Bacterial cellulose synthesis mechanism of facultative anaerobe Enterobacter sp. FY-07

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Enterobacter sp. FY-07 can produce bacterial cellulose (BC) under aerobic and anaerobic conditions. Three potential BC synthesis gene clusters (bcsi, bcsiI and bcsiII) of Enterobacter sp. FY-07 have been predicted using genome sequencing and comparative genome analysis, in which bcsiI was confirmed as the main contributor to BC synthesis by gene knockout and functional reconstitution methods. Protein homology, gene arrangement and gene constitution analysis indicated that bcsiI had high identity to the bcsi operon of Enterobacter sp. 638; however, its arrangement and composition were same as those of BC synthesizing operon of G. xylinum ATCC35852 except for the flanking sequences. According to the BC biosynthesizing process, oxygen is not directly involved in the reactions of BC synthesis, however, energy is required to activate intermediate metabolites and synthesize the activator, c-di-GMP. Comparative transcriptome and metabolite quantitative analysis demonstrated that under anaerobic conditions genes involved in the TCA cycle were downregulated, however, genes in the nitrate reduction and gluconeogenesis pathways were upregulated, especially, genes in three pyruvate metabolism pathways. These results suggested that Enterobacter sp. FY-07 could produce energy efficiently under anaerobic conditions to meet the requirement of BC biosynthesis.

Bacterial cellulose (BC) is a water-insoluble extracellular polysaccharide with a simple structure. It comprises only one type of sugar (glucose), which are linked together through β-1, 4 linkages linearly. Bunches of β-1, 4 glucan chains are assembled into microfibrils in the same direction, and then crystallised into cellulose fibers. BC has many excellent physicochemical properties compared with plant cellulose, such as high purity, ultrafine reticulated structure, high crystallinity, high mechanical strength, high hydrophilicity and good biocompatibility. Consequently, BC has been widely used in the fields of paper, food, medicine, acoustic membranes, biomedical engineering and oil exploration.

To date, many BC producing bacteria have been identified, including gram-negative species such as, Gluconacetobacter xylinus, Achromobacter sp., Aerobacter sp., Agrobacterium sp., Enterobacter sp., Pseudomonas sp., Rhizobium sp., Salmonella sp. and Sarcina sp.; and gram-positive species, such as Gluconacetobacter hansenii. Among them, G. xylinus has been investigated extensively for its higher BC productivity. The BC biosynthesis mechanisms of G. xylinus have been determined at the biochemical and genetic levels. In G. xylinus, BC biosynthesis which needs precursor UDP-glucose (UDPG) and activator cyclic diguanylic acid (c-di-GMP) is accomplished by a cellulose synthase complex containing BcsA, BcsB, BcsC and BcsD subunits, which are encoded by genes within a single operon. BcsA and BcsB form the minimum complex required for cellulose synthesis. BcsA containing eight transmembrane helices, is the catalytic subunit of cellulose synthase complex, and belongs to the GT-2 glycosyltransferase family. BcsB is an auxiliary subunit that interacts with BcsA via its C-terminal transmembrane helix and regulates cellulose synthesis by interacting with c-di-GMP. Wong et al. proved that BcsC is essential for cellulose synthesis in vitro, but not required for β-1, 4 glucan synthesis in vivo. Saxena et al. suggested that the BcsC subunit might form a pore in the cell membrane.

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membrane for cellulose secretion using bioinformatic analysis. Mutants with an interrupted bcsD gene showed 40% decreased cellulose production, but produced both cellulose I and II, suggesting that BcsD controls the crystallisation of cellulose into nanofibrils27,28. The structure of BcsD showed an exquisite cylinder shape with a right-hand twisted dimer on the cylinder wall and formed a functional octamer unit suggesting that BcsD could provide a passageway for extruding the glucan chains29. UDP-glucose pyrophosphorylase (UGPase) which is encoded by galT30,31 converts glucose-1-phosphate (Glc-1-p) to UDPG. Phosphoglucomutase (PGM) which is encoded by pgm gene converts glucose-6-phosphate to Glc-1-p32,33. PGM bridges the BC polymerisation steps with the common cellular metabolite Glc-6-P34. As an allosteric activator of cellulose synthase, c-di-GMP is essential to activate cellulose synthase35,36. The c-di-GMP activator is synthesised from GTP by diguanylate cyclase, and degraded by two phosphodiesterases (PDE-A and PDE-B) to regenerate 5‘GMP, which may be used for GTP synthesis and completes the biosynthetic and degradative pathway cycle of c-di-GMP36,37. In addition to the above genes, cellulose synthesis-related genes are located both upstream and downstream. In the most well studied strain G. xylinus ATCC 53582, the upstream region contains the cmcax and ccpax genes38,39. Endo-β-1,4-gluanase encoded by gene cmcax has cellulose hydrolysing activity and can enhance cellulose synthesis40. The CcpAx protein is essential for cellulose production and plays a critical role in locating the cellulose synthesising complex on the cell membrane38. Sunagawa et al. suggested that the CcpAx protein functions as a mediator of protein-protein interactions41. The bglxA gene is located downstream of the BC synthesis operon and encodes a β-glucosidase; disruption of bglxA causes a decrease in cellulose production42.

Despite being a well-studied strain for BC biosynthesis research, G. xylinus is a strict aerobe that can only produce BC under aerobic conditions43. BC production of G. xylinus can only be accomplished through tray fermentation and is costly. Recently, our lab reported an Enterobacter sp. FY-07 that can produce BC under aerobic and anaerobic conditions44, Enterobacter sp. FY-07 has very close evolutionary relationship with Enterobacter sp. 638. Although possess two BC synthesis operon, Enterobacter sp. 638 only produce a little amount of BC as a component of biofilm44. To identify the BC synthesis gene cluster of Enterobacter sp. FY-07, comparative genomic analysis, gene knockout and functional reconstitution methods were performed. To investigate whether the different oxygen demands lead to the different BC synthesis mechanisms between G. xylinus ATCC 53582 and Enterobacter sp. FY-07 or why Enterobacter sp. FY-07 has the different BC production ability with Enterobacter sp. 638, we compared the characteristics of the BC synthesis gene cluster among Enterobacter sp. FY-07, G. xylinus ATCC 53582 and Enterobacter sp. 638. To clarify the differences in BC biosynthesis of Enterobacter sp. FY-07 under aerobic and anaerobic conditions, comparative transcriptome analysis and metabolite quantitative experiments were performed.

Results

Genome sequencing of Enterobacter sp. FY-07. Although a few BC-producing Enterobacter sp. have been reported45,46,47, there has been no report of their gene information and cellulose synthetic mechanism. The genome of Enterobacter sp. FY-07 is the first sequenced genome of an Enterobacter sp. strain which is characterized as highly effective BC producer. The genome of Enterobacter sp. FY-07 consists of one 5.12 Mbp chromosome (Genbank access number: CP012487) and three plasmids, pAKI40A (CP012488), pAKI40B (CP012489) and pAKI40C (CP012490) of 2324 bp, 3776 bp and 13955 bp, respectively. The GC content of the chromosome is 53.62%, whereas the three plasmids have GC contents of 51.81%, 44.65% and 56.47%. Overall 5019 “locus tags” were identified in the chromosomal sequence, among which 4916 are protein-encoding genes, 82 are tRNA-encoding genes, and 21(organized in 7 operons) are rRNA-encoding genes. From practical point of view, BC production is the most important feature of the Enterobacter sp. FY-07. Three bacterial cellulose synthase operons (bcsI, bcsII and bcsIII) were identified in the genome using comparative genome analysis (Fig. 1a). All three are structurally complete. Operon bcsI comprises seven genes encoding: hypothetical protein (AKI40_0196); YihQ (AKI40_0197); four bacterial cellulose synthase subunits: BcsA (AKI40_0198), BcsB (AKI40_0199), BcsC (AKI40_0200) and BcsD (AKI40_0201); and cellulase (AKI40_0202). Operon bcsII comprises six genes: yihR (AKI40_0206), yihQ (AKI40_0207), bcsA (AKI40_0208), bcsB (AKI40_0209), bcsZ (AKI40_0210) and bcsC (AKI40_0211). Operon bcsIII only comprises four cellulose synthase subunits encoding genes: bcsA (AKI40_0894), bcsB (AKI40_0893), bcsC (AKI40_0892) and bcsD (AKI40_0891). In addition, there is an operon with three genes in the opposite orientation between operon bcsI and operon bcsII. These three genes are bcsG (AKI40_0203), bcsF (AKI_0204) and bcsE (AKI_0205), which are considered related to bacterial cellulose synthesis.

Identification of operons and genes responsible for BC production in Enterobacter sp. FY-07. Gene knockout, gene complementation and functional reconstitution methods were used to determine which operon is essential for BC synthesis and whether this operon plays the same role in BC synthesis in Enterobacter sp. FY-07 under aerobic and anaerobic conditions. Seven gene knockout mutants were constructed and designated as FY-07 ΔbcsI, FY-07 ΔbcsII, FY-07 ΔbcsIII, FY-07 ΔbcsGFE, FY-07 ΔbcsA (I); FY-07 ΔbcsA (II) and FY-07 ΔbcsA (III). The locations of the gene knockouts are shown in Fig. 1a. Only the bcsIII-knockout mutants and the bcsA (III)-knockout mutants produced no cellulose under aerobic and anaerobic conditions (Fig. 1b,c). When a plasmid harboring the native bcsA or bcsIII was introduced into the corresponding mutant, the complemented strains recovered their BC production ability (Fig. 1b). The function of bcsIII operon was also proven using functional reconstitution experiment. The bcsIII operon was amplified and inserted between the EcoRI and HindIII sites of the pBAD30, yielding the bcsIII-expressing vector. The vector was transformed into E. coli DH5α, yielding E. coli DH5α (pBADIII). The functional reconstitution strain E. coli DH5α (pBADIII) can produce BC when induced by 0.2% L-arabinose (supplementary Fig. 5b). These results confirmed that the operon bcsIII is essential for cellulose production of Enterobacter sp. FY-07. To investigate whether the different oxygen demands lead to the different BC synthesis mechanisms between G. xylinus ATCC 53582 and Enterobacter sp. FY-07, we compared the characteristics of the BC synthesis gene cluster among Enterobacter sp. FY-07, G. xylinus ATCC 53582 and Enterobacter sp. 638. To clarify the differences in BC biosynthesis of Enterobacter sp. FY-07 under aerobic and anaerobic conditions, comparative transcriptome analysis and metabolite quantitative experiments were performed.

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Enterobacter sp. FY-07 or why Enterobacter sp. FY-07 has the different BC production ability with Enterobacter sp. 638, the protein homology, arrangement and constitution of the genes involved in the cluster were compared among Enterobacter sp. FY-07, Enterobacter sp. 638 and G. xylinum ATCC 53582. Protein sequence alignment was performed at the EMBL-EBI website (http://www.ebi.ac.uk/Tools/psa/) (Fig. 1a). The gene constitution and arrangement of Enterobacter sp. FY-07 BC synthesis gene cluster bcsIII, comprising bcsA, bcsB, bcsC and bcsD, are same as G. xylinum ATCC53582 but has much higher homology with Enterobacter sp. 638 than with G. xylinum ATCC53582, suggesting that the BC synthesis mechanism of Enterobacter sp. FY-07 is same to those of G. xylinum ATCC53582 and there exists limiting factor for BC production in Enterobacter sp. 638.

BC biosynthesis under aerobic and anaerobic conditions. We performed comparative transcriptome analysis to investigate whether BC biosynthesis by Enterobacter sp. FY-07 is in the same way under aerobic and anaerobic conditions. According to the reported BC biosynthesis pathway and protein homology analysis, the BC biosynthesis pathway of Enterobacter sp. FY-07 includes the following four reactions:26 Firstly, glucose and ATP are converted to glucose-6-phosphate and ADP by glucokinase (GK). Secondly, glucose-6-phosphate is catalysed by PGM to glucose-1-phosphate. Then, UTP and glucose-1-phosphate are catalysed to UDPG and ppi by UGPase. Finally, UDPG is polymerised to BC by a BC synthase complex encoded by the bcsIII operon (Fig. 2). The polymerisation needs c-di-GMP as an activator. On the basis of protein homology analysis, GK, PGM and BC synthase complex are encoded by AKI40_1504, AKI40_2159, and AKI40_0894~AKI40_0891 respectively. Protein homology of GK and PGM is 96.6% and 97.3% to corresponding orthologs in Enterobacter sp. 638 (ENT638_RS15075; ENT638_RS06320). UGPase can be encoded by three gene copies: AKI40_0889, AKI40_1747, and AKI40_3354; the protein homologies of the UGPases are 68.6%, 97% and 96.1% to corresponding orthologs in Enterobacter sp. 638 (ENT638_RS11900, ENT638_RS13725), respectively. Comparative transcriptome results also clearly illustrated that the same BC biosynthesis pathway was employed by Enterobacter sp. FY-07 under aerobic and anaerobic conditions.
anaerobic conditions. Transcriptome sequencing results have been submitted to NCBI and the SRA number of samples under aerobic and anaerobic conditions is SRR2183388 and SRR2183389, respectively. Transcription of Enterobacter sp. FY-07 genes encoding GK, PGM and UGPase was essentially the same under aerobic and anaerobic conditions (supplementary Table S2). In addition, transcription of BC biosynthesis genes was analysed. The results showed that gene transcription of operon bcsIII provided an absolute advantage under aerobic and anaerobic conditions and was consistent with the results of the gene knockout experiments (supplementary Table S2).

Energy metabolism. Analysis of the BC biosynthesis process of Enterobacter sp. FY-07 suggested that there is no oxygen included in these reactions directly; however, adenosine triphosphate (ATP) is required to activate glucose to UDPG. In addition, ATP is also essential for the biosynthesis of c-di-GMP, which is the activator of BC
It suggested that glucose was metabolised by the glycolytic pathway, pyruvate metabolism and TCA cycle. Metabolite quantitative analysis results were basically consistent with the results of transcriptome sequencing. The production of ATP has two patterns: substrate-level phosphate through glucose metabolism; and oxidative phosphate through NADH (sometimes FADH₂) produced by glucose metabolism transferred to electron acceptors.

The transcription for glucose metabolism, electron transport chain and nitrogen metabolism related genes were analysed under aerobic and anaerobic conditions (Fig. 2). Five genes encoding for three glycolytic pathway key enzymes, G6P (AKI40_1504), 6-phosphofructokinase (AKI40_4866; AKI40_2744) and pyruvate kinase (AKI40_2849; AKI40_3571) were not differentially expressed under anaerobic conditions (Fig. 2 and supplementary Table S2). The results indicated that the glycolytic pathway was not differentially regulated under anaerobic conditions. However, genes encoding for glucose-6-phosphate isomerase (AKI40_4682), triosephosphate isomerase (AKI40_4862) and enolase (AKI40_4050), were upregulated under anaerobic conditions. Upregulation of the three genes and the genes encoding for glucose-1, 6-bisphosphatase (AKI40_1324; AKI40_4472) suggested the activation of gluconeogenesis pathway under anaerobic conditions (supplementary Table S2). Genes encoding for glucose-6-phosphate dehydrogenase which is the rate-limiting enzyme of the pentose phosphate pathway was not differentially regulated under anaerobic conditions. The results indicated that the PPP metabolism did not change significantly under anaerobic conditions. The differentially expression of transketolase genes (AKI40_0825, AKI40_1411) and transaldolase (AKI40_4259, AKI40_1412) genes suggested that different genes contributed to reversible conversion among hexoses, pentoses and tetroses under aerobic and anaerobic conditions. Most TCA cycle genes, such as those encoding citrate synthase (AKI40_2176), isocitrate dehydrogenase (AKI40_2659), succinate dehydrogenase (AKI40_2177, AKI40_2179, AKI40_2180) and malate dehydrogenase (AKI40_0551, AKI40_3010) were downregulated, suggesting the repression of TCA cycle in Enterobacter sp. FY-07 under anaerobic conditions (Fig. 2 and supplementary Table S2). Transcriptions of genes encoding the key enzymes isocitrate lyase (AKI40_4699) and malate synthase (AKI40_4700) of the glyoxylate pathway were expressed at very low levels under aerobic and anaerobic conditions (supplementary Table S2), suggesting that although Enterobacter sp. FY-07 possesses genes for this metabolism, glyoxylate metabolism is inactivated.

Compared with aerobic conditions, the glycolytic pathway was not significantly changed, but the rate of TCA cycle was reduced under anaerobic conditions. Thus, pyruvate, as the final product of the glycolytic pathway, cannot be consumed by the TCA cycle. The results, combined with transcriptome sequencing and quantitative PCR analysis, suggested that Enterobacter sp. FY-07 possesses three significantly upregulated pyruvate metabolic pathways under anaerobic conditions (Fig. 2 and supplementary Table S2). The first pathway is the phosphotransacetylase-acetate kinase (Pta-AckA) pathway, which consists of three enzymatic reactions. Genes of the pathway were upregulated under anaerobic conditions. In this pathway, pyruvate is oxidized by the pyruvate dehydrogenase complex (AKI40_4164–AKI40_4167) or formate-lyase (AKI40_2388), resulting in the formation of acetyl coenzyme A. The conversion of acetyl-CoA to acetic acid is catalysed by acetyltransferase (AKI40_1562) and acetate kinase (AKI40_1564), and couples with substrate-level ATP production. The second pathway is the formate-lyase pathway, with formic acid as the intermediate metabolite, H₂, CO₂ and NADH as the end-products. Genes of this pathway encoding formate-lyase (AKI40_2388), formate dehydrogenase (AKI40_3156–AKI40_3158, AKI40_3242, AKI40_4599) and hydrogen enzymes (AKI40_0155, AKI40_0754–AKI40_0761, AKI40_1035) were upregulated under anaerobic conditions. The third pathway is the acetoin biosynthesis pathway. Pyruvate is catalysed by acetolactate synthase (AKI40_0023–AKI40_0024, AKI40_4203–AKI40_4204, AKI40_4818, AKI40_0779), resulting in the synthesis of acetolactate. The transformation of acetolactate to acetoin is catalysed by acetolactate decarboxylase (AKI40_0778). Transcriptions of the acetolactate synthase gene and the acetolactate decarboxylase gene were upregulated under anaerobic conditions. In addition, transcription of genes encoding alcohol dehydrogenase and lactate dehydrogenase were not regulated under anaerobic conditions. This results suggested that lactate fermentation and ethanol fermentation do not exist in Enterobacter sp. FY-07 under anaerobic conditions. The regulation of genes involved in the above pathways was confirmed by qRT-PCR analysis (supplementary Table S2).

To further understand the growth and metabolism of Enterobacter sp. FY-07 under aerobic and anaerobic conditions, we measured the cell growth, BC yield, and the concentrations of glucose, formic acid, acetic acid, acetoin, lactic acid and ethanol in the culture broth (Fig. 3). The BC production of Enterobacter sp. FY-07 is similar under aerobic and anaerobic conditions (Fig. 3a). As shown in Fig. 3b, the concentration of acetic acid and formic acid under anaerobic conditions were higher than those under aerobic conditions. In addition, a higher concentration of acetoin was detected under anaerobic conditions than aerobic conditions. The other fermentation end-products such as lactic acid and ethanol were not detectable under aerobic and anaerobic conditions. Metabolite quantitative analysis results were basically consistent with the results of transcriptome sequencing. The results suggested that glucose was metabolised by the glycolytic pathway, pyruvate metabolism and TCA cycle, with carbon dioxide (CO₂) as the main final metabolite of Enterobacter sp. FY-07 under aerobic conditions. Under anaerobic metabolism, glucose was incompletely metabolised by the glycolytic pathway and pyruvate metabolism to form more formic acid, acetic acid, acetoin and hydrogen than aerobic conditions as the products.
Electron transfer. Oxygen is the electron acceptor under aerobic conditions. Electrons of NADH and FADH₂, generated from the glycolytic pathway and TCA cycle, are transferred to oxygen through the electron transfer chain. Energy production is mainly based on oxidative phosphorylation, supplemented by substrate-level phosphorylation under aerobic conditions. However, only a few ATP molecules are produced by the glycolytic pathway. To ensure the energy production for BC biosynthesis, the response of Enterobacter sp. FY-07 to anaerobic conditions was studied. According to the transcriptome sequencing and qRT-PCR analysis, transcriptions of genes encoding alcohol dehydrogenase (AKI40_3245, AKI40_3351, and AKI40_4427) and lactate dehydrogenase (AKI40_1686) were not differentially expressed under anaerobic conditions, suggesting that the NADH produced by the glycolytic pathway cannot be re-oxidised by fermentation. The substance functioning as the electron acceptor under anaerobic conditions was identified using transcriptome sequencing. Transcriptions of genes in nitrogen metabolism encoding dissimilatory nitrate reductase (AKI40_3370–AKI40_3367), nitrite reductase (AKI40_0420, AKI40_0421), nitrite transporter (AKI40_0419) were upregulated under anaerobic conditions (Fig. 2 and supplementary Table S2), proving that nitrate is the electron acceptor under anaerobic conditions. Nitrate accepting the electrons transported through the electron transport chain is reduced to nitrite. Nitrite is then reduced to ammonia. The results suggested that substrate-level phosphorylation and oxidative phosphorylation are both energy production modes under anaerobic conditions. In addition, transcriptions of genes encoding assimilative nitrate reductase (AKI40_1028, AKI40_1029) were downregulated under anaerobic conditions. These results suggested that Enterobacter sp. FY-07 has different pathways for nitrate utilisation under aerobic and anaerobic conditions. Enterobacter sp. FY-07 undergoes nitrate respiration under anaerobic conditions. Anaerobic nitrate respiration is an effective energy production mode and can supply energy for BC biosynthesis. All the results were confirmed by qRT-PCR analysis (supplementary Table S2).

In order to verify the results, the concentration of sulfate, nitrate and nitrite in the medium were determined under aerobic and anaerobic conditions (Fig. 4). The concentration of sulphate decreased slightly under aerobic and anaerobic conditions. However, nitrate was consumed rapidly under aerobic and anaerobic conditions, being consumed completely at 12th hour and 20th hour respectively. Although it was not added to the culture, a large amount of nitrite was detected in the culture medium after 16th hour under anaerobic conditions. Nitrite was only detected at 8th hour under aerobic conditions. These results agreed with the upregulation of transcription of gene (AKI40_0419) encoding nitrite transporter under anaerobic conditions. All these results provided direct evidence for nitrate being the electron acceptor of Enterobacter sp. FY-07 under anaerobic conditions.
These results suggested that some limiting factors of the transcription of bcs I and ability. The transcriptions of bcs concentrations in the culture medium are shown. Values are the means of three experiments with standard deviations.

**Discussion**

In this study, the BC biosynthesis genes cluster of *Enterobacter* sp. FY-07 was identified using gene knockout, gene complementation methods and functional reconstitution experiment. bcsIII operon is the functional BC biosynthesis gene cluster under aerobic and anaerobic conditions. Protein sequence alignment proved that genes constitution and arrangement of bcsIII operon are the same as BC synthesis operon of *G. xylinus* ATCC 53582. The result suggested the same BC synthesis mechanism of *Enterobacter* sp. FY-07 as *G. xylinus* ATCC 53582. However, the flanking sequences of *Enterobacter* sp. FY-07 BC synthesis gene cluster are totally different from those of *G. xylinus* ATCC53582\(^2\) (Fig. 1a). The gene (AKI40_0895) upstream of bcsA (III) in *Enterobacter* sp. FY-07 encodes a hypothetical protein. When knocked out, the BC biosynthesis ability of mutant *Enterobacter* sp. FY-07 (Δhyp) decreased sharply (supplementary Fig. S1). In *G. xylinus* ATCC 53582, the gene in the location corresponding to AKI40_0895, encodes a cellulose complementing factor (CcpAx)\(^4\), which was essential for BC production. Although its function in BC synthesis remains unknown, the absence of the CcpAx protein causes a lack of BC production ability. Protein sequence alignment proved that there is very low similarity between the unknown function protein and CcpAx (40.5%) (Fig. 1a). The genes (AKI40_0896 and AKI40_0897) encoding hypothetical protein and diguanylate cyclase exist upstream of the AKI40_0895 gene in *Enterobacter* sp. FY-07. However, in *G. xylinus* ATCC 53582 the gene cmcAx encoding a cellulase, is upstream of ccpAx\(^4\). Downstream of the BC synthesis genes cluster in *Enterobacter* sp. FY-07 are genes (AKI40_0890, AKI40_0889, AKI40_0888) encoding diguanylate cyclase/phosphodiesterase, UTP-glucose-1-phosphate uridylyltransferase and a minor endoglycanase Y; however, in *G. xylinus* ATCC 53582 there is gene encoding β-glucosidase\(^2\). Sequence comparison revealed that the genetic constitution and arrangement of operons bcsI and bcsII are the same as those in *Enterobacter* sp. 638 (Fig. 1a). However, in *Enterobacter* sp. 638 there is no gene cluster similar to bcsIII. This is why *Enterobacter* sp. FY-07 produces large clumps of cellulose, but only a small amount of BC is produced as a component of the biofilm by *Enterobacter* sp. 638\(^4\). The high homology between *Enterobacter* sp. FY-07 bcsIII cluster and *Enterobacter* sp. 638 bcsI cluster suggested that *Enterobacter* sp. 638 also possess the BC biosynthesis ability. The transcriptions of bcsI and bcsII were very low in *Enterobacter* sp. FY-07 (supplementary Table S2). These results suggested that some limiting factors of the transcription of bcsI and bcsII cluster exist. Regulation of bcsI and bcsII cluster needs to be further studied. In *E. coli* 1094, bcsQ which also exists in the bcsI and bcsII cluster, is an essential component of the cellulose synthesis apparatus that localises at the bacterial cell pole\(^4\). In this study, knockout of clusters bcsI or bcsII did not affect the BC biosynthesis capacity in *Enterobacter* sp. FY-07. The functions of other genes, such as bcsF, bcsE and bcsG, have not been identified. *Enterobacter* sp. FY-07Δ bcsGFE retained the ability to synthesise BC.

According to the comparative transcriptome and metabolite quantitatively analyze, most of the pyruvate which cannot enter TCA cycle was used for producing a large amount of formic acid, acetic acid and a small amount of acetoine rather than lactic acid and ethanol under anaerobic conditions. The formation of acetic acid was coupled with ATP biosynthesis. Under anaerobic conditions, the concentration of formic acid was increased first and then reduced in the culturing process. This may be due to that formic acid is the intermediate metabolite and can be further utilized in the culturing process\(^6\). The further metabolism of formic acid is catalysed by formate dehydrogenase and couples with the generation of NADH. Small portion were utilized for generating acetoin which can avoid excessive acidification of the medium\(^7\). These suggested that the pyruvate metabolism in *Enterobacter* sp. FY-07 was tended to produce more ATP rather than consume NADH under anaerobic conditions. Deficiency of electron acceptor (oxygen) under anaerobic conditions results in cellular NADH accumulation. To adapt to such circumstances, the microorganism normally regulates certain metabolic pathways to re-oxidise NADH to NAD and supports catabolic mechanisms. Through comparative transcriptome analysis and metabolite quantitation experiment, *Enterobacter* sp. FY-07 was found to upregulate the transcription of genes of the respiratory nitrate reduction pathway and downregulated those of the TCA cycle to eliminate NADH.
Oilfield12. The resulting culture of FY-07 grown on slant medium (per litre, yeast extract 5 g, peptone 10 g, NaCl
37 °C. ampicillin (Ap, 100
and at 42 °C for Ts plasmid curing. The pBBR1MCS2 plasmid was used for gene complementation. When needed, the culture was grown at 30 °C for Ts plasmid retention until the medium became colourless, after which it was inoculated with the bacterial suspension within an anaerobic boiled to remove dissolved oxygen before sterilisation. After sterilisation, filter-sterilised resazurin (1 mg/l) and
grown with shaking at 30 °C. Cellulase (1 g/l) was added to the medium for the gene knockout experiment and
ated with the bacterial suspension was grown statically at 30 °C. For RNA isolation, the inoculated medium was
obic chamber. When used for determination of BC production and glucose concentration, the medium inocu-
Table 1.
| strains | description | reference |
| --- | --- | --- |
| E.coli s17 | recA pro hsdR RP4-2-Tc::Mu Km::Tn7 | This lab |
| E.coli DH5αs | F’-c80 lacZΔM15Δ(lacZYA-argF)U169 end A1 recA1 hsdR17(k'/μ-) supE44 Δthi-1 gyrA96 relA1 phoA | This lab |
| Enterobacter sp. FY-07 | wild-type strain | Ting Ma et al.12 |
| Enterobacter sp. FY-07 ΔbcsI | ΔbcsI deletion derivative of FY-07 | This work |
| Enterobacter sp. FY-07 ΔbcsII | ΔbcsII deletion derivative of FY-07 | This work |
| Enterobacter sp. FY-07 ΔbcsIII | ΔbcsIII deletion derivative of FY-07 | This work |
| Enterobacter sp. FY-07 ΔbcsGFE | ΔbcsGFE deletion derivative of FY-07 | This work |
| Enterobacter sp. FY-07 ΔbcsA(I) | ΔbcsA(I) deletion derivative of FY-07 | This work |
| Enterobacter sp. FY-07 ΔbcsA(II) | ΔbcsA(II) deletion derivative of FY-07 | This work |
| Enterobacter sp. FY-07 ΔbcsA(III) | ΔbcsA(III) deletion derivative of FY-07 | This work |
| Enterobacter sp. FY-07 ΔbcsC(II) | ΔbcsC(II) deletion derivative of FY-07 | This work |
| Enterobacter sp. FY-07 Δhyp | ΔAK140_0895 deletion derivative of FY-07 | This work |
| E.coli DH5α-pBABIII | E.coli DH5α containing pBAD-30-AKI40_0895–AKI40_0891 | This work |

**Table 1. Strains and plasmids used in this study.**

accumulation. In addition, transcription of genes encoding alcohol dehydrogenase and lactate dehydrogenase were not upregulated, which suggested that Enterobacter sp. FY-07 cannot re-oxidise NADH to NAD by fermentation to produce ethanol or lactate under anaerobic conditions.

A few Enterobacter species have been reported to produce BC, however, it is still little known about their BC biosynthetic machinery11,12,16. Our results demonstrated the BC biosynthetic mechanism, glucose metabolism and energy metabolism of Enterobacter sp. FY-07 under aerobic and anaerobic conditions. The present study contributes to the understanding of energy supply mechanism for BC production in facultative anaerobic bacteria under anaerobic conditions.

**Methods**

**Strains, plasmids, media and culture conditions.** The strains and plasmids used in the study are listed in Table 1. E. coli were grown on LB medium (per litre, 10 g peptone, 5 g yeast extract and 5 g NaCl) at 37 °C. Enterobacter sp. FY-07 is a highly effective bacterial cellulose (BC) producer that was isolated from a Jilin Oilfield12. The resulting culture of FY-07 grown on slant medium (per litre, yeast extract 5 g, peptone 10 g, NaCl 10 g, glucose 10 g and agar 15 g) was washed, re-suspended in sterile water (∼107 cells/ml). The bacterium suspension was inoculated into a 250 ml Erlenmeyer flask containing 100 ml fermentation medium (per litre, NH4NO3 2 g, KH2PO4 1 g, K2HPO4·3 H2O 0.5 g, MgSO4·7 H2O 0.25 g, glucose 25 g, pH 7.2) and grown at 30 °C under aerobic conditions. Anaerobic culture was performed as described previously12. Briefly, the medium was boiled to remove dissolved oxygen before sterilisation. After sterilisation, filter-sterilised resazurin (1 mg/l) and cysteine hydrochloride (0.5 g/l) were added to the medium. The resulting medium was charged with nitrogen gas until the medium became colourless, after which it was inoculated with the bacterial suspension within an anaerobic chamber. When used for determination of BC production and glucose concentration, the medium inoculated with the bacterial suspension was grown statically at 30 °C. For RNA isolation, the inoculated medium was grown with shaking at 30 °C. Cellulase (1 g/l) was added to the medium for the gene knockout experiment and in the determination of cell growth. A temperature-sensitive (Ts) plasmid named pTSK1, which was constructed from pKD46 (the replication region and bla gene) and pEX18Tc (sacB, multiple cloning site, Tc and oriT) was used for the gene knockout experiment. When needed, the culture was grown at 30 °C for Ts plasmid retention and at 42 °C for Ts plasmid curing. The pBRR1MCS2 plasmid was used for gene complementation. When needed, ampicillin (Ap, 100μg/ml), tetracycline (Tc, 15μg/ml), carbenicillin (Car, 50μg/ml) or kanamycin (Km, 34μg/ml) were added to the medium.

**DNA extraction and genomic sequencing.** Genomic DNA was extracted using a bacterial genomic DNA miniprep kit (Axygen Scientific, Union City, CA, USA), according to the manufacturer’s instructions. Genome sequencing was performed by the Shanghai Majorbio Bio-pharm Biotechnology Company (China). The genome sequencing procedure comprised DNA library preparation, emulsion-based clonal amplification (emPCR), sequencing by the Genome Sequencer FLX system and data synthesis. Firstly, genomic DNA was purified and fragmented to produce small pieces DNA of 400–800 bp. Single-stranded DNA (ssDNA) was recovered after end repair, modification of specific joint connection and denaturation treatment with NaOH. The ssDNA library was fixed on specially designed DNA capture beads to make the most of the beads carrying a unique
overnight, washed by PBS buffer, and then dehydrated for scanning electron microscopy observation. (G was used to construct the library and for RNA sequencing. The RNA was treated with the Ribo-zero magnetic Kit of Hind EcoR sp. FY-07 genome as the template. The upstream fragment including the original ribosomal binding site of Enterobacter sp. FY-07 genome was amplified independently in their own microreactor. Subsequently, bead-immobilised clonally amplified DNA fragments were recovered and purified for Roche 454 and Solexa sequencing experiments. Gap closing was performed using PCR and a 3730 sequencer. The genome was constructed from 1.2 G 454 reads, 0.8 G Solexa reads and gap-closing reads. The complete genome sequence was analysed by gene prediction and annotation, tRNA/rRNA prediction, COG/KEGG/GO analysis, comparative genome analysis and evolutionary genome analysis. Other methods for DNA manipulation, such as PCR, restriction enzyme reaction and ligation, were performed according to molecular cloning manual and the manufacturer’s instructions.

**Gene knockout and complementation.** To construct the Ts plasmid, the replication unit of pKD46 (≈3000 bp), containing the repA and bla genes, was amplified with primerstar pol using the primers p46-1Fw and p46-1Rv (supplementary Table S1). The backbone of pEX18Tc, containing sacB, tet, oriT and multiple cloning site (≈5000 bp), was amplified with primerstar pol using p18-1Fw and p18-1Rv (supplementary Table S1). The resulting fragments were subsequently digested with NcoI and Xhol and ligated, resulting in pTSK1 (supplementary Fig. S4a).

Gene knockout was performed by homologous recombination. To construct the gene knockout vector, the 1.5 kb upstream and downstream flanking sequences of the candidate gene were PCR amplified from the chromosome of Enterobacter sp. FY-07. The DNA fragments were connected by overlap PCR. The resulting fragment was treated with restriction enzymes and then ligated into pTSK1 treated with the same enzymes to generate the gene knockout vector. The gene knockout vector was introduced into Enterobacter sp. FY-07 (Car+) through conjugation transfer. Car+ and Tc+ transformants of Enterobacter sp. FY-07 were distinguished by PCR using p18-2Fw and p18-2Rv primers (supplementary TableS1). The correct transformant was incubated in LB medium at 37°C overnight for single-crossover and plasmid curing. The cultures were spread onto LB agar with Tc using the gradient dilution method and incubated overnight at 42°C. The correct single-crossover colony was identified by PCR and then incubated in LB medium overnight before being spread onto LB plates containing 5–10% sucrose. Two PCR reactions were performed to verify whether the colonies grown on LB with 5–10% sucrose plates were correct or not. The design principle of primers using for verifying the gene knockout mutants is shown in supplementary Fig. S4b. The successful gene knockout strain was complemented by introducing the vector with the amplified corresponding gene ligated into pBBR1MCS2. Cultures of Enterobacter sp. FY-07, the gene knockout mutant and its complement were spotted on the LB agar containing 20 mg/L Congo red, which has a strong affinity to cellulose. The degree of staining with this dye was used to estimate cellulose production ability.

**Functional reconstitution of bcsII operon in E. coli DH5a.** The bcsIII operon and the gene upstream of bcsIII operon encoding hypothetical protein (AKI40_0895-AKI40_0891) were amplified using Enterobacter sp. FY-07 genome as the template. The upstream fragment including the original ribosomal binding site (RBS) sequence was amplified and inserted between the EcoRI and HindIII sites of the pBAD302, yielding the bcsIII-expressing vector. The expression of bcsIII operon was induced by 0.2% L-arabinose at a cell density of OD600 0.5–0.7. Cells were further cultured at 30°C for 4–5 h. The cells and synthesized cellulose were harvested by centrifugation at 5000 g for 10 min. The precipitate cells and cellulose were fixed with 2.5% glutaraldehyde for overnight, washed by PBS buffer, and then dehydrated for scanning electron microscopy observation.

**RNA isolation.** Enterobacter sp. FY-07 cultures were collected, immediately frozen in liquid nitrogen and powdered to extract total RNA using Purezol reagent combined with RNAprep pure Cell/Bacteria Kit (Tiangen Biotech, Beijing, China), according to the manufacturer’s protocol. The cultures, harvested by centrifugation at the late logarithmic growth phase under aerobic and anaerobic conditions, were used to prepare transcriptome sequencing RNA samples. Residual genomic DNA contamination was removed by on-column DNase digestion (Takara, Dalian, China), according to the manufacturer’s instruction. RNA integrity was assayed by agarose gel electrophoresis. Sample concentration and quality were quantified at OD260 nm, OD280 nm and OD230 nm by a Bio-drop nano-spectrophotometer.

**Transcriptome sequencing and comparative transcriptome analysis.** Total RNA (>5 μg/sample) was used to construct the library and for RNA sequencing. The RNA was treated with the Ribo-zero magnetic Kit (G + G + Bacteria) (Epiconcentre, USA) to remove rRNA. mRNA enriched RNA was isolated using a Truseq® RNA sample prep Kit (Illumina, USA), according to the user’s manual. Double stranded cDNA was generated using the double-stranded cDNA Synthesis Kit (Invitrogen, Carlsbad, CA) and ligated with Illumina adaptors. dUTP, instead of dTTP was used to synthesise the second strand. Uracil-N-glycosylase (UNG) was added to digest the second strand cDNA. The library was enriched by 15 cycles of PCR, and recovered from 2% agarose gels. Paired-end 100 bp reads were generated by sequencing using the HiSeq2500 sequencing platform. RNA-Seq reads were mapped to the Enterobacter sp. FY-07 reference genome [GenBank accession number CP012487], using the Burrows-Wheeler Aligner. Raw reads data were determined on a per-genome basis and normalised against dividing by a size factor for each library. The normalised read counts, which were divided by the length of gene in kb pairs, were compared with the qRT-PCR data.

Pair-wise differential expression analysis between aerobic and anaerobic conditions was performed using the R package DESeq2. An unbiased variance estimator and a negative binomial model were used to test for differential expression by DESeq. To control the false discovery rate, the resulting p values were adjusted using the Benjamini and Hochberg procedure. Genes with a fold-change greater than 2 and an adjusted p value < 0.1 were determined as differentially expressed. The differentially expression genes were classified according to functional categories in the prokaryotic orthologous groups (COG) database (http://www.ncbi.nlm.nih.gov/COG). Genes
were determined to be significantly differentially expressed with a selection threshold of fold change \((\log_{2}^{\text{aerobic}} / \log_{2}^{\text{anaerobic}}) \geq 2\) (group 1), \(\leq 2\) and \(\geq 1\) (group 2), \(\leq -2\) (group 3) and \(-1 < \) and \(\geq 2\) (group 4). Genes associated with specific pathways were analysed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (http://www.genome.jp/kegg/).

**Quantitative real-time reverse transcription PCR (qRT-PCR).** qRT-PCR was carried out to access the RNA-sequencing results for the genes of interest. cDNA was synthesised using a Quantscript RT Kit (Tiangen, Beijing, China), following the manufacturer’s instructions. A total input of 500 ng of RNA and random hexamers were used in each reaction. The 16S rRNA gene was used as the endogenous reference gene. The ratio of expression was quantified by \(2^{-\Delta \Delta CT}\) method. The error of the R-values (\(\Delta R\)) was calculated by the Bio-rad qPCR software.

**Quantitative methods.** Cell growth was measured by monitoring the OD600 nm of cultures using an ultraviolet and visible spectrophotometer at 3-h intervals. After centrifugation, the supernatant of the fermentation broth was filtered by passage through a 0.22-μm membrane (Millipore) and applied to determine the concentration of glucose, sulphate, nitrate, nitrite, formic acid, acetic acid and lactic acid. The supernatant of the culture was extracted by ethyl-acetate, filtered by passage through a 0.22-μm membrane and then applied to determine the concentration of ethanol and acetoin. The concentration of glucose was quantified by the 3, 5-dinitrosalicylic acid (DNS) method, as described previously. Ion chromatography was used to measure the concentration of sulphate, nitrate and nitrite. High performance liquid chromatography (HPLC) was used to measure the concentration of formic acid, acetic acid and lactic acid. Gas chromatography (GC) was applied to quantify the concentration of ethanol and acetoin. To purify BC, the formed BC pellets were washed, boiled in 1% NaOH solution for 10 min, immersed in it for 24 h and then washed with distilled water until the pH of the water was neutral. The purified BC was dried to a constant weight at 80°C to measure the BC production. In the determination of BC production, all experiments were performed in triplicate cultures and the relative error of the replicates was less than 5%.

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