Brytella acorum gen. nov., sp. nov., a novel acetic acid bacterium from sour beverages

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Subject category

Taxonomic descriptions

ABSTRACT

Polyphasic taxonomic and comparative genomic analyses revealed that a series of lambic beer isolates including strain LMG 32668^T and the kombucha isolate LMG 32879 represent a novel species among the acetic acid bacteria, with *Acidomonas methanolica* as the nearest phylogenomic neighbor with a valid name. Overall genomic relatedness indices and phylogenomic and physiological analyses revealed that this novel species was best classified in a novel genus for which we propose the name *Brytella acorum* gen. nov., sp. nov., with LMG 32668^T (= CECT 30723^T) as the type strain. The *B. acorum* genomes encode a complete but modified tricarboxylic acid cycle, and complete pentose phosphate, pyruvate oxidation and gluconeogenesis pathways. The absence of 6-phosphofructokinase which rendered the glycolysis pathway non-functional, and an energy metabolism that included both aerobic respiration and oxidative fermentation are typical metabolic characteristics of acetic acid bacteria. Neither genome encodes nitrogen fixation or nitrate reduction genes, but both genomes encode genes for the biosynthesis of a broad range of amino acids. Antibiotic resistance genes or virulence factors are absent.

Keywords

Brytella; acetic acid bacteria; novel genus; sour beverages; kombucha; lambic beer

Introduction

Acetic acid bacteria (AAB) are Gram-stain-negative or Gram-stain-variable, non-spore-forming, catalase-positive, and predominately oxidase-negative bacteria. Their key defining characteristic is an unusual metabolism that is characterized by the oxidization of ethanol, carbohydrates, and sugar alcohols to produce the corresponding sugar acids or acetic acid, a process executed by primary dehydrogenases located on the periplasmic side of the cytoplasmic membrane (Matsushita et al, 2016). Such oxidation reactions are referred to as 'oxidative fermentation' reactions, because they result in incomplete oxidation of compounds. Incompletely oxidized products accumulate in the culture medium in an early growth phase but can be further assimilated -or overoxidized- in a later growth phase (Komagata et al, 2014; Lynch et al, 2019; Sainz et al, 2016). Not surprisingly, AAB are commonly isolated from sugar-rich environments (Marchetti et al, 2021; Seearunruangchai et al, 2004; Sombolestani et al, 2021). AAB are foremost known for their role in the production of vinegars and many other fermented foods worldwide (De Roos & De Vuyst, 2018; Laureys et al, 2020), yet they are also notorious for their capacity to spoil beer, wine, and non-alcoholic beverages by forming acetic acid, acetaldehyde, or other unwanted metabolites, such as acetoin (De Roos et al, 2018b). More recently, they are increasingly detected in, and isolated from, a range of insect gut microbiomes (Crotti et al, 2010; Guzman et al, 2019; Roh et al, 2008).

Lambic beers are among the oldest types of beers still brewed today and are the products of a spontaneous fermentation process which traditionally mature on oak barrels between one and three years (Bongaerts et al, 2021; Bouchez & De Vuyst, 2022). The sour character of lambic beer originates from the metabolic activities of various yeasts, lactic acid bacteria, and AAB. In the frame of a long-term study on the lambic production process, multiple isolates with indistinguishable MALDI-TOF mass spectra remained unidentified after comparison of their mass spectra with those in a commercial and an *in-house* database (Li et al, 2017). Subsequent whole-genome sequence analysis of a randomly selected isolate revealed that it represented a unique line of descent within the AAB lineage, and that another recent unidentified AAB isolate from a kombucha sample represented the same species. The present study provides a polyphasic characterization of this novel taxon.

Materials and methods

Isolation and ecology

Three production batches of lambic beer were sampled longitudinally in a traditional lambic brewery located in the Senne river valley southwest of Brussels (Belgium), starting in November and December 2019. The three batches were produced following the same recipe

and raw materials, except for the wheat varieties used. Samples were taken from two wooden casks of each batch after 1 h, 24 h, three days, two weeks, three weeks, and ten weeks of fermentation and transported to the laboratory under cold conditions. The samples were then ten-fold serially diluted in 0.9% (m/v) saline and inoculated onto modified deoxycholate–mannitol–sorbitol (mDMS, consisting per liter of 10 g bacteriological peptone, 3 g yeast extract, 5.8 ml lactic acid, 6.33 ml ethanol, 1 g glucose, 1 g sorbitol, 1 g mannitol, 1 g potassium hydrogen phosphate, 0.1 g sodium deoxycholate, 7 g sodium acetate x $3H_2O$, 20 mg magnesium sulphate x 7 H2O, 30 mg bromocresol purple, and 18 g agar; pH 4.5) and acetic acid medium (AAM, consisting per liter of 10 g D-glucose, 15 g bacteriological peptone, 8 g yeast extract, 5 ml ethanol, 3 ml acetic acid, and 18 g agar; pH 3.5) agar media. Furthermore, an enrichment culture was inoculated in enrichment medium 3 (E3, consisting per liter of 20 g mannitol, 5 g bacteriological peptone, and 3 g yeast extract; pH 3.5), and incubated for three days at 28°C under aerobic conditions, after which the enrichment broth was serially diluted and plated on E3 agar as described above. All dilutions were plated in triplicate and inoculated agar plates were incubated for seven days.

In addition, an active ferment sample of a commercial kombucha fermentation (Oudenaarde, Belgium), arising from the aerobic fermentation of sucrose-sweetened green and oolong tea maintained between 22 to 28°C, was filtered by means of a cellulose-acetate membrane (0.45 µm) filter, whereby the membrane was directly placed on Wallerstein Laboratory Nutrient agar (consisting per liter of 4.0 g yeast extract, 5.0 g tryptone, 50 g glucose, 0.55 g potassium dihydrogen phosphate, 0.425 g potassium chloride, 0.125 g calcium chloride, 0.125 g magnesium sulfate, 0.0025 g ferric chloride, 0.0025 g manganese sulfate, 0.022 g bromocresol green, and 15.0 g agar) and incubated for 96 h at 28 °C under aerobic conditions.

MALDI-TOF MS analyses

Following primary isolation, lambic isolates were subcultivated on their respective isolation medium, and sample preparation and data acquisition using a Bruker Microflex™ LT/SH smart instrument (Bruker Daltonik, Bremen, Germany) were performed as previously described (Dumolin et al, 2019). The mass spectra obtained were compared to those in the commercial Bruker library BDAL and *in-house* databases (Li et al, 2017), yielding an identification score. We considered a score value > 2.3 as a highly probable species level identification, whereas samples with lower scores could not be reliably identified at the species level and required further identification steps (Normand et al, 2017). All mass spectra were subsequently dereplicated by means of the SPeDE software (Dumolin et al, 2019).

In the frame of the present taxonomic study, the lambic beer isolates LMG 32668^T, R-83025, R-83056, and R-83282, and the kombucha isolate LMG 32879, along with *Acidomonas methanolica* LMG 1668^T were re-cultivated on AAM agar and third generation subcultures were used to generate biological replicate MALDI-TOF mass spectra as described above. All cell extracts were spotted in duplicate as technical replicates. The profiles were visualized with MBT Compass Explorer 4.1 software (Bruker Daltonik) (Strohalm et al, 2010).

Whole-genome sequence analysis

Genomic DNA of LMG 32668^T, R-83025, R-83056, R-83282, and LMG 32879 was extracted using a Maxwell RSC instrument and the Maxwell RSC Cultured Cells DNA kit (Promega, USA). The resulting DNA was treated with RNase (2 mg ml^{-1}, 5 µl per 100 µl extract) and incubated at 37 °C for 1 h. The DNA integrity was evaluated on a 1.0 % agarose gel and the DNA concentration was quantified by the QuantiFluor ONE dsDNA System (Promega). Draft genomes were sequenced at the MiGS center (Pittsburgh, USA) for the lambic isolates, and at the Oxford Genomics Centre (University of Oxford, United Kingdom) for the kombucha isolate LMG 32879, using the Illumina NextSeq550 (PE150) and NovaSeq 6000 (PE150) platforms, respectively. Quality reports were created with FastQC version 0.11.8. Prior to assembly, reads were trimmed (Phred score >Q30) and filtered (length >50 bp) with fastp 0.20.0 (Chen et al, 2018) with the correction option enabled. The assembly was performed with Shovill version 1.1.0 (https://github.com/tseemann/shovill), with SPAdes genome assembler 3.14.0 (Prjibelski et al, 2020) at its core with read error correction disabled and default settings. Contigs shorter than 500 bp were removed from the final assembly. Quality of the final assembly was verified with the Quality Assessment Tool for Genome Analysis (QUAST) (Gurevich et al, 2013). Finally, the assemblies were checked for completeness and contamination using CheckM version 1.1.2 (Parks et al, 2015) and *Acetobacteraceae* as the marker lineage.

To verify taxonomy, genomes were submitted to the Type Strain Genome Server (TYGS) (Meier-Kolthoff & Goker, 2019), and digital DNA-DNA hybridization (dDDH, formula d4) values were calculated using the Genome-to-Genome Distance Calculator (GGDC 2.1). The average nucleotide identity (ANI) values were calculated by using OrthoANIu software (Yoon et al, 2017b). The average amino acid identity (AAI) values were calculated using the EzAAI pipeline (Kim et al, 2021). The percentage of conserved protein (POCP) values were calculated using pocp (https://github.com/hoelzer/pocp) (Qin et al, 2014).

The whole-genome sequence of strains LMG 32668^T and LMG 32879, and of type or reference strains of type species of other AAB genera were used to construct a phylogenomic tree based on the analysis of 107 single-copy core genes using BcgTree (Ankenbrand & Keller, 2016) with

Tanticharoenia sakaeratensis NBRC 103193^T as an outgroup. Visualization and annotation of the tree were performed using iTOL (Letunic & Bork, 2016). For completeness, a complete 16S rRNA gene sequence was extracted from the strain LMG 32668^T draft genome using the BAsic Rapid Ribosomal RNA Predictor software (Barrnap) (Seemann, 2018) and was submitted to the EzBiocloud identification service (Yoon et al, 2017a) to determine the closest phylogenomic neighbors.

Gene calling and basic annotation of the LMG 32668^T and LMG 32879 genomes was done using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) (Tatusova et al, 2016) and EggNOG-mapper v2.1.8 (Cantalapiedra et al, 2021). The EggNOG database v5.0.2 was used for functional annotation, i.e. assigning EC numbers, KEGG identifiers, gene names and descriptions. The KEGG annotation was used for the calculation of the KEGG module completeness fraction (mcf) by using the KO_mapper script from MicrobeAnnotator (Ruiz-Perez et al, 2021). KEGG mapper (Kanehisa & Sato, 2020) was used to reconstruct and visualize individual KEGG pathways and modules. In some cases, blastp or tblastn were used to verify absence of proteins. ABRicate v1.0.1 (https://github.com/tseemann/abricate) was used to detect antibiotic resistance and virulence genes by screening against ncbi, vfdb, card, resfinder and plasmidfinder databases. AntiSMASH v6.1.1 was used to detect secondary metabolites using the GenBank files produced by PGAP as input (Blin et al, 2021).

Finally, an analysis was conducted using Protologger (Hitch, 2021) to assess the occurrence of 16S rRNA gene sequences with high identity values with strain LMG 32668^T in 19,000 amplicon sequencing data sets and a collection of >49,000 metagenome-assembled genomes corresponding to different habitats (Lagkouvardos et al, 2016).

Metagenetics

To provide more information on the relative abundance of the novel taxon in the lambic beer microbial ecosystem, amplicon-based high-throughput sequencing was applied targeting the full-length 16S rRNA gene as described earlier (Decadt et al, 2023). More precisely, two fermenting beer samples were considered in the current study, namely a 1-h and 24-h sample taken from batch 2, cask 2. Briefly, after a total DNA extraction on the samples, the 16S rRNA gene was amplified using the primers 27F (AGRGTTYGATYMTGGCTCAG) and 1492R (RGYTACCTTACGACTT) (Integrated DNA technologies, Leuven, Belgium) and the KAPA HiFi DNA Polymerase (Hot Start and Ready Mix formulation; Roche, Basel, Switzerland). The primers were tagged with 5' sample-specific barcodes to allow for multiplexed sequencing, according to the manufacturer's instructions (PacBio, Menlo Park, California, USA). Next, the length distribution and concentration of the amplicons were checked using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) and a Qubit 2.0 fluorometer (Thermo Fisher

Scientific, Waltham, MA, USA), respectively. After equimolar pooling of the amplicon sets and ligation of circular sequencing adaptors, the library was sequenced using a PacBio Sequel system (PacBio, Menlo Park, CA, USA) in circular consensus mode in a commercial facility (VIB Nucleomics Core Facility, Leuven, Belgium). The obtained sequences were then clustered into amplicon sequence variants (ASVs) using the DADA2 R software package (version 1.14.1) (Callahan et al, 2019). Finally, the taxonomy of each ASV was assigned using the SILVA database (version 138) (Quast et al, 2013) to which the 16S rRNA gene sequence of the novel species was manually added.

Physiology

Physiological characteristics were analyzed in triplicate and included Gram-stain reaction, catalase and oxidase activity, growth on different carbon sources (i.e. 0.3% [m/v] D-fructose, D-glucose, D-mannitol, D-sorbitol, dulcitol, ethanol, glycerol, maltose, methanol, and sucrose), ketogenesis from glycerol, growth in 30% D-glucose, and production of 2-keto-D-gluconic acid and 5-keto-D-gluconic acid, as described before (Sombolestani et al, 2020). Cell morphology was examined using a light microscope (BX41, Olympus) after growth on AAM agar at 28 °C under aerobic conditions for 72 h. The hanging drop method was used to evaluate motility.

Results and discussion

Taxonomy and habitat

Lambic beer isolates were selected from agar media corresponding to the lowest possible dilutions with well-separated colonies to increase the probability to pick numerically less abundant AAB. At each sampling moment about 10% of well-separated colonies were picked from the three AAB isolation media, which yielded a total of 903 isolates (data not shown). All isolates were re-streaked at least twice to check purity and were preserved in 15% (v/v) glycerol at -80°C until further analysis.

MALDI-TOF MS-based dereplication of all isolates yielded numerous operational isolation units (Dumolin et al, 2019) (data not shown), including one that comprised spectra of 12 indistinguishable isolates: LMG 32668^{T} was isolated after 24 h of fermentation from batch 1, cask 1; LB528 was isolated after three weeks of fermentation from batch 1, cask 1; R-83056 was isolated after 1 h of fermentation from batch 1, cask 2; LB211 and LB520 were isolated after 24 h of fermentation from batch 2, cask 1; R-83025, LB297 and LB299 were isolated after 1 h of fermentation from batch 2, cask 2; R-83282 and LB276 were isolated after 24 h of fermentation from batch 2, cask 2; LB356 was isolated after three days of fermentation from batch 2, cask 2;

and LB519 was isolated after three days of fermentation from batch 3, cask 2. All isolates were selected from AAM isolation media, except for LB519 that was picked from an mDMS agar medium. The spectra of all isolates remained unidentified (identification scores below 1.70) when compared to the Bruker BDAL MSP-6309 and *in-house* databases.

Four lambic isolates, i.e., LMG 32668^T, R-83025, R-83056, and R-83282, were randomly selected for draft genome sequence analysis to perform taxonomic and phylogenomic analyses. OrthoANIu and dDDH analyses were performed between each pair of genomes and revealed values of >99.99% and 100%, respectively, suggesting all four isolates were clonal derivatives of a single strain that was universally present in each of the casks examined in this lambic brewery.

Upon comparison of the LMG 32668^T genome sequence with other unclassified AAB in our research group, the kombucha-derived strain LMG 32879 shared high OrthoANIu (99.77%) and dDDH (98.0%) values with strain LMG 32668^T, which demonstrated it represented the same species. Strain LMG 32879 had been picked as a single colony from a Wallerstein Laboratory Nutrient agar medium and was subcultivated on TSA (Oxoid) at 28°C under aerobic conditions. Its MALDI-TOF mass spectra too remained unidentified when compared to the Bruker BDAL MSP-6309 and *in house* databases, but subsequently showed a high similarity with the MALDI-TOF mass spectrum of LMG 32668^T upon adding the MALDI-TOF mass spectrum of the latter as a new entry to the database (log score 2.480). OrthoANIu and dDDH values of strains LMG 32668^T and LMG 32879 towards all established AAB species were below the species delineation thresholds (Table 1; data not shown), demonstrating that they represented a single novel species (Meier-Kolthoff et al, 2013; Yoon et al, 2017b).

The LMG 32668^T draft genome contained 3,321,354 bases in 42 contigs and had a percentage G+C content of 60.82% G+C. The N50 (kb), the number of coding DNA sequences and coding density were 407,188 bp, 2,952 and 88.9%, respectively. CheckM analysis revealed that the genome assembly was 99.69% complete and contamination was very low (0.60%). The LMG 32879 draft genome contained 3,523,780 bases in 85 contigs and had a percentage G+C content of 60.62% G+C. The N50 (kb), the number of coding DNA sequences and coding density were 150,828 bp, 3,185 and 90.4%, respectively. CheckM analysis revealed that the genome assembly was essentially complete (99.68%) and contamination was very low (1.10%).

A BcgTree analysis revealed that strains LMG 32668^T and LMG 32879 represented a novel lineage within the *Acetobacteraceae* with *Acidomonas methanolica* DSM 5432^T as nearest phylogenomic neighbor among the present AAB with valid names (Figure 1). The degree of divergence was considerably larger than that observed between other closely related AAB genera, such as *Swaminathania, Acidicaldus,* and *Asaia*, or between *Gluconacetobacter* and

Nguyenibacter (Figure 1). ANI and AAI analyses also identified *Ac. methanolica* DSM 5432^T as nearest phylogenomic neighbor (80.71% OrthoANIu and 73.57% AAI) (Table 1; data not shown). Similarly, percentage of conserved protein analysis identified *Ac. methanolica* DSM 5432^T as nearest neighbor organism (POCP value of 70.04%). In contrast, comparison of the complete 16S rRNA gene sequence extracted from the strain LMG 32668^T draft genome yielded *Ameyamaea chiangmaiensis* AC04^T as nearest neighbor organism (97.94% sequence identity) (Table 1; data not shown). The percentage 16S rRNA gene sequence identity towards *Ac. methanolica* DSM 5432^T was 95.88%.

Figure 2 presents the MALDI-TOF mass spectra of the lambic isolates LMG 32668^T, R-83025, R-83056, and R-83282, and the kombucha strain LMG 32879, along with *Ac. methanolica* LMG 1668^T as reference. As expected from an analytical tool that provides mainly species level differentiation, the mass spectra of LMG 32668^T, R-83025, R-83056, R-83282, and LMG 32879 were highly similar and clearly distinct from that of *Ac. methanolica* LMG 1668^T.

The use of Protologger revealed no metagenome-assembled genomes matching the query genomes. Operational taxonomic unit sequences present in public 16S rRNA gene amplicon sequence datasets matching (i.e. at least 97% sequence identity, 80% sequence coverage) the LMG 32668^T 16S rRNA gene sequence occurred at low percentages in insect gut, human skin, wastewater, plant and freshwater metagenome data sets (2.7%, 2.5%, 2.5%, 1.6%, and 1.6%, respectively) but consistently at very low mean relative abundances (1.53%, 0.26%, 0.18%, 0.15%, and 0.05%, respectively).

Microbial ecosystem analysis of two lambic beer fermentation samples relying on metagenetics targeting the full-length 16S rRNA gene revealed that the novel taxon was present in low abundances: in the samples of batch 2, cask 2 after 1 h and 24 h of fermentation, a total of 21,625 and 14,714 sequence reads were obtained, resulting in 245 and 177 ASVs, respectively. Most of these ASVs were assigned to enterobacteria (data not shown). In both samples, *Enterobacter* and *Raoultella* were the two most prevailing genera, with a combined relative abundance of 53.0 % after 1 h of fermentation and 27.7 % after 24 h of fermentation. In both samples, also one ASV was assigned to the novel taxon represented by strain LMG 32668^T. However, only 0.1 % of the total number of reads in both samples belonged to this ASV.

Genome features and metabolism

All genes for a modified tricarboxylic acid cycle were present in both strains LMG 32668^T and LMG 32879, and included succinyl-CoA:acetate CoA-transferase (EC 2.8.3.18) and malate:quinone oxidoreductase (EC 1.1.5.4) (Pelicaen et al, 2019), which confers acetic acid

resistance to AAB (Mullins et al, 2008). Also, the pentose phosphate, pyruvate oxidation, and gluconeogenesis pathways were complete. Neither strain encoded 6-phosphofructokinase, which rendered the glycolysis pathway non-functional. The Entner–Doudoroff pathway was absent. The glycogen biosynthesis pathway was present, but pathways for pectin, galacturonate, gluconate, and glucuronate degradation and trehalose biosynthesis were incomplete.

The basic aerobic respiration system of AAB consists of a membrane-bound transhydrogenase, an NADH:ubiquinone oxidoreductase, cytochrome *bo*3 ubiquinol oxidase, and cytochrome *bd* quinol oxidase (Lynch et al, 2019). Both strains had a complete set of genes for transhydrogenase, NADH:quinone oxidoreductase, succinate dehydrogenase, cytochrome *bo3* ubiquinol oxidase, cytochrome *bd* ubiquinol oxidase, and F-type ATPase. In addition, AAB have another metabolic option, referred to as oxidative fermentation, that is characterized by a rapid oxidization of substrates (typically carbohydrates, sugar alcohols, and ethanol) to the corresponding sugar acids or acetic acid. This incomplete oxidation of substrates is executed by primary dehydrogenases located on the periplasmic side of the cytoplasmic membrane and leads to a rapid acidification of the immediate cell environment, thus providing a competitive advantage (Lynch et al, 2019). Both strains encoded a range of dehydrogenase/reductase genes, which included among others alcohol dehydrogenases (EC 1.1.1.1, EC 1.1.1.2, and EC 1.1.5.5), aldehyde dehydrogenase (EC 1.2.1.3), D-lactate dehydrogenase (EC 1.1.2.4, but not EC 1.1.2.5), L-lactate dehydrogenase (EC 1.1.2.3), malate dehydrogenases (EC 1.1.5.4 and EC 1.1.1.38), UDP-glucose 6-dehydrogenase (EC 1.1.1.22), ribitol 2-dehydrogenase (EC 1.1.1.56), dihydroorotate dehydrogenase (EC 1.3.5.2), D-xylulose reductase (EC 1.1.1.9), and sorbose reductase (EC 1.1.1.289).

Neither strain encoded nitrogen fixation or nitrate reduction genes. Both strains had a full set of genes for the biosynthesis of arginine, histidine, lysine, ornithine, cysteine, proline, threonine, serine, isoleucine, valine, leucine, tryptophan, and spermidine, whereas the biosynthesis of putrescine was only complete in strain LMG 32668^{T}. Yet, whereas strain LMG 32879 lacked arginase, it could convert ornithine into putrescine. In addition, the biosynthesis of methionine (likely synthesized via MetZ instead of EC 2.5.1.48 [MetB]), tyrosine (likely synthesized via EC 2.6.1.1 and/or EC 2.6.1.9 instead of EC 2.6.1.57) and phenylalanine (likely synthesized via EC 2.6.1.1 and/or EC 2.6.1.9 instead of EC 2.6.1.57) likely proceeds through different reactions compared to those registered in the corresponding KEGG modules.

ABRicate analysis revealed no antibiotic resistance genes or virulence factors. AntiSMASH predicted a total of six secondary metabolite gene clusters in strain LMG 32668^{T} (data not shown). These included type terpene, arylpolyene, redox-cofactor, RiPP-like (i.e. another unspecified ribosomally synthesized and post-translationally modified peptide), and NRPS

(i.e. a non-ribosomal peptide synthetase) gene clusters. Strain LMG 32879 encoded another RiPP-like cluster and a type resorcinol as two additional secondary metabolite gene clusters compared to the six clusters that were predicted in strain LMG 32668^T.

Conclusion

We performed polyphasic taxonomic and comparative genomic analyses, which revealed that strains LMG 32668^T and LMG 32879 represented a new species within the AAB, which was present in very low relative abundances in source samples. BcgTree, average nucleotide identity, and average amino acid identity analyses identified *Ac. methanolica* DSM 5432^T as nearest phylogenomic neighbor, with orthoANIu, AAI, and percentage of conserved protein values above proposed genus level delineation thresholds (Goris et al, 2007; Konstantinidis & Tiedje, 2005), yet 16S rRNA gene sequence analysis identified *Am. chiangmaiensis* AC04^T as nearest neighbor organism, again with a percentage identity value above proposed genus level delineation thresholds (Qin et al, 2014; Yarza et al, 2014) (Table 1). Unlike the species delineation threshold for which a general consensus exists, there are no sharp genus delineation boundaries that are broadly applied in bacterial taxonomy when using ANI, AAI, or POCP values (Rodriguez-R et al, 2018; Wirth & Whitman, 2018), and not unexpectedly, delineation of taxonomic ranks across the tree of life is uneven (Parks et al, 2018). *Ac. methanolica* and the species represented by strains LMG 32668^T and LMG 32879 differ in genomic percentage G+C content (64.4 *vs*. 60.82, respectively) and the use of multiple single carbon sources for growth: the former utilizes methanol and ethanol, but not D-fructose, D-sorbitol, or D-mannitol as sole carbon sources for growth, while the latter does not utilize methanol and ethanol, and utilizes D-fructose, D-sorbitol, and D-mannitol as sole carbon sources for growth) (Urakami et al, 1989). Finally, *Ac. methanolica* is oxidase positive, an usual feature among AAB, while oxidase activity of both strains LMG 32668^T and LMG 32879 was weak and delayed after cultivation on AAM for 4d at 28°C, and negative after cultivation on medium M404 (containing per liter 50 g glucose, 10 g yeast extract, and 15 g agar). Given their position in the phylogenomic tree (Figure 1) and their physiological differences, and in analogy with the classification of several other AAB genera we propose to classify the new species represented by strains LMG 32668^T and LMG 32879 in a novel AAB genus and species for which we propose the name *Brytella acorum* gen. nov., sp. nov. below (Table 1).

Like in most AAB, the *B. acorum* energy metabolism is driven by both aerobic respiration and oxidative fermentation. Like some other AAB, *B. acorum* strains LMG 32668^T and LMG 32879 have a modified tricarboxylic acid cycle that confers acetic acid resistance. It is unclear how aerobic respiration and oxidative fermentation, or more precisely, cytosolic and periplasmic

dehydrogenases partition the use of reduced electron carriers to generate energy, but environmental pH, oxygen availability, and life cycle status may be involved (Deppenmeier & Ehrenreich, 2009; Lynch et al, 2019). 16S rRNA gene sequences highly similar to those of *B. acorum* strains were present at low percentages in several host-associated and environmental metagenome data sets, albeit at very low mean relative abundances. We collected most isolates of the present study from AAM agar media, which is a very commonly used primary isolation medium for AAB (De Roos et al, 2018a; De Roos et al, 2018b; Spitaels et al, 2015a). The absence of *Brytella* isolates in earlier studies of the lambic beer production process (De Roos et al, 2018b; Spitaels et al, 2014; Spitaels et al, 2015b), the sporadic isolation of small numbers of isolates at different sampling moments and the low read numbers detected at two sampling points of batch 2, cask 2, all suggest that only the analysis of a sufficiently large number of isolates in the frame of high-throughput cultivation efforts facilitated the isolation of *B. acorum,* which confirms that standard plating techniques have not yet been fully deployed for the cultivation of the so-called 'uncultivated majority' (Browne et al, 2016; Lewis et al, 2021).

Table 1. Pairwise OrthoANIu, AAI, dDDH, POCP, and 16S rRNA sequence identity values (%) between *B. acorum* LMG 32668^T, and *B. acorum* strain LMG 32879 and type strains of near neighbour taxa.

Strain	**OrthoANIu**	**AAI**	**dDDH**	**POCP**	**16S rRNA**
B. acorum LMG 32879	99.77	99.99	98.0	91.34	100
Ac. methanolica DSM 5432^T	80.71	73.57	24.8	70.04	96.03
Am. chiangmaiensis AC04^T	71.96	66.60	22.4	63.11	97.94

380 **Table 2.** Description of *Brytella* gen. nov. and *Brytella acorum* sp. nov.

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Guiding Code for Nomenclature [req]	ICNP	ICNP
Nature of the type material [req]	Species	Strain
Genus name [req]	*Brytella*	
Species name [req]		*Brytella acorum*
Genus status [req]	gen. nov.	
Genus etymology [req]	Bry.tel'la Gr. n. *brytos,* beer; N.L. fem. dim. n. *Brytella*, from beer, i.e. named after the source of the first isolates of this genus	
Type species of the genus [req]	*Brytella acorum*	
Specific epithet [req]		*acorum*
Species status [req]		sp. nov.
Species etymology [req]		a.co'rum. L. gen. pl. n. *acorum*, of sour substances
Designation of the Type Strain [req under ICNP]		LMG 32668^{T}
Strain Collection Numbers [req under ICNP]		LMG 32668^{T} = CECT 30723^{T}
Designated Genome, MAG or SAG [req under SeqCode]		JARBJP000000000

Genome status [opt]		Incomplete
Genome size [opt]		3.32 Mbp
GC mol% [opt]		60.82
16S rRNA gene accession nr. [req]		ON876751
Description of the new taxon and diagnostic traits [req]	Cells are Gram-negative rods. Oxidase-variable. Catalase-positive. No ketogenesis from glycerol. Growth on D-glucose. Production of 5-keto-D-gluconic acid from glucose. The genome sequence of the type strain of the type species revealed ubiquinones as major respiratory quinone.	Cells are Gram-negative non-motile straight rods, approximately 0.8 µm wide and 1.8-2.5 µm long, appear in single form, in pairs or chains. Beige, round, slightly raised, and opaque colonies with an approximate size of 1 mm in diameter after growing on agar medium AAM 3 at 28 °C for 3 days. Oxidase-delayed weakly positive (i.e. after about 1 min.) after cultivation on AAM for 4d at 28°C, but negative after cultivation on medium M404 (containing per liter 50 g glucose, 10 g yeast extract, and 15 g agar). Catalase-positive. No ketogenesis from glycerol. Growth in the presence of 30% D-glucose. Brown pigmentation. Growth on D-glucose. Growth on D-fructose, D-mannitol, D-sorbitol, and glycerol, but not dulcitol, maltose, ethanol, and methanol. Production of 5-keto-D-gluconic acid from glucose, but no 2-keto-D-gluconic acid.
Country of origin [opt]		Belgium
Region of origin [opt]		Pajottenland
Date of isolation (dd/mm/yyyy) [opt]		2019
Source of isolation [opt]		Lambic beer production

Sampling date (dd/mm/yyyy) [opt]		20/11/2019
Latitude (xx°xx'xx"N/S) [opt]		-
Longitude (xx°xx'xx"E/W) [opt]		-
Number of strains in study [opt]		>10 isolates; two genomically distinct strains
Information related to the Nagoya Protocol [req]		Flanders gives free access to genetic resources

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Nucleotide sequence accession numbers

The 16S rRNA gene and whole-genome sequences of strain LMG 32668^T have been deposited at DDBJ/ENA/GenBank in BioProject PRJNA853562 under the accession numbers ON876751 and JARBJP000000000, respectively. The 16S rRNA gene and whole-genome sequence of strain LMG 32879 has been deposited at DDBJ/ENA/GenBank in BioProject PRJEB58032 under the accession numbers OX437062 and GCA_949775715.1. The metagenetics data sets have been deposited at DDBJ/ENA/GenBank in BioProject PRJEB62700 under the accession numbers ERR11512390 and ERR11512391.

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Author Contributions

Atena Sadat Sombolestani: Investigation, Methodology, Software, Formal Analysis, Visualization, Project Administration, Writing-review & editing; **Dries Bongaerts:** Investigation, Methodology, Writing-review & editing; **Eliza Depoorter:** Investigation, Methodology, Software, Formal Analysis, Data Curation, Visualization, Writing-Original Draft; **Ilse Cleenwerck:** Investigation, Methodology, Software, Formal Analysis, Writing-review & editing; **Anneleen D. Wieme:** Investigation, Methodology, Software, Formal Analysis, Writing-review & editing; **Scott J. Britton:** Investigation, Methodology, Writing-review & editing; **Stefan Weckx**: Methodology, Writing-review & editing; **Luc De Vuyst:** Supervision, Project Administration, Funding Acquisition, Writing-review & editing; and **Peter Vandamme:** Conceptualization, Methodology, Writing-Original draft, Supervision, Project Administration, Funding Acquisition.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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603 Figure Legends

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605 **Figure 1.** Phylogenomic tree based on 107 single-copy core genes showing the phylogenetic
606 relationships of *Brytella* gen. nov. among type or reference strains of validly named genera in
607 *Acetobacteraceae* family. The sequence accession numbers are given between parentheses.
608 Bootstrap percentages (≥70 %) are shown next to the branch points. Bar, 0.1 changes per
609 nucleotide position. The tree topology was statistically analyzed using 1000 bootstrapping
610 replications.

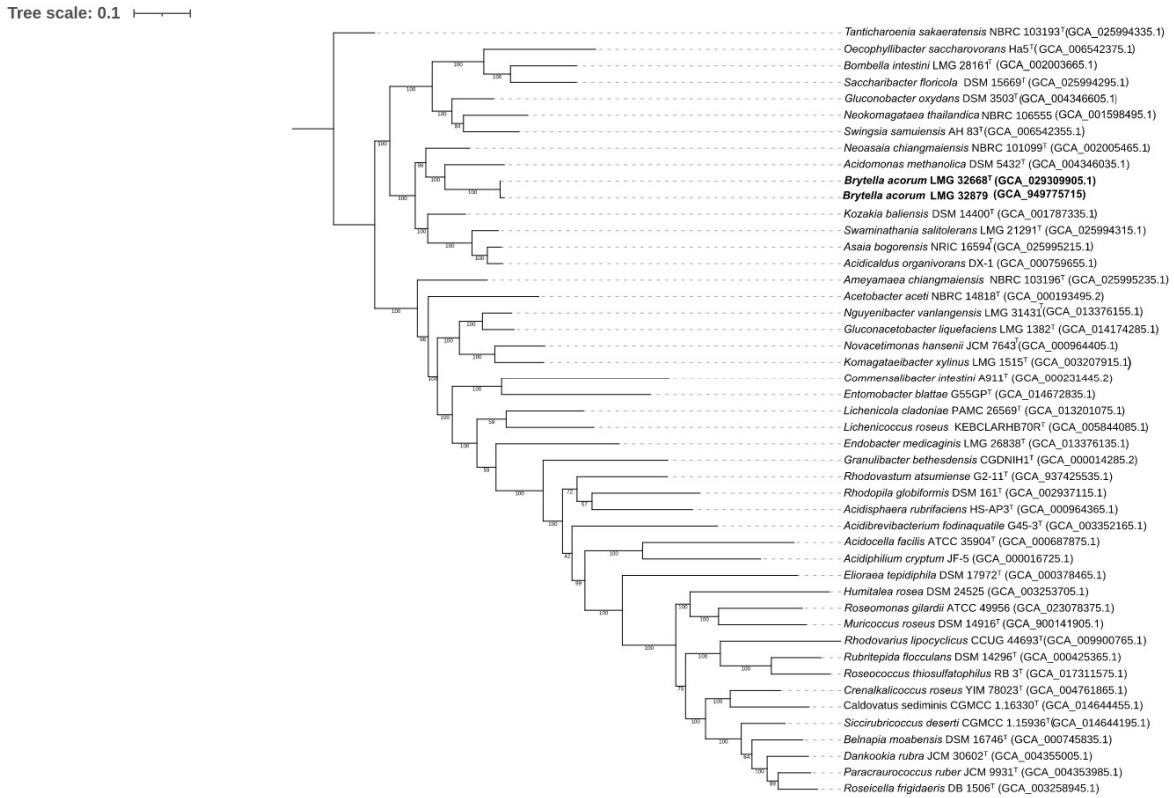

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Figure 2. MALDI-TOF mass spectra of the lambic beer isolates (1) LMG 32668^T, (2) R-83025, (3) R-83056, and (4) R-83282, (5) the kombucha isolate LMG 32879, and (6) *Ac. methanolica* LMG 1668^T.