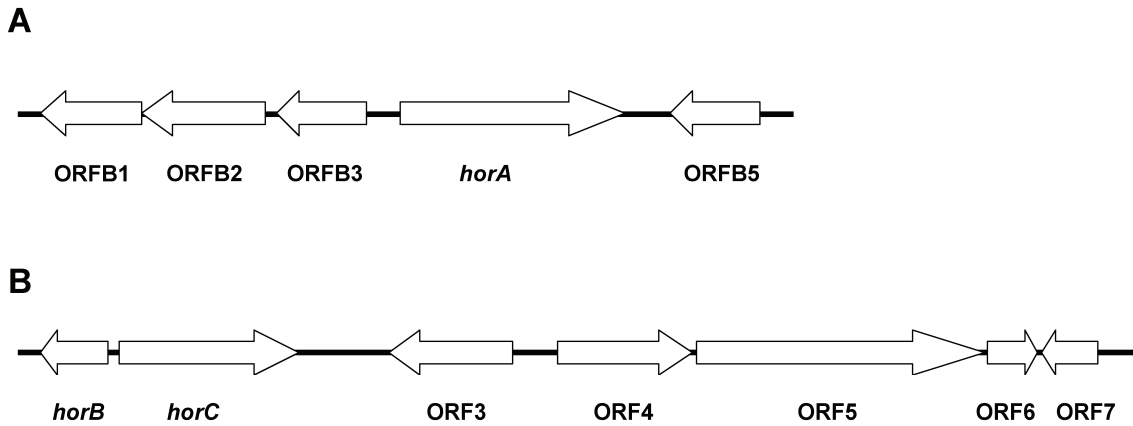


Fig. 1. ORF structures of the conserved DNA regions. A: *horA* and flanking regions (ca. 5.6 kb); B: *horC* and flanking regions (ca. 8.2 kb)

Table VI. Nucleotide sequence identities of *horA*- and *horC*-containing regions found in *P. inopinatus* LA20 and *L. backi* LA21 and LA22¹.

Region	Strains	Length (bp)	Whole region	ORFB1	ORFB2	ORFB3	<i>horA</i>	ORFB5			
<i>horA</i>	<i>P. inopinatus</i> LA20	5585	98.9%	98.7%	98.5%	99.2%	99.5%	98.9%			
<i>horA</i>	<i>L. backi</i> LA22	5581	98.9%	98.7%	98.5%	99.2%	99.5%	98.9%			
									<i>horB</i>	<i>horC</i>	ORF3
<i>horC</i>	<i>L. backi</i> LA21	8228	99.4%	99.7%	96.9%	99.8%	100%	99.8%	100%	100%	100%
<i>horC</i>	<i>L. backi</i> LA22	8230	99.4%	99.7%	96.9%	99.8%	100%	99.8%	100%	100%	100%

¹ Approximately 5.6 kb regions containing the *horA* homologs and 8.2 kb regions containing the *horC* homologs were sequenced and compared with the corresponding regions of *L. brevis* ABBC45 (DDBJ accession no. AB167897 and AB118106). The ORF designations were described previously^{23,24} and the nucleotide sequence identities with the corresponding regions of *L. brevis* ABBC45 are expressed as the percentage of the number of matched nucleotides in the aligned sequences. The *horA*-containing regions found in LA20 and LA22 and the *horC*-containing regions in LA21 and LA22 have been deposited in DDBJ under accession numbers AB279601, AB279602, AB279603 and AB279604, respectively.

lates identified as the established beer-spoilage species were carried out (Table V). Although the *horA* gene was not detectable in the two *L. lindneri* strains, all of the other strains were determined as *horA*-positive strains. On the other hand, all of the 34 beer-spoilage strains were found to possess *horC*. These results reconfirmed the usefulness of the species-independent genetic markers, *horA* and *horC*, for determining the beer-spoilage ability of established species.

Analysis of the *horA*-containing regions and the *horC*-containing regions

The *horA* and *horC*-specific methods have been supported by the horizontal gene transfer hypothesis of these genes^{17–19,22–25}. Although this hypothesis has not yet been demonstrated experimentally, the *horA* and *horC* genes have been speculated to transfer horizontally to nonspoilage LAB as part of composite transposons or plasmids, leading to the emergence of beer-spoilage LAB. Therefore, the potential applications of these genetic markers have been suggested to the comprehensive species-independent identification of beer-spoilage LAB strains. The horizontal transfer hypothesis of hop-resistance genes has been based on the sequence identities of the *horA* and *horC*-carrying DNA regions found in *L. brevis*, *L. lindneri*, *L. paracollinoides* and *P. damnosus*^{17,22–24}. Therefore we attempted to characterize the *horA* and *horC*-containing DNA regions in *P. inopinatus* and *L. backi*. In this study, *P. inopinatus* LA20, *L. backi* LA21 and *L. backi*

LA22 were chosen as representatives for *horA*-positive, *horC*-positive, and *horA*- and *horC*-positive strains, respectively. The sequenced *horA*- and *horC*-containing regions were compared with those of *L. brevis* ABBC45, a strain in which *horA* and *horC* genes have been originally identified^{14,17} (Fig. 1).

As a result, the analyzed 5.6 kb sequences of the *horA* and its flanking regions of *P. inopinatus* LA20 and *L. backi* LA22 showed approximately 99% identity with the corresponding DNA region of *L. brevis* ABBC45 (DDBJ accession no. AB167897) (Table VI). The ORF comparisons with those of *L. brevis* ABBC45 also revealed a similar level of identities. These results indicate that these genes are extraordinarily homologous. Notably, the 5.6 kb *horA*-containing regions found in *P. inopinatus* LA20 and *L. backi* LA22 are completely identical with each other, despite the distinct genus status of these strains. Together with the fact that these strains were isolated from the identical sampling site, the horizontal transfer of *horA*-carrying regions was suggested to have occurred in this particular brewery environment.

As was the case with the *horA* homologs and its flanking regions, the sequences of the *horC* homologs and its flanking regions of *L. backi* LA21 and *L. backi* LA22 showed about 99% identity with that of *L. brevis* ABBC45 (DDBJ accession no. AB118106) (Table VI). Therefore, it was speculated that the strains belonging to the newly assigned species, *L. backi*, also acquired the *horC* homolog and its flanking regions through horizontal gene transfer.

In this study, however, the identity of the *horC* homolog was somewhat low due to the lack of a 27 bp region of *L. backi* LA21 and LA22. The remaining regions of *horC* showed approximately 99% identity with that of *L. brevis* ABBC45. The identical 27 bp sequence was also found missing in the *horC* homologs of beer-spoilage *L. lindneri* DSM 20692 and *L. paracollinoides* JCM 11969^T. On the other hand, the *horC* homolog found in *P. damnosus* ABBC478 did not indicate such characteristics, and showed approximately 99% identity with that of *L. brevis* ABBC45. In fact, immediately after the missing 27 bp sequences, the identical 27 bp sequences were found in *L. backi* LA21 and LA22, suggesting this DNA region contains two 27 bp tandem repeat sequences in *L. brevis* ABBC45 and *P. damnosus* ABBC478. These insights further suggest that a duplication/deletion of the 27 bp sequence in the *horC* gene occurred in some LAB strains and that the *horC* and its homologs can be divided into two groups based on this particular region. Although it is unknown how such a duplication/deletion occurred, more detailed study on the sequence of the *horC* homologs among the other strains would provide interesting insights into the distribution and the origin of the *horC* gene.

Evaluation of *horA* and *horC* as species-independent genetic markers

We isolated *P. inopinatus* and *L. backi*, in addition to the well-established beer-spoilage *L. brevis*, *L. lindneri*, *L. paracollinoides* and *P. damnosus*, as candidates of beer-spoilage LAB from brewery environments and recycled containers. All isolated strains were shown to possess *horA* and/or *horC* genes, indicating that the use of hop resistance genes is useful for detecting beer-spoilage strains, including the previously uncharacterized LAB species (Tables IV, V). Furthermore it should be noted that *L. paracollinoides*, a newly proposed species in 2004, was the most frequently detected species in this study¹⁶. Until this study, we considered *L. paracollinoides* as a relatively rare beer-spoilage LAB species. In addition, three strains of *L. backi* were detected despite the fact that this is a new species just recognized in 2006. Some other reports have indicated the presence of previously uncharacterized beer-spoilage species^{6,9,10}. Judging from these insights, the *horA*- and *horC*-specific methods are considered to complement the conventional species-specific detection methods to detect beer-spoilage LAB comprehensively.

CONCLUSIONS

Beer-spoilage LAB were isolated from brewery environments and recycled containers. As a result, *P. inopinatus* and *L. backi* were isolated in addition to the well-established beer-spoilage LAB species, *L. brevis*, *L. lindneri*, *L. paracollinoides* and *P. damnosus*. Since at least one of the *horA* and *horC* genes was detected from all of the isolates, the *horA*- and *horC*-specific methods were demonstrated to be applicable to a wide variety of beer spoilage species of LAB. The *horA* and *horC* homologs found in *P. inopinatus* and *L. backi* showed extremely high identity with those of previously characterized strains. Therefore, it was presumed that the hop resistance genes and their flanking regions of *P. inopinatus* and *L. backi*

have been acquired through horizontal gene transfer, although this hypothesis remains to be tested with more direct experiments. In conclusion, the *horA* and *horC* genes are considered to be useful as species-independent genetic markers for evaluating the beer-spoilage ability of LAB.

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