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Characterization and detection of the glycosyltransferase (*gtf*) genes in ropy beer-spoilage lactic acid bacteria

Beer has been generally recognized as a microbiologically stable beverage. However, a limited number of lactic acid bacteria (LAB) species are able to grow in beer. Some strains of LAB produce exopolysaccharides (EPS) and form the encapsulation around the cells, which functions as a protective barrier. For instance, EPS-producing *Lactobacillus brevis* shows strong heat tolerance (up to 25 PU) and resist disinfectants used in the breweries, making them one of the most difficult beer-spoilage microorganisms to eradicate. Therefore it is important to distinguish EPS-producing *L. brevis* from other *L. brevis* strains. To achieve this purpose, we characterized the glycosyltransferase genes (*gtf*) in beer-spoilage L. brevis strains with the ropy phenotype. As a result of full sequencing, the *gtf* genes from ropy *L. brevis* exhibit approximately 98.5% identities with the *gtf* gene found in wine-spoilage *Pediococcus parvulus* 2.6, suggesting *gtf* genes have been horizontally acquired among spoilage LAB in alcoholic beverages. Based on this insight, new primers specific to *gtf* were designed. The newly developed *gtf*-specific PCR method was shown to detect not only EPS-producing *L. brevis* but also beer-spoilage *Ped. damnosus* and *Ped. claussenii* with ropy phenotype, suggesting that this PCR method is useful for species-independent identification of EPS-producing beer-spoilage LAB strains. No cross reactions have been observed with the non-ropy LAB strains in various species, as well as other nonspoilage brewery isolates. In conclusion, our newly developed PCR method allows specific and sensitive determination of the EPS-producing ability of beer-spoilage LAB.

Descriptors: lactic acid bacteria, EPS, beer spoilage, ropiness, PCR, horizontal gene transfer

1 Introduction

Beer has been generally recognized as microbiologically stable. However, a limited number of microorganisms are able to grow in beer and cause spoilage incidents [1, 2]. Among beer spoilage microorganisms, lactic acid bacteria (LAB) are known as predominant beer spoilers, and it has been reported that 60–90% of the microbiological incidents are caused by LAB [1, 3]. From incident reports published since 1980, 40–50% of incidents have been caused by one single species, namely *Lactobacillus brevis* [4]. Therefore, *L. brevis* has been recognized as the most frequent and threatening LAB species in the brewing industry. *L. brevis* typically causes visible turbidity when grown in beer, and confers acidity [2]. A few strains of *L. brevis* produce EPS, making the beer ropy and gelatinous [2, 5]. Among the beer-spoilage *L. brevis* strains, those with ropy phenotype are reported as particularly dangerous [2]. The ropy phenotype is characterized by the production of EPS (extracellular polysaccharides) and the concomitant formation of the encapsulation around the cell. EPS act as a protective barrier against various environmental stresses and promote biofilm formation [2, 3, 6]. Therefore *L. brevis* with ropy phenotype has been reported to show high resistance against heat and sanitizers. As for the heat resistance, it has been reported that ropy *L. brevis* may tolerate up to 25 pasteurization units [2]. Moreover they proliferate in fermentation tanks, as well as in gaskets, packing and valves, which are notoriously difficult to control by the cleaning in place (CIP) process [2]. Accordingly, they are one of the most difficult beer spoilers to eradicate. But this phenotype is relatively rare among *L. brevis* and EPS-producing gene in ropy beer-spoilage *L. brevis* has been uncharacterized. Therefore it is difficult to discriminate ropy *L. brevis* from ordinary *L. brevis* using a rapid molecular microbiological method.

In this study, we screened ropy beer-spoilage *L. brevis* strains and characterized the EPS-producing gene. Based on the DNA information obtained, we tried to develop a rapid discriminatory method for beer-spoilage LAB strains with ropy phenotype.

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2 Materials and methods

2.1 Bacterial strains and growth conditions

Bacterial strains used in this study were obtained from the National Institute of Technology Evaluation Biological Resource Centre (NBRC), the Japan Collection of Microorganisms (JCM), the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSM), and our original bacterial collections (ABBC, HC and AGYC).

Beer-spoilage LAB were grown anaerobically at 25 °C in de Man, Rogosa and Sharpe (MRS) agar (Merck, Darmstadt, Germany) or advanced beer-spoiler detection (ABD) medium [7]. Strains of non-spoilage environmental isolates were grown anaerobically at 30 °C in thioglycolate (TGC) agar (Nissui Pharmaceutical, Tokyo, Japan).

2.2 Screening of ropy beer-spoilage strains of LAB

For screening ropy strains from the culture collections of beer-spoilage LAB, the roping ability test was conducted [8]. The roping ability of the colonies was based on visual observation by touching them with an inoculation loop and assessing the occurrence of ropy filaments. Moreover, ropy character in the presence of brewery yeast was tested in MRS broth or degassed commercial pilsner-type beers (pH 5.0) to detect an increased level of the viscosity and ropy filaments.

2.3 Ribotyping

The ribotypes of each ropy *L. brevis* strain were obtained with *Eco*RI as a restriction enzyme using a RiboPrinter (Qualicon, Inc., Wilmington, USA) in accordance with the manufactures instructions [9–11].

2.4 Genomic DNA extraction

A single colony of each bacterial strains grown on the agar media was selected. Genomic DNA was extracted using the Prepman Ultra Reagent (Life Technologies Corporation, Carlsbad, USA), according to the manufacturer's instructions.

2.5 Analysis and characterization of the *gtf* genes

The *gtf* gene was amplified by degenerate PCR using the primers as described by Werning et al. [8]. The less stringent PCR conditions were adopted as follows. The amplification was carried out with an initial denaturation for 2.5 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 15 s, annealing at 48 °C for 30 s, and primer extension at 72 °C for 180 s, and a final extension at 72 °C for 5 min. PerfectShot Ex Taq (TaKaRa Bio Inc., Otsu, Japan) was used for PCR in this experiment.

For TA cloning, pCR2.1-TOPO vector (Invitrogen, Carlsbad, USA) was used as a vector and *Escherichia coli* TOP 10 (Invitrogen, Carlsbad, USA) was used as competent cells. Plasmids were extracted using the Prepman Ultra Reagent (Life Technologies Corporation, Carlsbad, USA) and, when better quality of plasmid extraction was needed, the Plasmid Mini Kit (Qiagen, Hilden, Germany). Sequencing was performed using the 3130xL GeneticAnalyzer (Life Technologies Corporation, Carlsbad, USA).

2.6 The *gtf*-specific primer design

For specific detection of the ropy LAB strains, primers GTS-BF (5'-CGTTTCCTGTTAATATGACAGC-3') and GTSBR (5'-AAGACCCATATAACAACATTG-3') were designed. The primer sequences were based on full sequencing data of the *gtf* gene of beer-spoilage *L. brevis*.

2.7 PCR and agarose gel electrophoresis

PCR mixture contained 25 µL of PerfectShot Ex Taq (TaKaRa Bio Inc., Otsu, Japan), 0.5 µL of each primer (100 µM), 5 µL of DNA extract. The final volume was brought to 50 µL with sterile distilled water. PCR was performed using the GeneAmp PCR system (model 9700, Life Technologies Corporation). Amplification was carried out with an initial denaturation for 2.5 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 15 s, and primer extension at 72 °C for 30 s, and a final extension at 72 °C for 3 min. A 5 µL portion of the PCR product was applied to 2.0 % (w/v) agarose gel electrophoresis in TAE buffer (pH 8.3) and visualized with SYBR Green® (Invitrogen, Carlsbad, USA). The 100 bp DNA ladder (TaKaRa Bio, Otsu, Japan) was used as the molecular size marker.

2.8 Evaluation of specificity and sensitivity

The specificity of our developed *gtf*-specific PCR method for ropy LAB strains was evaluated using 45 strains (Table 1, Table 2 and Table 3). To evaluate the sensitivity, the detection limit was examined by PCR tests with the DNA extracted from serially 10-fold diluted cells of target strains. In addition, the detection limit was also determined in the presence of brewery yeast in a similar manner, except that the concentration of yeast remains 10^7 cells/ ml.

Table 1 Evaluation of specificity for *Lactobacillus* strains

Species	Strain No.	Extracellular polysaccharide formation	Reactivity
Lactobacillus brevis	ABBC42	+	+
Lactobacillus brevis	ABBC633	+	+
Lactobacillus brevis	HC653	+	+
Lactobacillus brevis	ABBC45	–	–
Lactobacillus brevis	ABBC46	–	–
Lactobacillus brevis	ABBC100	–	–
Lactobacillus brevis	ABBC104	–	–
Lactobacillus brevis	JCM1059^T	–	–
Lactobacillus brevis	ABBC400	–	–
Lactobacillus paracollinoides	DSM 11969^T	–	–
Lactobacillus paracollinoides	ABBC90	–	–
Lactobacillus paracollinoides	ABBC516	–	–
Lactobacillus lindneri	DSM 20690^T	–	–
Lactobacillus lindneri	DSM 20691	–	–
Lactobacillus lindneri	DSM 20692	–	–
Lactobacillus casei	ABBC72	–	–
Lactobacillus casei	ABBC96	–	–
Lactobacillus plantarum	ABBC55	–	–
Lactobacillus plantarum	ABBC80	–	–
Lactobacillus plantarum	JCM 1149^T	–	–
Lactobacillus coryniformis	JCM 1164^T	–	–
Lactobacillus backi	DSM 18080^T	–	–

Table 2 **Evaluation of specificity for other non-spoilers**

Species	Strain no.	Extracellular polysaccharide formation	Reactivity
Lactobacillus brevis	ABBC42	+	+
Lactobacillus brevis	ABBC633	+	+
Lactobacillus brevis	HC653	+	+
Serratia marcescens	HC367	–	–
Citrobacter freundii	HC417	–	–
Enterobacter cloacae	HC432	–	–
Staphylococcus warneri	HC437	–	–
Pantoea agglomerans	HC453	–	–
Paenibacillus jamilae	HC466	–	–
Staphylococcus epidermidis	HC475	–	–
Clostridium beijerinckii	HC491	–	–
Klebsiella oxytoca	HC534	–	–
Lactococcus lactis	HC560	–	–
Bacillus thuringiensis	HC384	–	–
Paenibacillus validus	HC699	–	–
Saccharomyces cerevisiae	AGYC192	–	–

Table 3 **Evaluation of specificity for *Pediococcus* strains**

Species	Strain No.	Extracellular polysaccharide formation	Reactivity
Pediococcus damnosus	ABBC500	+	+
Pediococcus claussenii	DSM 14800	+	+
Pediococcus damnosus	NBRC3889	–	–
Pediococcus damnosus	NBRC3896	–	–
Pediococcus damnosus	JCM 5886	–	–
Pediococcus damnosus	ABBC2	–	–
Pediococcus damnosus	ABBC15	–	–
Pediococcus damnosus	ABBC16	–	–
Pediococcus damnosus	ABBC17	–	–
Pediococcus damnosus	ABBC18	–	–

2.9 Dection of the *horA* and *horC* genes

The strains were subjected to the *horA*- or *horC*- specific PCR as described previously [12].

3 Results and discussion

3.1 Screening of ropy beer-spoilage *L. brevis* strains

The screening test for ropy phenotype was carried out to identify ropy beer-spoilage *L. brevis* strains from our culture collection. A total of 50 strains belonging to beer-spoilage *L. brevis* were examined by this test. Consequently three strains with ropy phenotype were identified. These strains induced viscosity and formed long ropy filaments in liquid media. Sometimes the presence of brewing yeast in liquid media is necessary to observe the ropy phenotype, suggesting the interaction with brewing yeast enhance the roping ability of beer-spoilage *L. brevis*. Therefore the roping ability test condition should be studied more extensively in future investigation for more rapid and efficient identification of ropy strains. The ribotyping of these strains revealed that they were not identical on the strain level (data not shown).

3.2 Characterization of the glycosyltransferase genes

To characterize EPS-producing gene in beer-spoilage *L. brevis,* previous studies were examined for reference. Two genes, *gtf* and *dps*, were reported as responsible for EPS production in LAB, and the presence or absence of these genes was found to be highly correlated with ropy phenotype in wine-spoilage LAB [8, 13, 14]. They have been reported to be located on plasmids [8, 14]. The *gtf* gene has been proposed to have a glycosyltransferase activity. Glycosyltransferases are known as an enzyme that assembles monosaccharide moieties into linear and branched glycan chains and macromolecules including cell wall components and extracellular polysaccharides. On the other hand, the *dps* gene has been proposed as a putative glucan synthase gene. After preliminary tests following previous studies, it was suggested that the presence of *gtf* gene was correlated with the ropy phenotype of beer-spoilage *L. brevis* (data not shown).

To further analyze the EPS-producing gene of beer-spoilage *L. brevis*, the cloning of the entire *gtf* gene (1.7 kb) was attempted by the degenerate PCR, on the basis of the DNA sequences of *gtf* described by the previous studies of wine-spoilage LAB [8, 13]. This is because the DNA information from wine-spoilage LAB

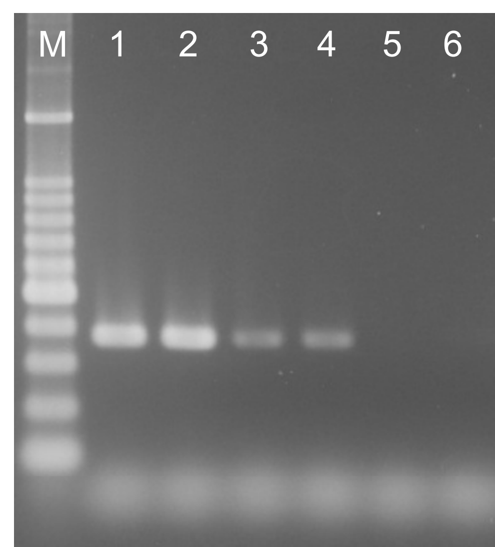

Fig. 1 **Evaluation of sensitivity in the presence of brewery yeast. The cell suspensions of ropy *L. brevis* (ABBC42) were serially diluted and added to brewery yeast to give 1×10$^{2–3}$ cells/ml *L. brevis* suspensions in the presence of 3×10^{7} cells/ml brewery yeast. After the centrifugation of 1ml suspensions and the subsequent DNA extraction, the detection limits were determined (N = 2); ropy *L. brevis* (1×10^{3} cells/ml) with brewery yeast (lanes 1–2), ropy *L. brevis* (1×10^{2} cells/ml) with brewery yeast (lanes 3–4) and only brewery yeast (lanes 5–6). Lane M indicates the 100 bp DNA ladder**

may help the cloning of *gtf* from beer-spoilage *L. brevis*, even if the DNA sequences of *gtf* genes are somewhat different from each other. As a consequence, PCR bands were observed at the approximate position of 1.7 kb, indicating putative *gtf* genes were successfully cloned from *L. brevis*. The full sequencing of the PCR products showed that the putative *gtf* genes are > 99.5 % identical among three beer-spoilage *L. brevis* strains investigated in this study.

3.3 Development of the *gtf*-specific PCR method for ropy beer-spoilage *L. brevis*

To develop the *gtf*-specific PCR method, *gtf*-specific primers were designed based on full sequencing data of *L. brevis*. The specificity of the designed primers was confirmed using 22 *Lactobacillus* strains, such as those belonging to *L. brevis*, *L. paracollinoides*, *L. lindneri* (Table 1). Among the strains used in this study, all the three ropy strains were positively identified and no cross reactions were observed for the non-ropy *Lactobacillus* strains. In addition, the specificity for 13 other non-spoilers that do not belong to lactic acid bacteria, as well as the specificity for brewery yeast, was evaluated (Table 2). As a result, no cross reactions were observed in these non-LAB strains. It was thus demonstrated that our developed *gtf*-specific PCR method is able to distinguish ropy *L. brevis* from non-ropy LAB strains in various species, as well as other non-spoilage brewery isolates. Furthermore, the specificity and sensitivity for ropy *L. brevis* in the presence of brewery yeast was evaluated. This is because ropy *L. brevis* often threatens the quality of semi-products in fermentation and maturation processes, where brewery yeast is present. As figure 1 indicates, our developed *gtf*-specific PCR was shown to detect ropy *L. brevis* strains even if brewery yeast coexisted. It was also found that the detection limit was ca 1×10^2 cells/ml, which was considered sufficient for practical applications to the brewery QC tests.

3.4 Specificity of our developed *gtf*-specific PCR method for ropy beer-spoilage *Pediococcus* strains and characterization of EPS-producing gene

It was demonstrated that our developed *gtf*-specific PCR method is able to distinguish ropy *L. brevis* from ordinary *L. brevis* rapidly. However, some strains of other beer-spoilage LAB species also produce EPS and potentially exhibit enhanced tolerance against heat and sanitizers [2, 15]. Under these backgrounds, it is desirable to distinguish ropy strains from non-ropy counterparts within the identical LAB species. To determine whether our method is applicable to other ropy LAB species, ropy beer-spoilage *Pediococcus* strains were screened by the roping ability test from our culture collection as described before. Consequently, two ropy *Pediococcus* strains, belonging to *Pediococcus damnosus* and *Pediococcus claussenii*, were found. The specificity for 10 *Pediococcus* strains including two ropy strains was evaluated (Table 3). As a result, it was demonstrated that our developed *gtf*-specific primers for ropy *L. brevis* were shown to detect ropy *Ped. damnosus* and *Ped. claussenii* with no cross reactions. Since the primers specific to ropy *L. brevis* were reacted positively with ropy *Pediococcu*s strains, the full sequencing of the *gtf* genes in ropy *Pediococcu*s strains and the subsequent homology study were conducted. The putative *gtf* genes in *Pediococcu*s were cloned by a degenerate PCR approach in a manner similar to the case with *L. brevis*, and PCR bands were successfully obtained at the approximate position of 1.7 kb. As a result of full sequencing, the putative *gtf* genes from ropy *Pediococcus* exhibit approximately 98.5 % identities with the *gtf* gene found in ropy beer-spoilage *L. brevis*. These findings suggest that *gtf* genes have been horizontally acquired across the genera in beer-spoilage LAB. The exact mechanisms underlying the horizontal gene transfer of *gtf* are unknown, but unusually high nucleotide sequence identities strongly suggest that the horizontal transfer of *gtf* genes is involved in the emergence of ropy beer-spoilage LAB in the brewing environments [16–19]. To further discuss this point, the horizontal gene transfers are quite common in the world of microbiology. The acquisitions of resistance against various environmental stresses through the horizontal gene transfers are often observed with bacteria living under harsh conditions. This is also true for those surviving in the brewing environments. For instance, hop-resistance genes, *horA* and *horC*, appear to have been acquired through horizontal gene transfer. The *horA* and *horC* genes were reported as excellent species-independent genetic markers for determining the beer spoilage ability of detected LAB [12, 18, 20]. Interestingly, the *horA* or *horC* genes were also detected in ropy beer-spoilage *L. brevis, Ped. damnosus* and *Ped. claussenii* (data not shown). This implies that the identification of ropy beer-spoilage LAB strains is possible in a species-independent manner. Therefore, after DNA extraction, the simultaneous identification methods for the *gtf* and hop-resistance genes allow more accurate assessment of harmfulness in detected LAB strains.

3.5 Implication of the horizontal gene transfer of *gtf*

To further discuss horizontal gene transfer of the *gtf* genes and its implications, the comparative studies between beer-spoilage LAB and wine-spoilage LAB were carried out. As a result of homology analysis, the *gtf* genes from beer-spoilage LAB exhibit approximately 98.5 % identities with the *gtf* genes found in wine-spoilage LAB, *Ped. parvulus* 2.6 [8]. From this finding, it is suggested that ropy LAB in alcoholic beverage emerged through horizontal gene transfer of the *gtf* gene. As described earlier, EPS act as a protective barrier and also promote biofilm formation. Biofilm is the aggregate of various microorganisms. Both of these abilities make it possible for LAB to tolerate various stress factors, including ethanol, and enhance the growth ability in alcoholic beverages. Incidentally EPS-producing LAB are more likely to survive the suboptimal CIP process due to the elevated resistance to heat and sanitizers. Thus this is most likely a survival strategy for spoilage LAB living under harsh environments including beer and wine. On the basis of this hypothesis, it is plausible that the *gtf* genes will further spread among spoilage LAB in alcoholic beverages, leading to the emergence of new EPS-producing LAB species. Indeed, *L. rossiae*, which has been recently reported as a new beer-spoilage LAB species, exhibits ropy phenotype [21]. This genetic marker, presumably spreading through horizontal gene transfer, was found to be applicable to ropy LAB species other than *L. brevis*. Therefore, our developed method will be useful for the detection and identification of as yet uncharacterized LAB species with ropy phenotype. This is the strength of species-independent method.

4 Conclusions

In this study, several ropy strains of beer-spoilage LAB were screened, and the *gtf* genes in these LAB strains were examined. As a consequence, the *gtf* genes found in *L. brevis, Ped. damnosus* and *Ped. claussenii* were shown to exhibit approximately 98–99 % identities with the wine-spoilage LAB strain, *Ped. parvulus* 2.6. Therefore it was suggested *gtf* genes have been horizontally acquired among spoilage LAB in alcoholic beverages, although this hypothesis remains to be tested with more direct experiments. Our newly developed PCR method allows specific and sensitive determination of the EPS-producing ability of beer-spoilage LAB. This method is potentially useful for the detection and identification of ropy spoilage LAB in the alcoholic beverage industry.

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