



Functional and genomic characterization of *Komagataeibacter uvaceti* FXV3, a multiple stress resistant bacterium producing increased levels of cellulose



Francisco X. Nascimento^{a,*}, Cristiana A.V. Torres^b, Filomena Freitas^b, Maria A.M. Reis^b, Maria T.B. Crespo^{a,c}

^a iBET, Instituto de Biologia Experimental e Tecnológica, Apartado 12, 2781-901, Oeiras, Portugal

^b UCIBIO-REQUIMTE, Department of Chemistry, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516, Caparica, Portugal

^c Instituto de Tecnologia Química e Biológica António Xavier, Universidade Nova de Lisboa, Av. da República, 2780-157, Oeiras, Portugal

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ABSTRACT

Bacterial cellulose is one of the most promising biomaterials for the development of a wide array of novel biotechnological solutions. Nevertheless, the commercial production of bacterial cellulose is still a challenge and obtaining novel strains presenting increased cellulose biosynthesis and stress resistance properties is of extreme importance.

This work demonstrates the increased stress resistance, cellulose production abilities, and overall genomic properties of *Komagataeibacter uvaceti* FXV3, a novel cellulose-producing and stress resistant strain isolated from a fermented grape must.

K. uvaceti FXV3 was able to grow under several stress conditions, including the presence of high concentrations of ethanol (up to 7.5 % v/v), a trait that is not observed in the model strain *K. xylinus* CECT 7351^T. Moreover, *K. uvaceti* FXV3 produced increased concentrations of cellulose (4.31 mg/mL, 7 days after inoculation-DAI) when compared to *K. xylinus* CECT 7351^T (1.42 mg/mL, 7 DAI). Moreover, the detailed analysis of strain FXV3 genome revealed the presence of several genes involved in cellulose and acetan biosynthesis, quorum-sensing and quenching mechanisms, carbohydrate, amino acid, alcohol and aldehyde metabolism, as well as several other genes involved in stress resistance. Additionally, comparative genomic analysis revealed the increased prevalence of stress resistance genes in *K. uvaceti* FXV3 when compared to *K. xylinus* CECT 7351^T.

Ultimately, this study reveals the increased biotechnological potential of *K. uvaceti* FXV3 and brings new insights into the genetics behind *Komagataeibacter* stress resistance and cellulose production abilities.

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1. Introduction

Bacterial cellulose presents unique properties such as high purity, increased mechanic resistance, high-water capacity, biodegradability, and increased biocompatibility, hence, prompting its use in the development of a wide range of novel products for biomedical, cosmetic and other industrial applications [1–3]. This exceptional material is mostly produced by selected strains belonging to the *Komagataeibacter* genus, members of the acetic-acid bacteria (Acetobacteraceae), a group of bacteria that is known to synthesize acetic acid from ethanol oxidation. As a

result, most *Komagataeibacter* strains are found associated with fermented fruits and are involved in the production of several fermented products such as vinegar, kombucha or nata de coco [4].

The *Komagataeibacter* genus is currently composed of sixteen different species, including *K. xylinus* which is one of the most studied bacterial cellulose producers and a model organism in the study of the genetic and biochemical mechanisms behind bacterial cellulose biosynthesis [5,6].

Bacterial cellulose is synthesized through the expression of the cellulose synthase gene clusters (*bcsABCD*, *bcsZ* and *ccpA*) that encodes the enzymes directly involved in the synthesis and transport of cellulose across the membrane (reviewed in [7]). Furthermore, several other mechanisms impacting bacterial cellulose biosynthesis have been identified, including the regulation of cellulose synthase by cyclic di-3',5' guanylate (c-di-GMP),

* Corresponding author.

E-mail address: francisco.nascimento@ibet.pt (F.X. Nascimento).

the metabolism of carbon and nitrogen sources, quorum-sensing and quenching, motility, exopolysaccharide (acetan) biosynthesis, among others [7–12].

Despite the great properties of bacterial cellulose, its commercialization is still a challenge [13]. Some of the solutions aiming to decrease bacterial cellulose production costs reside on the use of cheaper substrates for bacterial cellulose production (e.g. residues from the agro-food industry) [2]. Importantly, these alternative substrates may contain increased levels of sugars and toxic compounds such as alcohols and other aromatic compounds, thus, impacting bacterial cellulose biosynthesis. Hence, obtaining *Komagataeibacter* strains presenting both increased stress resistance and increased cellulose biosynthesis properties is of great importance.

In an effort to obtain stress-resistant and cellulose-producing bacteria, several naturally fermented grape musts were obtained and bacteria were isolated, including *Komagataeibacter* sp. strain FXV3 that produced cellulose-containing films. Based on this data, strain FXV3 showed a great potential for the development of novel fermentation processes aiming at increased cellulose biosynthesis.

In this work, *Komagataeibacter* sp. strain FXV3, as well as *K. xylinus* CECT 7351^T, were characterized for their ability to produce bacterial cellulose in several substrates and to resist several stress conditions (e.g. low pH, high ethanol concentrations, high osmolarity). In addition, the genome of strain FXV3 is presented and analyzed in detail, bringing new insights into its phylogeny, the genetic mechanisms behind its stress resistance and increased cellulose production abilities.

2. Materials and methods

2.1. Inoculum preparation

Strain FXV3 and *K. xylinus* CECT 7351^T were routinely grown in Hestrin and Schramm medium (HS) (Yeast extract, 5 g/L; Peptone, 5 g/L; Na₂HPO₄, 2.7 g/L; citric acid, 1.15 g/L; pH 6) supplemented with 20 g/L glucose (original HS formula) or 20 g/L of other carbon sources (e.g., fructose, glycerol, mannitol).

In order to prepare the bacterial inoculum for use in the cellulose biosynthesis assays, strains were firstly grown in HS (glucose) agar plates for three days at 28 °C. After this period, three loops (~10 µl) of the bacterial culture were inoculated in a sterile 50 mL Falcon tube containing 5 mL of liquid HS (glucose) medium and vortexed at maximum speed for 1 min in order to release bacteria from cellulose aggregates. One milliliter of each supernatant was collected and used for the determination of OD₆₀₀. The bacterial inoculum was adjusted to OD₆₀₀ = 0.5 and used in further experiments.

2.2. Bacterial cellulose biosynthesis assays under normal conditions

The bacterial ability to produce cellulose containing films was tested in HS medium, pH 6, supplemented with 20 g/L of different carbon sources (glucose, fructose, glycerol or mannitol) and ethanol, a known inducer of cellulose biosynthesis in several *Komagataeibacter* strains [10,14]. The final concentration of ethanol used in the growth media was 3% (v/v).

The assays were conducted in 60 mm plates (static conditions) or 500 mL flasks (agitated conditions, orbital, 125 rpm). Each plate received 7.5 mL of selected HS medium and 75 µL of the bacterial suspension (OD₆₀₀ = 0.5), and each of the flasks received 100 mL of selected HS medium and 1 mL of the bacterial suspension (OD₆₀₀ = 0.5). The plates and flasks were incubated at 28 °C for seven days. Each experiment consisted of three replicates.

After the incubation period, the obtained cellulose films were washed by immersion in a 0.1 M NaOH solution and incubation

overnight at 50 °C, and afterwards washed with distilled water. After the washing process, the films were freeze dried and weighted in an analytical scale.

2.3. Bacterial cellulose biosynthesis assays under stress conditions

Bacterial cellulose biosynthesis was also tested in HS medium (20 g/L glucose, 3% ethanol) under different stress conditions, such as acidic pH (3.5), alkaline pH (9.0), increased ethanol concentrations (5%, 7.5% and 10% v/v, pH 6) or increased osmolarity (50, 100, 150 g/L glucose, pH 6, 3% ethanol). The experiments were conducted in plates (static conditions) as described above. Bacterial cellulose production was measured qualitatively (presence of cellulose-containing films).

2.4. Strain FXV3 genome sequencing and analysis

Genomic DNA was isolated from a centrifuged bacterial inoculum (prepared from plates as previously described) using the PureLink™ Genomic DNA Mini Kit (Invitrogen, USA) following the manufacturer's instructions. The obtained DNA was quantified and sent to the Microbes NG company (<https://microbesng.com/>) for genome sequencing. Genomic DNA libraries were prepared using Nextera XT Library Prep Kit (Illumina, San Diego, USA) and were sequenced on the Illumina HiSeq using a 250bp paired-end protocol developed by Microbes NG. Reads were adapter trimmed using Trimmomatic 0.30 [15] with a sliding window quality cutoff of Q15. A total of 288,115 paired-end reads were obtained, representing a genome coverage of approximately 39X, and the *de novo* assembly was performed using SPAdes v. 3.7 [16]. The assembly resulted in 208 contigs (N50 = 35440) that were reordered using progressive alignments in MAUVE version 2.4.0 [17], using the complete genome sequence of *K. europaeus* SRCM101446 (NZ_CP021467.1) as guideline. The genome sequence of *K. uvaceti* FXV3 can be found in the NCBI database with the accession number VWPI00000000.

Genome annotation was performed using the NCBI Prokaryotic Genome Annotation Pipeline [18] and the functional genome annotation was performed using BlastKOALA [19]. Secondary metabolite production clusters were predicted using antiSMASH [20].

Comparative genomic analysis between *K. uvaceti* FXV3 and *K. xylinus* LMG1515^T was conducted using BLASTp, using a maximum e value threshold of 1e⁻²⁰. The analysis was conducted using the Geneious 9.1 software [21].

The genomic data presented in supplementary tables relates to the functional annotation obtained from BLASTKOALA analysis and BLASTp analysis (standard parameters) conducted against the UNIPROT database [22].

2.5. Phylogenetic analysis

The genomic sequences from all *Komagataeibacter* type strains were obtained from the NCBI database (<https://www.ncbi.nlm.nih.gov/>).

The multilocus sequence analysis (MLSA) was conducted based on the concatenated sequences of *recA*, *atpD*, *groL*, *dnaK* and *rpoB* genes from all *Komagataeibacter* type strains. The concatenated sequences were aligned using MUSCLE [23], and used in the phylogenetic analysis conducted using MEGA X [24].

Genomic average nucleotide identity (ANI) analysis was conducted using OrthoANI [25]. The dendrogram generated from OrthoANI contained the ANI values of the ten most closely related genomes of type strains, when compared to the genome of strain FXV3.

3. Results

3.1. Strain FXV3 produces increased levels of cellulose

The cellulose biosynthesis abilities of strain FXV3 and *K. xylinus* CECT 7351^T were tested in HS media supplemented with different carbon sources (20 g/L, glucose, fructose, glycerol and mannitol) and 3% ethanol (inducer of cellulose biosynthesis), under static and agitation conditions.

Under static conditions, strain FXV3 formed dense and homogeneous cellulose-containing films in all tested substrates (Fig. 1A). Still, cellulose biosynthesis was higher when the strain was cultivated in HS medium supplemented with glucose or glycerol (4.31 and 4.27 mg/mL, respectively), than with fructose (3.63 mg/mL) or mannitol (2.8 mg/mL) (Fig. 1B). Moreover, strain FXV3 cellulose production was higher (~3 fold) than that obtained for the type strain *K. xylinus* CECT 7351^T in any of the tested substrates (0.90–1.42 mg/mL), under similar cultivation conditions (Fig. 1 A, B).

On the other hand, when grown under agitation (orbital, 125 rpm), strain FXV3 produced cellulose-containing pellets (Fig. 2A) and the cellulose production was severely affected (<0.38 mg/mL) (Fig. 2B). Interestingly, *K. xylinus* CECT 7351^T was unable to grow under agitation conditions, indicating a Cel-phenotype for this strain.

3.2. Strain FXV3 presents increased stress tolerance abilities

Strain FXV3 was able to produce cellulose containing films when grown in a variety of stress conditions, including acidic pH (3.5), alkaline pH (9.0) (only in the presence of glucose, but not glycerol), ethanol concentrations up to 7.5% (v/v), and increased glucose concentrations (up to 150 g/L glucose and glycerol, maximum tested) (Table 1). The pH and ethanol induced stress

conditions negatively affected cellulose biosynthesis when compared to the control conditions, leading to the formation of clumps, thinner and less homogenous cellulose-based pellicles (Fig. 3). Moreover, these tests also revealed the increased stress resistance of strain FXV3 when compared to *K. xylinus* CECT 7351^T (Table 1). In this sense, *K. xylinus* CECT 7351^T was unable to form cellulose or grow at alkaline pH (9.0), nor in the presence of ethanol concentrations of 5 and 7.5% (v/v).

3.3. Strain FXV3 genome main features

The genome of strain FXV3 presented a size of approximately 3.61 Mbp and an average GC content of 60.9%. A total of 3386 CDS were predicted, of which 57 are related to RNA biosynthesis.

Genes encoding a putative plasmid replication initiator protein (F1542_16235), a ParA family protein (F1542_16220) and a ParB/RepB/Spo0J family partition protein (F1542_16210) were found together in the same replicon, further suggesting that strain FXV3 carries a plasmid.

BLASTKOALA analysis led to the functional annotation of 51.7% of the CDS, with most of the CDS being classified to genetic Information processing (375 CDS), signaling and cellular processes (216 CDS), carbohydrate metabolism (195 CDS), metabolism of cofactors and vitamins (119 CDS), environmental Information Processing (111 CDS) and amino acid metabolism (102 CDS) functions (Table 2).

The sec (secretion) and tat (twin-arginine translocation) secretion system genes were found in the genome, as well as genes encoding several lcm/Dot type IV secretion system proteins and Trb type IV secretion system proteins.

AntiSMASH analysis revealed the presence of gene clusters involved in the production of secondary metabolites such as bacteriocins, homoserine lactone, terpene and a terpene-saccharide (hopene).

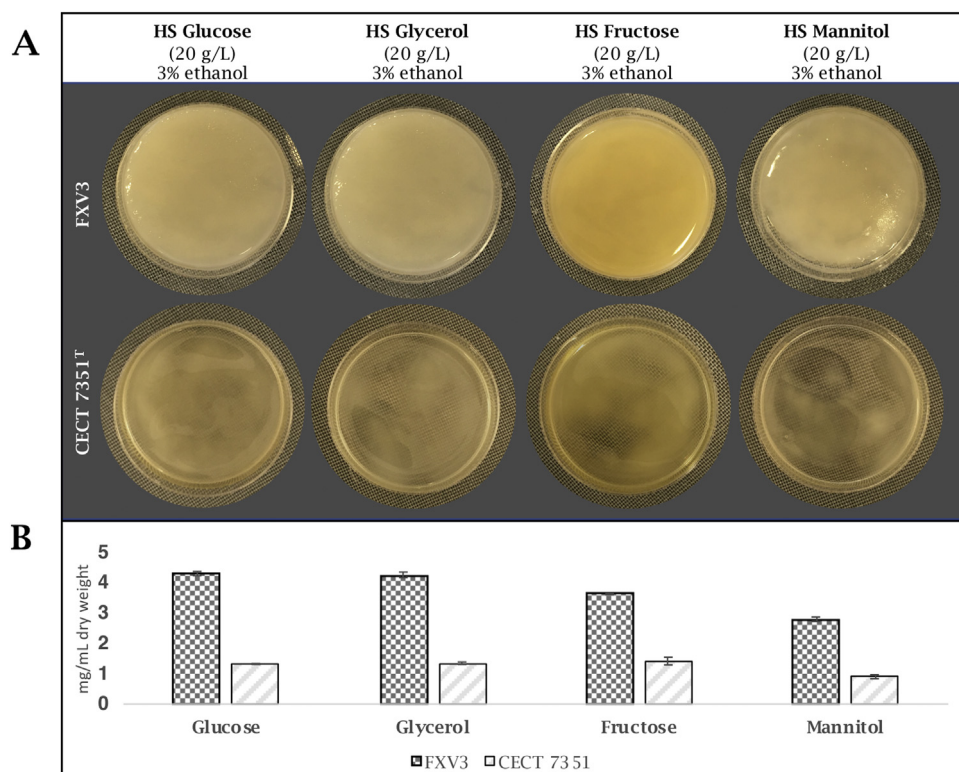


Fig. 1. Bacterial cellulose production by *K. uvaceti* FXV3 and *K. xylinus* CECT 7351^T strains under normal conditions (28 °C, pH 6, 7 days incubation) using several substrates. A) cellulose pellicles formed by the strains in the different substrates; B) dry weights of cellulose pellicles formed by the strains in the different substrates.

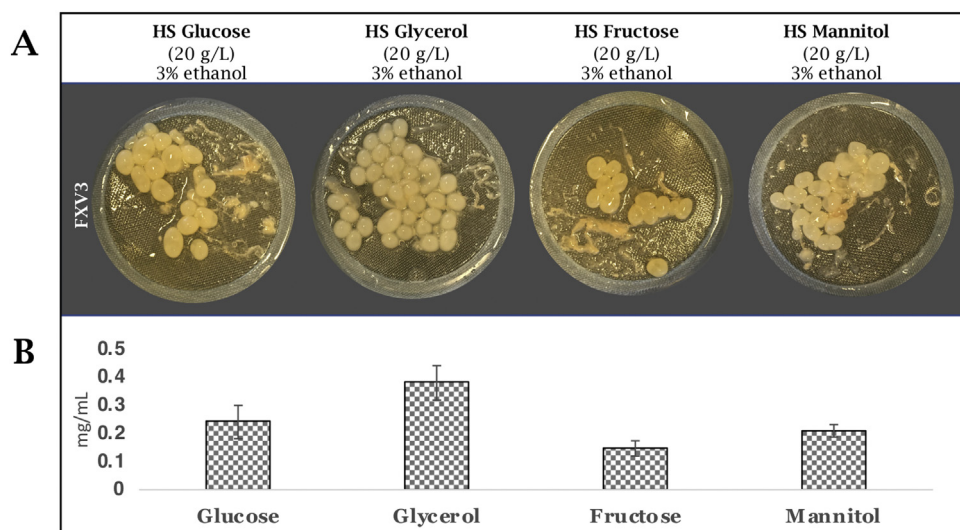


Fig. 2. Bacterial cellulose production by *K. uvaceti* FXV3 under agitation conditions (125 rpm, 28 °C, pH 6, 7 days incubation). A) cellulose pellets formed by the FXV3 strain in the different substrates; B) dry weights of cellulose pellets formed by the FXV3 strain in HS media supplemented with the different substrates.

Table 1
Growth of *K. uvaceti* FXV3 and *K. xylinus* CECT 7351^T strains under stress conditions.

Growth conditions	<i>K. uvaceti</i> FXV3	<i>K. xylinus</i> CECT 7351 ^T
20 g/L glucose, pH 3.5, EtOH 3%	+	+
20 g/L glycerol, pH 3.5, EtOH 3%	+	+
20 g/L glucose, pH 9, EtOH 3%	+	-
20 g/L glycerol, pH 9, EtOH 3%	-	-
20 g/L glucose, pH 6, EtOH 5%	+	-
20 g/L glycerol, pH 6, EtOH 5%	+	-
20 g/L glucose, pH 6, EtOH 7.5%	+	-
20 g/L glycerol, pH 6, EtOH 7.5%	+	-
20 g/L glucose, pH 6, EtOH 10%	-	-
20 g/L glycerol, pH 6, EtOH 10%	-	-
50 g/L glucose, pH 6, EtOH 3%	+	+
100 g/L glucose, pH 6, EtOH 3%	+	+
150 g/L glucose, pH 6, EtOH 3%	+	+

(+) production of cellulose film.
(-) no production of cellulose film.

3.4. MLSA and phylogenomic analysis

The Multilocus Sequence Analysis showed that strain FXV3 grouped closer to the *K. europaeus* LMG 18890^T type strain, however, it formed a distinct and independent cluster (Fig. 4A).

This result was in agreement with the data obtained from the genomic ANI analysis (Fig. 4B), which revealed that *K. europaeus* LMG 18890^T is the closest relative of strain FXV3 (93.37% ANI), followed by *K. swingsii* LMG 22125^T (91.35% ANI), *K. dyospiri* MSK9^T (90.31% ANI), *K. intermedius* TF2^T (88.53% ANI), *K. oboediens* LMG 18849^T (88.49% ANI), *K. kakiaceti* JCM 25156^T (84.11% ANI), *K. rhaeticus* LMG 22126^T (83.56% ANI), *K. sucrofermentans* LMG 18788^T (83.37% ANI), *K. medellinensis* NBRC 3288^T (83.23% ANI), *K. xylinus* LMG 1515^T (83.22% ANI), *K. nataicola* LMG 1536^T (83.24% ANI), *K. saccharivorans* LMG 1582^T (82.49% ANI), *K. maltaceti* LMG 1529^T (78.45% ANI), *K. entanii* LTH 4560^T (78.47% ANI), *K. cocois* WE7^T (78.25% ANI), *K. pomaceti* T5K1^T (78.33% ANI) and *K. hansenii* LMG 23726^T (76.14% ANI). Nevertheless, all the obtained genomic ANI values are lower than the proposed value threshold (95–96% ANI) for the delimitation of bacteria belonging to the same species [26]. Since all *Komagataeibacter* type strains were used in the analysis and none presented more than 95–96% ANI to FXV3, then these results indicate that strain FXV3 belongs to a new species, hereby tentatively termed *Komagataeibacter uvaceti* FXV3 (uva = grape; aceti = acetic acid; producing acetic acid from grape fermentations).

Currently, there is no other available bacterial genome presenting more than 95% ANI when compared to the genome of strain FXV3.

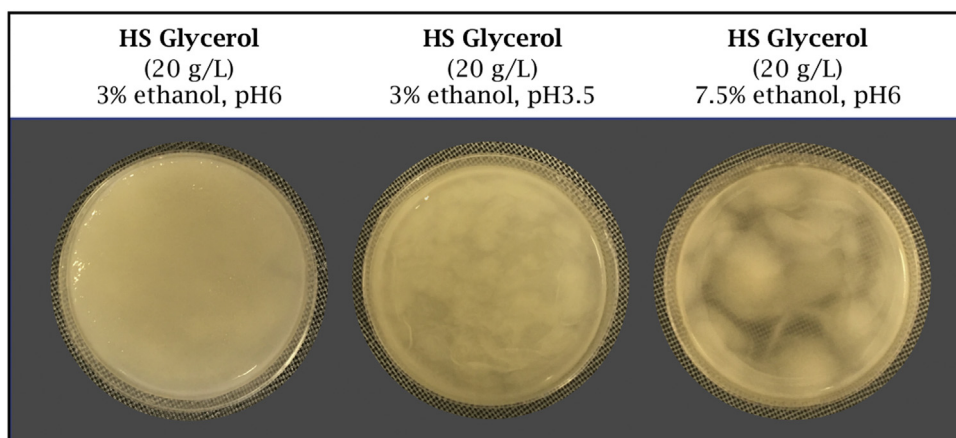


Fig. 3. Bacterial cellulose pellicles formed by *K. uvaceti* FXV3 under stress conditions.

Table 2
Main genomic characteristics of *K. uvaceti* FXV3 and *K. xylinus* LMG 1515^T.

Genome characteristics	<i>K. uvaceti</i> FXV3	<i>K. xylinus</i> LMG 1515 ^T
Genome size (Mbp)	3.618	3.661
Genome GC%	60.9	62.2
CDS	3386	3533
RNA related genes	57	54
BLASTKOALA Functional annotation: Number of CDS		
Signaling and cellular processes	215	206
Genetic Information processing	204	205
Carbohydrate metabolism	195	176
Metabolism of cofactors and vitamins	119	108
Environmental Information Processing	111	110
Amino acid metabolism	102	100
Energy metabolism	93	60
Nucleotide metabolism	74	68
Cellular processes	43	51
Lipid metabolism	34	28
Metabolism of other amino acids	27	24
Glycan biosynthesis and metabolism	23	23
Metabolism of terpenoids and polyketides	25	18
Xenobiotics biodegradation and metabolism	9	7

3.5. Genomic insights into *Komagataeibacter uvaceti* FXV3 cellulose production abilities, metabolism and stress-resistance mechanisms

3.5.1. Cellulose biosynthesis

The genome of strain FXV3 harbors multiple genes involved in cellulose biosynthesis, including three copies of the cellulose synthase gene, *bcsA* (Table S1). The *bcsA1* gene (F1542_14325) was found in a typical cellulose biosynthesis cluster containing *bcsZ* (endoglucanase), *ccpA* (cellulose-complementing protein), *bcsB* (cellulose synthase subunit B), *bcsC* (cellulose synthesis tetratripeptide repeat domain protein), *bcsD* (cellulose synthesis

periplasmic protein) and a *bglX* (beta-glucosidase) gene (Table S1); the *bcsA2* gene (F1542_08375) was detected in a cluster containing *bcsX* (SGNH/GDSL hydrolase family protein), *bcsY* (membrane-bound transacylase), *bcsC*, and an uncharacterized gene encoding a SGNH/GDSL hydrolase family protein (Table S1); the *bcsA3* gene (F1542_06685) was found with no other cellulose biosynthesis genes in its direct vicinity.

The genes involved in the biosynthesis of the bacterial cellulose precursor, uridine-5'-diphosphate- α -D-glucose (UDP-glucose), were also found (Table S1). In this sense, a glucokinase encoding gene (F1542_15525) responsible for the conversion of D-glucose to D-glucose-6-phosphate; a gene encoding a phosphoglucomutase (F1542_04970), responsible for the conversion of D-glucose-6-phosphate to D-glucose-1-phosphate ; and a UTP-glucose-1-phosphate uridylyltransferase encoding gene (F1542_04965), involved in the direct biosynthesis of UDP-glucose, were found present in the FXV3 genome.

In addition, the FXV3 genome also harbors several genes encoding enzymes involved in the biosynthesis (diguanylate cyclase, three genes) and catabolism (phosphodiesterase, eight genes) of c-di-GMP (Table S1), a known regulator of cellulose biosynthesis [27]. The three diguanylate cyclases and the eight phosphodiesterases found in the FXV3 genome presented increased identity to the functional diguanylate cyclases and phosphodiesterases of *K. xylinus* 1306–21 [27] (Table S2; Figs. S1 and S2).

3.5.2. Biosynthesis of acetan

Komagataeibacter strains are known to synthesize other extracellular polysaccharides besides cellulose. One of such polysaccharides is acetan, a water-soluble polysaccharide presenting homology to xanthan that plays a role in cellulose biosynthesis and cell protection against stress [12]. The acetan biosynthesis gene cluster has been described in *K. sucrofermentans* DSM 15973 [28] and identified in other *Komagataeibacter* strains, such as *K. xylinus* E25, *K. intermedius* AF2, *K. europaeus* LMG 18890^T, among others [4].

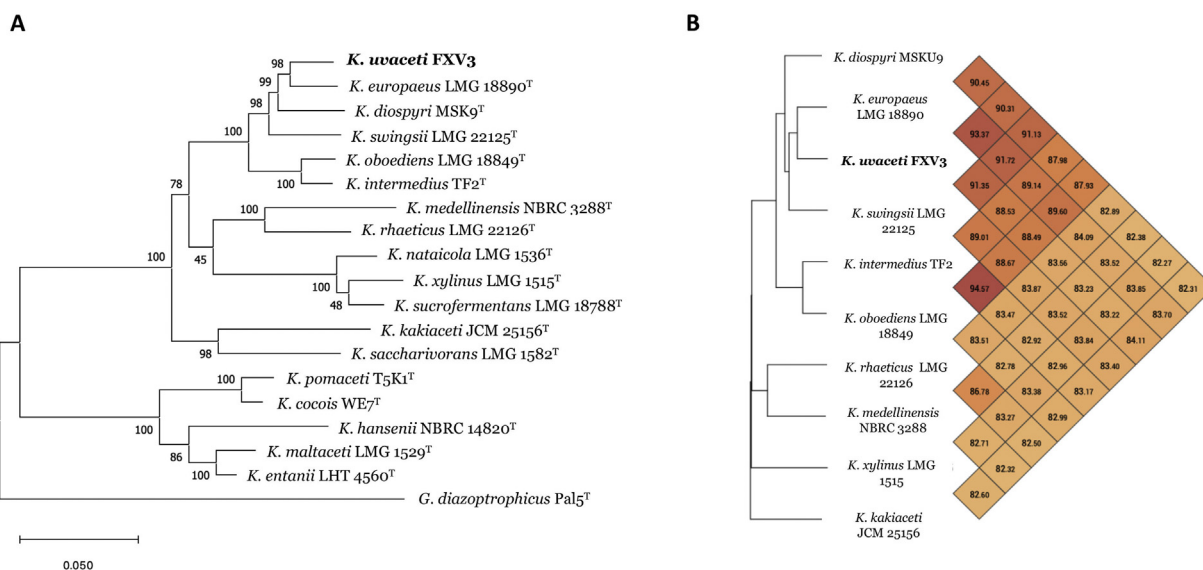


Fig. 4. Phylogenetic analysis of the *Komagataeibacter* genus. **A)** Phylogram based on MLSA. The evolutionary history was inferred by using the Maximum Likelihood method and General Time Reversible model. The tree with the highest log likelihood (-50179.58) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Joining and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2120)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 36.01% sites). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. *Gluconacetobacter diazotrophicus* Pal5^T was used as an outgroup. **B)** Dendrogram constructed based on genomic average nucleotide identity of selected *Komagataeibacter* strains. The phylogram was constructed using OrthoANI.

Genomic analysis revealed the presence of several acetan biosynthesis genes in the genome of strain FXV3. These genes were found in a cluster of approximately 34 Kb, containing *ace* gene homologs, as well as other associated glycosyltransferases (**Table S3**).

GDP-alpha-D-mannose is one of the acetan building blocks [29], and its biosynthesis is accomplished by the action of mannose-6-phosphate isomerase (*manA*), phosphomannomutase (*manB*), and GDP-D-mannose pyrophosphorylase (*aceF/xanB*). Curiously, the genome of strain FXV3 contains *manA*, two copies of *manB* and two *aceF/xanB* homologs (one in the acetan cluster, and other found far from the acetan gene cluster) (**Table S3**), suggesting the strain's ability for increased production of GDP-mannose.

3.5.3. Quorum-sensing and quorum-quenching mechanisms

Some *Komagataeibacter* strains may produce N-acyl-homoserine-lactones (AHLs) that are involved in quorum-sensing mechanisms. For example, *K. intermedius* NCI1051 produces N-decanoyl-L-homoserine lactone, N-dodecanoyl-L-homoserine lactone, and an N-dodecanoyl-L-homoserine lactone, through the expression of the *GinI/GinR* quorum-sensing system [30]. Moreover, the *ginI/ginR* genes are involved in the repression of oxidative fermentation, including acetic acid and gluconic acid fermentation, and may play a role in the regulation of c-di-GMP [31].

Genomic analysis indicated the presence of an acyl-homoserine-lactone synthase encoding gene (F1542_06620) and an associated gene encoding a *luxS*-like transcriptional regulator (F1542_06615) in the genome of strain FXV3. BLASTp analysis revealed that the F1542_06620 encoded protein presents 82.3% identity to *K. intermedius* NCI1051 *GinI* protein, and the F1542_06615 gene product presents 92.4% identity to *K. intermedius* NCI1051 *GinR* protein, which are involved in the synthesis of AHLs [30]. In addition, the FXV3 genome also harbors a gene (F1542_04870) encoding a protein that presents high identity (97.5%) to *K. europaeus* CECT 8546 *Gqqa* protein involved in bacterial quorum-quenching and cellulose formation [8].

Altogether, the obtained results suggest that strain FXV3 is able to synthesize and metabolize AHLs that regulate important cellular functions including fermentation and cellulose biosynthesis.

3.5.4. Carbohydrate metabolism and transport

The genes encoding the enzymes involved in the Pentose Phosphate, Entner-Doudoroff and De Ley-Doudoroff (via D-galactonate) pathways were detected in *K. uvaceti* FXV3 genomic sequence (**Table S4**). Most of the genes involved in glycolysis were also detected in the genome, however, the phosphofructokinase gene was not found, thus suggesting an incomplete metabolic pathway.

In addition, genes involved in the metabolism of other sugars such as fructose, ribose, sucrose, trehalose, glycogen, D-xylulose, L-sorbose, sulfoquinovose and glucosides; sugar acids, such as gluconate (and several of its keto forms), D and L-lactate, D-galactonate; and sugar alcohols such as glycerol, D-sorbitol, galactitol, xylitol and L-idoitol, were also detected (**Table S4**).

Additionally, the FXV3 genome contains several genes encoding proteins and transport systems involved in the transport of sugars, including a glucose ABC transporter permease protein homolog, four galactose:H⁺ symporters, a fructose/mannose PTS system, three gluconate:H⁺ symporters, a polyol transport system, and three glycerol uptake facilitator proteins (**Table S4**).

3.5.5. Amino acid and 4-aminobutyrate (GABA) metabolism

Amino acid transport and metabolism genes were detected in the genome sequence of strain FXV3 (**Table S5**), thus, suggesting the use of several amino acids (e.g. L-aspartate, L-asparagine, L-glutamine, L-histidine, glycine) as carbon and nitrogen sources.

In addition, genomic analysis revealed that the genome of strain FXV3 is rich in genetic elements involved in the transport and metabolism of the non-proteinogenic amino acid, 4-aminobutyrate (GABA). This includes four *gabP* genes encoding GABA permease homologs, involved in the transport of GABA: *gabT*, encoding a 4-aminobutyrate-2-oxoglutarate transaminase responsible for the conversion of GABA to succinate semialdehyde and glutamate; and six *gabD* gene homologs, encoding the succinate-semialdehyde dehydrogenase [NADP(+)] enzyme that catalyzes the NADP⁺-dependent oxidation of succinate semialdehyde to succinate (**Table S5**). GABA is commonly found in fermented food and beverages, including grape must, and fermentative yeasts are able to use it as sole carbon and nitrogen sources [32]. The obtained results suggest that, like wine yeasts, *K. uvaceti* FXV3 is able to use GABA as sole carbon (via succinate) and nitrogen (via glutamate) sources possibly potentiating its colonization activities.

3.5.6. Alcohol and aldehyde metabolism

Komagataeibacter strains are known to efficiently metabolize ethanol, and, consequently, produce acetic acid that serves as a mechanism of protection against other bacteria (high acidity), as well as a potential carbon source used for cell growth. The metabolism of ethanol and synthesis of acetic acid is accomplished by the action of alcohol dehydrogenase and aldehyde dehydrogenase enzymes [33].

Two *adhA* genes encoding the membrane-associated quinone-dependent alcohol dehydrogenase, and one *adhB* gene encoding a cytochrome c subunit, (both involved in the conversion of ethanol to acetaldehyde) were detected (**Table S6**). Moreover, several *aldH* genes, encoding acetaldehyde dehydrogenase responsible for the conversion of acetaldehyde to acetic acid, are also present in the genome of strain FXV3 (**Table S6**). These results indicate that strain FXV3 possesses all the genetic mechanisms necessary for ethanol oxidation and acetic acid biosynthesis.

Additionally, the FXV3 genome also contains a gene (F1542_01330) encoding an alcohol dehydrogenase presenting homology to *Pseudomonas putida* AlkI enzyme involved in the oxidation of 1-butanol, 1-hexanol, and 1-octanol [34]; a gene (F1542_05515) encoding a class-3 alcohol dehydrogenase known to oxidize long-chain primary alcohols and S-(hydroxymethyl) glutathione [35]; genes (F1542_05850; F1542_13140) encoding enzymes that present similarity to *Geobacillus stearothermophilus* NAD⁺-dependent alcohol dehydrogenase involved in methanol, ethanol, propan-1-ol, butan-1-ol and butan-2-ol oxidation [36]; and a gene (F1542_08460) encoding an alcohol dehydrogenase presenting high identity to *Escherichia coli* alcohol dehydrogenase YqhD that metabolizes several alcohols [37].

Several other aldehyde dehydrogenase-encoding genes were also detected in the genome (**Table S6**). These include a gene (F1542_03780) that encodes a glutathione-independent formaldehyde dehydrogenase that catalyzes the NAD⁺-dependent oxidation of formaldehyde and acetaldehyde [38]; a gene (F1542_00605) encoding an enzyme presenting identity to *Bacillus subtilis* benzaldehyde dehydrogenase that acts on substrates with 3- and 4-hydroxy and methoxy substitutions (e.g. vanillin) [39]; the *paoCBA* genes encoding a periplasmic aldehyde oxidoreductase system that oxidizes aromatic aldehydes (e.g. cinnamaldehyde) to the corresponding carboxylic acids [40]; and a gene that encodes a coniferyl aldehyde dehydrogenase homolog that catalyzes the NAD⁺-dependent oxidation of coniferyl aldehyde to ferulic acid [41].

Overall, the obtained data suggests that *K. uvaceti* FXV3 is able to metabolize a wide range of alcohols and aldehydes, including aromatic alcohols and aldehydes known to act as inhibitors of bacterial growth. The increased prevalence of these genes in the

genome is consistent with strain FXV3 increased resistance to high ethanol concentrations.

3.5.7. Metabolism of acetic acid and other organic acids

Genes involved in the transport and metabolism of major organic acids (acetic acid, citrate, fumarate, oxaloacetate, malate, succinate) were detected (Table S7). Amongst these were genes encoding a succinate-acetate/proton symporter homolog involved in the uptake of acetic acid and succinic acid, as well as a succinyl-CoA:acetate CoA-transferase encoding gene (F1542_05590) that is involved in the conversion of acetic acid into acetyl-CoA through the transfer of the CoA moiety from succinyl-CoA to acetate. BLASTp analysis revealed that the F1542_05590 encoded enzyme presents 67.3 % identity to the succinyl-CoA:acetate CoA-transferase of *Acetobacter aceti* that is required for growth on acetic acid, and for the resistance to high concentrations of acetic acid [42].

In addition, an acetate kinase encoding gene, *ackA* (F1542_11010), involved in the conversion of acetic acid to acetyl phosphate was also found in the FXV3 genome. Curiously, a gene encoding a phosphoketolase homolog (F1542_11005) that uses acetyl phosphate as substrate was found in the direct vicinity of *ackA*, suggesting the channeling of acetate into the carbohydrate metabolism pathways.

3.5.8. Resistance to stress conditions

In addition to the efficient metabolism of toxic products (e.g. acetic acid, ethanol) *Komagataeibacter* and related acetic acid bacteria also employ other mechanisms in order to cope with stress conditions, including the modification of membranes (e.g. composition and the content of components such as lipids, polysaccharides and terpenoids), efficient efflux systems, production of osmolytes and oxidative stress resistance mechanisms [33,43–45].

3.5.8.1. Membrane modifications. In the presence of high concentrations of acetic acid, *Komagataeibacter europaeus* V3 increases the concentration of cis-vaccenic acid and phosphatidylglycerol in its membranes. Moreover, this strain also produces a spongy layer and modifies its cell form (from short to long rods) in order to cope with increased levels of acetic acid [45], thus, indicating that overall membrane modifications play a role in the adaptation to acid stress.

Genes involved in fatty acid biosynthesis, terpenoid biosynthesis (hopanoid), glycerolipid and glycerophospholipid metabolism were abundantly found in the genome of strain FXV3 (Table S8). Moreover, genes encoding enzymes involved in sphingolipid and capsular polysaccharides biosynthesis (possibly involved in the production of the spongy layer), were also detected (Table S8). This data suggests that strain FXV3 is able to synthesize and metabolize several membrane-associated compounds, consequently modifying its membrane organization and fluidity to adapt and resist to increased concentrations of toxic compounds such as acetic acid and ethanol.

3.5.8.2. Efflux systems and transporters. Acetic acid bacteria possess efficient efflux systems that allow the transportation of toxic metabolites from the cell to the external environment, thus, facilitating the resistance against toxic compounds (e.g. acetic acid) [33].

Genomic analysis revealed that *K. uvaceti* FXV3 possesses an increased number of multiple efflux system genes as well as other transporter genes (Table S9), including several compound:H⁺ transporters involved in the removal of H⁺ from the cell and the balancing of cell pH (Table S9).

3.5.8.3. Production of compatible solutes. Bacteria are known to synthesize a wide range of intracellular compounds (known as

compatible solutes), such as trehalose, glycogen, glycine-betaine, proline, glutamine, and polyols, that play a role in the resistance to osmotic stress as well as other stress conditions [46]. *K. uvaceti* FXV3 is not only resistant to increased levels of ethanol and acetic acid but it is also able to grow in the presence of increased levels of sugars (150 g/L glucose), indicating a strong osmotic stress resistance. These results are consistent with the increased presence of genes involved in the biosynthesis and metabolism of trehalose, glycogen, glutamate, glutamine and proline in the genome sequence of *K. uvaceti* FXV3 (Table S10), suggesting an increased production of several compatible solutes.

3.5.8.4. Oxidative stress resistance. Stress conditions and the exclusive aerobic and oxidative metabolism of *Komagataeibacter* leads to increased levels of oxidative damage. As a result, the genome of *K. uvaceti* FXV3 contains several genes encoding enzymes involved in the oxidative stress response (Table S11), including one catalase, a superoxide dismutase [Cu-Zn], a superoxide dismutase [Fe], peroxiredoxin OsmC, peroxiredoxin bcp, other peroxiredoxins, cytochrome c peroxidase, Dye-decolorizing peroxidase YfeX, four alkyl hydroperoxide reductases and glutathione S-transferase. Additionally, one gene encoding a nitric oxide dioxygenase involved in the resistance against nitrosative stress was also found in the genome (Table S11).

3.6. Comparative genomics between *K. uvaceti* FXV3 and *K. xylinus* LMG 1515^T (=CECT 7351^T)

The genomes of *K. uvaceti* FXV3 and *K. xylinus* LMG 1515^T present different characteristics (Table 2), which resulted in a differential functional annotation and a value of only 83.22% ANI between the genomes. Comparative genomic analysis revealed that *K. uvaceti* FXV3 and *K. xylinus* LMG1515^T share 2676 CDS, however, 710 CDS were uniquely found in *K. uvaceti* FXV3. Interestingly, most of the CDS that were found in *K. uvaceti* FXV3 but absent in *K. xylinus* LMG 1515^T genome are involved in several processes related to detoxification and stress resistance mechanisms (Table S12).

The genes uniquely found in *K. uvaceti* FXV3 encode proteins mainly involved in oxidative stress resistance (e.g. glutathione-S-transferase, chloroperoxidase), acid stress resistance (e.g. YdeP, cytochrome bd-I ubiquinol oxidase), DNA repair (e.g. UmuC, LigD), membrane modifications (e.g. dihydroceramide fatty acyl 2-hydroxylase, ethanolamine ammonia-lyase, CDP-diacylglycerol pyrophosphatase), biosynthesis of AHLs, glucans and opines (e.g. AHL synthase, cyclic beta-(1,2)-glucan synthase, agropine reductase), metabolism of alcohols and aldehydes (e.g. AlkJ, PaoABC), carbohydrate metabolism (e.g. L-sorbose, fructose, D-galactonate), amino acid metabolism (e.g. histidine ammonia-lyase), and xenobiotics metabolism (e.g. methylamine dehydrogenase, alkanesulfonate monooxygenase, nitronate monooxygenase). These results are in agreement with the observed increased stress resistance of *K. uvaceti* FXV3 when compared to *K. xylinus* LMG 1515^T.

Since *K. uvaceti* FXV3 also presented an increased cellulose biosynthesis when compared to *K. xylinus* LMG1515^T, we also analyzed the cellulose biosynthesis operons of both strains (Fig. 5A, B). The cellulose biosynthesis operons presented similar organizations, still, sequence alignments revealed some level of diversification. In this case, sequence alignments revealed that the *bcsABCD* and *bcsAXYC* gene clusters of FXV3 present 80.4% and 71.7% identity, respectively, to those of *K. xylinus* LMG 1515^T. Curiously, the *K. xylinus* LMG 1515^T *bcsA2XYC2* operon does not contain a transcriptional regulator, instead it contains a transposase gene in its direct vicinity, thus, suggesting that this operon is possibly less functional [47]. On the other hand, in *K. uvaceti* FXV3 a TetR/AcrR family transcriptional regulator is present in the direct vicinity of the *bcsAXYC* operon.

Genomic analysis also revealed that both strains possess three diguanylate cyclase encoding genes, still, *K. uvaceti* FXV3 possesses more copies of phosphodiesterase genes than *K. xylinus* LMG 1515^T (8 vs 6). In addition, *K. uvaceti* FXV3 possesses the GinI/GinR quorum-sensing system that is absent in *K. xylinus* LMG 1515^T.

4. Discussion

Genomic and functional analysis revealed that strain FXV3 is a novel stress resistant and efficient cellulose-producing *Komagataeibacter* species of increased biotechnological interest.

Phylogenomic and phylogenetic analysis showed that strain FXV3 formed an independent cluster and presented <95% ANI (the threshold for defining bacteria belonging to the same species [48]) values when compared to all *Komagataeibacter* species described to date. These results indicate that strain FXV3 represents a novel species within the *Komagataeibacter* genus, hereby, tentatively termed *K. uvaceti*.

K. uvaceti FXV3 produced cellulose in growth media supplemented with glucose, glycerol, fructose or mannitol, indicating a diverse metabolic potential. Still, the maximal production of cellulose by *K. uvaceti* FXV3 occurred when using glucose and glycerol as carbon sources. This data is in agreement with previous results obtained from other *Komagataeibacter* strains such as *K. xylinus* E26, *K. xylinus* BCRC 12334, *K. hansenii* ATCC 53582, *K. rhaeticus* PG2 or *K. nataicola* RZS01, that presented increased cellulose production abilities when grown in media supplemented with glucose or glycerol, but lower with fructose or mannitol [49–51]. Nevertheless, some other *Komagataeibacter* strains (e.g. *K. xylinus* K2G30 or *K. xylinus* K1G4) presented maximal cellulose production when using mannitol as sole carbon source [52,53], further indicating that the optimal carbon source for increased cellulose production is variable and strain dependent.

The maximal concentrations of cellulose produced by *K. uvaceti* FXV3 (4.31 g/L) were comparable and within the range (~4–8 g/L in 6–15 days of cultivation) of those of other efficient cellulose-producing *Komagataeibacter* strains (e.g. *K. xylinus* E26, *K. xylinus* K2G30, *K. xylinus* K1G4, *K. rhaeticus* P1463) [49,52–54], and increased when compared to the model type strain *K. xylinus* CECT 7351^T (1.42 g/L, this study).

Genomic analysis revealed that *K. uvaceti* FXV3 presented the *bcsZ*, *ccpA*, *bcsABCD* and *bcsAXYC* operons involved in cellulose biosynthesis, as well as several other genes that are known to impact cellulose biosynthesis, such as those encoding proteins involved in quorum-sensing, quorum-quenching, acetan biosynthesis and c-di-GMP biosynthesis and degradation. Interestingly, many of these genes were less abundant or even not present in *K. xylinus* CECT 7351^T genome, which may explain the observed discrepancy in cellulose biosynthesis abilities between both strains. For instance, *K. uvaceti* FXV3 presented an increased number of phosphodiesterase genes (8 vs 6), when compared to *K. xylinus* CECT 7351^T, thus, suggesting a differential biosynthesis of c-di-GMP, a known regulator of cellulose biosynthesis [27]. Moreover, *K. uvaceti* FXV3 possesses the GinI/GinR system involved in the biosynthesis of AHLs, which was not present in *K. xylinus* CECT 7351^T. Quorum-sensing mechanisms are involved in the regulation of *Komagataeibacter* fermentative metabolism and possibly in the regulation of c-di-GMP levels, thus, potentially impacting cellulose biosynthesis [27,28].

The analysis of the *K. uvaceti* FXV3 and *K. xylinus* CECT 7351^T *bcsABCD* and *bcsAXYC* cellulose biosynthesis operons also revealed differences between the strains. Both strains presented similar *bcsABCD* operons (organization and topology), however, *K. xylinus* CECT 7351^T presented a transposase gene in the direct vicinity of the *bcsAXYC* operon that was not detected in *K. uvaceti* FXV3 (this strain possessed a TetR/AcR transcriptional regulator in the direct vicinity of *bcsAXYC*). Previous studies have demonstrated that

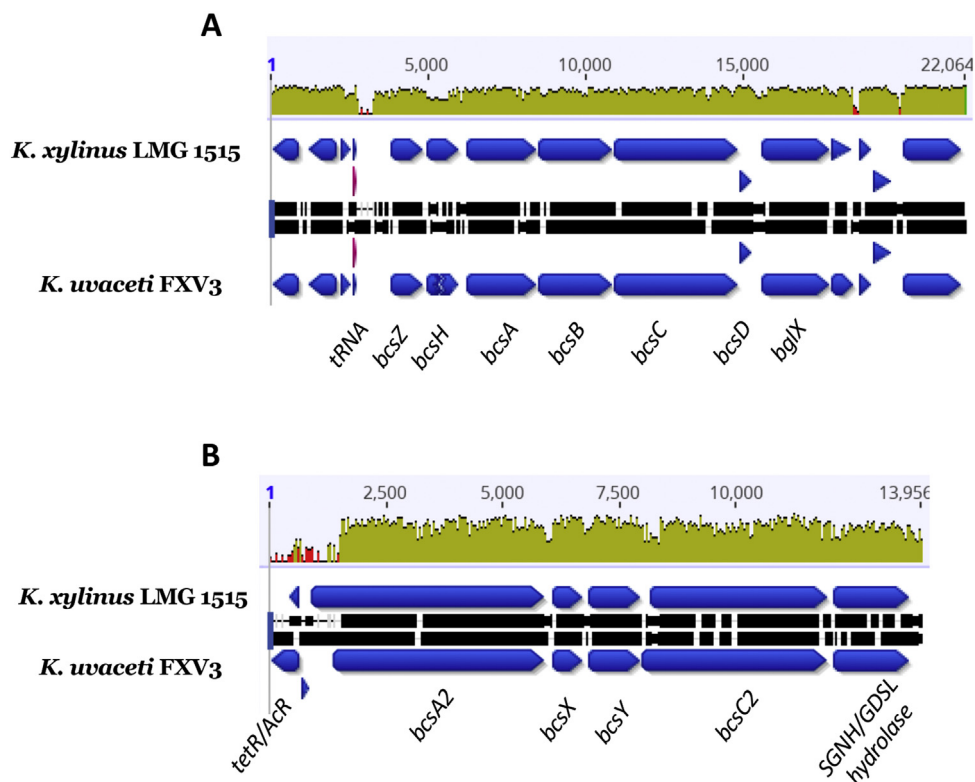


Fig. 5. *K. uvaceti* FXV3 and *K. xylinus* LMG 1515^T=CECT 7351^T cellulose biosynthesis genes sequence alignments. A) alignment of the *bcsABCD* gene cluster; B) alignment of the *bcsA2XYC2* gene cluster.

transposase genes are often detected in the cellulose biosynthesis operons of *Komagataeibacter* strains that present a Cel⁻ phenotype and therefore are unable to synthesize cellulose [56–58]. Curiously, our results showed that *K. xylinus* CECT 7351^T presented a Cel⁻ phenotype when grown under agitation conditions.

One of the major factors that limit bacterial cellulose commercialization is its high production costs. In order to overcome this problem several authors have used alternative and cheaper growth media/substrates, such as fruit juices, molasses, agro, food and industrial wastes [59,60]. Nevertheless, these cheaper substrates are often of complex chemical composition, and several of these contain not only C sources but also a wide range of toxic metabolites such ethanol and other alcohols, aldehydes and organic acids that may hinder cellulose production. Hence, in order to maximize cellulose production using cheaper substrates, *Komagataeibacter* strains presenting both efficient cellulose production and stress resistance abilities are desirable.

K. uvacetii FXV3 presented a remarkable resistance to stress conditions, especially high ethanol concentrations. This strain was able to form cellulose films in media presenting pH ranging from 3.5 to 9, increased osmolarity (150 g/L glucose) and ethanol concentrations up to 7.5% (v/v). Curiously, many of these traits (cellulose film biosynthesis in pH9 and in media containing >3% ethanol) were not observed in *K. xylinus* CECT 7351^T. To our knowledge, this is the first report of bacterial cellulose production in the presence of >5% ethanol (v/v) in the growth media. The observed results are in agreement with the genomic data analysis, which showed an increased prevalence of stress resistance genes in the genome of *K. uvacetii* FXV3, including multiple alcohol and aldehyde dehydrogenase encoding genes (involved in the metabolism of ethanol and other alcohols and aldehydes), as well as genes involved in the modification of membranes, efficient efflux systems, production of osmolytes and oxidative stress resistance mechanisms, all of which play a role in the adaptation and tolerance to a wide range of stress conditions [33,43–45]. Moreover, the comparative genomic analysis between *K. uvacetii* FXV3 and *K. xylinus* CECT 7351^T showed that most of the FXV3 unique genes encoded enzymes involved in stress resistance, and some were directly involved in alcohol-aldehyde metabolism/stress-resistance, such as AlkJ (alcohol dehydrogenase) [34] and PaoABC (aldehyde dehydrogenase) [40]. These results suggest that *K. uvacetii* FXV3, likely evolved in environments containing high concentrations of alcohols and aldehydes and consequently, developed efficient mechanisms to deal with the stress conditions imposed. This is consistent with the fact that strain FXV3 was isolated from a fermented grape must (wine), known to contain increased ethanol levels.

5. Conclusions

Ultimately, this study demonstrated that *K. uvacetii* FXV3 is a bacterium of increased biotechnological interest given its ability for the synthesis of cellulose using diverse substrates and its resistance to extreme stress conditions. These characteristics make it a suitable candidate for the development of novel fermentation strategies for bacterial cellulose production. Its detailed genomic analysis may also contribute to the development of novel tools to obtain genetically modified strains with increased stress resistance and cellulose biosynthesis abilities.

Author statement

All authors have made substantial contributions to the conception and design of the study, acquisition of data, analysis and interpretation of data, drafting the article and revising it critically, and approved the final version of the article to be submitted.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.btre.2021.e00606>.

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