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ENVIRONMENTAL BIOTECHNOLOGY

Applied microbiology and biotechnology uncovering the biosynthetic pathway of polysaccharide-based microbial flocculant in *Agrobacterium tumefaciens* F2

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Abstract

The low yield as bottleneck problem limits the application of microbial flocculant in water treatment. However, genetic information of microbial flocculant-producing strains can guide the regulation of microbial flocculant production, but it remains unknown. *Agrobacterium tumefaciens* F2 produced polysaccharide-based microbial flocculants in the fermentation medium but none in Luria Bertani medium; hence, the transcriptome was used to analyze the potentially associated genes with the production of microbial flocculants. Glucose, mannose, rhamnose, and galactose are the main sugar monomers, and genes (*manA*, *glmM*, *manC*, *rfb* genes, *exo* genes, etc.) with changed expression levels related to sugar monomers metabolism potentially participated in the biosynthesis of polysaccharide-based microbial flocculants. *exoC*, *exoP*, and *manC* were confirmed to participate in the biosynthesis via constructing the mutants F2-d*exoC*, F2-d*exoP*, and F2-d*manC*. An exo_{F2} gene cluster was annotated due to the high percentage of matches between the genome sequences of strains F2 and C58, and *exo* genes in their genome sequences showed the similarity of 86~92%. The hypothetical pathway for the biosynthesis of polysaccharide-based microbial flocculants in strain F2 was proposed, laying the basis for the production yield regulation.

Key points

- *An exo_{F2} gene cluster in the polysaccharide biosynthesis was annotated.*
- *exoC, exoP, and manC genes participated in the polysaccharide biosynthesis.*
- *A hypothetical biosynthesis pathway of polysaccharide in flocculant was proposed.*

Keywords *Agrobacterium tumefaciens* F2 · Polysaccharides · Microbial flocculants · Transcriptome · Biosynthetic pathway · *exo* genes

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Introduction

Natural microbial flocculants are attractive due to their ubiquitous and environment friendly nature, biodegradability, and high application value (Salehizadeh et al. 2018; Yang et al. 2016). In general, microbial flocculants can be divided into polysaccharide-, protein-, nucleic acid-, and lipid-based microbial flocculants. Notably, the extensive application of microbial flocculants produced by bacterial strains in aqueous environments renders it a potential biomaterial (Salehizadeh et al. 2018). However, the low yield of microbial flocculants is the bottleneck, limiting their large-scale industrial production. Genetic studies regarding microbial flocculant-producing bacteria and the biosynthetic mechanism of microbial flocculants can solve this problem. Reports suggest that genetic

modification and alteration of biosynthetic or regulatory pathways improve exopolysaccharide yield (Ruffing and Chen 2006). Thus, investigations regarding the genetics of the biosynthetic pathway of microbial flocculant-producing bacteria may assist in improving the yield of polysaccharide-based microbial flocculants.

The microbial flocculant-producing bacterium, *Agrobacterium tumefaciens* F2, was isolated from the sludge of sewage treatment plants, and polysaccharide is the main component of its microbial flocculant products (Wu et al. 2015). Polysaccharide-based microbial flocculants produced by strain F2 have been used for pollutant treatment in the aqueous environment, but the genetics underlying their biosynthetic pathway are not yet understood. Fortunately, the relevant genes and pathways associated with the production of major bacterial exopolysaccharides by *Agrobacterium* have been widely studied. Succinoglycan is a common polysaccharide biosynthesized by bacteria of family from *Rhizobiaceae*, such as *Rhizobium* and *Agrobacterium* (Zevenhuizen 1997). Several studies have reported succinoglycan production by *Agrobacterium* (Cangelosi et al. 1987; Chouly et al. 1995; Evans et al. 2000; Stredansky and Conti 1999; Stredansky et al. 1998), while the succinoglycan biosynthetic pathway has been more widely proposed in *Sinorhizobium meliloti* (Becker 2015; Glucksmann et al. 1993; Janczarek 2011; Reuber and Walker 1993; Skorupska et al. 2006).

Wood et al. (2001) observed extensive similarity in the genome sequences of *A. tumefaciens* C58 and *S. meliloti* (Wood et al. 2001), and subsequent studies have also reported that strain C58 utilized an identical succinoglycan biosynthesis pathway (Wu et al. 2016). In general, the polysaccharide biosynthetic pathway can be classified into three main steps, including biosynthesis of nucleotide sugars, assembly of the repeat unit and polymerization, and export (Rehm 2010; Schmid et al. 2015). Succinoglycan is a branched polysaccharide consisting of glucose and galactose in the ratio of 7:1, with substituent additions of succinate, pyruvate, and acetate (Chouly et al. 1995; Reinhold et al. 1994; Schmid et al. 2015; Wang et al. 1999). In the succinoglycan biosynthetic pathway, *exoC*, *exoB*, and *exoN* are essential for sugar precursor biosynthesis, whereas *exoY* and *exoF* are responsible for galactose addition to the lipid carrier. Glucose residues are added by glycosyltransferases (GTs) encoded by *exoALMOUW* genes, which are also responsible for glycosidic bond formation. *exoZ*, *exoH*, and *exoV* are involved in the substituent modifications of the growing exopolysaccharides, adding acetyl, succinyl, and pyruvyl groups to the sugar chain. Polymerization of repeating units and secretion of exopolysaccharides are accomplished by proteins encoded by *exoPQT* (Becker 2015; Reuber and Walker 1993; Schmid et al. 2015; Skorupska et al. 2006; Wu et al. 2016). These studies have laid the foundation for an in-depth investigation regarding the biosynthetic pathway of polysaccharide-based microbial flocculants in strain F2.

However, certain information regarding polysaccharide-based microbial flocculant biosynthesis is still lacking, including (a) the homology in the genome sequence and the similarity between the *exo* genes of strains F2 and C58, (b) the sugar monomer composition of polysaccharide-based microbial flocculants, and (c) the key genes that participated in the biosynthesis of polysaccharide-based microbial flocculants. This has impeded uncovering of the biosynthetic pathway of polysaccharide-based microbial flocculants. Thus, the present study aims at investigating the above-mentioned unknown aspects to guide constructing the biosynthesis pathway of polysaccharide-based microbial flocculants in strain F2, and thereby guiding yield improvement of polysaccharide-based microbial flocculants and promoting their industrial application.

Materials and methods

Strain and medium

The *A. tumefaciens* F2 was isolated by our laboratory and deposited in the China General Microbiological Culture Collection Center (CGMCC no. 10131). This strain can produce polysaccharide-based microbial flocculants (Li et al. 2011; Wu et al. 2015). The fermentation medium (g/L) contained glucose, 10; urea, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; KH_2PO_4, 2; K_2HPO_4, 5; NaCl, 0.1, and yeast extract, 0.5, with the initial pH of 7.2~7.5; it was sterilized at 112 °C for 30 min. All components in medium were purchased from the Sinopharm Chemical Reagent Co., Ltd.

Comparative analysis of the genome

The MUMmer software was used to compare the ortholog distribution among the genomes in *A. tumefaciens* F2 (GenBank No. AFSD00000000.1), *Agrobacterium radiobacter* K84 (CP000628), *Agrobacterium vitis* S4 (CP000633), and *A. tumefaciens* C58 (AE007869). Genome clustering analysis and collinear analysis were completed using the SiLiX and BSR methods. Protein functions were analyzed using Gene Ontology (GO), Clusters of Orthologous Group, Kyoto Encyclopedia of Genes and Genomes (KEGG), and InterPro Database.

Analysis of gene expression by RNA-seq and qRT-PCR

Strain F2 was first pre-cultured in Luria Bertani (LB) medium and was then inoculated into LB and fermentation medium, respectively. The samples used for transcriptome analysis were collected after the bacterial strain was cultured until 24 h. Total RNA was extracted from the strain F2 using the RNeasy mini kit (Qiagen, Germany) together with DNA

digestion using the RNase-free DNase set (Qiagen, Germany), per the manufacturer's instructions. RNA concentration and quality were estimated using a NanoDrop 8000 spectrophotometer (Thermo Scientific, USA). Ribosomal RNA removal was performed with the Ribo-Zero rRNA kit removal kit. Upon mixing with the fragmentation buffer, the mRNA was fragmented into short fragments. Then, cDNA was synthesized using the mRNA fragments as templates. Short fragments were purified and dissolved with EB buffer for end reparation and single nucleotide A (adenine) addition. Afterwards, the short fragments were connected with the adapters. After agarose gel electrophoresis, the suitable fragments were selected for PCR amplification as templates. During the QC steps, the Agilent 2100 Bioanalyzer and ABI StepOnePlus Real-Time PCR System were used for the quantification and qualification of the sample library.

The primary sequencing data produced by Illumina HiSeq™ 2000, which are called raw reads, were subjected to QC to determine if a resequencing step is needed. After QC, the raw reads were filtered into clean reads, which were then aligned to the reference sequences with SOAPaligner/SOAP2. The alignment was subjected to QC to determine if resequencing is needed. The alignment data were utilized to calculate the distribution of reads on reference genes and perform coverage analysis. Gene expression was analyzed after the alignment result passed the QC. Differentially expressed genes (DEGs) were screened using statistical cutoff of $|\log_2 \text{ratio}| \geq 1$ and FDR ≤ 0.001. All raw RNA sequencing data are deposited and are accessible in NCBI (PRJNA557413).

The samples were also used for quantitative real-time PCR (qRT-PCR) analysis. Total cDNA was obtained using the PrimeScript™ RT reagent kit with gDNA eraser (Takara Biotechnology, Co., Ltd., Dalian, China). The obtained cDNA was used for further qRT-PCR analysis using SYBR® Premix Ex Taq™ (Tli RNaseH Plus) on an ABI7500 (Applied Biosystems, USA), per the manufacturer's instructions in a 50 μL reaction mixture. PCR was performed in triplicate under the following conditions: 95 °C for 30 s, followed by 40 cycles at 95 °C for 5 s, 56 °C for 45 s, and 72 °C for 40 s, with a fixed melting curve analysis at 95 °C for 15 s, 60 °C for 1 min, 95 °C for 15 s, and 60 °C for 15 s. Reactions without the cDNA template were used as negative controls. The mRNA levels were analyzed by comparing strain F2 cultured in the fermentation medium and in LB medium after normalization to the reference gene *trpE* using the $2^{-\Delta\Delta Ct}$ method. All samples were measured in triplicate, and the average values of these measurements were used to calculate mRNA expression. In addition, the samples in the two media were also collected and analyzed using qRT-PCR under the above-mentioned conditions except that the collection time was at 9 h instead of at 24 h. All primers are listed in Table S1.

Gene disruption and complementation in *A. tumefaciens* F2

A 500-bp DNA fragment containing the middle part of *exoC* was amplified from the genomic DNA of strain F2 with the restriction sites of *Sac*I and *Pst*I. The purified PCR product was excised via restriction digestion with both *Sac*I and *Pst*I and inserted into the same sites in the pJQ200SK vector, yielding the plasmid pJQ-d*exoC*, with gentamicin resistance as a selectable marker. The plasmid pJQ-d*exoC* was introduced into *Escherichia coli* Top10 and then transferred into strain F2 cells via helper strain *E. coli* HB101 (pRK600). The *exoC* disruption was confirmed via PCR using the d*exoC*-F and d*exoC*-R primers, followed by sequencing. Meanwhile, the expression of the disrupted gene in the recombinant bacteria was analyzed using reverse transcription PCR (RT-PCR). RNA and cDNA were obtained using the same procedure as mentioned previously. The cDNA products were amplified in 50 μL RT-PCR mixtures with 1 μL cDNA as the template using PrimeSTAR® Max DNA polymerase (Takara Biotechnology, Co., Ltd., Dalian, China), followed by PCR and sequencing.

Two oligonucleotide primers were designed to amplify *exoC* containing the native promoter from the genomic DNA of strain F2 via PCR. The DNA fragment was amplified with 20 bp homologous arms and inserted into the broad-host-range plasmid pBBR1-MCS2, yielding the plasmid pBB-*exoC* with neomycin resistance as a selectable marker. The plasmid was transferred into *E. coli* DH5α cells and then introduced into F2-d*exoC* with the help of *E. coli* HB101 (pRK600). The complementation strain containing the pBB-*exoC* plasmid was designated as F2-d*exoC*-C. The respective recombinant strains with *exoP* and *manC* gene disruption (F2-d*exoP* and F2-d*manC*) and complementation (F2-d*exoP*-C and F2-d*manC*-C) were constructed using the same procedures as mentioned previously. The strains and plasmids used for genetic engineering are shown in Table 1 (Kovach et al. 1995; Quandt and Hynes 1993).

Microbial flocculant production

The wild type and recombinant strains were first pre-cultured in the fermentation medium at 30 °C and 160 rpm for 24 h. Subsequently, the seed culture was inoculated into the fermentation medium and cultured under the same conditions. The fermentation liquid was centrifuged at 10,000 ×*g* for 20 min to remove the cell pellets. Two volumes of cold ethanol was added to the supernatant for extracting microbial flocculants, and the microbial flocculant-producing capacity of the strains was first evaluated based on the ability to produce white flocs. The microbial flocculant-producing capacity of strains was further evaluated based on the polysaccharide content. The collected flocs were dialyzed, freeze-dried, and then redissolved in the original volume using ultrapure water.

Table 1 Strains and plasmids used in this study

Strains or plasmids	Description	Source
Strains		
Agrobacterium tumefaciens F2	Str^r, Km^r; wild type; microbial flocculant-producing strain; G^-	CGMCC no. 10131
F2-d*exoC*	Km^r, Gm^r; *exoC*-disruption mutant of F2	This study
F2-d*exoP*	Km^r, Gm^r; *exoP*-disruption mutant of F2	This study
F2-d*manC*	Km^r, Gm^r; *manC*-disruption mutant of F2	This study
F2-d*exoC*-C	Str^r, Km^r, Gm^r, Neo^r; F2-d*exoC* containing pBB-*exoC*	This study
F2-d*exoP*-C	Str^r, Km^r, Gm^r, Neo^r; F2-d*exoP* containing pBB-*exoP*	This study
F2-d*manC*-C	Str^r, Km^r, Gm^r, Neo^r; F2-d*manC* containing pBB-*manC*	This study
Escherichia coli		
Top10	Host strain for gene disruption plasmid	Invitrogen
HB101(pRK600)	Conjugation helper strain; Cm^r	Takara
DH5α	Host strain for gene complementation plasmid	Takara
Plasmids		
pJQ200SK	Gm^r; suicide plasmid	Quandt and Hynes (1993)
pBBR1-MCS2	Neo^r; broad-host-range cloning plasmid	Kovach et al. (1995)
pJQ-d*exoC*	Gm^r; *exoC* gene disruption plasmid; the middle 500 bp regions of *exoC* gene fused into *Sac*I/*Pst*I-digested pJQ200SK	This study
pJQ-d*exoP*	Gm^r; *exoP* gene disruption plasmid; the middle 500 bp regions of *exoP* gene fused into *Sac*I/*Pst*I-digested pJQ200SK	This study
pJQ-d*manC*	Gm^r; *manC* gene disruption plasmid; the middle 500 bp regions of *manC* gene fused into *Sac*I/*Pst*I-digested pJQ200SK	This study
pBB-*exoC*	Neo^r; *exoC* gene complementation plasmid; the *exoC* gene recombined into pBBR1-MCS2	This study
pBB-*exoP*	Neo^r; *exoP* gene complementation plasmid; the *exoP* gene recombined into pBBR1-MCS2	This study
pBB-*manC*	Neo^r; *manC* gene complementation plasmid; the *manC* gene recombined into pBBR1-MCS2	This study

Str^r, streptomycin resistant; Gm^r, gentamicin resistant; Neo^r, neomycin resistant; Km^r, kanamycin resistant; Cm^r, chloramphenicol resistant

Polysaccharide concentration was determined using the phenol-sulfuric acid assay with glucose as the standard. In addition, OD_{660} was determined after every 3 h to analyze bacterial growth status.

Sugar monomer components

Polysaccharide-based microbial flocculants (2 mg) was dissolved into 1 mL of anhydrous methanol solution containing 1 M HCl, followed by sealed using N_2, hydrolyzed at 80 °C for 16 h, and dried by air pump. Then, 2 M trifluoroacetic acid (TFA, 1 mL) was added and hydrolyzed at 120 °C for 1 h. A small amount of ethanol was added and dried by water bath at 60 °C, which was repeated for 3–5 times until complete evaporation of TFA.

1-Phenyl-3-methyl-5-pyrazolone (PMP) solution (0.5 mL) and NaOH (0.3 M, 0.5 mL) were added into the treated sample until fully dissolved. Next, 0.1 mL of the sample was performed with water bath at 70 °C for 30 min. Then, 0.3 M HCl (0.05 mL) and distilled water (0.05 mL) were added and thoroughly mixed after centrifugation at 10,000 rpm for 5 min. Trichloromethane (1 mL) was added and mixed well to extract the remaining PMP reagent. The obtained sample in the water layer was filtered through 0.22 μm filter membrane and diluted by distilled water for HPLC measurement with mannose, rhamnose, glucuronic acid, galacturonic acid, glucose, galactose, xylose, arabinose, and fucose as standards.

Parameters included Shimadzu HPLC system with LC-10ATvp pump and SPD-10AVD ultraviolet detector and DIKMA InertsiL ODS-3 (4.6 × 150 mm). The mobile phase is PBS (0.1 M, pH 7.0) and acetonitrile with 82:18 (*v*/*v*). The flow rate was 1.0 mL min^{-1} with the sample volume of 20 μL, and the wavelength was 245 nm.

Results

General features and genome comparison of *Agrobacterium*

Comparison of the genome of strains F2, C58, S4, and K84 showed that the four strains shared a common core genome of

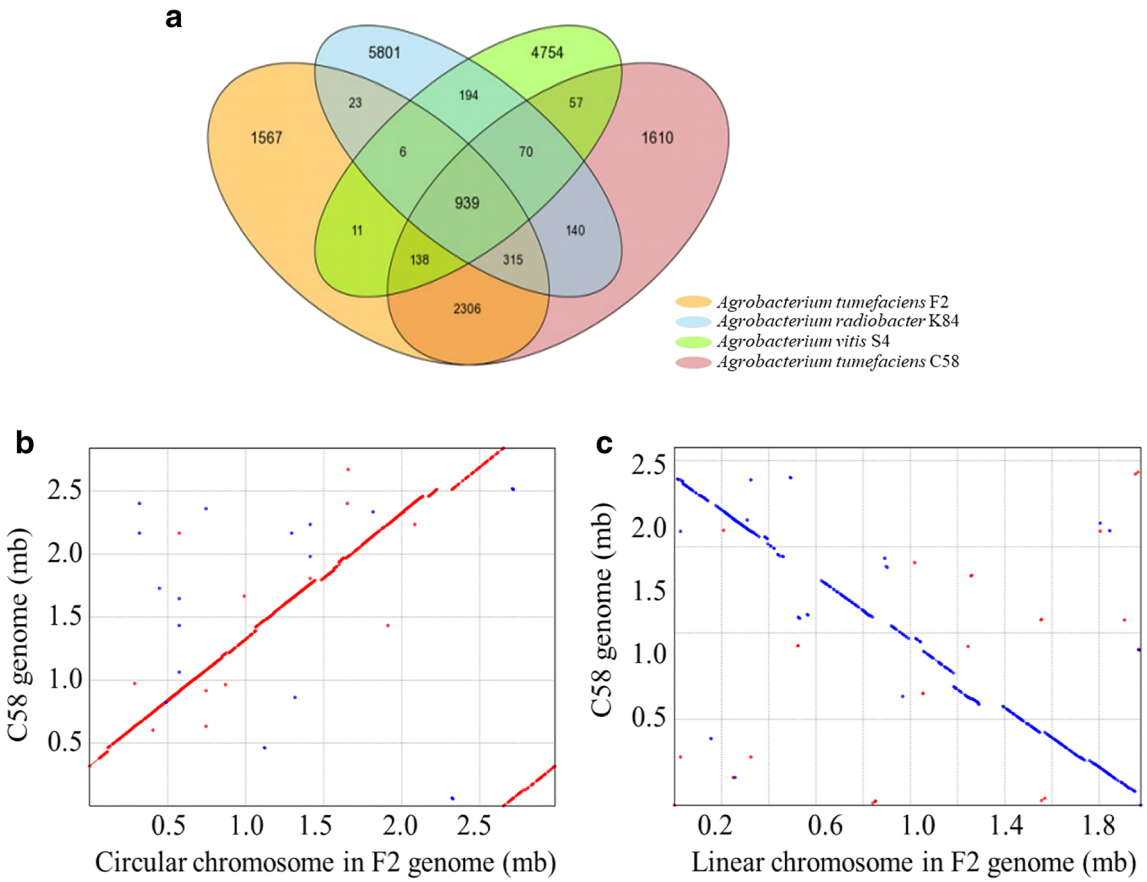

Fig. 1 Genome comparison and collinear analysis. (**a**) Genome comparative analysis among *Agrobacterium* strains. (**b**) Genome collinear analysis between circular chromosome in strain F2 genome and strain C58. (**c**) Genome collinear analysis between linear chromosome in strain F2 genome and strain C58

approximately 939 genes, while strains F2 and C58 were highly similar as they shared the most coding sequences (Fig. 1a). The genome size of the strain F2 is roughly equal to that of strain C58 but smaller than those of strains S4 and K84. The collinear analysis showed high match between the genome sequences of strains F2 and C58 (Fig. 1b and Fig. 1c). Thus, strain F2 was widely collinear with strain C58, exhibiting good sequence conservation. Strain C58 can biosynthesize succinoglycan using *exo* genes, and thus an exo_{F2} gene cluster responsible in strain F2 was annotated on the basis of genome information (Fig. 2). Homologous sequence alignment showed that the *exo* genes in strain F2 were similar (86~92%) to that of strain C58 (Table S2). Thus, more information regarding the biosynthesis of polysaccharide-based microbial flocculants in strain F2 can be obtained based on the succinoglycan biosynthesis pathway.

Production of polysaccharide-based microbial flocculants

Bacterial growth and bioflocculant production of strain F2 cultured in LB and fermentation medium were analyzed, respectively. Strain F2 showed higher biomass in LB medium than when cultured in fermentation medium (Fig. 3a), but it could not produce bioflocculants in LB medium. In the fermentation medium, bioflocculant production paralleled cell growth in strain F2 during logarithmic and stationary phase (Fig. 3b). The polysaccharide content was approximately 303.93 mg/L in microbial flocculants produced by strain F2 cultured in the fermentation medium until 24 h. Furthermore, component analysis showed glucose (48.24%), mannose (27.60%), rhamnose (13.53%), and galactose (6.66%) to be the main sugar monomer components of polysaccharide-based microbial flocculants. Thus, transcriptome was used to analyze the gene expression difference of strain F2 when cultured in respective LB and fermentation medium at 24 h, on which time point the microbial flocculants were largely produced by strain F2 when cultured in the fermentation medium, whereas no microbial flocculants were produced in the LB medium.

Transcriptional analysis related to polysaccharide biosynthesis

RNA-seq was used to determine the changes in the expression of genes associated with polysaccharide-based microbial

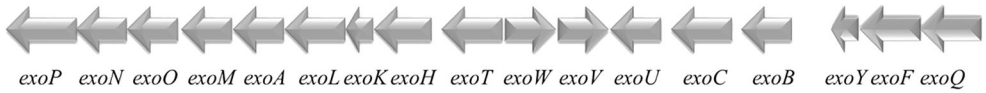

Fig. 2 The exo_{F2} gene cluster of polysaccharide biosynthesis in *A. tumefaciens* F2

flocculant production under flocculating and non-flocculating conditions in strain F2. Gene expression analysis revealed significant changes in several genes involved in glycolysis and TCA cycle (Table S3), and the change profiles are shown in Fig. S1. Thus, perturbed energy metabolism was observed during polysaccharide-based microbial flocculant biosynthesis. Compared with that during no flocculation production, significant changes in the expression level of genes related to sugar metabolism were observed during microbial flocculant biosynthesis in strain F2 (Table S4). Along with the significant changes in the expression levels of *exo* genes in the exo_{F2} gene cluster, the expression levels of *manA*, *glmM*, and *manC* (related to mannose metabolism) and *rfbA* and *rfbB* (related to rhamnose metabolism) were also significantly up-regulated. Furthermore, the expression levels of some other genes, such as *exoB*, *exoO*, *exoU*, *exoW*, and *exoT*, also changed, but not as significantly as those of the above-mentioned genes. qRT-PCR confirmed the changes in the expression level of certain genes, including *exoP*, *exoA*, *exoL*, *exoM*, *exoO*, *exoU*, *exoH*, *exoN*, *exoC*, *exoY*, *exoF*, and *manA* (Fig. 4a). The trend in the changes in gene expression level at 24 h was in accordance with the RNA-seq results, thereby confirming the accuracy of the RNA-Seq analysis.

In addition, qRT-PCR showed that these genes in strain F2 cultured in the fermentation medium for 9 h were up-regulated compared with when cultured in the LB medium for 9 h (Fig.

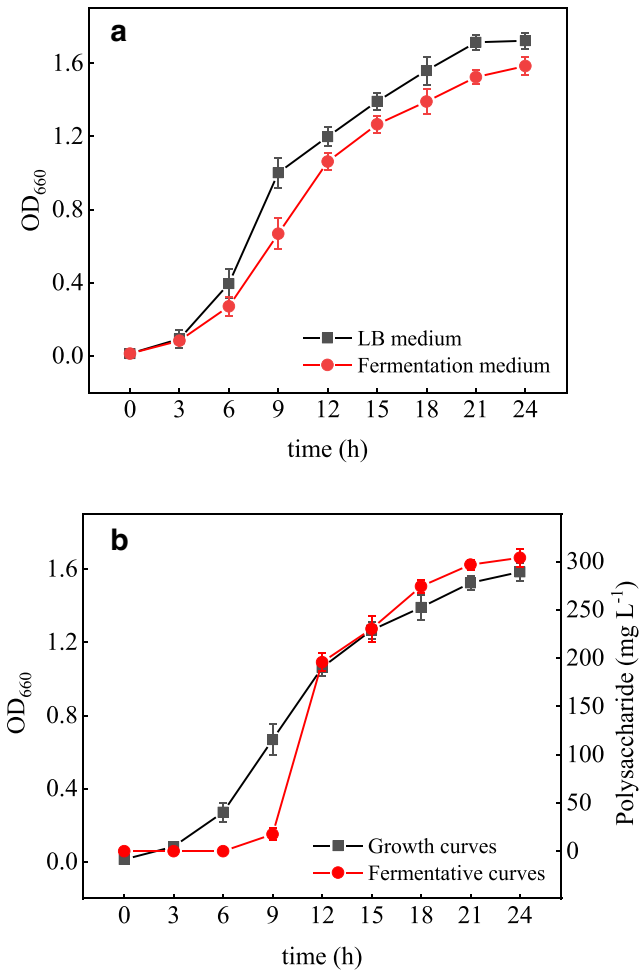

Fig. 3 Growth and fermentative curves of *A. tumefaciens* F2. (**a**) Growth curves of strain F2 cultured in LB and fermentation medium. (**b**) Growth and fermentative curves of strain F2 cultured in fermentation medium

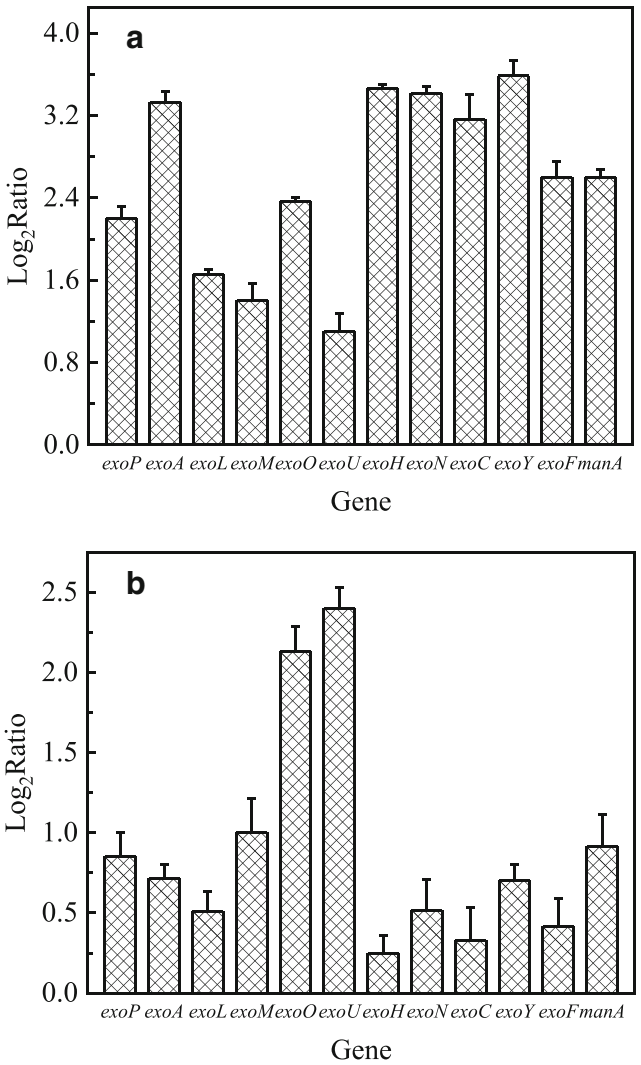

Fig. 4 Expression level change of genes in *A. tumefaciens* F2 cultured in the fermentation medium compared with LB medium based on qRT-PCR. (**a**) Samples collected at 24 h. (**b**) Samples collected at 9 h

4b). Results demonstrated that the production of microbial flocculants paralleled cell growth in strain F2 during the logarithmic and stationary phases, as well as the expression level of *exo* genes changed along with the production of microbial flocculants in strain F2.

Verification of the potential key genes

To verify whether *exoC* was involved in polysaccharide-based microbial flocculant production in strain F2, *exoC* was disrupted via a single-crossover event. Thus, the *exoC*-disrupted mutant F2-d*exoC* was constructed, in which *exoC* was not transcribed, as observed using RT-PCR analysis. The microbial flocculant-producing ability of the mutant F2-d*exoC* was determined and compared with that of the wild-type strain F2 as the control. Results showed that the wild-type strain F2 secreted microbial flocculants with polysaccharide content of 303.93 mg/L, whereas F2-d*exoC* did not produce microbial flocculants. Thus, the *exoC* mutation hindered microbial flocculant production by strain F2. In addition, the complementation strain F2-d*exoC*-C was constructed, which showed restoration of the wild-type phenotype and exhibited nearly identical microbial flocculant-producing capacity (Fig. S2a). Growth curves showed that the disruption and complementation of *exoC* in strain F2 did not hinder bacterial growth (Fig. S2b). Thus, the participation of *exoC* in the biosynthesis of polysaccharide-based microbial flocculants in strain F2 was confirmed.

Similarly, the mutant F2-d*exoP* was also obtained, in which *exoP* was not transcribed. No microbial flocculant was produced by the mutant F2-d*exoP*, although the complementation strain F2-d*exoP*-C restored microbial flocculant-producing capacity to the level of the wild-type strain F2 (Fig. S2c). Furthermore, the disruption and complementation of *exoP* in strain F2 did not hinder bacterial growth (Fig. S2d). Thus, the participation of *exoP* in the biosynthesis of polysaccharide-based microbial flocculants in strain F2 was confirmed.

Furthermore, the recombinant strain F2-d*manC* was constructed, in which *manC* was not transcribed. Unlike the mutants F2-d*exoC* and F2-d*exoP*, the mutant F2-d*manC* produced polysaccharide-based microbial flocculants at levels identical to that of the wild-type strain F2 (Fig. S2e). Further structure analysis showed the main sugar units of the microbial flocculants produced by the mutant F2-d*manC* to be glucose (65.8%), mannose (11.3%), rhamnose (6.7%), and galactose (10.2%). Compared with glucose (48.24%), mannose (27.60%), rhamnose (13.53%), and galactose (6.66%) in microbial flocculants produced by wild strain F2, the proportion of mannose and rhamnose unit decreased, whereas the increased ratio of glucose and galactose unit was also observed. *manC* disruption and complementation in strain F2 did not hinder bacterial growth (Fig. S2f). Thus, *manC* was also confirmed to participate in the biosynthesis of polysaccharide-based microbial flocculant by strain F2.

Discussion

Difference of exopolysaccharide production in strain F2 when cultured in the LB medium and fermentation medium was also observed in other reports, namely, microorganisms may overproduce exopolysaccharides in glucose-rich medium but were unable to produce them in the LB medium (Ferreira et al. 2011; Zlosnik et al. 2008). A possible explanation is that high concentration of glucose and high carbon/nitrogen ratio in the fermentation medium may activate and regulate intracellular metabolic pathways in strain F2 to divert them into polysaccharide biosynthesis (Yu et al. 2015; Yu et al. 2017). RNA-Seq analysis revealed active expression of genes related to sugar metabolism during biosynthesis of microbial flocculants, such as the *exo* genes. Of them, either *exoC* or *exoP* disruption resulted in the no production of microbial flocculants in strain F2, so both *exoC* and *exoP* were confirmed to participate in the biosynthesis of microbial flocculants. Combined with the results of genome match and homologous sequence alignment between strains F2 and C58, results could indicate the similarity in the polysaccharide biosynthesis pathways using *exo* genes between strains F2 and C58. Thus, the functions of some of the *exo* genes were inferred based on their function in succinoglycan biosynthesis (Table 2).

The main sugar monomers of polysaccharide-based microbial flocculants are glucose, mannose, rhamnose, and galactose. Changes in the expression levels of certain genes related to mannose and rhamnose metabolism were also detected during the production of polysaccharide-based microbial flocculants, such as *manA*, *glmM*, *manC*, *rfbA*, and *rfbB*. Among them, *manC* was verified to be involved in the polysaccharide-based microbial flocculant biosynthesis. However, the effect of *manC* gene disruption on the biosynthesis of microbial flocculants in strain F2 is different from *exoC* and *exoP* gene disruption. Mutant with *manC* gene disruption showed unchanged production level of microbial flocculants but changed the proportion of sugar unit. The reason for the effects of *manC* gene disruption needs to be further clarified. But in any case, *manC* also participated in the biosynthesis of microbial flocculants. Except *exo* genes, the functions of other genes related to sugar metabolism were proposed according to genome annotation and KEGG database analysis (Table 2).

Furthermore, glycolysis/gluconeogenesis and tricarboxylic acid (TCA) cycle mainly provided the monomer and energy for polysaccharide biosynthesis as observed by the changes in the levels of gene expression. Glycolysis and TCA cycle are the main pathways related to energy metabolism, which can produce a large amount of energy-rich compounds (such as ATP, NADH, and $FADH_2$) and precursors for useful compound biosynthesis, such as polysaccharides. Energy metabolism is also closely related to microbial activity and biosynthesis of biopolymer, such as polysaccharide. Significant changes of several genes involved in glycolysis and TCA

Table 2 Gene function in the hypothetical pathway

Gene locus	Gene name	Gene function
Agau_RS17155	*exoC*	Phosphoglucomutase
Agau_RS17035	*exoN*	UTP-glucose-1-phosphate uridylyltransferase
Agau_RS17590	*exoB*	UDP-glucose 4-epimerase
Agau_RS01650	*rfbA*	Glucose-1-phosphate thymidylyltransferase
Agau_RS01655	*rfbB*	dTDP-glucose 4,6-dehydratase
Agau_RS22570	*manA*	Phosphomannose isomerase
Agau_RS15190	*glmM*	Phosphomannomutase
Agau_RS19670	*manC*	Mannose-1-phosphate guanylyltransferase
Agau_RS22495	*exoY*	Undecaprenyl-phosphate galactose phosphotransfer
Agau_RS22500	*exoF*	Undecaprenyl-phosphate galactose phosphotransfer precursor
Agau_RS17050	*exoA*	Glycosyltransferase
Agau_RS17055	*exoL*	Glycosyltransferase
Agau_RS17045	*exoM*	Glycosyltransferase
Agau_RS17040	*exoO*	Glycosyltransferase
Agau_RS17085	*exoU*	Glycosyltransferase
Agau_RS17075	*exoW*	Glycosyltransferase
Agau_RS17030	*exoP*	Polysaccharide polymerization protein
Agau_RS17070	*exoT*	Polysaccharide transport protein

cycle accelerated energy metabolism are observed along with polysaccharide biosynthesis, due to bacterial cells requiring more energy to ensure growth and metabolism (Guan et al. 2018). These observations may enable construction of the biosynthetic pathway of microbial flocculant.

On the basis of the reported succinoglycan biosynthesis pathway (Becker 2015; Skorupska et al. 2006; Wu et al. 2016) and the common pathway for biosynthesis of the nucleoside sugar of rhamnose and mannose (Freitas et al. 2011; Giraud and Naismith 2000; Schmid et al. 2015), the hypothetical pathway of polysaccharide biosynthesis in the polysaccharide-based microbial flocculant production in strain F2 was finally proposed (Fig. 5). Microbial flocculants often have the complex structure, while polysaccharides are the main components in the microbial flocculants produced by strain F2 in this study. The proposed pathway highlights the

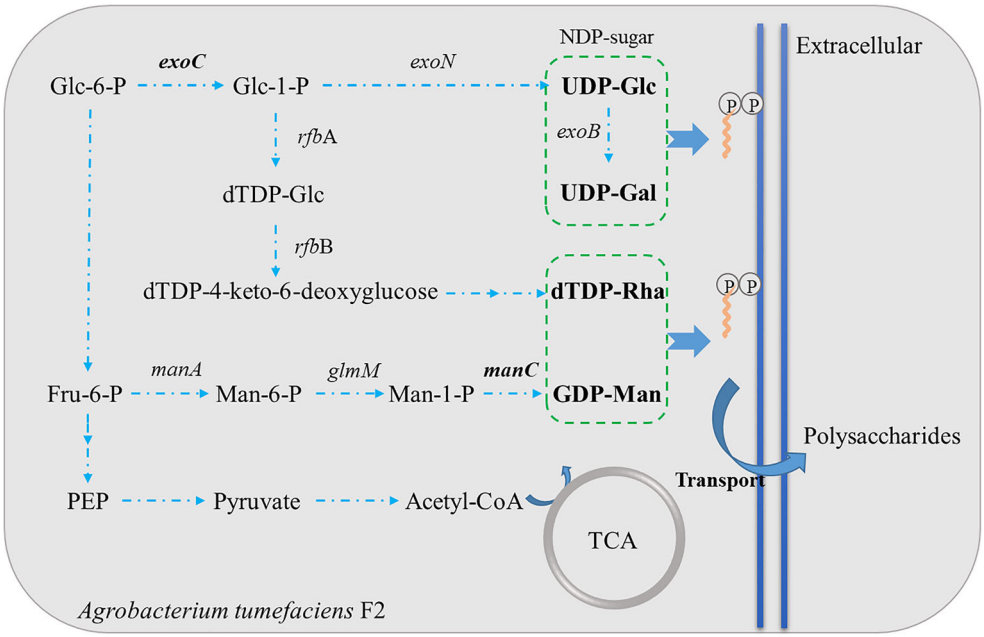

Fig. 5 Hypothetical biosynthesis pathway of polysaccharide in polysaccharide-based microbial flocculant produced by *A. tumefaciens* F2. Glc-6-P, glucose-6-phosphate; Glc-1-P, glucose-1-phosphate; UDP-Glc, uridine-5′-diphosphate glucose; UDP-Gal, uridine-5′-diphosphate galactose; dTDP-Glc, deoxythymidine diphosphate glucose; dTDP-Rha, deoxythymidine diphosphate rhamnose; Fru-6-P, fructose-6-phosphate; Man-6-P, mannose-6-phosphate; Man-1-P, mannose-1-phosphate; GDP-Man, guanosine-5′-diphosphate mannose

biosynthesis of the main components (i.e., polysaccharides) in the microbial flocculants produced by strain F2 that uses glucose as the substrate. The production process mainly included the formation of nucleoside sugars, assembly of sugar repeating units and polymerization, and export of the polysaccharides. UDP-Glc and UDP-Gal were formed by the enzymes encoded by *exoC*, *exoN*, and *exoB*, while dTDP-Rha formation mainly required *rfbA* and *rfbB*. GDP-Man was formed by the enzymes encoded by *manA*, *glmM*, and *manC*. After the first nucleoside sugar was added to the lipid linker, other sugar residues were added to the extending sugar chain by glycosyltransferases (GTs), such as *exoA*, *exoL*, and *exoM*. Finally, the sugar chains were polymerized and secreted out of the bacterial cell by polysaccharide polymerization and export protein, such as enzyme coded by *exoP* and *exoT*.

In summary, the present study provided new information regarding microbial flocculant biosynthesis in strain F2. The glucose-based biosynthetic pathway of polysaccharides in microbial flocculants in strain F2 was proposed, including three steps, namely, formation of nucleotide sugars, assembly of repeating units and polymerization, and export of the polysaccharide. The findings were expected to provide guidance for improving the yield of microbial flocculants. In future, research is still required to further confirm the biosynthesis pathway (e.g., by using metabolic flux analysis) and yield improvement of microbial flocculants that can be realized by enhancing the expression level of key genes in the biosynthetic pathway.

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Author contribution statement A. Li and F. Ma conceived and designed the research. S.S. Pi and L. Feng conducted the experiments. J.G. Qiu and H.P. Zhao contributed new reagents or analytical tools. S.S. Pi and D. Wu analyzed the data. S.S. Pi wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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