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Research article

Genetic diversity of exopolysaccharides from acetic acid bacteria isolates originating from apple cider vinegars

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Abstract

Acetic acid bacteria (AAB) produce acetic acid but are also gaining importance as safe microorganisms for producing extracellular polysaccharides (EPSs). The best-known homopolysaccharides among them are cellulose and levan. In addition, acetic acid bacteria also produce heteropolysaccharides, water-soluble acetans. Isolates from the broth of organic and conventional apple cider vinegar production were screened for biofilm production. Phenotypic and genomic diversity of EPS-producing isolates was assessed. The diversity of phenotypically different EPSs of apple cider vinegar isolates was investigated at the gene level for the following novel strains: *Komagataeibacter* (*K.*) *melomenusus* SI3083, *K. oboediens* SI3053, *K. pomaceti* SI3133, and *Gluconacetobacter* (*Ga.*) *entanii* SI2084. Strain *K. melomenusus* SI3083 possesses cellulose operons *bcs1*, *bcs2*, and *bcs4* together with the type I acetan cluster in the absence of the levan operon, strain *K. oboediens* SI3053 has the operons *bcs1*, *bcs2*, *bcs3*, and *bcs4*, the levan operon, and the acetan cluster (type I), and the strains *K. pomaceti* SI3133 and *Ga. entanii* SI2084 both contain recently described novel ace-type II cluster in addition to the incomplete operon *bcs1*. A comparison of the genetic diversity of these EPSs to those of the reference strains suggests that the studied EPSs are not species-descriptive. The results of this study deepen our understanding of the genetic variability of the EPS genes in AAB, thereby enabling us to better characterize and exploit the various insoluble and soluble exopolysaccharides produced by AAB for biotechnological applications in the future.

Keywords: Acetic acid bacteria genomes, Apple cider vinegar microbiota, Biofilm production, Bacterial cellulose, Acetan, *Acetobacter*, *Komagataeibacter*

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Introduction

Exopolysaccharides (EPSs) are produced by various organisms from all taxonomic domains. In particular, several microorganisms have been identified as microbial EPS producers. EPSs serve various biological functions, such as adhesion of cells to surfaces, gliding motility, mitigation of desiccation, and biofilm formation (Knirel and Van Calsteren, 2021). The overwhelming structural diversity of bacterial exopolysaccharides originates from their homopolysaccharide and heteropolysaccharide chemical composition. Homopolysaccharides contain only one type of monomer, and the most common homopolymers are glucans (Moradali and Rehm, 2020).

On the other hand, heteropolysaccharides are composed of different monomers and are complex with branched structures and therefore have interesting physicochemical and rheological properties. The production of exopolysaccharides can be strain-specific, and some bacteria can simultaneously produce different EPS types (Moradali and Rehm, 2020; Knirel and Van Calsteren, 2021). The diverse viscoelastic properties of non-chemically synthesized exopolysaccharides appeal to modern consumers with high environmental and health consciousness.

The diversity of current and potential future applications ranges from the food industry (thickeners, stabilizers, antimicrobial bio-based materials) to personal care products and the medical and pharmaceutical industries (Hussain et al., 2017; Mohd Nadzir et al., 2021). The most common EPSs with homopolymer structures are cellulose (Römling and Galperin, 2015), levan (Öner et al., 2016), and curdlan (Chen and Wang, 2020); heteropolymer structures have alginic acid, gellan, xanthan, dextran. Numerous applications have been identified for several decades, but few have been commercialized because modern production costs exceed market value. However, as far as human applications are concerned, EPS producers must comply with the status GRAS (generally recognized as safe), as is

the case with acetic acid bacteria, so further research is not compromised.

Acetic acid bacteria have long been used in human nutrition because of their ability to oxidize ethanol to acetic acid in the presence of oxygen, making them the most important vinegar producers (Trček et al., 2015, 2016). We have recently begun to investigate the polysaccharides that these bacteria secrete in the environment, forming a protective layer around the cells. Floating biofilms also allow acetic acid bacteria better access to oxygen and thus faster growth (Augimeri et al., 2015; Trček and Barja, 2015). Acetic acid bacteria produce homopolysaccharides (cellulose and levan) and heteropolysaccharides (acetan and acetan-like polysaccharides) (Dutta and Gachhui, 2006; Deppenmeier and Ehrenreich, 2009).

Most of the attention in molecular analysis of genes and/or operons required for exopolysaccharide synthesis in acetic acid bacteria has been devoted to elucidating cellulose and levan synthesis, whereas acetan synthesis is poorly understood. Recently, we are also gaining knowledge about various acetan-like polysaccharides and the possibility of obtaining new EPSs with new potentials. A specific (industrial) environment where a high diversity of acetic acid bacteria is expected (Štornik et al., 2016; Coton et al., 2017; Nie et al., 2017; Gaggìa et al., 2018) and represents a source of diverse EPS producers.

This work aimed to characterize novel isolates of already known acetic acid bacteria species from a large-scale apple cider vinegar production as EPS producers and their genetic diversity for EPS production.

Material and Methods

Sampling vinegar and isolation of microorganisms

Two apple cider vinegar production broths of industrial submerged bioreactors (a 500-liter bioreactor contained organic apple cider vinegar and a 1000-liter bioreactor contained conventional apple cider substrate) at the vinegar production facility plant of Simonič Farm (Zgornja Ščavnica, Slovenia) were sampled in November 2020. In the 500-liter submerged bioreactor, exclusively used for the production of organic apple cider vinegar, the substrate for organic apple cider vinegar production was obtained from apples harvested from orchards with organic certification.

The apples used to produce organic apple cider vinegar were traditional old Slovenian apple varieties such as bobovec, krivopecelj, and štajerski mošancelj, which are organically grown and subject to strict restrictions on the selection of additives and auxiliaries that may be used. Conventional apple cider vinegar was produced in another 1000-liter acetator. At both sampling points during the oxidation cycle, multiple dilutions of the unfiltered vinegar were immediately aseptically streaked onto the reinforced acetic acid and ethanol (RAE) medium and aerobically incubated for 3 days at 30°C and 100% relative humidity (Sokollek et al., 1998; Štornik et al., 2016). Twenty colonies from each sample's countable plates were randomly selected and streaked at least three times on the same type of media to obtain pure cultures. Morphologically different colonies were preserved in an RAE medium containing 20% glycerol at –80°C.

Analyses of acetic acid and ethanol

The acidity of the sample was determined by titrating the samples with 0.1 M NaOH in the presence of phenolphthalein. The acetic acid in vinegar accounts for 98% of acids (Sanarico et al., 2003); therefore, total acidity can be used as a measure of acetic acid concentration. At the time of sampling, the acetic acid concentration was 33 g/L for the organic apple cider vinegar broth and 28 g/L for the conventional apple cider vinegar.

Phenotypic analysis

The selected strains were cultured in 10 mL test tubes filled with 3 mL RAE media at 30°C under static conditions for 5 days. We examined the biofilm morphology directly in the test tube under a Leica M205C stereo microscope with a Leica MC 190 HD camera and processed them with LasX software. Microscopic images of the test tubes under a 45° angle were taken with a Nikon SMZ800 stereo microscope with a Nikon DS-Fi2 digital camera and processed with NIS -Elements D 4.20 software.

Growth in the presence of different concentrations of ethanol and acetic acid was tested in liquid RAE medium containing 1% acetic acid and 1.0, 3.0, 6.0, 9.0 and 12.0% ethanol or RAE medium containing 1% ethanol and 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0% acetic acid. The cultures were incubated at 180 rpm and 30°C for 7 days.

Taxonomic analysis

For identification, isolates were revitalized from –80°C on an RAE medium containing 1% ethanol and 1% acetic acid. A single colony from each culture was inoculated on the same medium and grown for 3 days to obtain sufficient biomass for DNA isolation. After harvesting the biomass, DNA was isolated using the GeneJET Genomic DNA Purification Kit (Thermo Scientific, Waltham, MA, USA). The regions of the 16S-23S rRNA gene ITS were amplified with the primers SpaFw (5'-TGCGG(T/C)TGGATCACCTCCT-3') and SpaRev (5'-GTGCC(A/T)AGGCATCCACCG-3') (Trcek and Teuber, 2002).

The PCR products were purified using the GenJet PCR Purification Kit (Thermo Scientific, Waltham, Massachusetts, USA). PCR products were sequenced using the Sanger method at Microsynth (Vienna, Austria). For more detailed analysis, genomic sequencing of the selected strains *K. pomaceti* SI3133, *K. oboediens* SI3053, *K. melomenusus* SI3083, and *Ga. entanii* SI2084 was carried out.

Genome sequencing, assembly, and annotation

Genomic DNA was extracted using the NucleoSpin Tissue Kit (Macherey-Nagel, Dueren, Germany) according to the manufacturer's protocol. Whole-genome sequencing analysis was performed at the Department for

Microbiology, Faculty of Medicine, University of Maribor. First, paired-end libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocol.

Sequencing was performed using Illumina MiSeq (2x300 cycles) (Illumina, San Diego, California, USA). Fastq reads were quality checked and trimmed using Trimmomatic, followed by genome assembly using SPAdes (v. 3.11.1). These Whole Genome Shotgun projects have been deposited at DDBJ/EMBL/GeneBank under BioProject accession number PRJNA739075. The assemblies of the other genomes described in this study were downloaded from NCBI (https://www.ncbi.nlm.nih.gov/, accessed October 2021).

Bioinformatics

For each sequenced genome of *K. pomaceti* SI3133, *K. oboediens* SI3053, *K. melomenusus* SI3083, and *Ga. entanii* SI2084, contigs longer than 500 bp were extracted and used to calculate the average nucleotide identity (ANI) using the JSpecies program (Richter et al., 2016). Alignment of these contigs against the four reference draft genomes (*K. pomaceti* T5K1^{T}, *K. oboediens* LMG 18849^{T}, *K. melomenusus* AV436^{T} and *Ga. entanii* LTH 4560^{T}, respectively) was made with Mauve Aligner (Progressive Mauve) using default parameters. All unaligned contigs were searched using BLAST (Altschul et al., 1990) and do not match sequences from portions of EPS production addressed in the study.

The genome distances between newly isolated four strains and the type strains of the species of *Komagataeibacter/Gluconacetobacter* were calculated using the Genome-to-Genome Distance Calculator 3.0 (Meier-Kolthoff et al., 2022), and the OrthoANI calculator was also used for all strains (Lee et al., 2015). Genes and operons important for EPS production and ecology of *Komagataeibacter/Gluconacetobacter*, such as operons for extracellular polysaccharide synthesis, alcohol and aldehyde dehydrogenase genes (*adhA*, *aldh*), and acetic acid-resistant genes (*AarC*, *Azr1*, *AatA*) were searched using BlastP (Camacho et al., 2009). Prophage prediction was made with the online service PHASTER using unannotated chromosome sequences (Arndt et al., 2016). Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) were predicted in all analyzed genomes using the web tool CRISPRFinder (Grissa et al., 2007).

Results

Identification of isolates from vinegar

Among the randomly selected 20 bacterial isolates from the broth of organic apple cider vinegar production, where we expected the highest diversity based on a previous study, namely acetic acid concentration at 33 g/L (Štornik et al., 2016), the species *Komagataeibacter pomaceti*, *K. oboediens*, *K. melomenusus*, and *Acetobacter pasteurianus* were present in descending order of percentage (40, 25, 20 and 15%, respectively) (Table 2). All isolates of the same species are genotypically homogeneous in the 16S-23S rDNA gene sequence ITS.

Interestingly, under comparable oxidation cycle and acidity conditions, *Ga. entanii* was detected in 18 out of 20 isolates in conventional apple cider vinegar broth (Table S1).

The genus *Komagataeibacter* has already been demonstrated as the most abundant genus of acetic acid bacteria in most submerged processes and summarized (Barja et al., 2016), including apple cider vinegar (Fernández-Pérez et al., 2010). The species *K. pomaceti*, *K. oboediens*, *K. melomenusus* and *A. pasteurianus* have already been identified in apple cider vinegar (Fernández-Pérez et al., 2010; Štornik et al., 2016; Marič et al., 2020). In particular, *K. pomaceti* and *K. melomenusus* were first isolated from the apple cider broths of different vinegar plants in Slovenia (Škraban et al., 2018; Marič et al., 2020). On the other hand, it was surprising that 90% of isolates from conventional production cider broth turned out to belong to the species *Ga. entanii*, as it was previously described as the dominant species in spirit vinegar fermentation broths (Schüller et al., 2000).

Phenotypic and genomic diversity of the selected EPS-producing isolates from the apple cider vinegar broth

For the selection of isolates of the different species, the first selection criterion in our study was biofilm production (Table 2). The isolates producing EPS were randomly selected for further analysis. The results show that two different species (*K. melomenusus* and *K. oboediens*) produced a thick layer of floating biofilm when grown in a test tube. One of the *K. melomenusus* strains (SI3133) and one of the *K. oboediens* strains (SI3053) were randomly selected for further analysis. For both strains, the production of EPS (mainly insoluble cellulose) was previously described in the type strain *K. melomenusus* AV436^{T} (Marič et al., 2020) and in different isolated strains of *K. oboediens* (Sokollek et al., 1998; Ryngajłło et al., 2019; Taweecheep et al., 2019; Orlovska et al., 2021).

Only two of eight isolates of *K. pomaceti* produced a visible pellicle of a biofilm when grown in a test tube (Table 2). One of the two biofilm-producing isolates (SI3133) was selected for further analysis. The type strain *K. pomaceti* T5K1^{T} is reported to produce various EPS, including cellulose (Škraban et al., 2018). A further study of isolates of *A. pasteurianus* was omitted as no visible EPS production was observed, supporting most studies on *A. pasteurianus*, with the exception of the recently published strain RSV-4 that produces cellulose (Thakur et al., 2020). For comparative study also, one non-EPS producing strain isolated from conventional vinegar production broth was used, namely *Ga. entanii* SI2084. The inclusion of the randomly selected *Ga. entanii* strain in this study was mainly due to its unexpectedly high dominance in (conventional) apple cider vinegar and the fact that previous research in *Ga. entanii* has not assessed the genetic potential of this species to produce EPSs (Schüller et al., 2000).

For selected isolates, growth was tested in different concentrations of ethanol and acetic acid, up to

Table 1: List of isolates from organic apple cider vinegar.

Identification (accession no.)	Isolate designation	Biofilm formation
K. pomaceti (MZ725319)	SI3133[1]	Pellicle biofilm
K. pomaceti (MZ758918)	SI3113	Pellicle biofilm
K. pomaceti (MZ758902)	SI3023	-
K. pomaceti (MZ758913)	SI3093	-
K. pomaceti (MZ758915)	SI3153	-
K. pomaceti (MZ758916)	SI3013	-
K. pomaceti (MZ758917)	SI3172	-
K. pomaceti (MZ759774)	SI3183	-
K. oboediens (MZ725322)	SI3053[1]	A thick layer of floating biofilm
K. oboediens (MZ758903)	SI3043	A thick layer of floating biofilm
K. oboediens (MZ758912)	SI3063	A thick layer of floating biofilm
K. oboediens (MZ758920)	SI3103	A thick layer of floating biofilm
K. oboediens (MZ758921)	SI3033	A thick layer of floating biofilm
K. melomenusus (MZ725320)	SI3083[1]	A thick layer of floating biofilm
K. melomenusus (MZ758901)	SI3166	A thick layer of floating biofilm
K. melomenusus (MZ758910)	SI3088	A thick layer of floating biofilm
K. melomenusus (MZ758908)	SI3075	A thick layer of floating biofilm
A. pasteurianus (MZ725321)	SI3123	-
A. pasteurianus (MZ759775)	SI3192	-
A. pasteurianus (MZ759776)	SI3203	-

[1]Strains selected for further analysis.

the highest concentration of 12% ethanol or acetic acid in the presence of 1% of the other respective component (Table 1). The ethanol-adaptation ability of *K. oboediens* in certain industrial broths showed loss of cellulose production, which may suggest previous exposure to various substrates (Taweecheep et al., 2019). In publications, *Ga. entanii* LTH 4560^{T} was shown to grow at total concentrations greater than 6% acetic acid and ethanol (Schüller et al., 2000), *K. pomaceti* T5K1^{T} grew on RAE medium in the presence of 3% ethanol and 4% acetic acid (Škraban et al., 2018), and *K. melomenusus* AV436 grew on the same medium in the presence of 3% ethanol and 5% acetic acid (Marič et al., 2020).

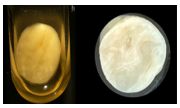
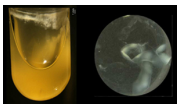
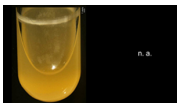
Thermal and ethanol adaptation enhancement studies showed tolerance to 1% acetic acid in combination with 5.5% ethanol by an adaptive method for *K. oboediens* strain MSKU 3, while growth on 3% ethanol in the presence of up to 8% acetic acid was observed for *K. oboediens* LTH 2460T (Sokollek et al., 1998). All isolates from this study showed a high ability to grow in liquid media containing up to 9.0% ethanol (Table 1). The isolate *Ga. entanii* SI2084 was able to grow in the presence of 12.0% ethanol in synthetic RAE media, while the growth of *K. melomenusus* SI3083, *K. oboediens* SI3053 and *K. pomaceti* SI3133 was inhibited at 12.0% ethanol.

The highest tolerance to ethanol, observed in *Ga. entanii* SI2084, was probably enhanced in the submerged process in a 1000-L acetator, using various substrates (wine, apple cider and occasionally spirit), where vinegars are yielding higher concentration of acetic acid (up to 80-90 g/L) and also initial ethanol concentrations are higher than in a 500-liter acetator, where exclusively organic apple cider vinegar is produced.

Further phenotypic analysis revealed resistance to acetic acid. The highest resistance was found in the isolated strains of *K. oboediens* and *Ga. entanii* (Table 1). Resistance to acetic acid concentration is comparable to previous results for type strains (Sokollek et al., 1998; Schüller et al., 2000; Škraban et al., 2018; Marič et al., 2020) and is lower in synthetic media than in production broths yielding higher acetic acid concentrations (isolation points of certain species/isolates), suggesting inducible and transient molecular mechanisms towards this weak lipophilic molecule, which can easily diffuse through the cytoplasmic membrane and, with the protons released, lowers the internal pH of the cytoplasm by dissociation (Barja et al., 2016).

Because strains were isolated from vinegar where the genetic determinants of antimicrobial resistance could be transferred to human pathogens as well as to environmental bacteria, selected isolated strains were also tested for antimicrobial resistance. *K. pomaceti* SI3133, *K. melomenusus* SI3083, and *K. melomenusus* SI3083 were tested for resistance to gentamicin (GMN), ampicillin (AMP), chloramphenicol (CHL), ciprofloxacin (CIP), erythromycin (ERY), and trimethoprim (TMP). The growth of all the tested strains was inhibited around the antibiotic disc containing ERY and TMP. In addition, for *K. pomaceti* SI3133 and *K. oboediens* SI3053, no growth inhibition was detected in the presence of CIP; and for strain *K. melomenusus* SI3053, no inhibition of growth was detected even when exposed to AMP. The methodology and detailed results are presented elsewhere (Cepec and Trček, 2022).

Table 2: Phenotypic and genomic characteristics of the selected strains from the production broth. Biofilm formation: Comparison of EPS production under static conditions in RAE medium; liquid medium tilted at 45-degree angle (left panel) and images of membranes (right panel; not applicable for *Ga. entanii* SI2084); Resistance to acetic acid/ethanol growth under different conditions in RAE medium.

Isolate	Biofilm formation		Resistance to acetic acid (%) (+1% ethanol)	Resistance to ethanol (%) (+1% acetic acid)
K. melomenusus SI3083		A thick layer of floating biofilm	4.0	9.0
K. oboediens SI3053		A thick layer of floating biofilm	5.0	9.0
K. pomaceti SI3133		Pellicle biofilm	2.5	9.0
Ga. entanii SI2084		None	4.0	12.0

To understand the genetic basis of the observed phenotypic differences between the three EPS-producing strains from the same production broth and non-EPS producing strain from the conventional apple cider vinegar, their genomes were sequenced using Next-generation sequencing (NGS) technology. Different methods were used to identify the isolates, such as in-silico DNA-DNA hybridization, average nucleotide identity (ANIb & ANIm, and OrthoANI), which showed a precise match with the known species (Table 3).

Analysis of putative operons for EPS production

To describe the genetic organization of genes related to EPS production, the genomes were sequenced, annotated, and explored. Organization of putative operons for EPS production, namely cellulose, acetan, and levan, was compared to genome sequences of type strains of the corresponding species, where more than type strain genome sequences were available, various representative strains were included in the comparisons.

Therefore, putative operons for EPS production of *K. melomenusus* SI3083 were compared with the type strain *K. melomenusus* AV436^{T} (Marič et al., 2020), first isolated also from apple cider vinegar. The putative EPS operons of *K. oboediens* SI3053 were compared to the type strain *K. oboediens* LMG 18849^{T} (Sokollek et al., 1998) and in addition also to the available genome sequences of *K. oboediens* MSKU 3 (Taweecheep et al., 2019), *K. oboediens* IMBG 314/IMBG 311/BSL-kmcMAG003 (Orlovska et al., 2021), *K. oboediens* 172Bp2 (Andrés-Barrao et al., 2011), and *K. oboediens* AV371 (in-house strain, unpublished data, deposited at DDBJ/EMBL/GenBank under BioProject accession: PRJNA739075, BioSample: SAMN19771103).

The background of isolation or use of specific strains of *K. oboediens* varies. Strain 172Bp2 was isolated from industrial submerged spirit vinegar, strain LMG 18849^{T} from the fermentation of red wine vinegar, strain MSKU 3 from banana (Naloka et al., 2018), strain BSL-kmcMAG003 from kombucha (Podolich et al., 2019), and strain AV371 from industrial submerged apple cider vinegar broth (in-house strain). It was reported that strain MSKU 3 of *K. oboediens* grows well at 37°C and produces a large amount of acetic acid at this temperature. It has been used as a parental strain for heat and ethanol adaptation studies (Taweecheep et al., 2019).

On the other hand, strain BSL-kmcMAG003 was used as a parental strain that survived under simulated Mars-like conditions in the multimicrobial kombucha culture (Podolich et al., 2019). Subsequently, strain IMBG 311 was exposed to a Mars-like environment simulated outside the International Space Station, and strain IMBG 314 was used as a control sample on the ground (Orlovska et al., 2021). The genetic background of EPS operons of the isolate *K. pomaceti* SI3133 was compared with *K. pomaceti* T5K1^{T} and strain *K. pomaceti* AV446 (Škraban et al., 2018). All strains were isolated from the apple cider vinegar from different vinegar facilities in Slovenia. For a comparison, strain *Ga. entanii* SI2084 was selected and sequenced from the conventional production broth, which showed no visible biofilm production (Table 2). The genome sequences of *Ga. entanii* strain LTH 4560^{T} (isolated from high acid spirit vinegar) and strain AV429 (isolate of apple cider vinegar, in-house strain) were used for comparison.

Bacterial cellulose, poly-β-(1,4)-D-glucose, is biosynthesized intracellularly by cellulose synthase and undergoes extracellular self-assembly. Cellulose

Table 3: Genetic description of *K. melomenusus* SI3083, *K. oboediens* SI3053, *K. pomaceti* SI3133, and *Ga. entanii* SI2084 strains isolated from apple cider vinegar.

Item	***K. melomenusus* SI3083**	***K. oboediens* SI3053**	***K. pomaceti* SI3133**	***Ga. entanii* SI2084**
		General info		
Biofilm formation	A thick layer of floating biofilm	A thick layer of floating biofilm	Pellicle biofilm	None
Assembly number	GCF_019083825.1	ASM1905277v1	ASM1908381v1	ASM1979333v1
BioSample	SAMN19771101	SAMN19771100	SAMN19771102	SAMN20927474
BioProject	PRJNA739075	PRJNA739075	PRJNA739075	PRJNA739075
Genome acc. no.	GCA_019083825.1	GCA_019052775.1	GCA_019083815.1	GCA_019793335.1
GenBank	JAHRDS010000009.1	JAHQRM010000001.1	JAHRDT010000001.1	JAILXQ000000000
Length (bp)	3.677.350	3.666.777	3.554.913	3.644.056
GC (%)	62.7	61.69	62.5	62.6
Number of contigs	228	185	207	326
Contig N50	69.931	92.421	83.842	25.894
Contig L50	13	15	14	43
		Taxonomy check		
Closely related species	*K. melomenusus* AV436^{T}	*K. oboediens* LMG 18849^{T}	*K. pomaceti* T5K1^{T}	*Ga. entanii* LTH 4560^{T}
ANIb (%)	99.94	97.71	98.02	97.42
ANIm (%) 99.99	98.59	98.43	98.24	
GGDC–DDG (%)	99.90	86.40	85.50	83.00
GGDC–Prob. DDH>=70%)	98.28	94.38	93.98	92.64
OrthoANI (%)	99.97	98.46	98.44	98.13
		Mobile elements		
No. of prophage regions	1: 0 intact, 1 incomplete	2: 2 intact	5: 1 intact, 3 incomplete, 1 questionable	2: 2 intact
CRISPR arrays	2	0	0	0
Caspr CRISPS	3 sequences with CRISPR 1 sequence with Cas cluster	0 sequences with CRISPR /Cas clusters	0 sequences with CRISPR /Cas clusters 1 sequence with CRISPR	0 sequence with Cas cluster
		Putative EPS operons		
Cellulose operons				
bcs1	Complete bcs1	Complete *bcs1*	Incomplete *bcs1*	Incomplete *bcs1*
bcs2	Complete *bcs2*	Complete *bcs2*	Complete *bcs2*	None
bcs3	None	Complete *bcs3*	Complete *bcs3*	None
bcs4	Complete *bcs4*	Complete *bcs4*	None	None
Acetan clusters				
ace type I	Complete ace I	Complete ace I	None	None
ace type II	None	None	Complete ace II	Complete ace II
Levan operon				
lsdA & lsdB	None	Complete	None	None
		Important AAB proteins		
adhA-1	MBV1831866	MBV1823757	MBV1834749	MBY4639374
adhA-2	None	None	None	MBY4638629
AdlH	MBV1829365	MBV0887286	MBV1833811	MBY4640835
AarC	MBV1831235	MBV0890023	MBV1834866	MBY4641232
Azr1	MBV1831903	MBV0889292	MBV1835087	MBY4640444
AatA	MBV1830652	MBV0888496	MBV1834189	MBV4640259

synthase, a membrane-embedded glycosyltransferase, consists of a series of subunits: *BcsA*, *BcsB*, *BcsC*, and *BcsD*. Briefly, the *BcsA* and *BcsB* subunits form the catalytic core of cellulose synthase, while the *BcsC* and *BcsD* subunits are involved in the export of glucan molecules and their packaging at the cell surface. In acetic acid bacteria, the cellulose synthase genes are organized into a main operon (*bcs1*) and additional operons *bcs2*, *bcs3*, and *bcs4*. The content and structure of operons belong to class I of cellulose-related operon organization, which was first described in *Komagataeibacter xylinus* (Römling and Galperin, 2015; Liu et al., 2018; Ryngajłło et al., 2019).

Figure 1 shows that in the first *bcs* operon (*bcs1*), the *bcsZ* and *bcsH* genes are found at the upstream position, while *bglX* is found at the downstream position. These genes encode accessory proteins: *bcsZ* encodes endo-β-1,4-glucanase, the product of *bcsH* is thought to be involved in the structural organization of the terminal complex and supports the arrangement of glucan chains in crystalline ribbons, cooperating with *BcsD*, and *bglX* encodes β-glucanase and also appears

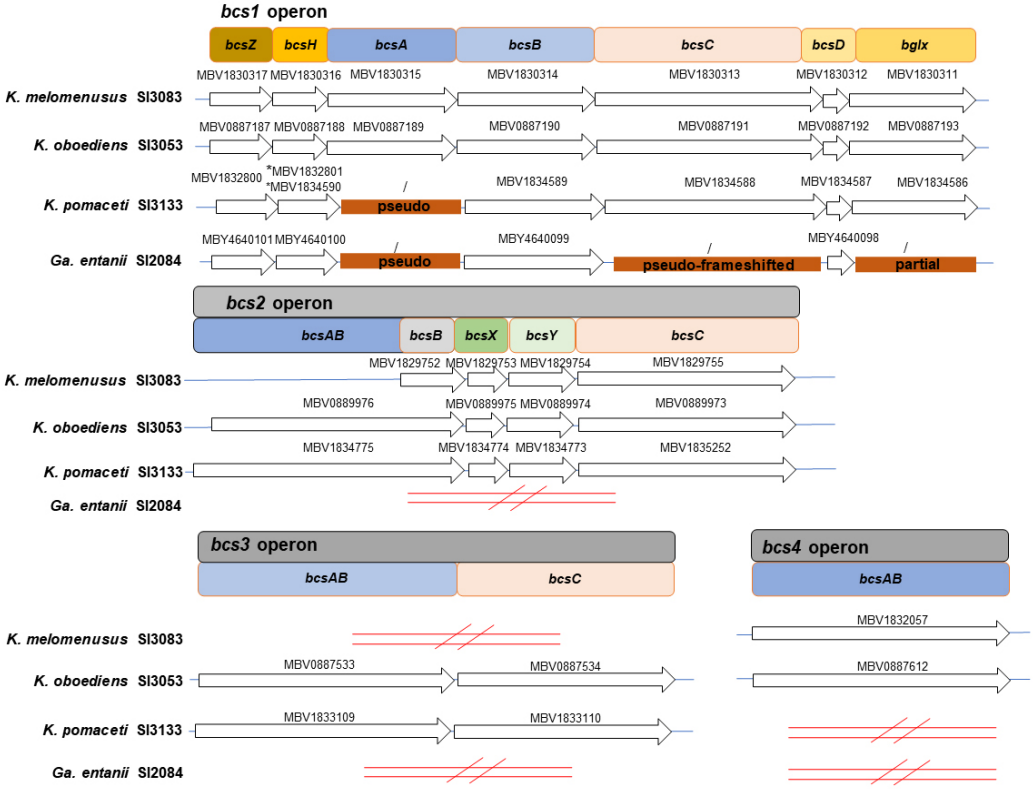


Figure 1: Arrangements of cellulose operons among selected strains *K. melomenusus* SI3083, *K. melomenusus* SI3083, *K. pomaceti* SI3133, and *Ga. entanii* SI2084, isolated from apple cider vinegar. The gene sizes are presented relative to each other. GeneBank accession numbers are given above the genes.

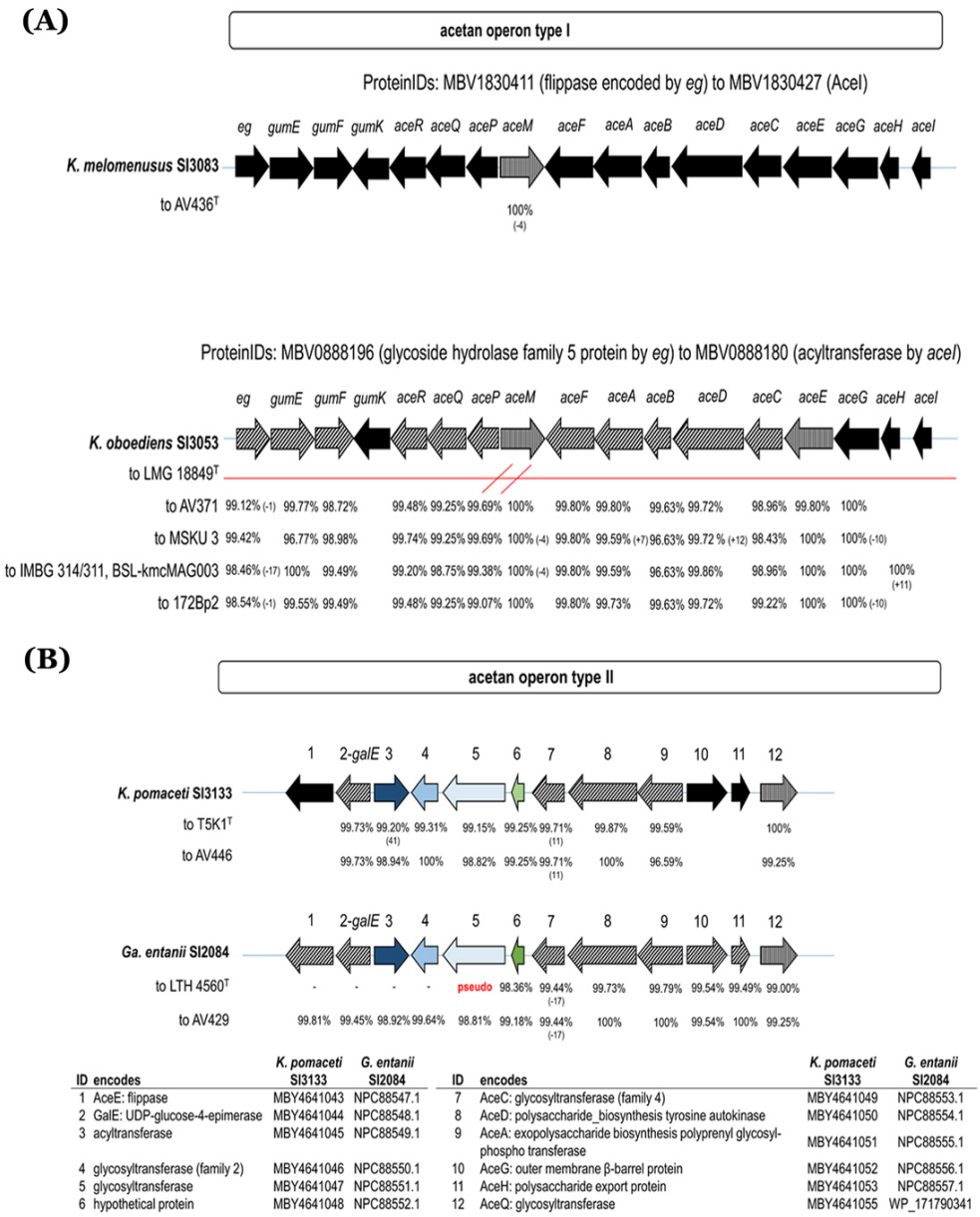
to have glucosyltransferase activity (Sunagawa et al., 2013; Römling and Galperin, 2015; Gullo et al., 2019). A second bcs operon (*bcs2*) consists of *BcsAB*, *bcsX*, *bcsY*, and *BcsC*. The genes encoding the *BcsA* and *BcsB* subunits are fused and referred to as *BcsAB*, and the gene encoding *BcsC* is separated from *BcsAB* by two other genes (*bcsX* and *bcsY*).

Apart from the *bcsX* product, whose function is unclear, *bcsY* is predicted to be a transacylase (involved in the production of acetyl cellulose). In additional bcs operons (*bcs3* and *bcs4*), the genes encoding *BcsA* and *BcsB* are also fused into *BcsAB*, and the *bcs3* operon also contains the gene encoding the *BcsC* subunit of cellulose synthase. In addition, the variable copy number of the bcs operon was detected in different AAC bacteria. For example, more than one copy of the cellulose operon was found in Komagataeibacter oboediens strain 172Bp2, where the *bcs2* operon was present in four identical copies (Ryngajłło et al., 2019).

All four isolated strains *K. melomenusus* SI3083, *K. melomenusus* SI3083, *K. pomaceti* SI3133, and *Ga. entanii* SI2084, were found to possess genes for cellulose synthase. However, the differences between the strains may explain the presence or absence of insoluble biofilm production, as shown in Table 1. In *K. melomenusus* SI3083, phenotypically characterized as a producer of the conspicuous insoluble EPS, the cellulose operons *bcs1*, *bcs2*, and *bcs4* were found. The operon *bcs3* is not present, and in the operon *bcs2*, only *BcsB* is present instead of *BcsAB* as in the typical scheme (Figure 1). When the sequences of the putative cellulose operons of *K. melomenusus* SI3083 were compared with the type strain AV436^{T} (Marič et al., 2020), almost complete homology was observed. A total of two differences in proteins involved in cellulose synthesis were found between *K. melomenusus* strains SI3083 and AV436^{T}.

In strain SI3083, the gene *BcsC* of operon *bcs1* encoding putative *BcsC* (MBV1830313) has a protein homology of 99.92% (gap of one amino acid in SI3083) when compared to AV436^{T}'s *BcsC* (NPC65869), and the second difference was found in operon *bcs4*, where the fused gene *BcsAB* (MBV1832057) of the strain SI3083 contains additionally 13 a.a. and therefore shows 99.93% protein homology to AV436^{T}'s protein NPC67849. In addition to the thick biofilm production of *K. melomenusus* SI3083, a thick layer of floating biofilm was also observed in the isolated strain *K. oboediens* SI3053. At the genomic level, NGS sequencing of *K. oboediens* SI3053 detected all 4 putative cellulose operons (*bcs1*, *bcs2*, *bcs3*, and *bcs4*). *K. oboediens* SI3053 essentially follows the organization of the bcs operons found in other strains of the same species (Figure S1& Figure S2).

The most unusual organization of *bcs* operons and



ID	encodes	*K. pomaceti* SI3133	*G. entanii* SI2084
1	AceE: flippase	MBY4641043	NPC88547.1
2	GalE: UDP-glucose-4-epimerase	MBY4641044	NPC88548.1
3	acyltransferase	MBY4641045	NPC88549.1
4	glycosyltransferase (family 2)	MBY4641046	NPC88550.1
5	glycosyltransferase	MBY4641047	NPC88551.1
6	hypothetical protein	MBY4641048	NPC88552.1
7	AceC: glycosyltransferase (family 4)	MBY4641049	NPC88553.1
8	AceD: polysaccharide_biosynthesis tyrosine autokinase	MBY4641050	NPC88554.1
9	AceA: exopolysaccharide biosynthesis polyprenyl glycosyl-phospho transferase	MBY4641051	NPC88555.1
10	AceG: outer membrane β-barrel protein	MBY4641052	NPC88556.1
11	AceH: polysaccharide export protein	MBY4641053	NPC88557.1
12	AceQ: glycosyltransferase	MBY4641055	WP_171790341

Figure 2: Schematic structure of two types of ace clusters. The gene sizes are shown relative to each other. (A) Ace type I cluster and (B) Ace type II cluster. The degree of identity is indicated below the corresponding gene; if gene has the full homology to all strains compared, is presented in black, otherwise the number of modified amino acids is indicated in brackets underneath. Genes in blue have no homology to ace type I genes. In green is hypothetical protein. Schematic structures of other EPS-related operons can be found in the supplementary material.

bacterial cellulose-related genes among *K. oboediens* strains is found in type strain LMG 18849^{T}, in which the organization of operons for cellulose synthesis consists of *bcs1*, *bcs3* with only the *BcsAB* gene, and *bcs4* (Figure S1) and no *bcs2* operon was found. When strain SI3053 was compared with other strains in this study (AV371, IMBG 314, IMBG311, BSL-kmcMAG003, and 172Bp2), the putative operons related to bacterial cellulose synthesis were found to be more similar to all of these strains than to strain LMG 18849^{T} because all of these strains have *bcs1*, *bcs2*, *bcs3*, and *bcs4* (Figure S1).

In *K. oboediens* MSKU3, the *BcsC* gene, which encodes a protein required for cellulose export, is in the *bcs2* operon in the reverse orientation, which was not observed in the other strains. In all *K. oboediens* strains examined in this study, the *BcsD* gene in the *bcs1* operon was found to be fully conserved (Ryngajłło et al., 2019). In addition, *BcsA* is also fully conserved the operon *bcs1*, while other putative proteins have at least 96% amino acid homology (Figure S1.

In Table 1, the isolated strain *K. pomaceti* SI3133 produces different EPS under static conditions mainly water-soluble EPS with lower proportions of floating pellets. Deciphering the genetic basis of EPS production reveals the presence of *bcs* operons: incomplete *bcs1* with pseudo-*BcsA*, complete operons *bcs2* and *bcs3*. The genes of operon *bcs1* have the same organization strategy (Figure S1, only the *BcsA* sequence is incomplete because of the missing C-terminus. The

pseudo-*BcsA* in the *bcs1* operon and all other ORFs of the putative *K. pomaceti* SI3133 *bcs2* and *bcs3* are of similar size and nearly identical to the proteins encoded by *bcs2* and *bcs3* of *K. pomaceti* T5K1^{T} and *K. pomaceti* AV446. A genetic basis of the putative cellulose operons for strains *K. pomaceti* T5K1^{T} and *K. pomaceti* AV446 was previously discovered (Škraban et al., 2018), and although cellulose production was reported for these two strains, cellulose production was not detected in strain SI3133 in this study, even though it has a similar cellulose operon organization.

For comparison, in an isolate from conventional apple cider vinegar without visible biofilm production, namely strain *Ga. entanii* SI2084, NGS sequencing of EPS-related operons revealed the presence of only one incomplete *bcs1* operon and no other bcs operons. The impeded cellulose production could be due to the presence of pseudo-*BcsA* present in the manner described above and the frame-shifted position of *BcsC* and *bglX* with missing N terminus. When the sequences encoding putative cellulose operons were compared and searched in the type strain LTH 4560^{T}, the main difference from strain SI2084, in addition to the presence of an incomplete *bcs1*, was the presence of an incomplete *bcs2* operon with frame-shifted position of *BcsAB* and the presence of pseudo-*BcsC* (the products of *bcsX* and *bcsY* are intact).

In addition, LTH 4560^{T} also has pseudo-*BcsA* and the shifted position of *BcsC* in the *bcs1* operon, and controversially, *bglX* encodes the intact protein PYD64535. In addition, there is another strain of *Ga. entanii*, the in-house strain AV429, in which biofilm production has been exploited (unpublished data). In strain AV429, the gene *BcsC* in the *bcs1* operon encodes the protein NPC87918 (a frame-shifted position was observed in comparable strains) and the *bcs2* operon is intact (*BcsAB*, *bcsX*, *bcsY*, and *BcsC*) and encode NPC90592, NPC90591, NPC90590, and probably *BcsC* (end of contig), respectively (Figure S4).

Levan, poly-β-(2,6)-linked polyfructan, an EPS produced from extracellular sucrose, is biosynthesized by levansucrase, as first reported for acetic acid bacteria in Komagataeibacter xylinus strain I-2281 (Kornmann et al., 2003). The levansucrase gene (*lsdA*) is clustered with levanase (*lsdB*). Levansucrase (*lsdA*) catalyzes the transfer of D-fructosyl residues from sucrose to a growing fructan chain by transfructosylation. After the complete degradation of sucrose, levanase (*lsdB*) facilitates the hydrolysis of levan to free fructose (cleaving the β-(2,6)-bonds of the newly formed levan chain, gradually releasing the terminal fructose until a branching point is reached) (La China et al., 2018; Ryngajłło et al., 2019).

Although it was suspected that the majority of Komagataeibacter strains do not synthesize levan (Ryngajłło et al., 2019), one levan producer was found among our isolates - *K. oboediens* SI3053 possesses *lsdA* (MBV0888382) and *lsdB* (MBV0888383). No levan cluster was found in both *K. melomenusus* strains (SI3083 and AV436^{T}) and in all *K. pomaceti* strains (SI3133, T5K1^{T}, AV446) with available genome sequence. Surprisingly, the levan cluster is present in 6 of 8 strains of *K. oboediens*. The operon for levan synthesis was not found in strains *K. oboediens* AV371 and 172Bp4. In addition, strain *Ga. entanii* AV429 has the levan operon, which is not present in strains SI2084 and LTH 4560^{T} (Figure S2).

Acetan, a complex anionic branched heteropolysaccharide, was first described in *K. xylinus*. The main chain of acetan consists of β-1,4-linked D-glucose residues, with every second glucose branched with pentasaccharide. The monosaccharide composition of this water-soluble polysaccharide revealed the presence of rhamnose, glucose, mannose, and glucuronic acid (Couso et al., 1987; Trček et al., 2021). The genetic basic of acetan production in *K. xylinus* E25 revealed the presence of together 17 genes in a cluster (Figure 1). Briefly, the acetan biosynthesis is divided into the following steps: growing intermediate chain of repeat units, polymerization, and export out of the cell.

The biosynthesis of the growing intermediate chain of acetan repeat units is mediated by specific enzymes, with specific activated monosaccharides added in the following order by the glycosyltransferases *AceA*, *AceB*, *AceC*, *AceK* (new proposed name for GumK in (Trček et al., 2021)), *AceQ*, *AceP*, and *AceR*. *AceA* is encoded by *AceA* and first transfers glucosyl-1-phosphate from uridine diphosphate-glucose (UDP-Glc) to the diphosphate-polyprenol lipid to form glucose-diphospho-polyprenol. Subsequently, the enzymes encoded by *AceB*, *AceC*, *AceK*, *AceQ*, and *AceP* add additional glucosyl, mannosyl, glucuronyl, two glucosyl residues.

Finally, rhamnosyl residue as the final sugar residue is catalyzed by the product of *AceR*. The polymerization and transport of acetan out of the cell appears to follow the Wzx/Wzy pathway because of the presence of polysaccharide copolymerase (*AceD*), flippase (*AceE*), β-barrel porin (*AceG*), and outer membrane transport protein (*AceH*) encoded by *AceD*, *AceE*, *AceG*, and *AceH*, respectively. In the acetan cluster of *K. xylinus* E25, genes for the synthesis of glycosylhydrolase (gene eg), acetyl/acyltransferases (genes *gumF*, *aceI*), and enzymes involved in the synthesis of nucleotide sugars (encoded by *aceF* and *aceM*) are also present. The product of *gumE* is thought to be involved in the polymerization or export process.

The organization of the acetan cluster does not follow the steps of acetan synthesis and is shown schematically in Figure 1. Bioinformatic analysis of several strains of Komagataeibacter revealed that many strains of *K. europaeus*, *K. xylinus*, *K. intermedius*, *K. medellinensis*, and *K. oboediens* share all or the majority of genes in the acetan cluster (Ryngajłło et al., 2019). Recently, genome analysis of *K. hansenii* strains and phylogenetically close species revealed the presence of another acetan-like locus, termed the ace-type II cluster (Ryngajłło et al., 2019; Trček et al., 2021). This newly described region was shown to harbor the necessary genes that enable the synthesis of enzymes involved in the initiation of polysaccharide chain growth, polymerization, and export of the polysaccharide. Ho-

mology with glycosyltransferases (*AceA*, *AceC*, *AceQ*), a nucleotide sugar epimerase (*galE*), flipasse (*AceE*), and proteins responsible for export encoded by *AceD*, *AceG*, and *AceH*, of ace type I was confirmed (Trček et al., 2021), in addition to the presence of putative glycosyltransferases and acyltransferases that were not known to exist in the acetan cluster that was first discovered (Figure 1). The presence or absence of specific glycosyltransferases may lead to the production of an acetan-like but uncharacterized EPS with heteropolysaccharide structure.

In all the isolated and sequenced strains, production of acetan or acetan-like heteropolysaccharides is proposed—the acetan biosynthesis cluster type I was found in *K. melomenusus* SI3083 and *K. oboediens* SI3053. A newly described ace-type II cluster is present in the isolated strain *K. pomaceti* SI3133, which produces different EPS under static conditions than strains *K. melomenusus* SI3083 and *K. oboediens* SI3053. The newly described ace-type II cluster was also found in *Ga. entanii* SI2084.

The acetan operon of *K. melomenusus* SI3083 is completely identical in 16 genes of *K. melomenusus* AV436^{T}, except for the gene *aceM* encoding putative glucose/GDP-mannose dehydrogenase (MBV1830418), which is 12 bp longer than in *K. melomenusus* AV436^{T} (Figure 2). In *K. oboediens* already the presence of acetan cluster was shown in strain 172Bp2 (Ryngajłło et al., 2019) and our isolate also shows the same structure of the acetan cluster. Surprisingly, only in the type strain LMG 18849^{T} the acetan cluster was not found (Figure 2).

Moreover, the isolated strain *K. pomaceti* SI3133, where deciphering the genetic basis of EPS production revealed the presence of bcs operons (incomplete *bcs1* with pseudo-*BcsA*, complete *bcs2* and *bcs3*), no levan operon, revealed the fully operational proposed ace cluster of type II. Figure 2 graphically shows the organization of the acetan cluster type II and additional information on the organization of these clusters in all 3 strains of *K. pomaceti*. In all *K. pomaceti* strains with available genome sequence, the sequences encoding the flippase (*AceE* homolog, MBY4641043 in strain SI3133) and the proteins involved in export (*AceG* and *AceH*, MBY4641052 and MBY4641053, respectively, in strain SI3133) are fully conserved.

High conservation and homology to the ace type I cluster was also observed for *AceD* (MBY4641050 in strain SI3133), *AceA* (MBY4641051), and the two glycosyltransferases *AceC* (MBY4641049) and *AceQ* (MBY4641055). The ace type II cluster also contains genes for two other glycosyltransferases (MBY4641046 and MBY4641047 in *K. pomaceti* SI3133) that do not show homology to the ace type I glycosyltransferases. Part of the ace type II cluster is also a short hypothetical protein and epimerase. Hypothetical protein (MBY4641048 in *K. pomaceti* SI3133) is also present in other species, mainly in *Acetobacteraceae*. The epimerase *galE* (MBY4641044 in *K. pomaceti* SI3133) and the acyltransferase (MBY4641045 in *K. pomaceti* SI3133) are part of this cluster in both species. All described proteins are also present in *K. pomaceti* strains T5K1^{T} and AV446.

For comparison, the strain *Ga. entanii* SI2084 NGS sequencing of EPS-related operons revealed the presence of the ace-type II cluster (incomplete *bcs1* operon was already described). The organization of the ace-type II cluster of *Ga. entanii* SI2084 is comparable to the ace-type II of *K. pomaceti* (Figure 2). Moreover, the new putative glycosyltransferases NPC88550 and NPC88551 from this strain show high homology to the glycosyltransferases from *K. pomaceti* strain SI3133 and no homology to ace-type I glycosytransferases. When comparing the sequences encoding all putative EPS operons with the type strain LTH 4560^{T}, the main difference apart from cellulose operon disruptions is an incomplete ace-type II cluster. Surprisingly, the absence of *AceE*, *galE*, and the genes for acyltransferase and 2 glycosyltransferases (one of which is pseudo) was detected in the ace cluster. In strain AV429, the ace-type II cluster is comparable to strain SI2084.

Although the number of strains with the available genome sequence of the species isolated in this study is imperceptible, the highest homology of putative operons for EPS production of the new strain and the type strain is shared between *K. melomenusus* SI3083 and *K. melomenusus* AV436^{T}. This high conservation rate (100% identical amino acid sequences) between strains was also confirmed when protein sequences for PQQ-*adhA*-1 & AdlH, acetic acid tolerance proteins (*AarC*, *Azr1*, and *AatA*) were compared (Škraban et al., 2018; Marič et al., 2020) (Figure S3).

In addition, we also focused on the mobile part of the sequenced genomes of the isolates (namely prophages and CRISPR-CAS systems). The presence of prophage sequences is usually associated with short-term strain variation and is a common element in bacterial genomes. However, in *K. oboediens* SI3053, *K. pomaceti* SI3133, and *Ga. entanii* SI2084, at least one intact prophage region is present, whereas only one incomplete region was found in strain *K. melomenusus* SI3083. This finding is comparable to the stable Acetobacter pasteurianus strain 386B and can be interpreted as an indicator of genome stability (Ryngajłło et al., 2019).

Surprisingly, in the same isolate *K. melomenusus* SI3083, the presence of CRISP-CAS class 1 clusters was predicted. This genome contains one locus of the cas cluster consisting of 12 palindromic repeats of 29 bp separated by spacers. When searching the NCBI nucleotide database with the spacers, only one spacer showed moderate sequence similarity to plasmid pAP1468-2 from *A. pasteurianus* subsp. *pasteurianus* strain SRCM101468. However, the isolated strains show low speculation of foreign DNA invasion events.

Discussion

As for the synthesis of the different EPS in a given community, this study shows the genetic diversity of the strains we were able to isolate and study in pure culture from the broth of apple cider vinegar production. This comparative genomic approach was carried out

after a random selection of isolates from cider production broth. As the isolation process via cultivation is still a golden rule in microbiology for extraction of important isolates to be used further, the indeed species diversity remains unclear.

Further metagenomics studies could reveal the representativeness of the present species list of acetic acid bacteria isolates, as the culture-based approaches may be biased due to species remaining refractory in culture due to experimental design (i.e., use of certain media, use of non-enrichment media for some subdominant species, slow growth, non-cultivable states, subdominant populations), but are nevertheless unavoidable for the study of specific traits, as shown here. Apple cider vinegar production is not a species-dependent process, but it has already been shown that *Komagataeibacter* species predominate over *Acetobacter* species in apple cider vinegar broths (Fernández-Pérez et al., 2010; Štornik et al., 2016).

However, this study focused on EPS acetic acid bacteria producers and revealed several genomic bases for predicting EPS production. The most comprehensive comparative study of the putative EPS operon between different strains was possible for *K. oboediens*. The basic framework of the putative EPS operon is shared among all investigated *K. oboediens* strains: four bcs operons (*bcs1*, *bcs2*, *bcs3*, and *bcs4*), ace-type I cluster, while levan operon is present in the majority of strains. All EPS related operons are found in one copy, only in strain 172Bp2, four identical *bcs2* operons were found. Newly isolated strain *K. oboediens* SI3053 confirms that *BcsD* is fully conserved in the *bcs1* operon between *Komagataeibacter* strains (Ryngajłło et al., 2019). In addition to *BcsD* in the same operon, *BcsA* is also fully conserved, while other putative proteins have at least 96% amino acid homology.

On the other hand, the presence and versatility of *BcsC*, encoding a subunit of cellulose synthase protein necessary for cellulose export, in *Komagataeibacter* strains have already been suggested as a target of evolutionary forces through perturbations of cellulose export (Ryngajłło et al., 2019). For *K. oboediens* strains, included in this study, gene *BcsC* in *bcs1* was present in all strains, in *bcs2* operon nonexistent in strain LMG 18849^{T} (as the whole missing second bcs operon together with *BcsAB*) and in a reverse direction in strain MSKU 3. For *K. oboediens* several studies reported stress-induced changes associated with cellulose production.

Strain MSKU3, which was reported to produce cellulose, was used for adaptation studies to higher ethanol concentrations and thermal conditions (Taweecheep et al., 2019). The authors found that thermo- and ethanol-adapted strains did not produce cellulose. In the ethanol-adapted strain, only in the *BcsC* gene alterations such as insertions were found. In the thermally adapted strain, *BcsC* gene deletions and transposon insertions were found. Surprisingly, it was also reported that the cellulose production was then recovered by static culture. On the other hand, sequencing of the *K. oboediens* strain exposed to the Mars-like stressors (strain IMBG 311) revealed no change in topology or induction of mutation in any of the putative EPS operons compared to the parental strain (strain BSL-kmcMAG003) and the soil control (strain IMBG 314), although the authors reported lower cellulose production in the resuscitated bacteria compared to the ground control (Podolich et al., 2019; Orlovska et al., 2021).

Another important putative EPS operon, levan operon, is found in *K. oboediens* SI3053, LMG 18849^{T}, MSKU 3, IMBG 314, IMBG 311, and BSL-kmcMAG003 and absent in strains AV371 and 172Bp3. Levansucrase presence in *Komagataeibacter* is reported as the exception rather than the rule in the genus Komagataeibacter (Öner et al., 2016). Moreover, some authors suspect the presence of this operon from horizontal (T2) evolutionary lineages (Jakob et al., 2019), which was not found here. As described previously (Trček et al., 2021), we found another complete locus for putative EPS production in this species–ace-type I. The presence of the ace cluster was not found in the type strain *K. oboediens* LMG 18849^{T}. Dissection of different putative EPS operons in *K. oboediens* strains and comparison to published phenotypic data of different strains is confirming that the presence of any of EPS cannot be species-descriptive.

In *K. melomenusus* SI3083, the striking insoluble EPS production was observed. The genetic background shows that cellulose synthesis is encoded by cellulose operons *bcs1*, *bcs2*, and *bcs4*, and no *bcs3* operon was found. In addition, *K. melomenusus* SI3083 harbors the ace-type I cluster and no genes encoding enzymes involved in the biosynthesis of levan were found. When the sequences of the putative EPS operons and other major proteins of *K. melomenusus* SI3083 were compared with the type strain AV436^{T} (Marič et al., 2020), almost complete homology was found, suggesting that *K. melomenusus* is an evolutionarily young species (Cleenwerck and De Vos, 2008). In this study, all identified *K. melomenusus* strains are stable cellulose producers. As observed previously and reviewed (Ryngajłło et al., 2019; Trček et al., 2021), the presence of acetan or acetan-like water-soluble polysaccharides enhances the dispersion and porosity of insoluble cellulose.

The phenotypic difference between the two isolated strains with the distinct insoluble EPS production is that the biofilm produced by *K. oboediens* SI3053 slips off when a test tube is tilted at a 45-degree angle, whereas the biofilm of *K. melomenusus* SI3083 remains as a plug on the surface. Remarkably, *K. oboediens* SI3053 has levansucrase, which is also involved in EPS formation. The presence of such an operon probably explains the phenotypic observation of EPS diversity in contrast to the *K. melomenusus* SI3083 biofilm because the isolated strain of *K. oboediens* also possesses a type I acetan cluster with a similar structure to the *K. melomenusus* SI3083 strain.

Comparing the cellulose operons of *K. pomaceti* strains with the isolated strains of *K. melomenusus* and *K. oboediens*, the main difference is that all strains

of *K. pomaceti* possess a pseudo-*BcsA* gene in *bcs1* operon. *K. pomaceti*, which lacks the C-terminus and stop codon in *BcsA*, suggests common transcription with the downstream *BcsB* gene. Such a fusion could abolish the production of mainly cellulosic biofilms, although two other bcs operons (*bcs2* and *bcs3*) are present and could accomplish the functional cellulose synthase. Moreover, among the isolated strains from the production broth with organic apple cider vinegar, strain *K. pomaceti* SI3133 is the only one that possesses the ace cluster of type II (Figure 2). This observation suggests that the newly described acetan-like biosynthetic locus may be responsible for the production of acetan-like EPS and that further studies are needed (Trček et al., 2021).

Strain *Ga. entanii* SI2084 was selected and sequenced from conventional apple cider vinegar broth production and used as a comparator strain, which showed no visible biofilm production. The predominant presence of *Ga. entanii*, first identified in the fermentation of spirit vinegar (Schüller et al., 2000), in conventional apple cider vinegar broth was probably due to the adaptation of the "starter culture" in the same aerated fermenter for the submerged production of vinegar, frequently changing in campaigns with wine or cider substrate and only occasionally containing spirit as a substrate for vinegar production. Since this species was prevalent in spirit vinegar fermentation broths, it was expected that some adaptations to an ethanol-tolerant environment would also be reflected in the EPS-related operons (Taweecheep et al., 2019).

Closer examination of the EPS-related operons revealed the presence of an incomplete *bcs1* operon and the ace-type II cluster. Forces preventing cellulose production or its export were observed in strain SI2084 as complete loss of the *bcs2* operon, along with pseudo-*BcsA* and *BcsC* at frame-shifted positions in *bcs1* operon. Again, the complete presence of the II-type ace cluster was observed in strain *Ga. entanii* SI2084. In contrast, *Ga. entanii* AV429 was reported to produce a visible biofilm. At the genetic level, the main difference between strains SI2084 and AV429 is the presence of a complete *bcs2* operon and a functional *BcsC* protein encoded by the *bcs1* operon in strain AV429, producing visible EPSs.

The acetan-type II cluster was found in strains SI2084 and AV429 and was not completely present in strain LTH 4560^{T}, leading to the tentative conclusion that this type of acetan or acetan-like EPS is also not species-specific. The levan operon was not found in the isolated strain from conventional apple cider vinegar, although it was present in another *Ga. entanii* strain, isolated from the submerged bioreactor with the similar substrate – apple cider vinegar (strain *Ga. entanii* AV429) (Trček et al., 2021).

Consclusion

In this study, we demonstrate that apple cider vinegar broth is a suitable environment for the isolation of EPS-producing acetic acid bacteria and that the specificity of EPS compared to type strains is not species-descriptive. Through the analysis presented, we have provided genetic evidence for the phenotypically observed EPS production in selected isolates. Strain *K. melomenusus* SI3083 possesses the cellulose synthesis operons *bcs1*, *bcs2*, and *bcs4* together with the type I acetan cluster in the absence of the levan operon and is, therefore, an excellent producer of bacterial cellulose that could be useful in various biotechnological applications. Another analyzed strain *K. oboediens* SI3053 possesses the *bcs1*, *bcs2*, *bcs3*, and *bcs4* operons, the levan operon, and the acetan cluster (type I).

When the *K. oboediens* strains were compared with respect to EPS-related genes, many differences were found, especially the complete loss of operons (*bcs2* or acetan cluster) or alterations related to cellulose export (*BcsC*) in some strains, indicating that EPS production is not species-specific. The genetic basis of strains *K. pomaceti* SI3133 and *Ga. entanii* SI2084, both of which contain a novel ace-type cluster II in addition to the incomplete operon *bcs1*, suggests that there may be even greater diversity of acetans than previously thought. Gene comparisons of selected EPS-producing AAB strains performed by studies such as this one may also be used in the future to predict the possible monosaccharide composition of EPS synthesized by the newly isolated strains.

This study expands our understanding of the genetic variation of EPS genes in AAB, a tremendous potential source of biotechnologically important products. This and similar studies are therefore important as they may enable us to fully exploit this potential in the future.

Article Information

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Conflict of Interest. The authors declare no conflict of interest.

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Supplementary Data

Vajdič, T. Genetic diversity of exopolysaccharides from acetic acid bacteria isolates originating from apple cider vinegars. Ger. J. Microbiol. 2022. 2(1): 1-18. https://doi.org/10.51585/gjm.2022.1.0011

Table S1: List of isolates from conventional cider vinegar.

Identification (acc. no.	Isolate designation	Biofilm formation	Colony Morphology
Gluconacetobacter entanii (MZ735454)	SI2035	Pellicle biofilm	Entire margin, Medium-sized colony, mucoid, transparently orange
Gluconacetobacter entanii (MZ747108)	SI1075	Pellicle biofilm	Entire margin, small-sized colony, butyrous, white
Gluconacetobacter entanii (MZ758898)	SI2074	Pellicle biofilm	Entire margin, medium-sized colony, mucoid, transparently
Gluconacetobacter entanii (MZ758900)	SI2055	Pellicle biofilm	Entire margin, medium-sized colony, partially mucoid, white
Gluconacetobacter entanii (MZ758906)	SI1095	Pellicle biofilm	Entire margin, medium-sized colony, butyrous, transparently white
Gluconacetobacter entanii (MZ758907)	SI2043	Pellicle biofilm	Entire margin, medium-sized colony, partially mucoid, white
Gluconacetobacter entanii (MZ758911)	SI2064	Pellicle biofilm	Entire margin, medium-sized colony, partially mucoid, yellowish
Gluconacetobacter entanii (MZ758919)	SI2104	Thin layer of floating biofilm	Entire margin, medium-sized colony,butyrous, white
Gluconacetobacter entanii (MZ747101)	SI1063	-	Entire margin, small-sized colony, butyrous, transparently yellow
Gluconacetobacter entanii (MZ747102)	SI1013	-	Entire margin, flat, large colony, butyrous, white
Gluconacetobacter entanii (MZ747103)	SI1045	-	Entire margin, medium-sized colony, mucoid, transparently white
Gluconacetobacter entanii (MZ747104)	SI1084	-	Entire margin, rounded, small-sized colony, butyrous, transparently white
Gluconacetobacter entanii (MZ747106)	SI1033	-	Entire margin, medium-sized colony, butyrous, white
Gluconacetobacter entanii (MZ747107)	SI1053	-	Entire margin, medium-sized colony, butyrous, white
Gluconacetobacter entanii (MZ758899)	SI2014	-	Entire margin, medium-sized colony, butyrous, transparently white
Gluconacetobacter entanii (MZ758909)	SI2024	-	Entire margin, medium-sized colony, butyrous, yellowish
Gluconacetobacter entanii (MZ758914)	SI1103	-	undilate margin, medium-sized colony, butyrous, yellowish
Gluconacetobacter entanii (MZ758905)	SI2084	-	Entire margin, medium-sized colony, butyrous, transparently yellowish
Komagataeibacter oboediens (MZ758904)	SI2093	Pellicle biofilm	Entire margin, medium-sized colony, mucoid, transparently orange
Komagataeibacter pomaceti (MZ747105)	SI1023	Pellicle biofilm	Entire margin, medium-sized colony, butyrous, white

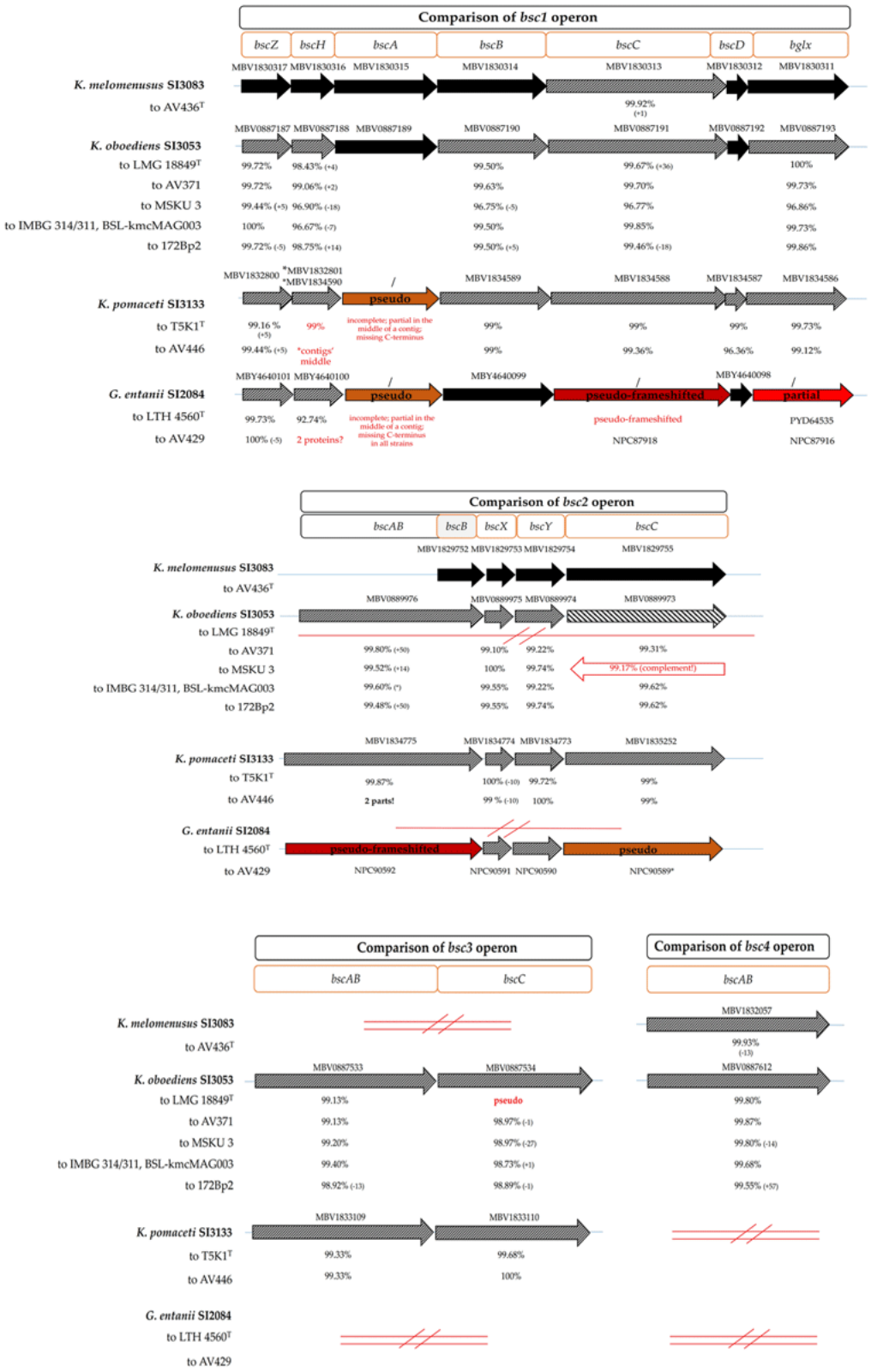


Figure S1: The arrangements of *bcs* operons.

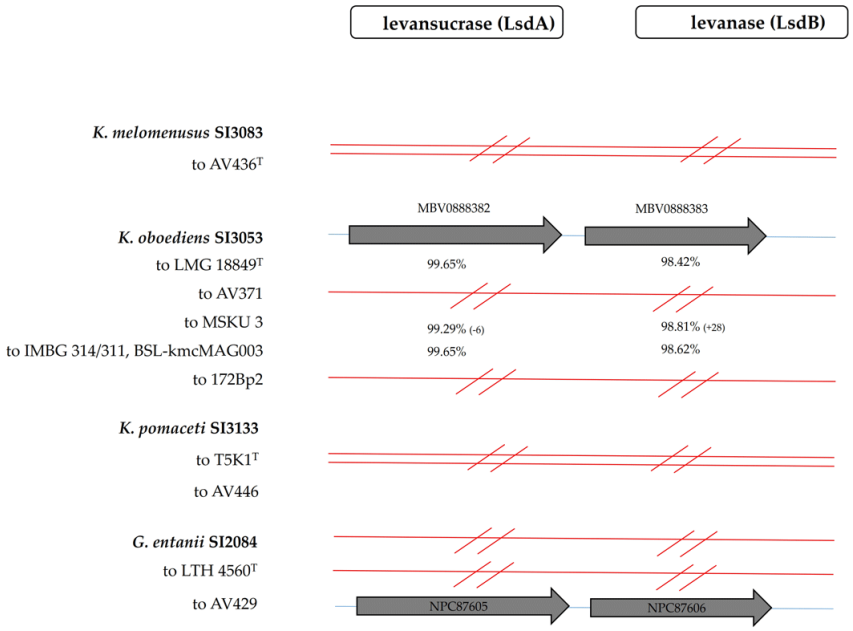


Figure S2: The arrangements of levan synthesis operon.

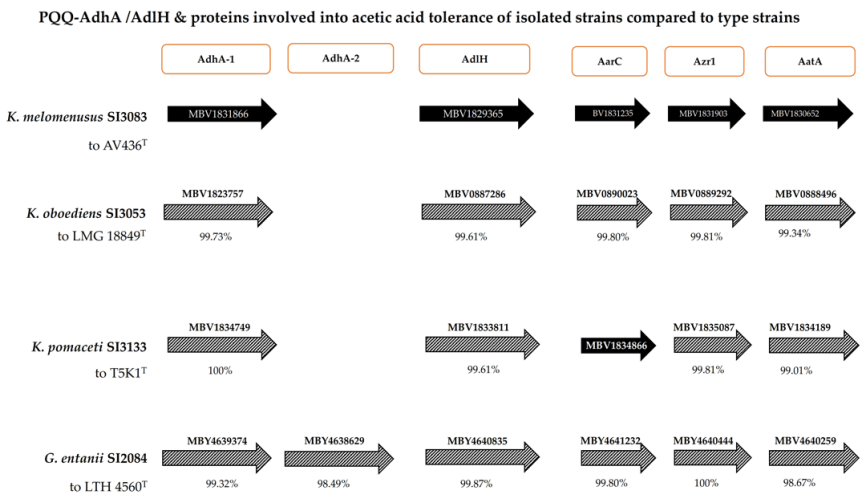
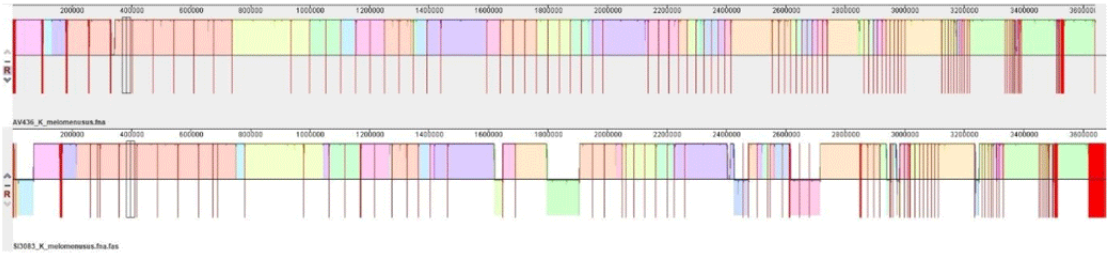
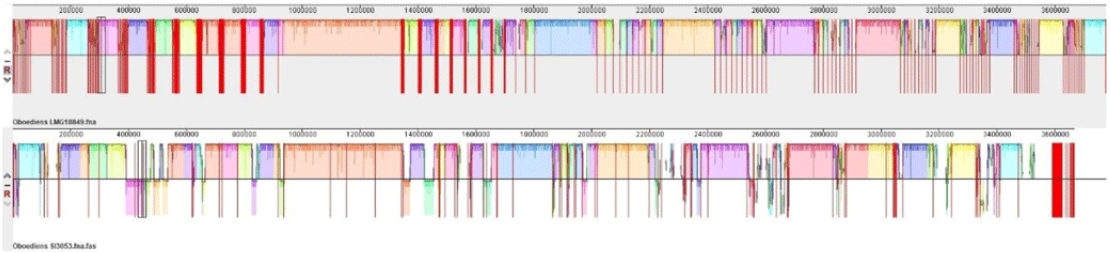


Figure S3: Presence of PQQ-AdhA/AdlH proteins and proteins (*AarC*, *Azr1* and *AatA*), involved into acetic acid tolerance in isolated strains compared to type strains.

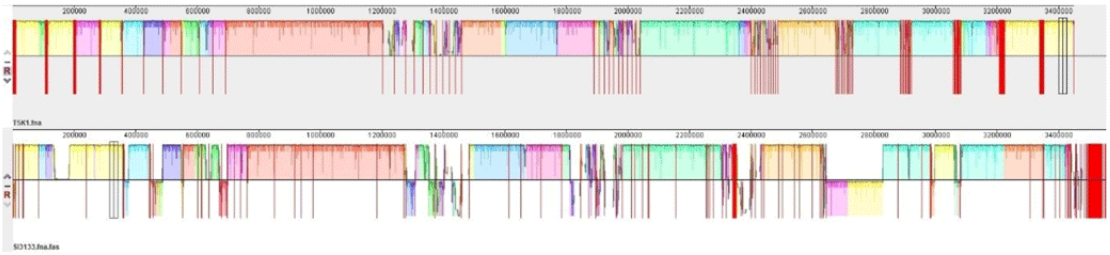
K. melomenusus AV436^{T} (on the top) vs. *K. melomenusus* SI3083



K. oboediens LMG 18849^{T} (on the top) vs. *K. oboediens* SI3053



K. pomaceti T5K1^{T} (on the top) vs. *K. pomaceti* SI3133



Ga. entanii LTH 4560^{T} (on the top) vs. *Ga. entanii* SI2084

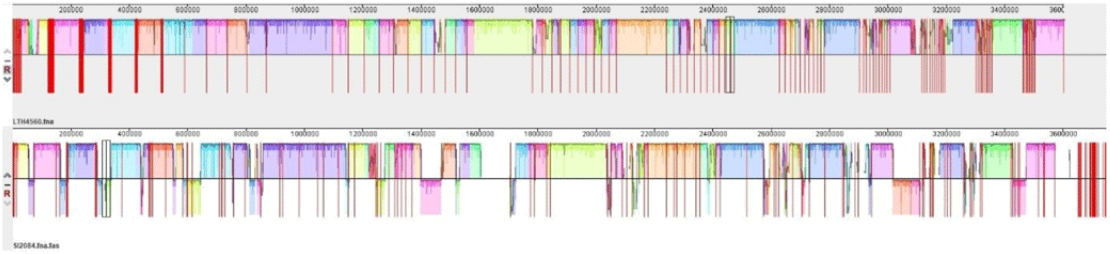


Figure S4: The alignments of the contigs of the sequenced strains from this study to reference genomes. On the top (in grey) are reference draft genomes, red lines indicate conting boundaries within the assembly. Sequences outside colored blocks do not have homologs in the other genomes.