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Description of *Lactobacillus backi* sp. nov., an Obligate Beer-Spoiling Bacterium

Lactic acid bacteria that are able to spoil beer comprise a limited number of bacteria of the genera *Lactobacillus* and *Pediococcus*. Five Gram-positive isolates of uncertain taxonomic position causing turbidity, sediments and decreasing pH-value were isolated from lager, pils and wheat beers from different breweries in Germany and Italy. Physiological and biochemical studies of these strains showed a homogeneous group of organisms. The 16S rDNA sequence analysis of three of the five isolates revealed that they formed a phylogenetically distinct line within the genus *Lactobacillus* and have clearly less than 97% 16S rDNA sequence similarities to any other species. All strains were homofermentative, producing D(L)-lactic acid.Based on the data presented in this study the new species *Lactobacillus backi* is proposed. The type strain of *L. backi* is DSM 18080^T (LMG 23555^T).

Descriptors: *Lactobacillus backi*, beer-spoiling bacteria, Lactic acid bacteria

1 Introduction

Only a limited number of species of the genus *Lactobacillus* are able to spoil beer. This is due to the selective conditions of beer such as low pH-values, CO_2-content (anaerobic atmosphere), alcohol-content, lack of nutrients and hop-content. According to Back [1,2] beer-spoiling bacteria can be subdivided into obligate and potential beer-spoiling bacteria. Thereby obligate beer-spoiling bacteria quickly lead to a deterioration of the product and show a very high beer-spoiling ability, regardless of the type of beer. Whereas the possibility of deterioration due to potential beer-spoiling bacteria depends on the ingredients of the beer and on the ability of the bacterium to adapt to those conditions.

So far only a few number of obligate beer-spoiling lactobacilli like *Lactobacillus brevis*, *Lactobacillus lindneri*, "*Lactobacillus brevisimilis*" and "*Lactobacillus frigidus*" are known. Strains of the *Lactobacillus casei* / *Lactobacillus paracasei* group, *Lactobacillus plantarum* and *Lactobacillus coryniformis* are known to be members within the group of potential beer-spoiling lactobacilli [1,2]. In the last years five *Lactobacillus* strains were isolated from lager beers (EBC BU 20 − 22), pils beers (EBC BU 32) and wheat beers (EBC BU 12 − 14) from different breweries in Germany and Italy (Table 1). These strains caused turbidity, sediments and decreased the pH-values of different types of beer. All strains showed a striking morphological resemblance to *L. coryniformis*, whereas in contrast to *L. coryniformis* physiological tests consistently showed a lack of maltose fermentation and diacetyl formation. These results in combination with the homofermentative behavior of these strains suggested that they belong to a new species of obligate beer-spoiling lactobacilli. Further studies based on 16S rDNA sequence analysis of three of the five strains revealed significant differences to other *Lactobacillus* sp.. On the basis of our results, the strains L1062, L1064 and L1065 should be classified as members of a novel species within the genus *Lactobacillus*. The name *Lactobacillus backi* is proposed for this organism. The type strain of *Lactobacillus backi* is strain L1062 (DSM 18080^T, LMG 23555^T).

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Tables and Figures see Appendix

2 Materials and Methods

Organisms and growth conditions

The origin of the *L. backi* strains studied is given in Table 1. The *L. backi* strains were cultivated on NBB-media (Döhler, Darmstadt, Germany) [3] at their optimum growth temperature of 28°C. Cultures were incubated in the presence of CO_2. Pure cultures were stored in 80% [v/v] glycerol at –20°C.

Physiological and biochemical tests

The physiological tests were carried out as described by BACK [4]. The determination of D- and L-lactic acid was carried out enzymatically according to the manufacturer´s instructions (UV method, Boehringer, Mannheim). Diacetyl was determined by the method of MEBAK [5]. The G+C content of the DNA was determined by HPLC analysis. [6, 7, 8, 9].

Cell walls were prepared and analyzed by the method of SCHLEIFER and KANDLER [10], MAC KENZIE [11] and GROTH et al. [12].

Phylogenetic analysis

The 16S rRNA gene sequences of *L. backi* L1062, L1064 and L1065 were determined. *In vitro* amplification and direct sequencing of 16S rRNA genes encoding DNA fragments were carried out as previously described [13].

Sequences were added to a 16S rRNA sequence database by the use of the program package ARB [14, 15]. The tool ARB_EDIT was used for sequence alignment. The alignment was checked visually and corrected manually. The 16S rRNA-based phylogenetic tree was constructed on the results of distance matrix analyses of a full set of more than 22 000 homologous full and partial primary structures available in ARB database (http://www.arb-home.de). The topology of the tree was evaluated by performing maximum parsimony and maximum likelihood analysis of the full data set

and subsets, respectively [16]. Alignment positions at which fewer than 50% of the sequences of the entire data set shared the same residues were excluded from the calculations. The phylogenetic positions of organisms presented by partial sequences were roughly reconstructed by applying the parsimony criteria without changing the overall tree topology.

Bootstrapping was performed (1000 resamplings) with the ARB_GDE phylogeny tool to estimate stability of the clusters formed.

Nucleotide sequence accession numbers

16S rDNA sequences were submitted to GenBank under the accession numbers DQ406860 to DQ406862.

3 Results

Phenotypic and physiological properties

The cell morphology was observed by dark field microscopy. The studied beer-spoiling lactobacilli enriched in NBB-Bouillon formed rods morphologically similar to *L. coryniformis*. The shape of the rods was irregular, whereas cells were mostly appearing in the form of cudgels, drops or hearts, with rounded ends. They were occurring singly, in pairs or in short chains (about 5 to 10 cells). Whereby their arrangement in chains was jagged. All strains grew well in beers up to 32 EBC BU and spoiled these beverages by causing turbidity and sediments. Due to acidification the pH-value was decreased between 0.1 to 0.2 pH units. The strains analysed in this study can be distinguished from their phylogenetic most closely related species by their narrow range of carbohydrate fermentation patterns. The phenotypic and physiological results obtained in this study are summarized in Table 2.

The main characteristics of *L. backi* are the hydrolysis of aesculin and the fermentation of fructose, glucose, D(–)–mannitol, D(+)–mannose, salicine and D(–)–sorbitol. Neither maltose, D(+)–melezitose, melibiose, D(–)–ribose, L(+)–rhamnose, amygdalin, D(+)–galactose, cellobiose, raffinose, sucrose, trehalose, L(+)–arabinose, D(+)–xylose, dextrin nor lactose are fermented. No gas is produced from gluconate, glucose and maltose. Ammonia is not formed from L-arginine. All strains produce D(L)–lactic acid. The peptidoglycan is of L-lysine-D-asparagine type. The G+C content is 37 mol%. The cell wall composition and the G+C content of the DNA was determined for the type strain (DSM 18080^T/ LMG 23555^T) of *Lactobacillus backi*.

16S rDNA sequence analysis

Due to the fact that all five strains of *L. backi* showed a homogenous phenotypic and physiological behavior (Table 2), the 16S rDNA sequence was exemplary determined for three of the five isolated strains.

Phylogenetic analysis of almost complete 16S rDNA sequences (1483 bp, 1419 bp and 1428 bp) confirmed the affiliation of the *L. backi* strains L1062 (Genbank accession no. DQ406862), L1064 (DQ406861) and L1065 (DQ406860) within the genus *Lactobacillus*. The three strains shared average 16S rDNA sequence similarity values of 99.3% to each other and formed a tight monophyletic group (Fig. 1).

The constructed consensus tree showed the position of *L. backi* within a cluster composed of *Lactobacillus coryniformis*, *Lactobacillus rennini* and *Lactobacillus bifermentans*. The position of this cluster within the consensus tree was confirmed by all applied treeing methods.

The phylogenetic most closely related species *Lactobacillus coryniformis* subsp. *coryniformis* (Genbank accession number M58813) and *Lactobacillus coryniformis* subsp. *torquens* (AJ575741) had 96.2 – 96.6% and 95.8 – 96.3% 16S rDNA sequence similarities to the new strains. All other species were more distantly related.

4 Discussion

Despite self-protecting properties beers like lager, pils or wheat beer can undergo microbial spoilage resulting in turbidity, sedimentation and decreased pH-value. During the last years we have isolated a variety of obligate beer-spoiling strains in breweries that were characterized by their ability to grow under highly acidic and anaerobic conditions. Physiological properties and phylogenetic analyses differentiated these organisms from hitherto known beer-spoiling organisms. Although showing a high morphological resemblance to *L. coryniformis* these new strains can be clearly distinguished physiologically from *L. coryniformis* by the lack of maltose and saccharose fermentation as well as by the lack of diacetyl formation. Moreover, the very low G+C content of 37 mol% (G+C content *L. coryniformis*: 45 mol%, [1]) and a homofermentative fermentation behavior underlines the differentiation. Their 16S rDNA sequence similarity values higher than 99% indicated their relationship at species level among themselves.

The 16S rDNA sequence similarities lower than 97% to the next related species *Lactobacillus coryniformis* justify that these three strains represent a new species within the genus *Lactobacillus* [18].

Description of *Lactobacillus backi* Bohak, Thelen and Beimfohr sp. nov.

Lactobacillus backi [bac.ki N.L. gen. n. of Back, named in honor to Werner Back, who contributed to the general classification of beer-spoiling bacteria and brewing microbiology]. Gram-positive, non spore-forming, non-motile, catalase-negative, mostly unregular rods with rounded ends, average size 0.7 to 2.0 µm. Cells are found singly, in pairs and occasionally in chains. Colonies on NBB-Agar are small (1 to 2 mm), flat or little exalted, white and smooth. They are facultatively anaerobic and can grow up to 36°C with an optimum at 28°C; no growth below 15°C. Optimum growth at pH 4.5 to 6.5, no growth above pH 8.0. Homofermentative, producing D(L)–lactic acid. Acid is only produced from aesculin, fructose, glucose, D(–)–mannitol, D(+)–mannose, salicine and D(–)–sorbitol. Neither acid nor gas are formed from gluconate and maltose. No gas is formed from glucose. Gelatine is not hydrolysed, arginine decarboxylase activity was not detected. Urease and H_2S are not produced; nitrate is not reduced to nitrite. The G+C content of the DNA is 37 mol%. Habitat: Isolated from spoiled beers of different breweries. The type strain is DSM 18080^T (LMG 23555^T).

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Appendix

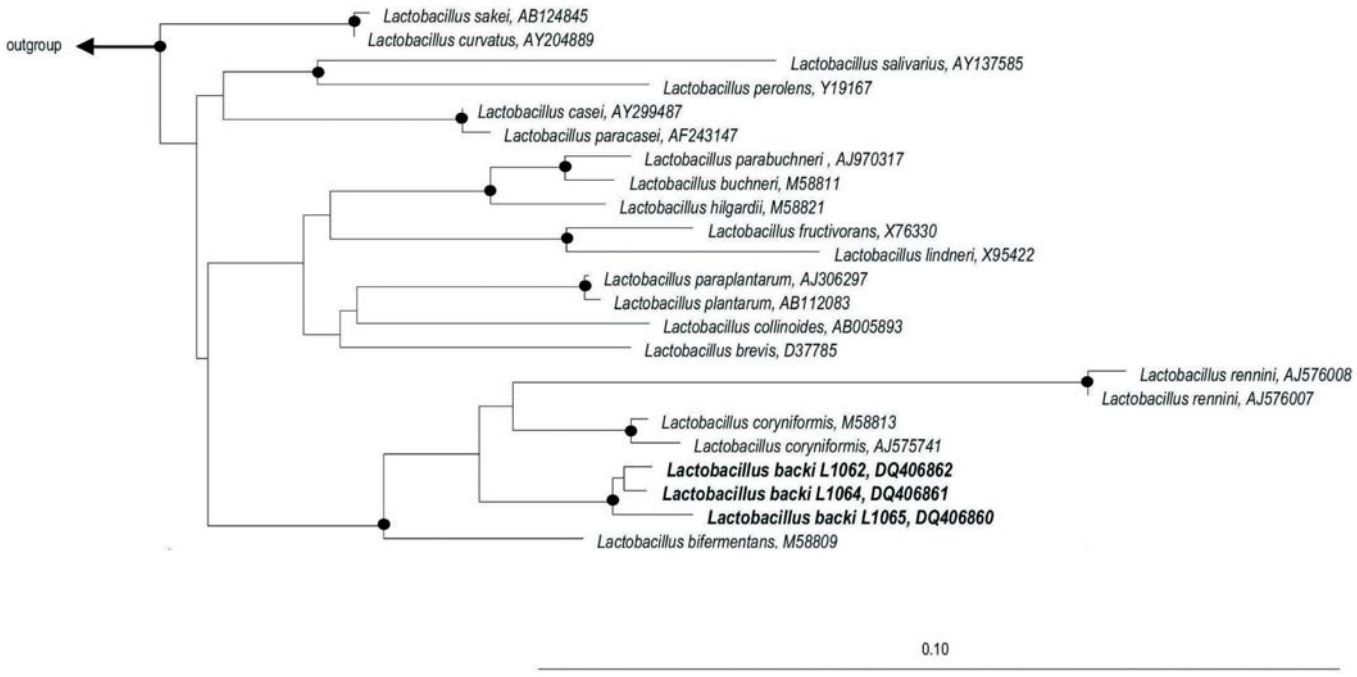

Fig. 1: Phylogenetic tree based on 16S rRNA sequences showing the position of *L. backi* among closely related species of the genus *Lactobacillus*. *Lactococcus lactis* was used as outgroup. The consensus tree topology was determined by maximum-likelihood analysis. Black points on tree nodes symbolize high parsimony bootstrap support above 90% based on 1000 replications. The scale bar indicates 0.1 estimated changes per nucleotide. Sequences retrieved in this study are marked in boldface.

Table 1 Origin of isolated *L. backi* strains

Strain[1]	Source	Year of isolation	Geographic location of the brewery
L1062 DSM 18080^T (LMG 23555^T)	Lager beer	2005	South Bavaria, Germany
L1064	Pils beer	2005	Hesse, Germany
L1065 (LMG 23556)	Lager beer	2005	Northern Italy
L700	Pils beer	1994	Hesse, Germany
L1002	Wheat beer	2002	Northern Bavaria, Germany

[1] DSMZ, Deutsche Sammlung für Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany
LMG, BCCMTM/LMG Bacteria Collection, Laboratorium voor Microbiologie, Gent, Belgium
L: Culture collection of the Lehrstuhl für Technologie der Brauerei I, TU Munich, Freising, Germany

Table 2 Phenotypic and physiological characteristics of *Lactobacillus backi* in comparison to the phylogenetic most closely related species *Lactobacillus coryniformis* and *Lactobacillus rennini*

	Lactobacillus backi					*Lactobacillus coryniformis* ssp. *coryniformis*[1]	*Lactobacillus coryniformis* ssp. *torquens*[1]	*Lactobacillus rennini*[2]
	L1062	L1064	L1065	L700	L1002			
Gas production from:								
Gluconate	–	–	–	–	–	+	+	–
Glucose	–	–	–	–	–	–	–	–
Maltose	–	–	–	–	–	–	–	ND
D,L-Lactate	D(L)	D(L)	D(L)	D(L)	D(L)	D(L)	D	L(D)
Growth at:								
5°C	–	–	–	–	–	ND	ND	–
15°C	+	+	+	+	+	+	+	w
36°C	w	w	w	–	w	+	+	ND
45°C	–	–	–	–	–	–	–	–
Acid production from:								
Amygdaline	–	–	–	–	–	–	–	–
L-Arabinose	–	–	–	–	–	–	–	w
D-Ribose	–	–	–	–	–	–	–	+/w
D-Xylose	–	–	–	–	–	–	–	w
D-Galactose	–	–	–	–	–	+	v	w
D-Mannose	+	+	+	+	+	+	v	+
L-Rhamnose	–	–	–	–	–	+	–	–
D-Sorbitol	w	+	w	+	+	v	–*	–
N-Acetylgluco-samine	ND	ND	ND	ND	ND	+	ND	+
Arbutine	ND	ND	ND	ND	ND	ND	–	–
D-Cellobiose	–	–	–	–	–	–	–	–
D-Melezitose	–	–	–	–	–	–	–	–
Starch	ND	ND	ND	ND	ND	ND	–	–
D-Trehalose	–	–	–	–	–	–	–	–
D-Mannitol	w	+	w	+	+	+	+	+
Lactose	–	–	–	–	–	v	+*	+
Melibiose	–	–	–	–	–	v	–	–
D-Glucose	+	+	+	+	+	+	+	+
Maltose	–	–	–	–	–	+	+*	+
Saccharose	–	–	–	–	–	+	+*	+
D-Fructose	+	+	+	+	+	+	+	+
Salicine	+	+	+	+	+	v	–*	–
Hydrolysation of:								
Aesculin	+	+	+	w	+	v	–	+
Arginin	–	–	–	–	–	–	–	–
Diacetyl formation	–	–	–	–	–	+	+	ND

Symbols:
+, all strains positive; –, all strains negative; w, weakly positive result; v, feature variable; *, exceptions possible; ND, no data

[1]Data of *Lactobacillus coryniformis* ssp. *coryniformis* and *Lactobacillus coryniformis* ssp. *torquens* were obtained by BACK ([1], p. 66).
[2]Data of *Lactobacillus rennini* were obtained by Chenoll et al. [17].