Multiplex PCR for Putative *Lactobacillus* and *Pediococcus* Beer-Spoilage Genes and Ability of Gene Presence to Predict Growth in Beer

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ABSTRACT

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Current methods of detecting *Lactobacillus* and *Pediococcus* isolates found in beer are time-consuming and do not differentiate between benign bacteria and those bacteria capable of growing in beer. Four putative beer spoilage-associated genes (*hitA*, *horA*, *horC*, and ORF5) have been suggested but have never been statistically correlated with the ability to grow in beer. We have designed a multiplex PCR to detect these putative spoilage-associated genes that includes the 16S rRNA gene as an internal control. In all, 133 *Lactobacillus* and *Pediococcus* isolates were screened using this multiplex PCR, and the results were compared with the ability of the isolates to grow in beer. We found that only *horA* was predictive of an organism's ability to grow in beer. Although *hitA* and *horC* were not predictive of an organism's ability to grow in beer, the presence of *hitA*, *horC*, or both in addition to *horA* was indicative of the ability to grow rapidly in beer. Statistical modeling based on our data indicates that assaying for the presence of *horA* is highly accurate in predicting the beer-spoilage potential of *Lactobacillus* and *Pediococcus* isolates. This multiplex PCR substantially reduces the time required to determine whether a *Lactobacillus* or *Pediococcus* isolate has a high probability of causing beer spoilage.

Keywords: Beer-spoilage genes, *horA*, *Lactobacillus*, Multiplex PCR, *Pediococcus*

RESUMEN

Los métodos actuales de detección de aislados de *Lactobacillus* y *Pediococcus* encontrado en la cerveza consumen mucho tiempo y no diferencian entre bacterias benignas y las bacterias capaces de crecer en la cerveza. Cuatro putativo genes asociados con la deterioración de la cerveza (*hitA*, *horA*, *horC*, y ORF5) se han sugerido, pero nunca han sido estadísticamente correlacionadas con la capacidad de crecer en la cerveza. Hemos diseñado un PCR múltiplex para la detección de estos putativo genes asociado con la deterioración de la cerveza que incluye el gen 16S rRNA como un control interno. En total, 133 aislados de *Lactobacillus* y *Pediococcus* fueron seleccionados utilizando este PCR múltiplex, y los resultados se compararon con la capacidad de los aislamientos de crecer en la cerveza. Se encontró que sólo *horA* fue predictivo de la capacidad de un organismo para crecer en la cerveza. Aunque *hitA* y *horC* no fueron predictivos de la capacidad de un organismo para crecer en la cerveza, la presencia de *hitA*, *horC*, o ambos, además con *horA* era indicativa de la capacidad de crecer rápidamente en la cerveza. Modelación estadística basada en nuestros datos indican que analizaron para detectar la presencia de *horA* es sumamente preciso en la predicción de la potencial de los aislamientos de *Lactobacillus* y *Pediococcus* para dañar cerveza. Este PCR múltiplex reduce sustancialmente el tiempo necesario para determinar si una aislado de *Lactobacillus* o *Pediococcus* tiene una alta probabilidad de dañar la cerveza.

Palabras claves: Genes asociados con deterioración de la cerveza, *horA*, *Lactobacillus*, PCR múltiplex, *Pediococcus*

Spoilage of beer by bacteria is a significant problem for the brewing industry. Although most gram-positive bacteria fail to grow in beer due to the presence of hop compounds, the presence of a specific resistance-associated gene or genes is believed to be associated with growth in beer (6,13). The most common beer-spoilers are lactic acid bacteria (LAB), i.e., select isolates within the gram-positive genera *Lactobacillus* and *Pediococcus* (5,6). These beer-spoilage LAB are able to withstand the bitter acid compounds (e.g., *trans*-isohumulone) derived from hop-extract α-acids that act as mobile carrier protonophores, effectively dissipating the cell's trans-membrane pH gradient (12).

Although not all *Lactobacillus* and *Pediococcus* isolates can grow in beer, some isolates of these genera have developed mechanisms that confer resistance to hop compounds (13), thus allowing growth in beer. The ability to grow in beer is not restricted by the boundaries of speciation (2) and, as such, spoilage-specific genetic markers must be identified. Although several genes have been claimed to be involved in hop resistance (1,3,7–11,14–17), isolates used in these studies often have been derived from a single source (i.e., one brewery), and supporting statistical evidence has not been provided. As such, the objective of the present study was to determine whether the presence of putative beer-spoilage genes can be used to predict the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer. To accomplish this goal, the putative beer-spoilage genes chosen as targets for multiplex PCR were *hitA* (3), *horA* (11) *horC*, and ORF5 (1,15).

The *horA* and *hitA* genes code for primary- and secondary-type multidrug transporters, respectively. The *horA* gene has homology to ATP-binding cassette-type multidrug resistance genes, using an ATP-binding transporter to export *trans*-isohumulone, preventing its accumulation in the intracellular space (7–11). It has been suggested that *hitA* is an integral membrane protein that functions as a divalent cation proton motive force transport system, counteracting the activity of *trans*-isohumulone (3). The *horC* and ORF5 genes code for proteins of unknown function with no homology to known proteins. *horC* and ORF5 were selected based on their hypothetical membrane localization, similarity to other membrane proteins, and potential correlation with hop resistance, as suggested previously by Suzuki et al (14,16). Independent studies identified two unique plasmids that both harbored *horC* and ORF5 (1,15), with *horC* corresponding to ORF2 and ORF9, as described by Suzuki et al (15) and Fuji et al (1), respectively, whereas ORF5 corresponds to ORF5 and ORF2, respectively, in the same two articles.

In this paper we describe a multiplex PCR that simultaneously detects these four putative spoilage-associated genes, as well as the 16S rRNA gene used as an internal positive control. This multiplex PCR was used to screen 133 *Lactobacillus* and *Pediococcus* isolates. Statistical analyses were used to delineate the relative roles these four genes play in the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer.

EXPERIMENTAL

Bacteria

The 133 isolates analyzed in this study were from diverse origins and included 83 lactobacilli and 50 pediococci, comprising 22

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known *Lactobacillus* spp., 4 putative new *Lactobacillus* spp., and 7 *Pediococcus* spp. (Table I). Growth of the bacterial isolates in two different kinds of beer involved adaptation of the bacteria to grow in beer using modified MRS medium (de Man, Rogosa, Sharpe medium modified by the omission of Tween 80) supplemented with incremental concentrations of beer (2). The identities of the isolates were confirmed pre- and postgrowth in beer by sequencing of the 16S rRNA gene as previously described (2). The ability of the 133 isolates to grow in beer was reported previously (2), and the results are incorporated into Table I for direct comparison with the results on presence or absence of each of the four putative beer-spoilage genes. Beer 1 was a filter-sterilized 4% (vol/vol) alcohol beer, aver-

TABLE I
Bacterial Strains, Presence of Genes, and Ability to Grow in Beer

Isolate[a]	Origin	PCR Result[b]					Growth[c]	
		hitA	*horA*	*horC*	ORF5	16S	Beer 1	Beer 2
Lactobacillus acetotolerans								
ATCC 43578[T]	Rice vinegar	–	–	–	–	+	–	–
L. acidophilus								
ATCC 521	Unknown	–	–	–	–	+	–	–
ATCC 4356[T]	Human	+	–	–	–	+	–	–
CCC B1209	Brewery	–	–	–	–	+	–	–
L. amylovorus								
ATCC 33198[d]	Hog intestine	–	–	–	–	+	–	–
ATCC 33620[T]	Corn silage	–	–	–	–	+	–	–
Field isolate[e]	Unknown	–	–	–	–	+	–	–
Ingledew I1[f]	Fuel alcohol	+	–	–	–	+	–	–
Ingledew I2	Fuel alcohol	+/+	+/+	–/+	–/–	+/+	+ (2)	+ (3)
T-13[g]	Poultry	–	–	–	–	+	–	–
L. brevis								
ATCC 4006	Unknown	–	–	–	–	+	–	–
ATCC 8007	Kefir grains	–	+(T)[h]	–	–	+	–	–
ATCC 14869[T]	Human feces	–	–	–	–	+	–	–
BSO 31[i]	Brewery	+/+	+/+	+/+	+/+	+/+	+ (5)	+ (5)
CCC 96S1L	Brewery	–/+	+/+	–/+	–/–	+/+	+ (5)	+ (5)
CCC 96S2AL	Brewery	–/+	+/+	–/+	–/–	+/+	+ (5)	+ (5)
CCC B1202	Brewery	+/+	+/+	+/+	+/+	+/+	+ (5)	+ (5)
CCC B1203	Brewery	+/+	+/+	+/+	+/+	+/+	+ (5)	+ (5)
CCC B1204	Brewery	+/+	+/+	+/+	+/+	+/+	+ (5)	+ (5)
CCC B1206	Brewery	+/+	+/+	+/+	+/+	+/+	+ (5)	+ (5)
CCC B1300	Brewery	–/–	+/+	–/–	+/+	+/+	+ (2)	+ (3)
ETS.1	Wine	–	–	–	–	+	–	–
ETS.2	Wine	–	–	–	–	+	–	–
L. casei								
ATCC 334[g]	Cheese	–	–	–	–	+	–	–
ATCC 4913[g]	Unknown	–	–	–	+	+	–	–
ATCC 25598[T]	Milking machine	–/–	–/–	–/–	–/–	+/+	+ (8)	+ (11)
CCC 95G1L	Brewery	+/+	–/–	–/+	–/–	+/+	+ (8)	+ (10)
CCC 95G2L	Brewery	–/–	–/+	–/–	+/+	+/+	+ (5)	+ (5)
CCC B9657	Brewery	–/–	–/+	–/–	–/–	+/+	+ (9)	+ (9)
CCC B1205	Brewery	–/+	–/+	–/+	–/+	+/+	+ (2)	+ (2)
CCC B1241	Brewery	–/–	–/+	–/–	–/–	+/+	+ (27)	–
Ingledew I3	Fuel alcohol	–	–	–	–	+	–	–
Ingledew 18C	Fuel alcohol	–/–	+/+	–/–	–/–	+/+	+ (27)	–
L. delbrueckii								
ATCC 4797	Corn mash	–	–	–	–	+	–	–
ATCC 9649[T]	Sour grain mash	–	–	–	–	+	–	–
ATCC 11842[T]	Bulgarian yogurt	+	–	–	–	+	–	–
ATCC 12315[T]	Cheese	+	–	–	–	+	–	–
CCC 95G3L	Brewery	–	–	–	–	+	–	–

(*continued on next page*)

[a] Isolate identity as determined by Haakensen et al (2), with type strains indicated. ATCC = American Type Culture Collection, Manassas, VA; BSO = Beer Spoilage Organism; CCC = Coors Brewing Company, Golden, CO; DSM = German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany; ETS = ETS Laboratories (T. Arvik), St. Helena, CA; Molson = Molson Breweries of Canada Limited, Montreal, PQ, Canada; R = ropy phenotype; and NR = nonropy phenotype.
[b] For bacterial isolates capable of growing in beer, the presence of genes was recorded as pre- or postgrowth in beer.
[c] + = visible turbidity in beer and growth upon subsequent subculture to 85/15 medium; – = no visible turbidity in beer and not capable of growing upon subculture to 85/15 medium; and S = static, no visible turbidity in beer, but capable of growing upon subculture to 85/15 medium. The number in parentheses following a + indicates the number of days required to attain visible growth in beer.
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[h] *horA* was sequenced, and the gene was found to be truncated by approx. 700 bp.
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aging 9.8 bitterness units (BU) and pH 4.2, whereas beer 2 was a pasteurized 5% (vol/vol) alcohol beer, averaging 11 BU and pH 3.8.

PCR Primers

A region of the bacterial 16S rRNA gene was amplified using the PCR primers 386F (*unpublished*) and 534R (4). The *horA*-specific primers h198F2 and h198R are as previously described (2). When designing PCR primer pairs specific to *hitA*, *horC*, and ORF5, attributes of each primer set were kept relatively similar (e.g., melting temperature and percent G+C content similar to that of the 16S rRNA gene and *horA* primers) to optimize their ability to function under a single set of PCR conditions. Primer pairs were

TABLE I
(*continued from preceding page*)

Isolate[a]	Origin	PCR Result[b]					Growth[c]	
		hitA	***horA***	***horC***	**ORF5**	**16S**	**Beer 1**	**Beer 2**
CCC B1044	Brewery	–	–	–	–	+	–	–
CCC B1240	Brewery	–	–	–	–	+	–	–
CCC B1262	Brewery	–	–	–	–	+	–	–
L. ferintoshensis								
ATCC 11307	Brewery	–	–	–	+	+	–	–
L. fermentum								
ATCC 9338[g]	Unknown	–/–	–/–	–/–	–/–	+/+	+ (9)	+ (11)
ATCC 14931[T]	Fermented beets	–	–	–	–	+	–	–
ATCC 14932[g]	Saliva	–	–	–	–	+	–	–
L. fructivorans								
ATCC 8288[T]	Unknown	–	–	–	–	+	–	–
L. helviticus								
ATCC 15009[T]	Cheese	–	–	–	–	+	–	–
CCC B1186	Brewery	–/–	+/+	–/–	–/–	+/+	+ (6)	+ (S)
L. hilgardii								
ATCC 8290[T]	Wine	–	–	–	–	+	–	–
ATCC 27305	Wine	–/–	–/–	–/–	–/–	+/+	+ (4)	+ (8)
ATCC 27306	Wine	–	–	–	–	+	–	–
L. homohiochii								
ATCC 15434[T]	Spoiled sake	–	–	–	–	+	–	–
L. jensenii								
ATCC 25258[T]	Human	+	+(T)	–	–	+	–	–
L. kefiri								
ATCC 35411[T]	Kefir grains	+	–	–	–	+	–	–
L. kefirgranum								
ATCC 51647[T]	Kefir grains	–	–	–	–	+	–	–
L. kefironofaciens								
ATCC 43761[T]	Kefir grains	–	–	–	–	+	–	–
L. paracollinoides								
ATCC 8291	Brewery	–/–	–/–	–/–	–/–	+/+	+ (7)	+ (11)
L. plantarum								
ATCC 8014	Unknown	–	–	–	–	+	–	–
ATCC 8041	Corn silage	+/+	+/+	+/+	+/+	+/+	+ (2)	+ (3)
ATCC 11305	Brewery	–	–	–	–	+	–	–
ATCC 12706	Cured meat	–	–	–	–	+	–	–
ATCC 14431[g]	Grass silage	–	–	–	+	+	–	–
ATCC 14917[T]	Pickled cabbage	–	–	–	–	+	–	–
BSO 92	Brewery	+/+	+/+	+/+	+/+	+/+	+ (2)	+ (2)
CCC 96M2BL	Brewery	+/+	+/+	+/+	–/–	+/+	+ (12)	+ (12)
CCC B1301	Brewery	–/–	+/+	–/–	–/–	+/+	+ (5)	+ (12)
L. reuteri								
ATCC 19371	Silage	–	–	–	–	+	–	–
ATCC 25744	Plants	–	–	–	–	+	–	–
ATCC 31282	Unknown	–/–	–/–	–/–	–/–	+/+	+ (13)	+ (13)
ATCC 43200	Cucumbers	–	–	–	+	+	–	–
RC-14[g]	Unknown	–	–	–	–	+	–	–
L. rhamnonsus								
ATCC 7469[T]	Unknown	+	–	–	–	+	–	–
ATCC 7469a[g]	Derived from ATCC 7469	–	–	–	–	+	–	–
ATCC 8530[g]	Unknown	–/–	–/–	–/–	–/–	+/+	+ (7)	+ (10)
ATCC 15820	Corn liquor	–	–	–	–	+	–	–
ATCC 21052[g]	Human feces	–	+(T)	–	–	+	–	–
L. sakei								
ATCC 15521[T]	Moto	+	–	–	–	+	–	–
ATCC 15578	Moto	+/+	+/+	–/+	–/–	+/+	+ (6)	+ (7)
L. zeae								
ATCC 393	Cheese	–	–	–	–	+	–	–
Lactobacillus unspeciated								
ATCC 4005	Tomato pulp	–	–	–	–	+	–	–

(*continued on next page*)

designed to produce amplicons with 15–30 bp differences to allow for differentiation upon visualization by 2% agarose gel electrophoresis. Specificity of the primers was confirmed in silico and by sequencing the amplicons of randomly selected isolates. The five primer pairs used in this multiplex PCR are described in Table II.

Multiplex PCR

DNA extractions were performed as previously described (2). Each multiplex PCR contained 2 U of Invitrogen Platinum *Taq* DNA polymerase, 1× PCR buffer (Invitrogen, Burlington, ON, Canada), 1.5 m*M* $MgCl_2$, 0.2 m*M* each of the four deoxynucleotide triphosphates, 0.2 μ*M* each primer (except for the 16S rRNA gene

TABLE I
(*continued from preceding page*)

		PCR Result[b]					Growth[c]	
Isolate[a]	**Origin**	***hitA***	***horA***	***horC***	**ORF5**	**16S**	**Beer 1**	**Beer 2**
ATCC 27054	Apple juice	–	–	–	–	+	–	–
ATCC 27304	Wine must	+/+	+/+	+/+	–/+	+/+	+ (3)	+ (3)
CCC L86	Brewery	–/–	+/+	+/+	+/+	+/+	+ (4)	+ (11)
Pediococcus acidilactici								
ATCC 8042	Brewery	–/–	+/+	–/–	–/–	+/+	+ (8)	–
ATCC 12697	Unknown	–	–	–	–	+	–	–
ATCC 25740	Plant	–	–	–	–	+	–	–
BSO 54	Brewery	–	–	–	–	+	–	–
BSO 77[i]	Brewery	–	–	–	–	+	–	–
Molson B77b	Brewery	–	–	–	–	+	–	–
Pac 1.0[j]	Unknown	–	–	–	–	+	–	–
P. claussenii								
CCC B962A	Brewery	–/–	–/–	+/+	–/–	+/+	+ (9)	+ (10)
CCC B1056R	Brewery	–/–	+/+	–/–	–/–	+/+	+ (14)	+ (14)
CCC B1056NR	Brewery	–/–	+/+	–/–	–/–	+/+	+ (13)	–
CCC B1098R	Brewery	–/–	–/–	–/–	–/–	+/+	+ (7)	+ (7)
CCC B1098NR	Brewery	–/–	+/+	–/–	–/–	+/+	+ (8)	+ (13)
CCC B1099R	Brewery	–/–	–/–	–/–	–/–	+/+	+ (12)	+ (14)
CCC B1099NR	Brewery	–/–	–/–	–/–	–/–	+/+	+ (7)	+ (7)
CCC B1100	Brewery	–/–	–/–	–/+	–/+	+/+	+ (8)	+ (S)
CCC B1208	Brewery	–	–	–	–	+	–	–
CCC B1260R	Brewery	–/–	–/–	–/–	–/–	+/+	+ (8)	+ (13)
CCC B1260NR	Brewery	–/–	–/–	–/–	–/–	+/+	+ (6)	+ (6)
ATCC BAA-344^{T} R	Brewery	–/–	+/+	–/–	–/–	+/+	+ (8)	+ (8)
ATCC BAA-344^{T} NR	Brewery	–/–	+/+	–/–	–/–	+/+	+ (6)	+ (6)
P. damnosus								
ATCC 11308	Brewery	–/–	–/–	–/–	–/–	+/+	+ (27)	–
ATCC 25248	Brewery	–	–	–	–	+	–	–
ATCC 25249	Brewery	–	+(T)	–		+	–	–
ATCC 25249a	Brewery	–	–	–	+	+	–	–
ATCC 29358^{T}	Brewery	–	–	+	+	+	–	–
Molson B48	Brewery	+/+	+/+	+/+	+/+	+/+	+ (7)	+ (7)
Molson 49	Brewery	–/–	+/+	+/–	+/–	+/+	+ (10)	+ (13)
Molson B76	Brewery	+/–	+/+	+/–	+/–	+/+	+ (11)	+ (12)
P. dextrinicus								
ATCC 33087^{T}	Silage	–	–	–	–	+	–	–
P. inopinatus								
ATCC 49902^{T}	Brewery	–	–	–	–	+	–	–
P. parvulus								
ATCC 43013	Wine	–/–	–/–	–/–	–/–	+/+	+ (13)	+ (13)
ETS.3	Wine	–	–	–	–	+	–	–
ETS.4	Wine	–	–	–	–	+	–	–
ETS.5	Wine	–	–	–	–	+	–	–
ETS.6	Wine	–	–	–	–	+	–	–
ETS.7	Wine	–	–	–	–	+	–	–
ETS.8	Wine	–	–	–	–	+	–	–
ETS.9	Wine	–	–	–	–	+	–	–
ETS.11	Wine	–	–	–	–	+	–	–
ETS.12	Wine	–	–	–	–	+	–	–
ETS.13	Wine	–	–	–	–	+	–	–
ETS.14	Wine	–	–	–	–	+	–	–
Spain 2.6R[k]	Cider	–	–	–	–	+	–	–
Spain 2.6NR[k]	Cider	–	–	–	–	+	–	–
P. pentosaceus								
ATCC 8081	Milk	–	–	–	–	+	–	–
ATCC 10791	Cucumber	+	–	–	–	+	–	–
ATCC 11309	Unknown	–	–	–	–	+	–	–
ATCC 29723	Horse urine	–	–	–	+	+	–	–
ATCC 33314	Sake mash	–	–	–	–	+	–	–
ATCC 33316^{T}	Brewery	–	–	–	–	+	–	–

primers, which were at 0.1 µ*M*), and 1 µL of bacterial DNA. Water was added to bring the total volume to 25 µL. The PCR program consisted of an initial denaturation step of 5 min at 95°C, followed by 35 cycles of 94°C for 45 sec, 52°C for 45 sec, and 72°C for 50 sec and a final extension step of 72°C for 5 min. Amplicons were detected by electrophoresis in 2.0% agarose gels containing ethidium bromide.

Statistics

Statistical analysis of data was performed using SPSS for Windows (version 12.0, SPSS Inc., Munich). For both binary logistic regression and *t* test for independent samples, the confidence interval and level of significance were set at 95% and $P = 0.05$, respectively. Binary logistic regression models were calculated for *Lactobacillus, Pediococcus,* and *Lactobacillus* and *Pediococcus* isolates combined, with the ability to grow in beer 1 and beer 2 as outcome variables. For binary logistic regression models, multiplex PCR results for the *hitA, horA, horC*, and ORF5 genes were included as covariates (0 = PCR negative and 1 = PCR positive). Binary logistic regression was performed using the backward stepwise (likelihood ratio) method and forward stepwise method, with the same results produced (data not shown). All binary logistic regression likelihood ratio χ^2 values had $P < 0.00025$.

RESULTS AND DISCUSSION

Gene Detection

The multiplex PCR protocol described here not only detects the presence of *hitA*, *horA*, *horC*, and ORF5 but also provides an internal control indicating the presence of bacterial DNA in the test sample through incorporation of primers that amplify a portion of the 16S rRNA gene. Because the PCR amplicon for each gene has a different length, a bacterial DNA sample should always have a minimum of one band (i.e., the 16S rRNA control amplicon) and may include as many as four additional bands when the PCR reaction is analyzed by agarose gel electrophoresis (Fig. 1). Table I provides the multiplex PCR results for each of the isolates analyzed.

To ensure that the h198F2/h198R primers were successful in detecting the *horA* gene whenever present, the 16 *horA*-negative, growth-positive isolates were subjected to PCR by additional PCR primer set combinations. All PCR primers were designed to be specific to *horA*, and the 16 *horA*-negative, growth-positive isolates were negative for all combinations of primer sets to *horA* (data not shown). The additional primer sets used included the previously described h297F/R primer set (2) and a primer set designed to a multiple sequence alignment of all GenBank *horA* sequences to span the full length of the *horA* gene (forward primer horAFullF located 110- to 96-bp upstream of the start codon and reverse primer horAFullR located at the 3′ end of the *horA* gene at bases 1,745–1,728). The horAFullF/R primers also were used in respective combinations with the h198F2/R primers to produce shorter, overlapping amplicons for DNA sequencing. The *horA* gene of each of the four *horA*-positive, growth-negative isolates (*L. brevis* ATCC 8007, *L. jensenii* ATCC 25258^T, *L. rhamnosus* ATCC 21052, and *P. damnosus* ATCC 25249; GenBank accession nos. EU223373, EU223372, EU223374, and EU223375, respectively) were sequenced and were found to be truncated by approx. 700 bp corresponding to the 3′ end of *horA* sequences deposited in GenBank. Despite this truncation, the sequenced regions of *horA* from these isolates were 97.9–99.7% identical compared with deposited *horA* sequences, and all coded for phenylalanine instead of serine in amino acid position 75.

Interestingly, four isolates were initially *horA* PCR negative prior to growth in beer but, after subsequent passage in beer, were positive for *horA*. This same phenomenon was observed three times for each *hitA* and ORF5, and six times for *horC*. Conversely, one isolate (*P. damnosus* Molson B76) was PCR positive for all four genes prior to growth in beer but, after passage in beer, was positive only for *horA* (Table I), and a second isolate (*P. damnosus* Molson B49) was PCR positive for *horA*, *horC*, and ORF5 prior to growth in beer but was only *horA* PCR positive after growth in beer. These findings suggest that previous studies may have incorrectly identified some bacterial isolates as being positive or negative for genes of interest because they did not screen for their presence after growth in beer (or a similarly appropriate adaptation step). Because the four genes of interest are plasmid localized, continuous passage of the bacteria in laboratory media can lead to gene loss. Therefore, it is important to screen for genes of interest after a small subset of bacteria harboring advantageous plasmids has been preferentially amplified by growth in a beer environment.

It has been suggested previously that *horC* and ORF5 are jointly plasmid localized (1,15). However, our data show that the presence of *horC* and ORF5 did not correlate (Table III). Not only were there numerous instances in which *horC* and ORF5 occurred independently of one another, but there were five isolates that demonstrated preferential selection for *horC* (i.e., *horC* PCR-negative pregrowth in beer or *horC* PCR-positive postgrowth in beer) while failing to maintain the presence of ORF5 (i.e., ORF5 PCR-positive pregrowth in beer or ORF5 PCR-negative postgrowth in beer) (Table I). This finding suggests that, although *horC* and ORF5 sometimes may be located on the same plasmid, these two genes can occur independently.

The widespread lateral gene transfer that can occur among LAB is demonstrated by the fact that a small or extremely small subset of bacteria harboring advantageous plasmids can be preferentially amplified by growth in a beer environment, as well as the additional

TABLE II
Locations and Sequences of PCR Primers

Target	Amplicon (bp)	PCR Primer[a]	Sequence
horA	210	h198F2	AAATCTTAACCCTGCCGG
		h198R	GCGGAACGGCGATAAACATA
hitA	179	28F	AGCGTAGCAGAAGAACCTAAG
		207R	CAATTACCAGGATCCATGTACC
16S rRNA	148	386F	CTACGGGAGGCAGCAAG
		534R	ATTACCGCGGCTGCTGG
ORF5	117	154F	GTACGGATCGTGGTAAACG
		270R	GACCATTTGTCTACAAGGCAG
horC	94	46F	CTTGTTGGAGCAATTATTGG
		139R	CGTTGACAAGTGCTACAGG

[a] With the exception of primers h198F2 and h198R, located at positions 318 and 521, respectively, the number in the primer name refers to its position within the gene. For each primer set, F and R indicate forward and reverse.

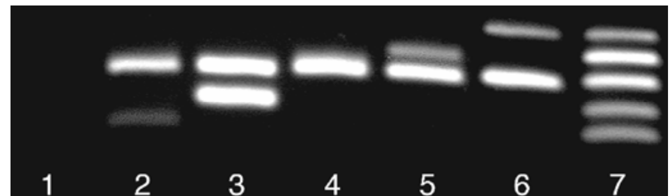

Fig. 1. Agarose gel electrophoresis of multiplex PCR for putative spoilage-associated genes. PCR-positive genes are listed from the bottom to top for each lane. Lane 1, negative control (no added DNA). Lane 2, *Pediococcus claussenii* CCC B962A–*horC* and 16S rRNA. Lane 3, *P. parvulus* ETS.4–ORF5 and 16S rRNA. Lane 4, *Lactobacillus brevis* ATCC 14869–16S rRNA only. Lane 5, *L. delbrueckii* ATCC 12315^T–16S rRNA and *hitA*. Lane 6, *P. claussenii* CCC B1056R–16S rRNA and *horA*. Lane 7, *L. brevis* CCC B1202–*horC*, ORF5, 16S rRNA, *hitA*, and *horA*.

finding that *horC* and ORF5 can be located either separately or together on the same plasmid. Not only does this reflect the genetic diversity and promiscuity of genetic material exhibited by *Lactobacillus* and *Pediococcus* isolates, it also reinforces the need for appropriate subculturing techniques to effectively maintain the presence of genes of interest for the purposes of quality control testing.

Correlation of Genes with Growth in Beer and Isolate Origin

Analysis of the distribution of each of the four putative beer-spoilage genes with respect to whether an isolate was a member of *Lactobacillus* or *Pediococcus* and whether an isolate could grow in beer is presented in Tables III and IV. The χ^2 analysis indicated that each of the four genes correlated with ability of lactobacilli to grow in beer (Table IV). In contrast, for pediococci, only the presence of *horA* was highly correlated with growth in beer, whereas ORF5 was negatively correlated with the ability to grow in beer (i.e., the presence of ORF5 was predictive of the inability of *Pediococcus* isolates to grow in beer). Because the number of lactobacilli exceeded that of the pediococci isolates studied (83 and 50, respectively), three of the four genes (ORF5 being the exception) were significantly correlated with growth in beer when the lactobacilli and pediococci data were combined (Table IV). This correlation is explained by the overlap in the occurrence of genes shown in Table III. In the majority of cases, when the *hitA*, *horC*, or ORF5 genes were found in bacteria able to grow in beer, *horA* also was present (15/16, 16/19, and 13/14 instances, respectively). This may explain why the *hitA*, *horC*, and ORF5 genes previously were erroneously thought to be predictive of the ability of an organism to grow in beer (3,6,8,14,16,17). For this reason, it was necessary that binary logistic regression analyses be performed to determine the actual contribution of each gene to the overall predictive model. Previous studies on *hitA*, *horC*, and ORF5 either failed to concurrently screen for the presence of *horA* or used a set of isolates originating from a single source, thereby skewing interpretation of the importance of a given gene. Moreover, statistical analyses were not performed in previous studies.

To determine whether a correlation exists between the origin of a LAB isolate and the presence of the genes of interest (Table I), a χ^2 test was used to compare the presence of putative spoilage-associated genes in brewery and nonbrewery isolates. Of *hitA*, *horA*, *horC*, and ORF5, only the presence of *horA* was significantly correlated with brewery origin for both *Lactobacillus* and *Pediococcus* isolates ($P < 0.0001$). This suggests that, of the four genes, only *horA* is positively selected for in the brewery environment, reaffirming that the presence of *horA* enhances the beer-spoilage potential of a LAB. Thus, environmental LAB introduced into a brewery could become vicious beer spoilers should even a few bacteria among the original population harbor the *horA* gene.

Predictive Abilities

To determine the actual contribution of each gene to the ability of an organism to grow in beer, binary logistic regression analyses were performed. The results of these analyses (Table V) indicate that only *horA* is a significant predictor of the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer. When the *Lactobacillus* and *Pediococcus* data were grouped for binary logistic regression analysis, *horA* and *horC* both were retained by the statistical model (data not shown). However, *horC* showed a contribution to the ability to grow in beer only when lactobacilli and pediococci data were combined; this was due to the three *horA*-negative, *horC*-positive isolates (two pediococci and one lactobacilli) that were capable of growing in beer, which created what we believe is a statistical anomaly. The inclusion of *horC* as a predictor in the binary logistic model actually caused a decrease in the odds ratio and only a small increase (<1.5%; data not shown) in the overall ability of the model to predict growth in beer. Although *horC* previously has been shown to confer some level of hop resistance (14), we found that this gene rarely occurred in the absence of *horA*. Meanwhile, *horA* was present in many growth-positive isolates that were *horC* negative. These factors show that *horC* is a much less effective marker than *horA* for the prediction of growth in beer; therefore, *horC* was discarded from the predictive model.

TABLE III
Presence of *hitA*, *horA*, *horC*, and ORF5 Genes and Bacterial Growth in Beer[a]

	Growth +			Growth –		
Genes Present	***Lactobacillus* spp.**	***Pediococcus* spp.**	**All**	***Lactobacillus* spp.**	***Pediococcus* spp.**	**All**
horA only	5	8	13	2	1	3
horA and *hitA*	0	0	0	1	0	1
horA and ORF5	2	0	2	0	0	0
horA, *hitA*, and *horC*	5	0	5	0	0	0
horA, *horC*, and ORF5	1	0	1	0	0	0
horA, *hitA*, *horC*, and ORF5	9	1	10	0	0	0
hitA only	0	0	0	7	1	8
horC only	0	1	1	0	0	0
ORF5 only	0	0	0	4	12	16
horC and ORF5	0	1	1	0	1	1
horC and *hitA*	1	0	1	0	0	0
No known genes	7	6	13	40	17	57

[a] Growth in beer defined as ability to grow in beer 1, or beer 1 and beer 2.

TABLE IV
χ^2 Correlation of Putative Beer Spoilage-Associated Genes with Growth in Beer

	Lactobacillus		*Pediococcus*		All Bacteria	
Gene	**Beer 1**	**Beer 2**	**Beer 1**	**Beer 2**	**Beer 1**	**Beer 2**
hitA	$P < 0.005$	$P < 0.005$	NS[a]	NS	$P < 0.005$	$P < 0.005$
horA	$P < 0.005$	$P < 0.005$	$P < 0.005$	$P < 0.005$	$P < 0.005$	$P < 0.005$
horC	$P < 0.005$	$P < 0.005$	NS	$P < 0.05$	$P < 0.005$	$P < 0.005$
ORF5	$P < 0.005$	$P < 0.005$	($P < 0.05$)[b]	($P < 0.05$)[b]	NS	NS

[a] Not significant ($P > 0.05$).
[b] Negatively correlated (i.e., presence of ORF5 is predictive of inability to grow in beer).

The binary logistic regression models in Table V report the predictive abilities generated based on the various groupings analyzed. The models show that *horA* detection has a significant capability for predicting the ability of an isolate to grow in beer. A *horA*-positive PCR result is 88.6% accurate in predicting that an organism will grow in beer (i.e., 31 of 35 *horA*-positive isolates grew in beer) (Table III). Conversely, a *horA*-negative PCR result is 83.7% accurate in predicting that an isolate will not be capable of growing in beer. The lower negative predictive ability was due to the 16 isolates in this study that were *horA*-negative yet capable of growing in beer (Table IV). Because 13 of these 16 isolates did not possess any known spoilage-associated genes, there must be other as yet undefined genetic mechanisms that allow bacterial growth in beer.

The odds ratios given in Table V are the ratio of the probability of growth in beer for *horA*-positive isolates to the probability of growth in beer for a *horA*-negative group. These ratios were normalized to one and, therefore, can be expressed as "times more likely." For example, a *horA*-positive *Lactobacillus* isolate is 53.4 times more likely to grow in beer 1 than a *horA*-negative *Lactobacillus* isolate. As indicated by the predictive abilities and odds ratios in Table V, the ability of *horA* to predict growth was lower for the pediococci than for the lactobacilli, implying that the genetic basis for the ability to grow in beer is currently less well defined for pediococci compared with lactobacilli.

Although *horA* is, overall, >80% accurate in predicting the ability of *Lactobacillus* and *Pediococcus* isolates to grow in beer, Nagelkerke's R^2 values for binary logistic regression confirmed the idea that there must be other mechanisms associated with these bacteria and their ability to grow in beer. As shown by the R^2 values in Table V, *horA* alone could account for only 23–59% of the variability seen in the ability of an organism to grow in beer, depending on the type of beer and the genus of the isolate involved. It must be stressed that these additional mechanisms are not represented by the *hitA*, *horC*, or ORF5 genes, because binary logistic regression analysis found no significant relationship between the presence of these genes and the ability to grow in beer. As such, correlation of these three genes with the ability of a bacterium to grow in beer (i.e., as shown by the χ^2 analyses in Table IV) results from the presence (irrespective of function) of these genes in bacteria also found to possess *horA*. Although *hitA*, *horC*, and ORF5 may somehow play an as yet undetermined role in hop resistance through synergy with *horA* or by other mechanisms (e.g., nutrient acquisition or ethanol resistance), the presence of these three genes cannot be used to predict the ability of an isolate to grow in beer.

Growth Rate in Beer

A *t* test for independent samples was performed to determine whether an organism's growth rate in beer was affected by the presence of any of the four genes (Table VI). Although the presence of *horA* predicted growth in beer, the additional presence of *hitA* or *horC* was associated with an average growth rate in beer that was more than doubled. Because *hitA* and *horC* occurred together in 15 of 16 instances in which either gene was found in the same isolate with *horA*, it was impossible to say whether *hitA*, *horC*, or both genes contributed to the increased growth rate seen in *horA*+/*hitA*+/*horC*+ isolates. Another possibility is that *hitA* and *horC* act as surrogate markers for as yet unknown genes present in these *horA*+/*hitA*+/*horC*+ isolates that actually are responsible for the increased growth rate in beer. Although *hitA* and *horC* were not accurate predictors of the ability to grow in beer by themselves, when multiplexed with *horA* they could serve to identify bacteria able to rapidly spoil beer.

CONCLUSIONS

The described multiplex PCR was effective in detecting the presence of a bacterium in beer and differentiating between *hitA*, *horA*, *horC*, ORF5, and 16S rRNA genes, producing five distinguishable bands (Fig. 1). As emphasized by the R^2 values and odds ratios in

TABLE V
Binary Logistic Regression of Putative Beer Spoilage-Associated Genes and Growth in Beer[a]

Bacterium	R^{2b}	Odds Ratio[c]	Predictive Ability of *horA* (%)[d]		
			Will Grow	Will Not Grow	Overall
Lactobacillus spp.					
Beer 1	0.59	53.4	88.0	87.9	88.0
Beer 2	0.50	29.1	80.0	87.9	85.5
Pediococcus spp.					
Beer 1	0.38	31.0	90.0	77.5	80.0
Beer 2	0.23	9.3	70.0	80.0	78.0
All bacteria					
Beer 1	0.50	39.7	88.6	83.7	85.7
Beer 2	0.40	18.7	77.1	84.7	82.7

[a] All putative spoilage-associated genes (*hitA*, *horA*, *horC*, and ORF5) were included in the analyses, but only *horA* was statistically significant.
[b] Nagelkerke's R^2 value indicates how much of the ability to grow in beer is accounted for by the independent variable *horA*.
[c] Ratio of the probability of growth with the presence of *horA* to the probability of growth in the absence of *horA*. All values were significant at $P < 0.0005$, except for *Pediococcus* in beer 2, which was significant at $P < 0.005$.
[d] Model's ability to predict growth in beer. Only *horA* was significant in each analysis, indicating the ability of *horA* to predict growth in beer.

TABLE VI
Days Required for Growth in Beer in Relation to Genes Present[a]

Genes Present	Beer 1			Beer 2		
	N^b	Mean	Range	N^b	Mean	Range
horA only	15	10.6	2–27	11	9.5	3–14
horA, *horC*, *hitA*	15	4.7	2–12	15	4.9	2–12

[a] The ORF5 gene was excluded as a variable because it had no correlation with the ability to grow in beer. Also, no *horA*+/*horC*–/*hitA*+ and only one *horA*+/*horC*+/*hitA*– growth-positive isolate was found. For beer 1 and beer 2, $P < 0.005$ and 0.0005, respectively, for independent sample *t* tests comparing growth-positive isolates from respective categories.
[b] Number of isolates possessing the gene or genes and capable of growing in beer.

Table V, there currently is a better understanding of the genetic basis for growth in beer for *Lactobacillus* isolates than for *Pediococcus* isolates. Although it is evident that there are other, as yet unknown, mechanisms involved in the ability to grow in beer (Tables IV and V), of the currently known putative beer-spoilage associated genes the only significant predictor of the ability to grow in beer was *horA* (Table V). However, the presence of *hitA*, *horC*, or both in addition to *horA* was predictive of increased growth rate in beer (Table VI).

We suggest that brewery quality control laboratories should, at a minimum, routinely screen lactobacilli and pediococci found in beer for spoilage potential using this multiplex PCR directed to the *horA* and 16S rRNA genes. In addition, although *hitA* and *horC* are not accurate predictors of the ability to grow in beer, incorporation of either gene into a multiplex PCR with *horA* could serve to identify isolates able to grow rapidly in beer (Table VI), thus providing a second crucial piece of information. Until the remaining gene or genes associated with bacterial beer spoilage are defined, the suggested *horA*, *hitA* or *horC*, and 16S rRNA gene multiplex PCR represents the best test available for quickly assessing whether a given *Lactobacillus* or *Pediococcus* isolate is capable of not only growing in, but also rapidly growing in, and spoiling beer.

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