Study on ATP Production of Lactic Acid Bacteria in Beer and Development of a Rapid Pre-Screening Method for Beer-Spoilage Bacteria

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ABSTRACT

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Three beer-spoilage strains of lactic acid bacteria (LAB), *Lactobacillus brevis* ABBC45, *L. lindneri* DSM 20690[T] and *L. paracollinoides* DSM 15502[T], exhibited strong ATP-yielding ability in beer. To investigate energy sources, these beer-spoilage strains were inoculated into beer. After the growth of the strains in beer, utilized components were determined by high performance liquid chromatography (HPLC). As a result, it was shown that citrate, pyruvate, malate and arginine were consumed by beer-spoilage LAB strains examined in this study. The four components induced considerable ATP production even in the presence of hop compounds, accounting for the ATP-yielding ability of the beer-spoilage LAB strains observed in beer. We have further examined the ATP-yielding ability of other strains of bacteria in beer. Beer-spoilage bacteria, including *Pectinatus frisingensis* and *P. cerevisiiphilus,* showed strong ATP-yielding abilities, whereas species frequently isolated from brewery environments exhibited low ATP-yielding abilities. Although some of the nonspoilage LAB strains produced substantial amount of ATP in beer, the measurement of ATP-yielding ability was considered to be useful as a rapid pre-screening method for potential beer-spoilage bacteria isolated from brewery environments.

Key words: ATP, beer-spoilage ability, energy source, lactic acid bacteria, *Lactobacillus.*

INTRODUCTION

Beer has been recognized as a beverage with high microbiological stability. Only a small number of bacterial species, predominantly lactic acid bacteria (LAB), represent the majority of beer-spoilage bacteria[1–3]. Therefore the species-identification method is widely adopted in the brewing industry to determine the beer-spoilage ability of detected bacteria[16,21,35,47,48]. Hop compounds added to confer bitter flavor on beer are reported to exert an antibacterial effect by acting as proton ionophores and dissipate the transmembrane pH gradient, which prevents Gram-positive bacteria including most LAB from growing in beer[29,30,32]. Hop-resistance ability has been known as a distinguishing character of beer-spoilage LAB strains[31,33,37,43] and appears to be conferred by multiple hop-resistance mechanisms[22].

So far, two genes, *horA* and *horC,* have been demonstrated to confer hop-resistance ability on LAB[11,23]. HorA acts as an ATP-dependent multidrug transporter and extrudes toxic hop compounds out of bacterial cells[23]. HorC, on the other hand, confers multidrug resistance to various drugs, including hop compounds, by presumably acting as a proton motive force (PMF)-dependent multidrug transporter[11,41]. On the basis of predicted transmembrane structures, HorC was considered to be a member of the resistance-nodulation-cell division (RND) superfamily, one of the major groups of PMF-dependent multidrug transporters[38]. In addition to these hop-resistance mechanisms, the proton translocating ATPase was suggested to play an important role in the hop-resistance ability of LAB by pumping out protons from the cells[24]. The extrusion of protons presumably counteracts the ionophoric effects of toxic hop compounds and helps beer-spoilage LAB strains maintain the transmembrane pH gradient. Other putative hop-resistance genes have also been reported for beer-spoilage LAB strains[9,10,36,39].

Given that these defense mechanisms are energy-consuming in nature, beer-spoilage LAB strains require substantial energy sources to grow in beer. Nevertheless beer is generally considered as a poor medium to support the growth of bacteria, because most of the nutrients have been depleted by brewer's yeast. Furthermore it has been reported that the ionophoric action of hop compounds inhibits the uptake of nutrients by bacteria[30]. Despite these disadvantages, beer-spoilage LAB strains are still capable of growing in beer. In this study, we investigated energy sources in beer that can be utilized by beer-spoilage LAB strains. On the basis of insights gained by the present study, we also developed a rapid pre-screening method to identify potential beer-spoilage bacteria isolated from brewery environments.

MATERIALS AND METHODS

Hop compounds and beer

Isomerized hop compounds were obtained from Simon H. Steiner Hopfen GmbH (Mainburg, Germany) as a concentrated hop extract. The iso-α-acid contents were determined, using high-performance liquid chromatography[6]. The concentration of hop compounds was expressed as the

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total iso-α-acid contents. Degassed commercial pilsner-type beers (hop compounds: 20 ppm, alcohol content: 5% (v/v), pH adjusted to 4.2) were used for the ATP measurements and the evaluation of beer-spoilage ability.

Bacterial strains and growth conditions

Strains of LAB were grown anaerobically at 25°C on MRS agar (Merck, Darmstadt, Germany), containing 25% (v/v) pilsner-type beer. The final pH of MRS medium was adjusted to 5.5 with HCl. Other strains isolated from brewery environments were cultured anaerobically at 25°C on TGC agar (Nissui Pharmaceutical, Tokyo, Japan). In this study, the 14 species most frequently isolated from our brewery environments were used[46]. Anaerobic conditions were generated by AnaeroPack (Mitsubishi Gas Chemicals, Tokyo, Japan). Cells were stored in respective broth media containing 20% glycerol at –80°C.

ATP measurements in beer

Approximately 10^7–10^8 cells of strains were washed with tartarate buffer (20 mM tartaric acid, 1 mM $MgSO_4$, 0.5 mM $MnSO_4$, pH adjusted to pH 4.2 with 320 mM K_2HPO_4 buffer) and resuspended in 50 µL tartarate buffer. Ten microliters of suspended cells were added to 190 µL degassed pilsner-type beer and incubated at 25°C for 60 min to allow the production of ATP in beer. Ten microliters of the incubated beers were added to 90 µl Reconstitution & Dilution Buffer supplied by Kikkoman Corporation (Chiba, Japan). The ATP production was measured by Lumat LB9501 (Berthold Technologies, Bad Wilbad, Germany) using the ATP measurement kit CheckLite 250 plus (Kikkoman Corporation). The ATP measurements were carried out in accordance with the manufacturer's recommendations. As a negative control, 10 µl of suspended cells were added to 190 µl tartarate buffer and incubated at 25°C for 60 min. Ten microliters of the incubated cells was added to 90 µL Reconstitution & Dilution Buffer and the ATP production was determined. The ATP-yielding abilities were defined as the amount of ATP produced in beer divided by the amount of ATP contained in the negative control.

Identification of utilized substrates in beer

Degassed pilsner-type beers (50 mL) were inoculated with 10^3 cells/mL of beer-spoilage LAB strains and incubated anaerobically at 25°C. After the growth was confirmed in beer, the contents of organic acids, fermentable carbohydrates and amino acids were determined by HPLC. The organic acid contents were analyzed using LC-VP Organic Acid Analysis System (Shimadzu, Kyoto, Japan). The analysis was performed in accordance with the manufacturer's protocols. The fermentable carbohydrate analysis was carried out by the method described in Analytica-EBC[7]. The amino acid contents were determined by Model L-8800 High Speed Amino Acid Analyzer (Hitachi, Tokyo, Japan) according to the manufacturer's instructions. For comparative purpose, the contents of these substrates in the uninoculated beer were also determined. The three beer-spoilage LAB strains, *L. brevis* ABBC45, *L. lindneri* DSM 20690^T and *L. paracollinoides* DSM 15502^T, were used in this experiment.

ATP production from the utilized substrates

Approximately 10^7–10^8 cells of strains were washed and resuspended in 50 µL tartarate buffer. Ten microliters of suspended cells was added to 190 µL tartarate buffer containing one of the following substrates: citrate 150 ppm, pyruvate 100 ppm, malate 60 ppm, maltotriose 1800 ppm, tyrosine 45 ppm and arginine 15 ppm. The concentrations of these substrates reflect the contents found in the pilsner-type beer used in this study. The suspended cells were incubated with each substrate at 25°C for 60 min in the presence or absence of 20 ppm hop compounds and subsequently the ATP production was measured. As a negative control, 10 µL of suspended cells was added to 190 µL tartarate buffer and incubated at 25°C for 60 min and the ATP production was determined. The ATP measurements were performed as described earlier. ATP yielding abilities were defined as the amount of ATP produced in tartarate buffer with the substrate divided by the amount of ATP produced in the negative control.

Detection of genetic markers

Genomic DNA was extracted from bacterial cells as described by Sami *et al.*[28]. The presence or absence of the two genetic markers, *horA* and *horC,* was examined by PCR, using the methods described previously[28,38]. The positively reacted PCR products were subjected to Southern blot analysis to determine whether these products are *horA* or *horC* homologs. The procedures for Southern blot analysis were previously described[38,44].

Evaluation of beer-spoilage ability

Degassed pilsner-type beers were adjusted to pH 4.2 with 5N NaOH. Ten mL of beer was dispensed into 15 mL sterile polypropylene tubes and inoculated with approximately 3×10^3 cells/mL of LAB 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 AND DISCUSSION

ATP-yielding abilities in three beer-spoilage strains and identification of utilized substrates in beer

ATP-yielding abilities in beer were measured, using three beer-spoilage strains, *L. brevis* ABBC45, *L. lindneri* DSM 20690^T and *L. paracollinoides* DSM 15502^T. As shown in Table I, these three strains exhibited strong ATP-yielding abilities, indicating the presence of energy sources in beer. Therefore we investigated the substrates that can be utilized by these beer-spoilage LAB strains. As a result, *L. brevis* ABBC45 was found to utilize citrate, pyruvate, malate, arginine and, to a lesser extent, tyrosine (Table II).

Table I. ATP-yielding abilities of beer-spoilage strains in beer.

	ATP-yielding ability
L. brevis ABBC45	11.3
L. lindneri DSM 20690^T	171.0
L. paracollinoides DSM 15502^T	11.2

Table II. Identification of the utilized substrates in beer[1].

	Substrates	*L. brevis* ABBC45	*L. lindneri* DSM 20690^T	*L. paracollinoides* DSM 15502^T	Control beer[2]
Organic acids (mg/L)	Phosphate	385	398	396	392
	Citrate	N.D.	19	154	153
	Pyruvate	N.D.	18	N.D.	102
	Malate	16	6	63	62
	Succinate	47	50	51	49
	Lactate	475	441	811	148
	Formate	4	4	3	4
	Acetate	317	227	337	122
	Pyroglutamate	130	145	148	133
Fermentable carbohydrates (g/100 mL)	Dextrin	2.51	2.48	2.46	2.50
	Maltotriose	0.18	0.18	0.13	0.18
	Maltose	0.06	0.06	0.06	0.06
	Glucose	0.01	0.01	0.01	0.01
	Fructose	0.02	0.02	0.03	0.03
Amino acids (mg/100 mL)	Asp	0.6	0.5	0.4	0.5
	Asn	0.1	0.1	0.0	0.1
	Thr	0.1	0.1	0.1	0.1
	Ser	0.1	0.1	0.1	0.1
	Met	0.2	0.1	0.1	0.1
	Lys	0.1	0.1	0.1	0.1
	Glu	0.2	0.1	0.4	0.3
	Gln	0.1	0.1	0.0	0.1
	Gly	1.6	1.3	1.4	1.3
	Val	2.1	2.1	1.9	2.1
	Ile	0.5	0.4	0.3	0.4
	Leu	0.5	0.5	0.4	0.5
	Ala	3.5	3.3	3.1	3.2
	Tyr	3.5	4.3	4.2	4.3
	Phe	2.7	2.7	2.4	2.7
	His	2.2	2.0	2.0	2.0
	Trp	2.1	2.1	2.1	2.0
	Arg	0.0	1.5	0.1	1.4
	Pro	29.0	28.5	28.9	28.4
	γ-ABA	5.1	5.0	5.1	5.0

[1] After the growth of each beer-spoilage strain, the contents of substrates in beer were determined by HPLC analysis. N.D. indicates that the substrate was not detectable.
[2] The uninoculated beer was used as a control.

L. lindneri DSM 20690^T utilized citrate, pyruvate and malate. *L. paracollinoides* DSM 15502^T was found to utilize pyruvate, arginine and, to a lesser extent, maltotriose. These substrates were considered as candidates for potential energy sources in beer.

ATP production from the utilized substrates

We examined ATP-yielding abilities of the beer-spoilage LAB strains using each utilized substrate (Table III). Only *L. brevis* ABBC45 yielded ATP with citrate and this was not substantially diminished by the presence of hop compounds, indicating citrate is a substrate that supplies an energy source for *L. brevis* ABBC45. Pyruvate was utilized for ATP production by all the strains examined in this study, regardless of whether hop compounds were present or not. These results indicate that pyruvate is one of the energy sources for beer-spoilage LAB strains in beer. In the case of malate, only *L. lindneri* DSM 20690^T used this substrate to yield ATP in the presence of hop compounds, showing malate provides an energy source for this strain in beer. Intriguingly, citrate and malate did not show strong ATP-yielding ability in *L. lindneri* DSM 20690^T and *L. brevis* ABBC45 respectively, although each substrate was utilized in beer by respective strains. It is conceivable that these substrates were used for purposes other than ATP production. Alternatively certain conditions may be necessary to induce the ability to produce ATP in *L. lindneri* DSM 20690^T and *L. brevis* ABBC45.

All the three beer-spoilage strains were able to yield ATP from maltotriose. Nevertheless ATP-yielding abilities were almost completely abolished in *L. brevis* ABBC45

Table III. ATP-yielding abilities of beer-spoilage strains with the utilized substrates.

	Citrate		Pyruvate		Malate	
Hop[1]	–	+	–	+	–	+
L. brevis ABBC45	10.1	4.9	6.2	5.0	1.1	0.4
L. lindneri DSM 20690^T	1.3	0.3	36.4	25.9	37.4	29.6
L. paracollinoides DSM 15502^T	1.1	0.2	26.6	10.6	0.8	0.2
	Maltotriose		**Tyrosine**		**Arginine**	
Hop	–	+	–	+	–	+
L. brevis ABBC45	11.5	1.1	1.1	0.5	13.4	7.6
L. lindneri DSM 20690^T	22.2	0.4	0.9	0.3	0.7	0.2
L. paracollinoides DSM 15502^T	30.6	5.8	1.2	0.3	21.6	3.7

[1] The ATP measurement was performed in the presence or absence of hop compounds. + indicates that 20 ppm hop compounds were added to tartarate buffer (pH 4.2) with the substrates shown in the table. – indicates that no hop compounds were added.

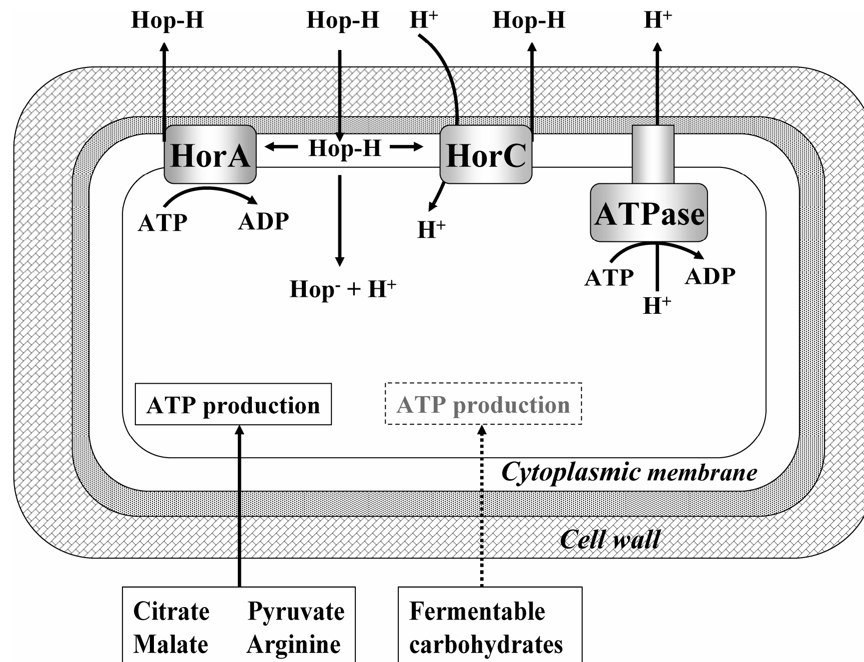

Fig. 1. The proposed mechanisms of hop resistance in beer-spoilage LAB. HorA and HorC are multidrug transporters that are driven by ATP and PMF respectively[11,23,38]. The undissociated hop compounds (Hop-H) intercalate into the cytoplasmic membrane and are pumped out by the multidrug transporters. Some portions of Hop-H escape the pumping activities of the transporters and enter the cytoplasm. In the cytoplasm, Hop-H dissociates into the anionic form (Hop^-) and H^+ due to the higher internal pH. H^+ also enters the cytoplasm in antiport with Hop-H by the action of HorC. To prevent the acidification of the cytoplasm and maintain the transmembrane pH gradient, proton translocating ATPase excretes H^+ across the membrane[24]. The energy sources for these hop-resistance mechanisms were supplied from citrate, pyruvate, malate and arginine. In some cases, fermentable carbohydrates, such as maltotriose, may be utilized to produce ATP (this study).

and *L. lindneri* DSM 20690^T with the addition of hop compounds, a phenomenon consistent with the inability of these strains to utilize maltotriose in beer (Table II). In contrast, ATP-yielding ability of *L. paracollinoides* DSM 15502^T was still observed with the addition of hop compounds, indicating that this strain can take up maltotriose in the presence of hop compounds. This result accounts for the partial utilization of maltotriose by *L. paracollinoides* DSM 15502^T in beer (Table II). The ionophoric action of hop bitter acids was reported to become stronger as the surrounding pH decreases[29,30]. The pH of beer, in which *L. paracollinoides* DSM 15502^T had grown, dropped from 4.2 to 3.8 due to the metabolic activities of this strain. The progressive decrease in pH is presumably accountable for the incomplete utilization of maltotriose in beer by *L. paracollinoides* DSM 15502^T.

As for arginine, this substrate was used to yield ATP by *L. brevis* ABBC45 and *L. paracollinoides* DSM 15502^T. ATP yielding abilities were still found in the presence of hop compounds, showing arginine supports ATP production for these strains in beer. In the case of tyrosine, no ATP yielding abilities were observed in the three beer-spoilage strains, suggesting this substrate is not utilized for ATP production in the strains examined in this study.

The metabolic pathways of beer-spoilage LAB strains are poorly understood but a number of studies in other areas of LAB have been reported. The occurrence of malolactic fermentation has been reported to be widespread in LAB[4,12,18]. In this pathway, L-malate enters the cells and is decarboxylated by malolactic enzyme to yield L-lactate and carbon dioxide, after which L-lactate and carbon dioxide leave the cells. During these processes, PMF is generated, resulting in the synthesis of ATP. The citrate metabolism in LAB has also been well studied[14,20]. The ATP production has been reported to occur by generation of PMF through uniport of anionic citrate and proton consumption in citrate metabolism. The metabolism of pyruvate also produces ATP. In LAB, pyruvate is known to be converted to acetylphosphate by pyruvate formate lyase, after which ATP is yielded through the conversion of acetylphosphate to acetate[15]. Pyruvate is also known to be a metabolic product of citrate[14]. It is therefore conceivable that this pathway is also utilized to yield additional ATP in citrate metabolism. As for arginine, the arginine deiminase pathway is reported to be widely distributed in LAB[13,18]. In this pathway, ATP is produced when arginine is metabolized to ornithine, carbon dioxide and ammonia. The presence of these metabolic pathways in beer-spoilage lactic acid bacteria is currently speculative but certainly an interesting area of research to explore.

Interestingly, the uptake of utilized substrates found in this study was not substantially inhibited in the presence of hop compounds. This may be important since hop compounds were shown to inhibit the uptake of nutrients by

Table IV. Relationship between ATP-yielding ability and beer-spoilage ability[1].

Strain no.[2]	Species	ATP-yielding ability	*horA*	*horC*	Beer spoilage ability
ABBC3	*L. brevis*	13.4	+	+	+
ABBC34	*L. brevis*	576.5	+	+	+
ABBC37	*L. brevis*	278.6	+	+	+
ABBC42	*L. brevis*	25.1	+	+	+
ABBC43	*L. brevis*	15.8	+	+	+
ABBC44	*L. brevis*	33.6	+	–	+
ABBC45	*L. brevis*	11.3	+	+	+
ABBC46	*L. brevis*	54.7	+	+	+
ABBC56	*L. brevis*	138.0	+	+	+
ABBC64	*L. brevis*	172.0	+	+	+
ABBC69	*L. brevis*	74.9	+	+	+
ABBC70	*L. brevis*	49.7	+	+	+
ABBC76	*L. brevis*	100.2	+	+	+
ABBC77	*L. brevis*	179.3	+	+	+
ABBC78	*L. brevis*	38.7	+	+	+
ABBC79	*L. brevis*	149.7	+	+	+
ABBC84	*L. brevis*	1571.4	+	+	+
ABBC85	*L. brevis*	635.4	+	+	+
ABBC86	*L. brevis*	632.3	+	+	+
ABBC99	*L. brevis*	95.6	+	+	+
ABBC100	*L. brevis*	234.4	+	+	+
ABBC104	*L. brevis*	40.6	+	+	+
ABBC400	*L. brevis*	36.6	+	+	+
ABBC402	*L. brevis*	38.2	+	+	+
ABBC403	*L. brevis*	23.0	+	+	+
ABBC404	*L. brevis*	212.0	+	+	+
ABBC405	*L. brevis*	201.4	+	+	+
ABBC407	*L. brevis*	25.2	+	+	+
ABBC408	*L. brevis*	288.8	+	+	+
ABBC4	*L. brevis*	16.7	+	–	–
ABBC12	*L. brevis*	20.0	–	–	–
ABBC36	*L. brevis*	33.8	+	–	–
ABBC65	*L. brevis*	26.5	+	–	–
ABBC67	*L. brevis*	33.9	+	–	–
ABBC406	*L. brevis*	10.7	+	–	–
JCM 1059^T	*L. brevis*	3.9	–	–	–
DSM 1267	*L. brevis*	7.2	–	–	–
DSM 2647	*L. brevis*	1.8	–	–	–
DSM 20556	*L. brevis*	3.3	–	–	–
DSM 15502^T	*L. paracollinoides*	11.2	+	+	+
LA3	*L. paracollinoides*	173.7	+	+	+
LA4	*L. paracollinoides*	230.3	+	+	+
LA7	*L. paracollinoides*	21.6	–	+	+
LA8	*L. paracollinoides*	32.0	–	+	+
LA9	*L. paracollinoides*	123.4	+	+	+
LA10	*L. paracollinoides*	246.2	+	+	+
LA11	*L. paracollinoides*	486.5	+	+	+
LA12	*L. paracollinoides*	122.0	+	+	+
LA13	*L. paracollinoides*	152.5	+	+	+
LA14	*L. paracollinoides*	172.5	+	+	+
LA15	*L. paracollinoides*	49.8	+	+	+
ATCC 8291	*L. paracollinoides*	23.2	–	–	–
DSM 20690^T	*L. lindneri*	171.0	+	–	+
DSM 20692	*L. lindneri*	854.8	+	+	+
HC92	*L. lindneri*	335.6	+	+	+
HC95	*L. lindneri*	537.3	+	+	+
HC98	*L. lindneri*	26.3	+	+	+
LA16	*L. lindneri*	1591.6	+	+	+
ABBC478	*Pediococcus damnosus*	12.5	+	+	+
ABBC500	*Ped. damnosus*	10.2	+	+	+
VTT E-76065	*Ped. damnosus*	195.2	+	+	+
DSM 20467^T	*Pectinatus cerevisiiphilus*	29.6	–	–	+
ABBC474	*P. cerevisiiphilus*	10.7	–	–	+
DSM 6306^T	*P. frisingensis*	40.9	–	–	+

(*continued on next page*)

[1] The results of the presence or absence of genetic markers were previously reported except for *Pectinatus* strains[38,44].

[2] ABBC, HC and LA: 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; VTT: culture collection obtained from Technical Research Center of Finland (VTT).

Table IV. (*continued*)

Strain no.[2]	Species	ATP-yielding ability	*horA*	*horC*	Beer spoilage ability
ABBC136	*P. frisingensis*	15.8	–	–	+
ABBC437	*P. frisingensis*	38.4	–	–	+
ABBC461	*P. frisingensis*	38.9	–	–	+
ABBC469	*P. frisingensis*	29.1	–	–	+
ABBC471	*P. frisingensis*	27.4	–	–	+
JCM 1123^T	*L. collinoides*	36.6	–	–	–
ATCC 27610	*L. collinoides*	6.8	–	–	–
ATCC 27611	*L. collinoides*	17.5	–	–	–
ABBC213	*L. plantarum*	0.4	–	–	–
ABBC219	*L. buchneri*	11.5	+	–	–
ABBC222	*L. paracasei*	4.2	–	–	–
ABBC228	*L. fermentum*	23.0	–	–	–
ABBC251	*L. coryniformis*	3.6	–	–	–
ABBC257	*L. fructivorans*	18.1	–	–	–
ABBC275	*L. rhamnosus*	12.0	–	–	–
ABBC279	*L. casei*	1.7	–	–	–
ABBC281	*L. delbrueckii*	10.8	–	–	–
JCM 8573^T	*L. parakefiri*	52.3	–	–	–
DSM 5707	*L. parabuchneri*	24.2	–	–	–
HC311[3]	*Lactococcus lactis*	0.5	–	–	–
HC367	*Serratia marcescens*	0.9	–	–	–
HC417	*Citrobacter freundii*	1.1	–	–	–
HC432	*Enterobacter cloaceae*	1.2	–	–	–
HC437	*Staphylococcus warneri*	0.4	–	–	–
HC440	*Propionibacterium acnes*	0.2	–	–	–
HC442	*Bacillus thuringiensis*	0.6	–	–	–
HC453	*Pantoea agglomerans*	0.7	–	–	–
HC459	*Paenibacillus amylolyticus*	1.0	–	–	–
HC466	*Paenibacillus jamilae*	1.8	–	–	–
HC472	*Clostridium beijerinckii*	1.4	–	–	–
HC475	*Staphylococcus epidermidis*	0.2	–	–	–
HC523	*Sporolactobacillus racemicus*	1.2	–	–	–
HC534	*Klebsiella oxytoca*	0.5	–	–	–

[3] Strains indicated as HC311–534 represent the most frequent environmental isolates[46].

abolishing the transmembrane pH gradient[30]. This phenomenon appeared to have occurred in the uptake of maltotriose in the presence of hop compounds. The transport of malate was reported to be carried out by malate/lactate antiport or uniport of malate[19,25]. It was also reported that the transport of citrate occurred via uniport of this ion[20]. Arginine was shown to be transported into the cells by arginine/ornithine antiporter[5,18]. These transport systems are presumably advantageous for the survival in beer because these systems are not dependent on the transmembrane pH gradient, which is diminished by the action of hop compounds. This hypothesis should be tested in future studies.

Multidrug transporters and proton translocating ATPase have been so far reported as the hop resistance mechanisms in beer-spoilage lactic acid bacteria[11,23,24]. These defense mechanisms undoubtedly require a considerable amount of energy to be maintained. The substrates identified in this study supply energy sources in beer. Beer is known to be a poor medium for the growth of bacteria. In this sense, the metabolic pathway of these substrates can be considered as additional hop resistance mechanisms for beer-spoilage LAB (Fig. 1).

Rapid pre-screening method for identifying potential beer-spoilage bacteria

The strong ATP-yielding abilities observed with the three beer-spoilage LAB strains prompted us to investigate potential applications of ATP-yielding ability to the identification of potential beer-spoilage bacteria. We examined a total of 97 strains to determine whether these strains exhibit ATP-yielding abilities in beer. All the beer-spoilage strains were found to exhibit ATP-yielding abilities in excess of five. In contrast, all of the 14 frequent brewery isolates did not show high ATP-yielding abilities, indicating rapid pre-screening of potential beer-spoilage strains is possible. Interestingly strains belonging to *Pectinatus frisingensis* and *P. cerevisiiphilus* also showed strong ATP-yielding abilities with the method used in this study. It appears that the exposure to oxygen during the measurement does not significantly inhibit ATP-yielding abilities for these strictly anaerobic bacteria. It is also of note that this pre-screening method can be used for strains which do not belong to LAB. This is in contrast with the recently proposed trans-species genetic markers, *horA* and *horC*[26–28,38,44]. These genetic markers were shown to be effective for a wide variety of beer-spoilage LAB species and expected to detect unencountered species of beer-spoilage LAB, which occasionally emerge in the brewing industry[8,17,34]. However, as shown in Table IV, these genetic markers were not detectable in *Pectinatus* strains. Therefore this rapid pre-screening method is considered to be potentially useful for identifying unencountered beer-spoilage strains, including those belonging to non-LAB species. Despite these possibilities, considerable ATP-yielding abilities were observed with many of the nonspoilage LAB strains, indicating these strains are able to produce ATP in beer. The alterations in experimental

conditions, such as measurement time or cell concentrations, were not effective for differentiating beer-spoilage and nonspoilage LAB strains, suggesting many nonspoilage LAB strains possess ATP-yielding abilities similar to those of beer-spoilage LAB strains.

Perplexingly, some of the nonspoilage strains with relatively high ATP yielding abilities possess *horA* homologs. However our previous study indicates these strains except *L. brevis* ABBC4 and *L. buchneri* ABBC219 are capable of growing in beers adjusted to pH 4.6[44]. This type of beer represents those with relatively weak microbiological stability and therefore these strains are considered weak beer-spoilage strains. As for *L. brevis* ABBC4 and *L. buchneri* ABBC219, functional *horA* products may not be expressed or alternatively the role of *horA* homologs is relatively small in the hop resistance of these strains. The other nonspoilage strains with high ATP-yielding abilities appear to lack effective hop-resistance mechanisms to grow in beer. It is therefore conceivable that the hop-resistance mechanisms and the energy-yielding abilities are both necessary for beer-spoilage bacteria to be capable of growing in beer. Nevertheless these nonspoilage strains should be considered as potential threats to brewers because the hop-resistance genes appear to be spreading among LAB strains through horizontal gene transfer[38–40,42,45]. The presence of nonspoilage LAB strains with high ATP-yielding abilities in brewery environments poses a threat to brewers since these strains may be transformed into beer-spoilage strains with the acquisition of hop-resistance genes, such as *horA* and *horC*. It is also conceivable that as yet uncharacterized hop-resistance genes could transform highly ATP-yielding strains into beer-spoilage strains. The identification of potential beer-spoilage strains by the present method is therefore useful to proactively eliminate the potential threats in brewery environments. Taken collectively, the ATP-yielding abilities in beer can be used as a rapid pre-screening method to identify potential beer-spoilage strains isolated from brewery environments.

CONCLUSIONS

Citrate, pyruvate, malate and arginine were identified as energy sources for beer-spoilage LAB in beer. The ATP-yielding abilities observed with these substrates were not substantially inhibited by the hop compounds, indicating beer-spoilage LAB strains preferentially utilize these substrates in beer. In contrast, the utilization of maltotriose was considerably suppressed by hop compounds. But it seems possible for beer-spoilage LAB to utilize this substrate, depending on the strains and the types of beer. The measurement of ATP-yielding abilities is also found to be useful for identifying potential beer-spoilage bacteria, including non-LAB strains. This is a clear advantage over the previously reported trans-species genetic markers that are effective only for beer-spoilage LAB strains.

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