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Gluconacetobacter hansenii subsp. nov., a High-Yield Bacterial Cellulose Producing Strain Induced by High Hydrostatic Pressure

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Abstract Strain M_{438}, deposited as CGMCC3917 and isolated from inoculums of bacterial cellulose (BC) producing strain screened in homemade vinegar and then induced by high hydrostatic pressure treatment (HHP), has strong ability to produce BC more than three times as that of its initial strain. It is the highest yield BC-producing strain ever reported. In this paper, M_{438} was identidied as *Gluconacetobacter hansenii* subsp. nov. on the basis of the results obtained by examining it phylogenetically, phenotypically, and physiologically–biochemically. Furthermore, the genetic diversity of strain M_{438} and its initial strain was examined by amplified fragment length polymorphism. The results indicated that strain M_{438} was a deletion mutant induced by HHP, and the only deleted sequence showed 99% identity with 24,917–24,723 bp in the genome sequence of *Ga. hansenii* ATCC23769, and the complement gene sequence was at 24,699–25,019 bp with local tag GXY_15142, which codes small multidrug resistance (SMR) protein. It can be inferred that SMR might be related to inhibiting BC production to a certain extent.

Keywords Phenotypically · Physiologically–biochemically · Phylogenetically · 16S rRNA genes · *Gluconacetobacter hansenii* subsp. nov. · Genetic diversity

Abbreviation
CGMCC China General Microbiological Culture Collection

Introduction

Cellulose, the most abundant biopolymer on earth, is usually obtained from plant cell walls by different pulping processes. But these processes always bring pollutants to the environment, such as polychlorinated dioxins, furans, sulfur oxides, nitrogen oxides, and carbon monoxide [1]. So, an eco-friendly cellulose-producing method is urgently required.

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Several kinds of bacteria have the ability to produce extracellular cellulose [2–6]. This kind of cellulose is called bacterial cellulose (BC) consisting of sets of parallel chains of β-1,4-D-glucopyranose units interlinked by intermolecular hydrogen bond and free of lignin, pectin, and hemicellulose [7], which has many unique properties comparing with that originating from the plant cell walls including high purity, high crystallinity, high water absorbing and holding capacity, high tensile strength, and strong biological adaptability [8–11].

Because of these unique properties, BC becomes a potential alternative to plant-derived cellulose for lots of special applications, such as biomedical field [8, 12–16], component for audio membranes [17], electronic paper [18], optically transparent composites [19], reinforcing agent for paper [20] and food additives [10, 11, 21]. As the range of the application of this biomaterial is expanded and the amount is increased, the strain with quite strong ability to produce BC is intensely demanded by the relevant BC industries. In order to meet the requirement of industrial application, BC mass production process should be developed by improving fermentation technology and BC-producing ability of strains [15]. A decade ago, researchers screened beneficial strains from mutants induced by physical and chemical methods [22, 23]. However, because these mutagenesis methods have radiation or toxicity which is rather harmful to human health, their applications are limited. So, it is meaningful to find new mutagenic treatments to increase BC yield of BC-producing bacteria.

In recent years, there were reports that *Photobacterium profundum*, a bacterium growing optimally in the deep ocean, adapted in metabolism and protein structure to high hydrostatic pressure (HHP) of its living environment, and this bacterium was so finely tuned to high-pressure life that atmospheric pressure triggered a stress response that activated distinct chaperones and DNA repair proteins [24, 25]. There were also reports that HHP treatment in laboratory could cause beneficial mutagenesis to beer yeast [26], *Esherichia coli* [27], *Ganoderma lucidum Karst*, and *Coprinus ovatus* [28]. All these reports gave us inspirations on whether or not HHP treatment could have beneficial mutagenic effects on the BC-producing strain.

In our previous research, a BC-producing strain J_2 was naturally isolated under static condition [29, 30]. It has been treated by HHP technology, and then a new strain M_{438} was obtained, which improved the yield of BC (dry weight) from 1.08 g in a 1,000-mL fermentation medium of J_2 to 3.58 g in the same volume of fermentation medium of M_{438} under the same static cultivation condition of at 30 °C for 8 days. Strain J_2, being the initial strain of M_{438}, was identified as *Gluconobacter oxydans* based on strain J_2's form and physiological and biochemical characteristics [30]. But by means of the next research, J_2 was reported as a *Gluconacetobacter xylinus* strain [31]. In this paper, the strain M_{438} was identified as *Gluconacetobacter hansenii* subsp. nov. based on phylogenetic analysis of 16S rRNA gene sequences, morphology, and physiological–biochemical characteristics. And J_2, which was found having the same phylogenetic relationships, characteristics of morphology, and physiology–biochemistry as M_{438}, should be re-identified as a varietas of *Ga. hansenii*.

In further study, the genetic diversity of M_{438} and initial strain J_2 were analyzed by amplified fragment length polymorphism (AFLP) technology, which is a powerful, highly reproducible and discriminatory tool for revealing genetic relationships in bacterial populations created by the scientists Zabeau and Vos of Dutch Keygene company in 1993 [32, 33]. It was found that strain M_{438} was a deletion mutant induced by HHP. The only deleted sequence was subjected to Basic Local Alignment Search Tool (BLAST) analysis and then appeared 99% identity with 24,917–24,723 bp in the genome sequence of *Ga.*

hansenii ATCC23769 (accession no. ADTV 01000064.1). The complement gene sequence of deleted sequence was at 24,699–25019 bp with local tag GXY_15142, which code small multidrug resistance (SMR) protein. Due to short length and broad substrate profile, SMR proteins are suggested to be the progenitors for larger α-helical transporters such as the major facilitator superfamily (MFS) and drug/metabolite transporter (DMT) superfamily [34]. Therefore, SMR proteins might be bound up with inhibiting BC production in some degree in the metabolic reactions. The differences between M_{438} and initial strain J_2 on proteomics were expected to be found in our advanced research, which could be a preliminary study of the mechanism underlying mutagenesis-induced BC-producing strain by HHP treatment, and supply a theoretical basis for increasing production mechanism of high-yield BC-producing strain and constructing BC-producing engineering bacteria.

Materials and Methods

Microorganism Preservation and Inoculation Preparation

The strain M_{438} used in this study was deposited as strain CGMCC3917 at China General Microbiological Culture Collection, Beijing, China, which was maintained on glucose agar slants containing (*w*/*v*): glucose 2%, yeast extract 0.5%, K_2HPO_4 0.1%, $MgSO_4{\cdot}7H_2O$ 1.5%, ethanol 1.5% (*v*/*v*), and agar 1.7% [29]. The strain was cultured for 12–18 h at 30 °C, then stored at 4 °C in a refrigerator and subcultured every 2 months for inoculum development or stored at −80 °C with 20% glycerol instead of agar for long-time storage. For producing inoculums, a loop of the strain was transferred from a slant culture into an Erlenmeyer flask (250 mL) containing 100 mL seed medium with the same components as glucose agar slants but without agar. The seed cultures were grown at 30 °C in a rotary shaker incubator at 150 rpm for 12–18 h until it reached the logarithmic growth phase.

Phylogenetic Analyses Based on 16S rRNA Gene Sequences

For genomic DNA extraction, M_{438} was agitatedly cultivated in 100 mL of seed medium with 150 rpm at 30 °C for 12 h. The genomic DNA was subsequently extracted by using MiniBEST Bacterial Genomic DNA Extraction Kit Ver.2.0 (TaKaRa, Japan) and analyzed by agarose gel electrophoresis (2% agarose, 1× TBE). The entire 16S rRNA genes were amplified by PCR with universal primers 9F and 1510R [35]. The PCR amplification was performed in a 50-μL solution containing 100 ng of DNA, 1.25 U of Ex Taq DNA Polymerase (TaKaRa, Japan), 200 μM dNTP Mix, 1× Taq buffer, 10 μM of each primer, then added sterile double distilled water to 50 μL. The cycling program started with initial denaturation of DNA at 94 °C for 3 min and continued with 30 cycles of 94 °C for 30 s, 58 °C for 40 s, and 72 °C for 1 min. At the end, a final extension at 72 °C for 5 min was performed, followed by cooling down to 4 °C. PCR products were verified by the agarose gel electrophoresis containing 1.5% agarose and 1× TBE at 110 V for 40 min, and purified by a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, USA). The purified 16S rRNA genes were sequenced directly with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit on an ABI PRISM model 310 Genetic Analyzer (Applied Biosystems, USA). The following primers were used for sequencing: 9F, 1510R, and 520F. The sequences were submitted to GenBank and subjected to BLAST analysis. Then, multiple sequence alignments were made with the program Molecular Evolutionary Genetics Analysis (MEGA; version 4.1). Sequence gaps and ambiguous bases

were excluded. Distance matrices were calculated by the two-parameter method of Kimura [36]. The neighbor-joining method was used for constructing phylogenetic trees [37]. In addition, the maximum parsimony method and the maximum likelihood method were used in constructing phylogenetic trees based on 16S rRNA gene sequences [38, 39]. Also with the program MEGA (version 4.1), robustness for individual branches was estimated by bootstrapping with 1,000 replications [40], and a phylogenetic tree was constructed by the maximum likelihood method, while pairwise sequence similarities (percentage) were calculated for strain M_{438} on 16S rRNA gene sequences by DNAMAN 6.0.

Phenotypic Characterization

Individual morphology of strain M_{438} was examined in its logarithmic growth phase in seed medium. Colony morphology was examined on plating medium and lawns on glucose agar slant for 72 h incubation at 30 °C in an incubator. Plating medium of M_{438} had the same components with glucose agar slants. Characteristics of physiology–biochemistry and the tests for compounds used as sole carbon source and as sole nitrogen source were carried out as mentioned by Dongand Cai [41], Buchanan and Gibbons [42], and George et al. [4], as well as acid produced from different kind of sugar as carbon source.

Differentiating Species in the Genus *Gluconacetobacter*

The phylogenetic trees of M_{438} based on 16S rRNA gene sequences and phenotypic characteristics indicated that M_{438} should be in the genus *Gluconacetobacter*. Therefore, the characteristics for differentiating species in *Gluconacetobacter* were detected as mentioned by Feng et al. [43] and George et al. [4].

AFLP Analysis

AFLP analysis was performed in duplicate from the two independent DNA extractions according to the manufacturer's instructions of AFLP Analysis System for Microorganisms AFLP Microorganism Primer Kit (Invitrogen). Adapter and primer sequences used in the AFLP analysis and AFLP reaction systems were shown as Tables 1 and 2, respectively. Genomic DNA extraction was the same as that in the step of phylogenetic analyses based on 16S rRNA gene sequences. Through the optimization study, the optimized conditions of

Table 1 Adapter and primer sequences used in the AFLP analysis

Restriction enzymes	*Mse*I	*Eco*RI
Adapters [47]	5′-GACGATGAGTCCTGAG-3′	5′-CTCGTAGACTGCGTACC-3′
	5′-TACTCAGGACTCAT-3′	5′-AATTGGTACGCAGTC-3′
Preamplification primers [47]	M+0:5′-GATGAGTCCTGAGTAA-3′	E+0:5′-GACTGCGTACCAATTC-3′
Selective amplification primers	M+0:5′-GATGAGTCCTGAGTAA-3′	E+0:5′-GACTGCGTACCAATTC-3′
	M+A:5′-GATGAGTCCTGAGTAAA-3′	E+A: 5′-GACTGCGTACCAATTCA-3′
	M+C:5′-GATGAGTCCTGAGTAAC-3′	E+C: 5′-GACTGCGTACCAATTCC-3′
	M+G:5′-GATGAGTCCTGAGTAAG-3′	E+G: 5′-GACTGCGTACCAATTCG-3
	M+T:5′-GATGAGTCCTGAGTAAT-3′	E+T: 5′-GACTGCGTACCAATTCT-3″

Table 2 AFLP reaction systems of J_2 and M_{438}

System of digestion reaction (μL)	
DNA (200 ng/μL)	3
ddH2O	16.75
BSA	0.25
Buffer4	2.5
*Eco*RI (10 U/μL)	0.5
*Mse*I (10 U/μL)	2
Total volume	25
System of ligase reaction (μL)	
Digestion products	25
ddH2O	3.5
T4 Buffer	3.5
Mse I adapter	1
*Eco*R I adapter	1
T4 ligase (400 U/μL)	1
Total volume	35
System of preamplification (μL)	
Ligation products	1
M+0 (10 μmol)	1
E+0 (10 μmol)	1
MgCl2 (50 mmol/L)	3
Taq DNA polymeras (5 U/μL)	1
dNTPs (2 mmol/L)	1
10×PCR buffer	5
ddH2O	12
Total volume	25
System of preamplification (μL)	
Preamplification products	1
Mse I primer (10 μmol)	1
*Eco*R I primer (10 μmol)	1
MgCl2 (50 mmol/L)	1.5
Taq DNA polymeras (5 U/μL)	0.5
10×PCR buffer	2.5
dNTPs (2 mmol/L)	0.5
ddH2O	17
Total volume	25

AFLP reaction suitable for strain M_{438} and its initial strain J_2 were obtained as follows: 600 ng genomic DNA which served as template was digested with *Eco*RI and *Mse*I endonucleases at 37 °C, and the resulting fragments were ligated to the adapter–linker sequencer with T4 DNA ligase for 8 h or overnight (more than 10 h) at room temperature. The ligation mix was amplified by PCR with the *Eco*RI/*Mse*I core primer (+0/+0) for preamplification. Then, the products of preamplification were diluted 500-fold and amplified by PCR with 24 different primer combinations (+0/+1, +1/+0, +1/+1) for selective amplification, and an equal volume (25 μL) of 6× loading buffer in the products of selective amplification was added and heated for 5 min at 95 °C then immediately placed

on ice. Finally, the products of selective amplification was examined by 5% polyacrylamide gel with 0.5-mm spacers and sharkstooth combs. The gel was pre-electrophoresed at a constant voltage (250 V) for 30 min, and 5 μl of the samples on the gel was loaded and electrophoresed at a constant voltage until slower dye was two thirds down the length of the gel. Finally, the gel was treated with the method of silver staining. The diversity fragments were purified by a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences, USA) and sequenced directly with an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit on an ABI PRISM model 310 Genetic Analyzer (Applied Biosystems, USA).

Results and Discussion

A total of 1,403 bases of the 16S rRNA genes (Fig. 1) of the strain M_{438} were amplified and sequenced. The sequence was submitted to GenBank with an accession no. GU227424. A phylogenetic tree (Fig. 2) was constructed by neighbor-joining method for 1,403 bases based on the BLAST results of the 16S rRNA genes of the strain M_{438} on National Center for Biotechnology Information (NCBI). M_{438} formed a cluster together with three *Ga. hansenii* strains NBRC14816, NBRC14817, and NBRC14820 in a lineage composed of the species *Gluconacetobacter kombuchae* LMG23726T, *Ga. kombuchae* RG3, and *Gluconacetobacter entanii* LTH4560. The phylogenetics branch between M_{438} and the three *Ga. hansenii* strains has almost the same length with that between the strains of the two *Ga. kombuchae* strains and *Ga. entanii*. Two phylogenetic trees were constructed by maximum parsimony and maximum likehood methods as well, showing similar clustering with the one constructed above (data not shown). Pairwise sequence similarities (percentage) of M_{438} in16S rRNA gene sequences for 1,403 bp were calculated to be 95.46, 97.27, 97.20, 96.11, 96.12, and 92.99, respectively, to the *Gluconacetobacter* species strains of *hansenii* NBRC14816, *hansenii* NBRC 14817, *hansenii* NBRC 14820, *kombuchae* LMG23726T, *kombuchae* RG3, and *entanii* LTH4560. According to the BLAST results of the 16S rRNA

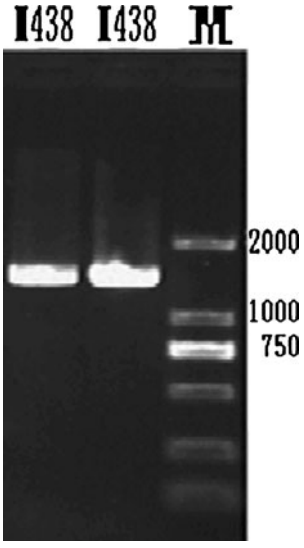

Fig. 1 Result of 16S rRNA genes of M_{438}. For estimation of 16S rRNA genes fragments, 2,000-bp DNA markers were used in agarose gel electrophoresis

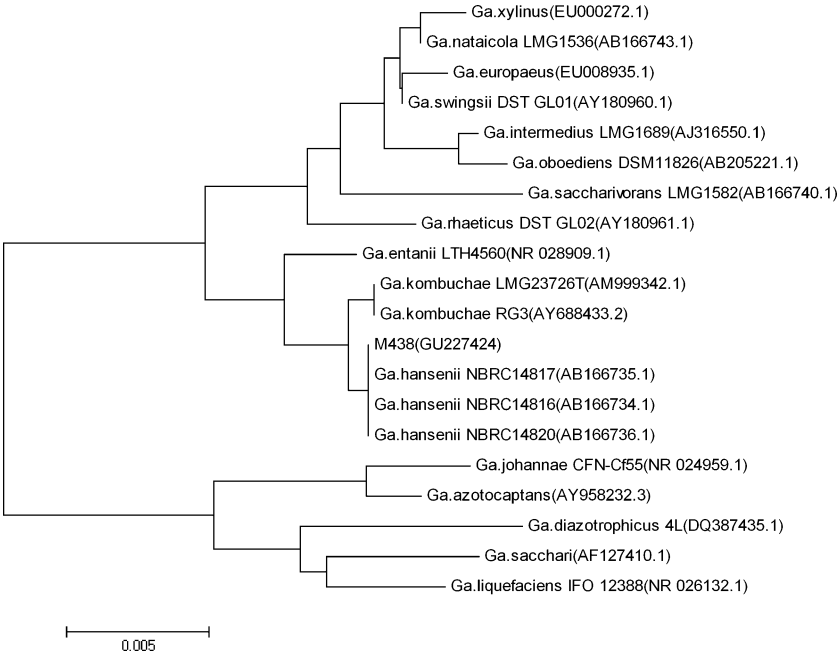

Fig. 2 Phylogenetic trees based on 16S rRNA gene sequences of M_{438}

genes of the strain M_{438} on NCBI and Fig. 2, all 16S rRNA gene sequences deposited in GenBank having high similarities (more than 70%) with those of M_{438} were *Gluconacetobacter* species, and 16S rRNA gene sequences of *Ga. hansenii* have the highest similarities with those of M_{438} as well.

The strain M_{438} was gram-negative rod, straight, or slightly curved singly or in pairs, 0.83–0.94×2.08–4.16 μm (Fig. 3). Colonies on plating medium were round, convex, and adiaphanous, 0.8–1.5 mm, with smooth surface (Fig. 4). Lawns on glucose agar slant were adiaphanous, filamentous, and smooth on the surface. Inoculums in seed medium for 3 days cultivation were free of bubbles and deposit, pellucid, and the color was unchanged, with pellicles on the surface.

As shown in Table 3, the strain could grow at pH 4.5 and 37 °C, but its optimal condition was 30 °C and it had natural pH. It presented positive in the tests of catalase, acetyl methyl carbinol, indole, and ketogenesis from glycerol, whereas negative in amylolysis, methyl red, gelatin liquefaction, halophilic test, nitrate reduction, and oxidation of acetic acid. M_{438} grew well in the media containing sole carbon source such as D-fructose, maltose, lactose or sucrose, and sole nitrogen source such as peptone, yeast extract or $(NH_4)_2HPO_4$, but poorly in the media containing D-xylose, methanol, propyl alcohol, KNO_3, or $NaNO_3$. M_{438} had the abilities to produce cellulose, 2-ketogluconic acid, and 5-ketogluconic acid from D-glucose, but did not produce H_2S, ammonia, and pigment on GYC medium. Additionally, M_{438} could produce acid from glucose and maltose but not from D-arabinose and inosite. M_{438} could oxidize ethanol, and need not niacin for growth. As mentioned by Buchanan and Gibbons [42], all these characteristics were also found in *Gluconacetobacter* strains. Differentiating species in the genus *Gluconacetobacter* was carried out and compared with the characteristics of the other 16

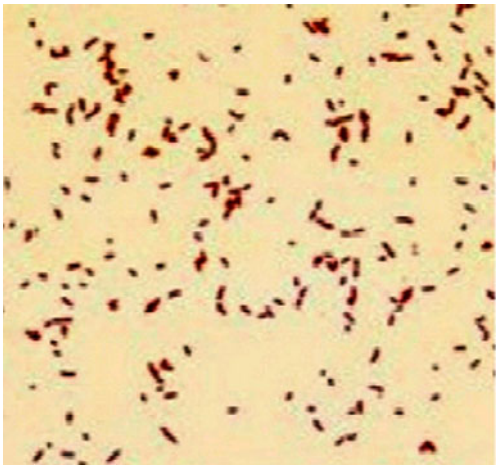

Fig. 3 Cell shape of bacterial strain M_{438}. The magnification of micrograph is 1,000 times

Gluconacetobacter species based on phylogenetic tree of M_{438} as described in Table 4. Strain M_{438} did not grow on 3% (*v*/*v*) ethanol in the presence of 5–8% acetic, methanol, or 0.01% malachite green agar, but on the medium of Carr and Passmore and carbon source ethanol. It could grow not only in the presence of acetic acid with ethanol and glucose, but in some other components as carbon source and nitrogen source. It presented weak positive for growth in the presence of 30% (*w*/*v*) D-glucose and had no need for acetic acid for growth. It could not fix nitrogen and produce 2,5-diketogluconic acid from D-glucose, but produce cellulose formation, 2-ketogluconic acid, and 5-ketogluconic acid from D-glucose. Major isoprenoid quinone was Q-10. M_{438} shared most characteristics with *Ga. xylinus* excluding the characteristics of being positive for growth on carbon source ethanol and week positive in the presence of 30% (*w*/*v*) D-glucose. M_{438} shared the secondmost characteristics with *Ga. hansenii* other than showing weak positive growth in the presence of 30% (*w*/*v*) D-glucose and having the abilities of producing cellulose and

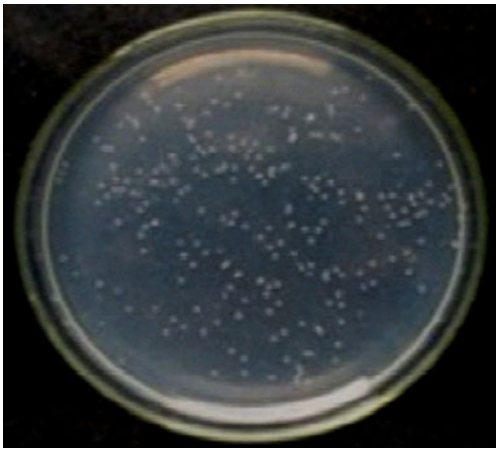

Fig. 4 Colony shape of bacterial strain M_{438}

Table 3 Physiological–biochemical characteristics of M_{438}

Tests[a]	Results	Tests	Results
Grow at pH 4.5	+	Formation from D-glucose of:	
Grow at 37 °C	−	2-ketogluconic acid	+
Catalase test	+	5-ketogluconic acid	+
Amylolysis	−	Requirement of niacin for growth	−
Oxidation of ethanol	+	Oxidation of acetic acid	−
Ammonia production	−	Utilization of ethanol	+
Ketogenesis from glycerol	+	H_2S production	−
Methyl red	−	Gelatin liquefaction	−
Acetyl methyl carbinol	+	Pigment production on GYC medium	−
Nitrate reduction	−	Halophilic test	−
Indole production	+	Nitrogen fixation	−
Cellulose formation	+		
Growth on compounds used as sole carbon source in the presence of yeast extract as nitrogen source[b]:			
D-Glucose	+	D-Fructose	++
D-Xylose	−	D-Arabinose	+
D-Mannitol	+	Inosite	+
Maltose	++	Lactose	++
Sucrose	++	Glycerol	+
Methanol	−	Ethanol	+
Propyl alcohol	−	Butanol	+
Citric acid	+		
Growth on compounds used as sole nitrogen source in the presence of glucose as carbon source[c]:			
L-Cysteine	+	L-Tryptophan	+
Carbamide	+	Peptone	++
Beef extract	+	Yeast extract	++
KNO_3	−	NH_4Cl	+
$(NH_4)_2SO_4$	+	$NaNO_3$	−
NH_4NO_3	+	$(NH_4)_2HPO_4$	++
Acid production from[d]:			
D-Glucose	+	D-Arabinose	−
Maltose	+	Inosite	−

Each test was carried out in triplicate

[a] + positive,− negative

[b] ++ better growth, + good growth,− no growth

[c] ++ better growth, + good growth,− no growth

[d] + positive,− negative

formatting 5-ketogluconic acid from D-glucose. However, it was reported that *Ga. hansenii* also has the ability to produce BC from the year 2003 [6, 44–46]. Therefore, there are eight species in *Gluconacetobacter* producing BC, not only *Gluconacetobacter europaeus*, *Ga. xylinus*, *Gluconacetobacter intermedius*, *Gluconacetobacter swingsii*,

Table 4 Comparison of physiological–biochemical characteristics between M_{438} and the other strains belonging to genus *Gluconacetobacter*

Strain	Characteristics of physiology and biochemistry													
	A	B	C	D	E	F	G	H	I	J	K	L	M	N
M_{438}	−	−	−	+	−	+	+	−	+	d	−	−	+	Q-10
Ga. liquefaciens	−	−	−	+	−	+	d	+	+	−	+	−	−	Q-10
Ga. azotocaptans	−	−	−	nd	−	nd	nd	nd	+	+	nd	+	−	Q-10
Ga. diazotrophicus	−	−	−	+	−	+	−	+	+	+	−	+	−	Q-10
Ga. entanii	+	+	+	−	−	−	−	−	+	−	nd	−	−	Q-10
Ga. europaeus	+	+	−	−	−	+	d	−	+	−	−	−	d	Q-10
Ga. hansenii	−	−	−	+	−	+	d	−	d	−	nd	−	−	Q-10
Ga. intermedius	+	−	−	+	−	−	−	−	+	+	nd	−	+	Q-10
Ga. johannae	−	−	−	nd	−	nd	nd	nd	+	+	nd	+	−	Q-10
Ga. oboediens	+	−	−	nd	−	+	−	−	+	+	nd	−	−	Q-10
Ga. Sacchari	−	−	−	+	−	+	+	+	+	+	+	−	−	Q-10
Ga. xylinus	−	−	−	+	−	+	+	−	d	−	−	−	+	Q-10
Ga. swingsii	+	−	nd	nd	nd	+	+	−	nd	+	nd	−	+	Q-10
Ga. rhaeticus	+	−	nd	nd	nd	+	+	−	nd	+	nd	−	+	Q-10
Ga. saccharivorans	nd	−	nd	nd	nd	+	−	−	nd	nd	nd	−	−	Q-10
Ga. nataicola	nd	−	nd	nd	nd	+	+	−	nd	nd	nd	−	+	Q-10
Ga. kombuchae	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	+	+	Q-10

The data for strains *Ga. liquefaciens*, *Ga. azotocaptans*, *Ga. diazotrophicus*, *Ga. entanii*, *Ga. europaeus*, *Ga. hansenii*, *Ga. intermedius*, *Ga. johannae*, *Ga. oboediens*, *Ga. sacchari*, and *Ga. xylinus* were from George et al. [4]. The data for strains *Ga. swingsii* and *Ga. rhaeticus* were from Dellaglio et al. [2]. For strain *Ga. saccharivorans* and *Ga. nataicola*, the data were taken from Lisdiyanti et al. [5]. And the data for *Ga. kombuchae* were from Dutta and Gachhui [3]

Letters from A to M represented the following test: *A* growth on 3% (*v*/*v*) ethanol in the presence of 5–8% acetic, *B* requirement of acetic acid for growth, *C* growth only in the presence of acetic acid and ethanol and glucose, *D* growth on the medium of Carr and Passmore, *E* growth on methanol, *F* formation of 2-ketogluconic acid from D-glucose, *G* formation of 5-ketogluconic acid from D-glucose, *H* formation of 2,5-diketogluconic acid from D-glucose, *I* growth on carbon source ethanol, *J* growth in the presence of 30% (*w*/*v*) D-glucose, *K* growth on 0.01% malachite green agar, *L* nitrogen fixation, *M* cellulose formation, *N* major ubiquinone. For M_{438}, + indicates more than 90% of strains belonging to the specific genus which are are positive in the tests, − means more than 90% are negative. And for the others, + indicates more than 90% of strains are positive, − means more than 90% are negative, *d* indicates 11–89% are positive, *nd* means the test was not carried out

Gluconacetobacter rhaeticus, *Gluconacetobacter nataicola*, and *Ga. kombuchae* [4, 43] but also *Ga. hansenii*.

With the optimal AFLP reaction and repeated experiments, the only one primer combination EcoRI+T/MseI+G was primer combinations (+0/+1, +1/+0, +1/+1) to analyze the diversity of M_{438} and its initial chosen among the 24 different strain J_2. And there was only one diversity locus on J_2 (Fig. 5), which was 213 bp. And the base sequence was shown as follows:

AGGCAATGCATGTCGGGAGCTCATTAGCTTTGGCCTTCTGTCTCTGGCT
ATGAAGTCACTGCCGTTGGGAACGGCTTACACTGTCTGGACAGGAATAGGC
GCGGTTGGGGCATTCCTGGTTGGGATAGTTATCCTGGGGGAAGCGT
TCACGCTGACCCGAGCTTTAGCTGCCATCTTCATTGTTTGTGGTCTCGTC
TTACTCAGGACTCATCA.

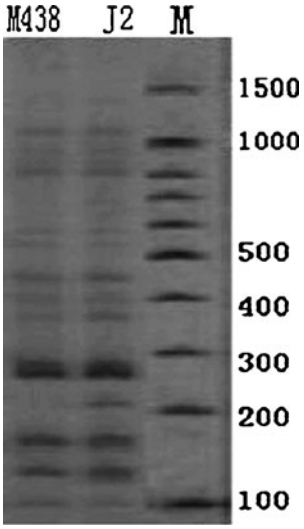

Fig. 5 Silver staining result of AFLP analysis of M_{438} and its initial strain J_2. For estimation of digestion fragments, 100-bp DNA markers were used in polyacrylamide gel

Subjected to GenBank and BLAST analysis, the sequence had 99% identity with 24,917–24723 bp in the genome sequence of *Ga. hansenii* ATCC23769 (accession no. ADTV 01000064.1), and the complement gene sequences were 24,699–25019 bp with local tag GXY_15142, which could be the deletion of M_{438} induced by HHP technology according to the inference. The deletion code SMR protein cl00910 is an integral membrane protein characterized by four α-helical transmembrane strands that confer resistance to a broad range of antiseptics and lipophilic quaternary ammonium compounds in bacteria and usually act as membrane transporters of cations and cationic drugs [34]. In addition, due to short length and broad substrate profile, several members in the SMR family may be evolving towards specific metabolite transport. SMR proteins were even recommended to be the progenitors for larger α-helical transporters such as the MFS and DMT superfamily [34]. They had been shown to export a range of toxins during the metabolic pathway, including ethidium bromide and quaternary ammonium compounds, through coupling with proton influx [34]. Therefore, it can be inferred that SMR might inhibit BC production to a certain extent. By extension, the toxins or something else produced by SMR might be the inhibitory actions of producing BC.

In conclusion, M_{438} was identified as *Ga. hansenii* subsp. nov. with the ability of high-yield BC production by being phylogenetically, morphologically, and physiologically–biochemically examined and distinguished from the other species in the genus *Gluconacetobacter*. The strain M_{438} is of great potential usage in medical, acoustic, and many other industries. The difference of BC yield between M_{438} and its initial strain might be caused by SMR protein cl00910.

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References

1. Keshk, S. M. A. S., Razek, T. M. A., & Sameshima, K. (2006). *African Journal of Biotechnology, 5*, 1519–1523.
2. Dellaglio, F., Cleenwerck, I., Felis, G. E., Engelbeen, K., Janssens, D., & Marzotto, M. (2005). *International Journal of Systematic and Evolutionary Microbiology, 55*, 2365–2370.
3. Dutta, D., & Gachhui, R. (2007). *International Journal of Systematic and Evolutionary Microbiology, 57*, 353–357.
4. Garrity, G. M., Bell, J. A., & Lilburn, T. G. (2004). Part C: Family II. *Acetobacteraceae* genus VIII. *Gluconacetobacter*. In M. Siever & J. Swings (Eds.), *Bergey's manual of systematic bacteriology* (Vol. 2, pp. 72–74). New York: Springer.
5. Lisdiyanti, P., Navarro, R. R., Uchimura, T., & Komagata, K. (2006). *International Journal of Systematic and Evolutionary Microbiology, 56*, 2101–2111.
6. Park, J. K., Park, Y. H., & Jung, J. Y. (2003). *Bioprocess E, 8*, 83–88.
7. Wan, Y. Z., Hong, L., Jia, S. R., Huang, Y., Zhu, Y., Wang, Y. L., et al. (2006). *Composites Science and Technology, 66*, 1825–1832.
8. Klemm, D., Schumann, D., Udhardt, U., & Marsch, S. (2001). *Progress in Polymer Science, 26*, 1561–1603.
9. Keshk, S., & Sameshima, K. (2006). *Enzyme and Microbial Technology, 40*, 4–8.
10. Nguyen, V. T., Gidley, M. J., & Dykes, G. A. (2008). *Food Microbiology, 25*, 471–478.
11. Hong, F., & Qiu, K. Y. (2008). *Carbohydrate Polymers, 72*, 545–549.
12. Backdahl, H., Esguerra, M., Delbro, D., Risberg, B., & Gatenholm, P. (2008). *Journal of Tissue Engineering and Regenerative Medicine, 2*, 320–330.
13. Svensson, A., Nicklasson, E., Harrah, T., Panilaitis, B., Kaplan, D. L., Brittberg, M., et al. (2005). *Biomaterials, 26*, 419–431.
14. Jonas, R., & Farah, L. F. (1998). *Polymer Degradation and Stability, 59*, 101–106.
15. Vandamme, E. J., Baets, S. D., Vanbaelen, A., Joris, K., & De-Wulf, P. (1998). *Polymer Degradation and Stability, 59*, 93–99.
16. Anicuta, S. G., Marta, S. F., Traian, Z., & Elena, G. (2007). *Nuclear Instruments and Methods in Physics Research B, 265*, 434–438.
17. Sutherland, I. W. (1998). *Trends in Biotechnology, 16*, 41–46.
18. Jung, R., Kim, H. S., Kim, Y., Kwon, S. M., Lee, H. S., & Jin, H. J. (2008). *Journal of Polymer Science. Part B, 46*, 1235–1242.
19. Nogi, M., & Yano, H. (2008). *Advanced Materials, 20*, 1849–1852.
20. Pommet, M., Juntaro, J., Heng, J. Y. Y., Mantalaris, A., Lee, A. F., Wilson, K., et al. (2008). *Biomacromolecules, 9*, 1643–1651.
21. Budhiono, A., Rosidi, B., Taher, H., & Iguchi, M. (1999). *Carbohydrate Polymers, 40*, 37–143.
22. De-Wulf, P., Joris, K., & Vandamme, E. J. (1996). *Journal of Chemical Technology and Biotechnology, 67*, 376–380.
23. Yu, X. B., Bian, Y. R., Quan, W. H., & Liu, W. (1999). *Journal of Cell Science and Technology, 7*, 63–66 (in Chinese).
24. Vezzi, A., Campanaro, S. D., Angelo, M., Simonato, F., Vitulo, N., Lauro, F. M., et al. (2005). *Science, 307*, 1459–1461.
25. Lauro, F. M., Tran, K., Vezzi, A., Vitulo, N., Valle, G., & Bartlett, D. H. (2008). *Journal of Bacteriology, 190*, 1699–1709.
26. Liu, F. Z., Zhang, H., & Mu, K. F. (2008). *Liquor-Making Science & Technology, 3*, 51–53 (in Chinese).
27. Gao, X., Li, J., & Ruan, K. C. (2001). *Acta Biochimica et Biophysica Sinica, 33*, 77–81 (in Chinese).
28. Wang, S. L., Wu, X. Z., Duan, X. C., & Sun, J. S. (2006). *Indian Microbiology, 36*, 31–35 (in Chinese).
29. Wu, R. Q., Du, S. K., Li, Z. X., Xing, X. H., Shao, D. Y., Fan, Y. L., et al. (2008). *Chinese Journal of Biotechnology, 24*, 1068–1074 (in Chinese).
30. Wu, R. Q., Li, Z. X., Shao, D. Y., Fan, Y. L., Zhang, X. L., Li, B., et al. (2008). *China Brewing, 10*, 37–38 (in Chinese).
31. Wu, R. Q., Li, Z. X., Yang, J. P., Xing, X. H., Shao, D. Y., & Xing, K. L. (2010). *Cellulose, 17*, 399–405.
32. Rosa, G. L., Carolis, E. D., Sali, M., Papacchini, M., Riccardi, C., Mansi, A., et al. (2006). *Microbiological Research, 161*, 150–157.
33. Vos, P., Hogers, R., Bleeker, M., Reijans, M., van de Lee, T., Hornes, M., et al. (1995). *Nucleic Acids Research, 23*, 4407–4414.
34. Bay, D. C., & Turner, R. J. (2009). *BMC Evolutionary Biology, 9*, 140–151.
35. Takahashi, M., Yukphan, P., Yamada, Y., Suzuki, K. I., Sakane, T., & Nakagawa, Y. (2006). *Journal of General and Applied Microbiology, 52*, 187–193.

36. Kimura, M. (1980). *Journal of Molecular Evolution, 16*, 111–120.
37. Saitou, N., & Nei, M. (1987). *Molecular and Biological Evolution, 4*, 406–425.
38. Felsenstein, J. (1981). *Journal of Molecular Evolution, 17*, 368–376.
39. Felsenstein, J. (1983). *Annual Review of Ecology and Systematics, 14*, 313–333.
40. Felsenstein, J. (1985). *Evolution, 39*, 783–791.
41. Dong, X. Z., & Cai, M. Y. (2001). *Systemic identification guide book for commonly isolated bacteria* (1st ed.). Beijing: Science Press (in Chinese).
42. Buchanan, R. E., & Gibbons, N. E. (1984). *Bergey's manual of determinative bacteriology* (8th ed.). Beijing: Science Press (in Chinese).
43. Feng, J., Shi, Q. S., Ouyang, Y. S., & Chen, Y. B. (2009). *Chemical Bioengineering, 26*, 10–13 (in Chinese).
44. Jung, J. Y., Park, J. K., & Chang, H. N. (2005). *Enzyme and Microbial Technology, 37*, 347–354.
45. Hutchens, S. A., Benson, R. S., Evans, B. R., O'Neill, H. M., & Rawn, C. J. (2006). *Biomaterials, 27*, 4661–4670.
46. Shehzad, O., Khan, S., Khan, T., & Park, J. K. (2009). *Korean Journal of Chemical Engineering, 26*, 1689–1692.
47. Li, H. X., Cao, Y. S., Fu, L. L., & Liu, X. H. (2005). *Journal of Microbiology, 25*, 50–53 (in Chinese).