Complete genome sequence of the cellulose-producing strain *Komagataeibacter nataicola* RZS01

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*Komagataeibacter nataicola* is an acetic acid bacterium (AAB) that can produce abundant bacterial cellulose and tolerate high concentrations of acetic acid. To globally understand its fermentation characteristics, we present a high-quality complete genome sequence of *K. nataicola* RZS01. The genome consists of a 3,485,191-bp chromosome and 6 plasmids, which encode 3,514 proteins and bear three cellulose synthase operons. Phylogenetic analysis at the genome level provides convincing evidence of the evolutionary position of *K. nataicola* with respect to related taxa. Genomic comparisons with other AAB revealed that RZS01 shares 36.1%–75.1% of sequence similarity with other AAB. The sequence data was also used for metabolic analysis of biotechnological substrates. Analysis of the resistance to acetic acid at the genomic level indicated a synergistic mechanism responsible for acetic acid tolerance. The genomic data provide a viable platform that can be used to understand and manipulate the phenotype of *K. nataicola* RZS01 to further improve bacterial cellulose production.

Acetic acid bacteria (AAB) are a group of microorganisms that belong to the family *Acetobacteraceae* of the class *Alphaproteobacteria*. These bacteria are widely found on fruits, flowers, and rotten food. Their metabolic uniqueness has been utilized in the industrial production of sorbose, vitamin C, dihydroxyacetone, D-gluconic acid, and bacterial cellulose (BC)2–5. Furthermore, AAB can also act as fermentative organisms, therefore, they are applied in beer, wine, and vinegar production6, 7. A remarkable feature of these strains is their ability to survive under extreme environments, such as high sugar concentrations and low pH values, which makes AAB suitable for various industrial applications8.

Currently, AAB include 14 genera, namely *Acetobacter*, *Glucobacter*, *Glucanacetobacter*, *Komagataeibacter*, *Granulibacter*, *Asaia*, *Acidomonas*, *Kozakia*, *Swaminathania*, *Saccharibacter*, *Neosaia*, *Tanticharoenia*, *Ameypaea*, and *Neokomagataeabac*1. Classification within the *Acetobacteraceae* depends on the ubiquinone type, including the Q-9 and Q-10 types. *Acetobacter* species use the Q-9–type ubiquinone, whereas the AAB genera of *Glucanacetobacter* and *Glucobacter* contain mainly the Q-10–type ubiquinone9. The genus *Komagataeibacter*, a gram-negative, obligately aerobic and rod-shaped acidophilic organism, was initially proposed by Yama in 2012, with 12 other strains on the basis of their taxonomic characteristics10. *Komagataeibacter* species possess the ability to secrete exopolysaccharides (EPS), especially BC14. Generally, *Komagataeibacter* species belong to the cellulose-nonproducing member of *Komagataeibacter* species, isolated from vinegar15. BC has been the focus of research owing to its excellent properties, such as high purity, predominant three-dimensional structure, high degree of crystallinity, and superior biocompatibility16–18. In view of its unique characteristics, we present a high-quality complete genome sequence of *K. nataicola* RZS01.
owing to its high resistance to ethanol and acetic acid, which makes it an ideal strain for industrial applications.22, 23. Considered to be the main producers of BC, particularly strains of the species *K. xylinus*, of which 19 demonstrated high yields of cellulose after cultivation. Species of the genus *Komagataeibacter* *K. rhaeticus* are characterized by their ability to synthesize high yields of cellulose. Maria José Valera et al. investigated the cellulose-producing ability of 77 strains of 35 species of AAB, of which 19 demonstrated high yields of cellulose after cultivation. Species of the genus *Komagataeibacter* are considered to be the main producers of BC, particularly strains of the species *K. xylinus*, *K. hansenii*, *K. swingsii*, and *K. nataicola*. In fact, *K. xylinus* is considered a model microorganism for BC production.29, 30.

**Results**

**General features of the *K. nataicola* RZS01 genome.** The complete genome of RZS01 is composed of 1 circular chromosome of 3,485,191 bp with a G + C content of 61.49%, and 6 plasmids ranging in size from 25,766 bp to 102,282 bp (Table 1; Fig. 1). Gene prediction and annotation of the RZS01 genome resulted in 3,609 open reading frames. The number of genes in the plasmids occupies 6.5% of the total genes in the genome, and genes with predicted function are assigned to 68.86% of the genome (2,485 genes). Furthermore, 5 ribosomal RNA operons (16S-23S-5S) were detected and 62 tRNA genes were predicted. From the genome data, we also identified 66 transposase genes, which might be responsible for the genetic instability leading to deficiencies in various physiological properties as reported in other AAB24. The complete genome has a total of 3,514 putative coding sequences, among which 2,485 are assigned a putative function, and 1,029 encode hypothetical proteins. The 6 plasmids have 228 coding DNA sequences (CDSs), of which approximately 47.8% encode hypothetical proteins. The remaining 42 CDSs encode mobile element proteins. Figure 2 summarizes the distribution of RZS01 proteins among 21 functional groups. Statistically, nearly 38% CDSs are completely uncharacterized, which is similar to the proportion of unassigned CDSs in some other sequenced bacterial genomes, such as in *Escherichia coli* (40%), *Haemophilus influenzae* (43%), and *Mycoplasma genitalium* (32%) 25. The 2 largest functional groups contain 277 and 231 proteins involved in carbohydrate and amino acid metabolism, respectively, which correspond to the physiological functions in RZS01.

**Phylogenetic analysis and comparative genomics.** Analysis of the most conservative sequence in the process of evolution, the 16S rRNA gene, is widely used in taxonomic resolution in bacteria. To ascertain the phylogenetic positions of RZS01 in *Acetobacteraceae*, a phylogenetic tree was constructed using 16S rRNA gene sequences (Fig. 3). The phylogenetic tree summarizes 13 other *Acetobacteraceae*, the genomes of which have been completely sequenced. The tree suggests that RZS01 is most closely related to *Komagataeibacter xylinus* E25, *Komagataeibacter medellinensis* LMG 1693, and *Gluconacetobacter hansenii* ATCC 23769. Furthermore, comparative analysis of RZS01 in relation to other members of the *Acetobacteraceae* family reveals that this strain has 36.1% shared genes with the human pathogen *Granulibacter bethesdensis* CGDNIH1 (Table 2). Approximately 40% of the genes are in common with members of the genus *Acetobacter*. Thus, RZS01 has more genes in common with *Komagataeibacter* species than with *Gluconacetobacter*, which corresponds with the phylogenetic relationship.

**Cellulose production.** *Acetobacteraceae* are characterized by their ability to synthesize high yields of cellulose. Maria José Valera et al. investigated the cellulose-producing ability of 77 strains of 35 species of AAB, of which 19 demonstrated high yields of cellulose after cultivation. Species of the genus *Komagataeibacter* are considered to be the main producers of BC, particularly strains of the species *K. xylinus*, *K. hansenii*, *K. swingsii*, and *K. nataicola*. In fact, *K. xylinus* is considered a model microorganism for BC production.29, 30.

On the RZS01 chromosome, we found the full set of cellulose synthase genes in the bcsI operon, which comprised the cellulose synthase genes bcsA1 (B0W47_12635), bscB1 (B0W47_12640), bscC1 (B0W47_12645), and bscD1 (B0W47_12650), as well as cmcA (B0W47_12625), ccpA (B0W47_12630) upstream, and bg(I)xA.
(B0W47_12655) downstream. cmcax encodes endo-β-1,4-glucanase, and bglxA encodes β-glucosidase, both of which have been suggested to assist cellulose biosynthesis by hydrolysing tangled glucan chains when there is a failure in chain arrangement, and all these genes are necessary for cellulose production31. In addition to the bcs1 operon, we found an additional bcs operon (bcs2) at the genomic position 363284–373685, and it differs in structure from the bcs1 operon. The bcs2 operon is composed of bcsA and bcsC, with 2 additional genes homologous to bcsX and bcsY in the middle of bcsA and bcsC. The protein encoded by bcsA contains the catalytically active subunit with a PilZ domain, which is responsive to c-di-GMP32. An additional bcsA (bcsA3) is also located on the reverse strand. Unlike the bcsA1 gene, the bcsA2 and bcsA3 genes do not contain a PilZ domain.

Generally, AAB possess the ability to secrete other polysaccharides, including capsular polysaccharides, lipopolysaccharides, and water-soluble EPS. One of the most studied polysaccharides is a xanthan gum-like EPS, named ‘acetan’, the structure of which is shown in Supplementary Information. The chemical repeat unit consists of a cellobiose unit solubilized by attachment of a charged pentasaccharide sidechain to one of the glucose

Figure 1. Overview of K. natacola RZS01 genome. (a) The circles represent (from the outside to the inside): circle 1, DNA base position (bp); circle 2, contig components; circle 3, protein-coding regions transcribed clockwise; circle 4, protein-coding regions transcribed anti-clockwise; circle 5, distribution of genes encoding ncRNA (black), tRNA (blue) and rRNA (red); circle 6, G + C content; circle 7, GC skew. (b) The compositions of 2 bcs operons, which differ from each other in gene content.

Figure 2. Subsystem category distributions of RZS01 proteins.
residues. Genetic analysis of RZS01 shows that the biosynthesis of acetan occurs via a pathway similar to that for BC biosynthesis. Because it is the composite of different monosaccharides, 2 CDSs are responsible for encoding UDP-glycosyl transferase (B0W47_01855, B0W47_03955, and B0W47_12635), GDP-mannosyl transferase (B0W47_09075, and B0W47_11925), and phosphomannose isomerase (B0W47_06245). The enzymes UDP-glycosyl transferase and GDP-mannosyl transferase transfer a glucose and mannose residues from UDP-Glc and GDP-Man, respectively, to one of the intermediates of acetan. However, not all genes from the gum operon are present in RZS01. Only 2 CDSs (B0W47_14415 and B0W47_14460) represent the genes of gumK and gumH, respectively. The genes for gumB, C, D, E, G, I, J, and M are absent in the RZS01 genome.

Regulation of cellulose biosynthesis. BC biosynthesis is regulated by the expression of bcs genes, which appear to be expressed in response to the second messenger cyclic-di-GMP (c-di-GMP). An earlier study revealed that cellulose biosynthesis could be stimulated almost 100-fold by c-di-GMP. The enzymes UDP-glycosyl transferase and GDP-mannosyl transferase transfer a glucose and mannose residues from UDP-Glc and GDP-Man, respectively, to one of the intermediates of acetan. However, not all genes from the gum operon are present in RZS01. Only 2 CDSs (B0W47_14415 and B0W47_14460) represent the genes of gumK and gumH, respectively. The genes for gumB, C, D, E, G, I, J, and M are absent in the RZS01 genome.

Table 2. Comparative analysis of Komagataeibacter nataicola RZS01 with members of the Acetobacteraceae family.

| Acetic acid bacterium strain | Number of shared genes | Percentage of shared genes |
|-----------------------------|------------------------|---------------------------|
| Gluconacetobacter diazotrophicus Pal5 | 5031 | 72.0 |
| Komagataeibacter medellinensis NBRC 3288 | 4742 | 75.0 |
| Acetobacter senegalensis 108B | 4339 | 61.7 |
| Komagataeibacter xylinus E2S | 4335 | 65.0 |
| Komagataeibacter hansenii ATCC 23769 | 3592 | 52.4 |
| Acetobacter pasteurianus IFO 3283 | 3216 | 51.1 |
| Acidiphilium multivorum AIU301 | 3098 | 44.5 |
| Gluconobacter oxydans 621H | 2993 | 50.3 |
| Acetobacter sp. SLV-7 | 2893 | 48.1 |
| Acetobacter baumanii 430A | 2774 | 46.0 |
| Asaia bogorensis NBRC 16594 | 2742 | 43.7 |
| Acidiphilium cryptum JF-5 | 2656 | 39.4 |
| Granulibacter bethesdenis CGDNIH1 | 2146 | 36.1 |

Genes for substrate metabolism. On the basis of the analysis of gene annotation for the complete genome, K. nataicola RZS01 possesses various transporters for the uptake of substrates and ions. We identified 49 ABC transporters, 10 symporters, and 47 permeases, which are responsible for the transportation of sugars (e.g. glucose and ribose), polyols (e.g. mannitol and sorbitol), sugar acids (e.g. gluconate and acetate), amino acids, purines, pyrimidines, phosphate, sulphate, NH4+, and metal ions. In addition, some components of the phosphotransferase system are also found. The most widely used substrate for industrial production, glucose could be taken up by a sugar symporter (B0W47_14370). Owing to the lack of the gene encoding phosphofructokinase (EC 2.7.1.11), RZS01 has an incomplete Embden-Meyerhof-Parnas pathway, as has been reported in Acetobacter.
K. medellinensis related to that of NBRC 3288, with 75.0% shared genes, which is congruent with the phylogenetic
of acidic stress during BC production.

Furthermore, the genome of RZS01 contains glucose dehydrogenase and glycerol dehydrogenase.

RZS01 possesses 1 membrane-bound PQQ-ALDH, whereas 5 other copies are dispersed in the cytoplasm.

The genes encoding PQQ-ADHs are present as 8 copies distributed throughout the chromosomes, and just is localized in the membrane. Moreover, vert alcohol into acetaldehyde and acetaldehyde into acetate, respectively. The genes encoding PQQ-ADHs and aldehyde dehydrogenase (PQQ-ALDH), which con-

nases depends on the cofactor pyrroloquinoline quinine (PQQ). The most representative dehydrogenases are

Membrane-associated primary dehydrogenases and the respiratory chain. K. nataicola RZS01 contains several genes that encode membrane-associated primary quinone reductases coupling substrate oxid-ation with quinone reduction, which can be classified into 2 groups (Table 3). The first group of dehydroge-

nases depends on the cofactor pyrroloquinoline quinine (PQQ). The most representative dehydrogenases are

PQQ-dependent alcohol dehydrogenase (PQQ-ADH) and aldehyde dehydrogenase (PQQ-ALDH), which

convert alcohol into acetaldehyde and acetaldehyde into acetate, respectively. The genes encoding PQQ-ADHs are present as 8 copies distributed throughout the chromosomes, and just is localized in the membrane. Moreover, RZS01 possesses 1 membrane-bound PQQ-ALDH, whereas 5 other copies are dispersed in the cytoplasm. Furthermore, the genome of RZS01 contains glucose dehydrogenase and glycerol dehydrogenase.

Adaptation to extreme conditions. K. nataicola RZS01 possesses the ability to tolerate low pH and high
centinations of organic acid in the environment. One strategy for this tolerance is that RZS01 has a cytosolic
acetate-assimilating detoxification pathway, whereby acetate is first converted into acetyl-CoA, which is cata-

lysed by acetyl-CoA synthetase (acn; B0W47_06505). The product is further oxidized by the citric acid cycle to
water and carbon dioxide. Overoxidation occurs in Acetobacter and Gluconacetobacter but not in Gluconobacter,
which exhibits relatively weak acetic acid resistance. The second mechanism for acid tolerance may involve
an ABC transporter gene, attA (B0W47_06270), which functions as an efflux pump of acetic acid.

In another similar efflux mechanism, a proton motive force-dependent efflux system occurs in the cytoplasmic mem-
brane, which is capable of exporting acetic acid and is dependent on the proton motive force but not on ATP.

Besides, there is also an operon involved in high resistance to acetic acid, a trait that can be a suitable target
for enhancement by breeding. This operon is composed of the genes groE (B0W47_13360 and B0W47_13365),
groS (B0W47_00055), and groL (B0W47_02405, B0W47_03785, and B0W47_09650), and the overexpression of
the groESL operon increases acetic acid resistance in Acetobacter. A previous thorough genome analy-

sis revealed the presence of a gene cluster encoding a urease (ureDABCEFG; B0W47_16420, B0W47_16425,
B0W47_16430, B0W47_16435, B0W47_16440, B0W47_16445, and B0W47_16450), a urea transporter (urtAB-
CE), B0W47_16480, B0W47_16485, B0W47_16490, and B0W47_16495), and an allopaphate
hydrolase (B0W47_14240, B0W47_14245, B0W47_16295, and B0W47_16300). These proteins transport urea
and catalyse it to ammonia, which enables the survival of RZS01 in an acidic environment, such as in vinegar
fermentation. This mechanism is also present in A. pasteurianus 386B and G. bethesdensis CGDNIH1 but is not
widespread among AAB strains.

Discussion

K. nataicola is widely distributed in nature and displays strong abilities of producing BC and tolerating acetic
acid. In this study, we sequenced, annotated, and analysed the complete genome sequence of K. nataicola RZS01
and found that RZS01 possesses a 3.5-Mb chromosome and 6 plasmids. The global overview of all genes provided

Table 3. Primary membrane-bound dehydrogenases in K. nataicola RZS01.

| Family                              | Cofactor | Genes                                                                 |
|-------------------------------------|----------|-----------------------------------------------------------------------|
| Glucose dehydrogenase               | PQQ      | B0W47_01230, B0W47_02520, B0W47_10950                                |
| Glycerol dehydrogenase              | PQQ      | B0W47_01005, B0W47_01010                                             |
| Alcohol dehydrogenase               | PQQ      | B0W47_13410                                                           |
| Aldehyde dehydrogenase              | PQQ      | B0W47_16410                                                           |
| 2-Keto-o-glucurate dehydrogenase    | FAD      | B0W47_11030, B0W47_11035, B0W47_11040                                |
| Gluconate 2-dehydrogenase           | FAD      | B0W47_05395, B0W47_05400, B0W47_13405                                |
relationship. On the basis of the genomic sequence of RZS01, we can now more extensively describe the process of cellulose biosynthesis and the physiological basis of the underlying pathways in this organism. For other AAB species whose genomes have been sequenced, 2 bcs operons are present in RZS01, which may explain the high cellulose productivity observed. However, the regulation of AcsAB activity by c-di-GMP may also play important roles. We identified 3 cdg operons containing a DGC and a c-di-GMP PDE. Four standalone c-di-GMP PDEs and 1 standalone DGC are also present in the genome. This regulatory mechanism has also been found in other bacteria.

K. nataicola RZS01 contains many membrane-bound dehydrogenases, which are responsible for the assimilation of substrates and contribution to acetic acid resistance, resisting the presence of high concentrations of acetic acid. The metabolism of several amino acids, such as threonine, glycine, and ornithine, produces a large amount of ammonia, which decreases the intracellular pH value. Certain other mechanisms also participate in acetic acid tolerance, including alcohol overoxidation, an acetate assimilation-related mechanism, an ABC transporter, and a proton motive force-dependent efflux system.

In summary, we uncovered global insights into BC production and acidic resistance mechanisms by genome analysis. These results provide useful information for further studies on evolution and genetic variation in Komagataea and provide a valuable resource for biological research. Furthermore, comparative genomics analysis and functional genomics analysis can also be performed to trace the origin and evolution of this organism.

**Methods**

**Bacterial strain and isolation of chromosomal DNA.** K. nataicola RZS01 (CGMCC number 10961) used in this study was isolated from rotten apples. For genomic DNA extraction, RZS01 was grown from a single colony in HZ medium, containing 20 g·L⁻¹ glucose, 6 g·L⁻¹ ammonium sulphate, 1 g·L⁻¹ monopotassium phosphate, 0.4 g·L⁻¹ magnesium sulphate, 3 g·L⁻¹ peptone, 2.25 g·L⁻¹ yeast extract, and 0.4 g·L⁻¹ carboxymethyl-cellulose sodium at 30 °C for 24 h. The produced cellulose was digested by adding 0.2% cellulase and maintained at 30 °C for 2 h. After the cells were harvested, chromosomal DNA was isolated using the QiaAmp DNA Mini kit (Qiagen, Valencia, Germany) following the manufacturer’s instructions. The quality of the DNA was evaluated using a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) after isolation.

**Genome sequencing and assembly.** The whole genome of K. nataicola RZS01 was sequenced at HangZhou GeneRui Biotechnology Co., Ltd. (Hangzhou, China) using a PacBio RS DNA sequencer. The average insert size was 550 bp. Data from 515-Mb paired-end reads were delivered by Baseclear as 2 fastq files. The FastQC procedure was used to verify the raw read data, and QC metrics including insert-sizes, mapped reads, unmapped reads and reads that align with a deviated pattern were examined. The sequencing errors were discarded using the error-correction module of Allpaths-LG. For single-molecule real-time sequencing (SMRT), a SMRT bell DNA template library with an insert size of 2–40 kb was prepared. Subsequently, the fragmented DNA was end-repaired and ligated into hairpin adapters. Sequence reads have been deposited in the ENA Sequence Read Archive (EMBL: ERS550016). Sequencing reads were corrected using the HGAP pipeline. Genomic sequences were assembled de novo using PacBio analysis RS_HGAP_Assembly.3 (Pacific Biosciences, Menlo Park, CA, USA).

**Bioinformatics analysis.** Automatic gene prediction and annotation were performed using Glimmer3.0. The genome was visualized using Circos (http://circos.ca/tutorials/lessons/). 16S rRNA phylogeny was constructed by the neighbour-joining method of MEGA5.1 at default settings. Metabolic enzymes were identified from UniProt (http://www.uniprot.org/) and BLASTp analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to identify orthologues in the genomes.

**Nucleotide sequence accession numbers.** The accession numbers of the complete sequences of K. nataicola RZS01 determined in this study and of the 6 plasmids from NCBI can be found in GenBank (http://www.ncbi.nlm.nih.gov) under the accession no. CP019875 to CP019881.

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