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Enhanced production of L-sorbose in an industrial *Gluconobacter oxydans* strain by identification of a strong promoter based on proteomics analysis

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Abstract *Gluconobacter oxydans* is capable of rapidly incomplete oxidation of many sugars and alcohols, which means the strain has great potential for industrial purposes. Strong promoters are one of the essential factors that can improve strain performance by overexpression of specific genes. In this study, a pipeline for screening strong promoters by proteomics analysis was established. Based on the procedure, a new strong promoter designated as P_{B932_2000} was identified in *G. oxydans* WSH-003. The promoter region was characterized based on known genome sequence information using BPROM. The strength of P_{B932_2000} was further assessed by analysis of enhanced green fluorescent protein (*egfp*) expression and comparison with *egfp* expression by two commonly used strong promoters, $P_{E.\ coli\_tufB}$ and $P_{G.\ oxydans\_tufB}$. Both quantitative real-time PCR and fluorescence intensities for *egfp* gene expression showed that P_{B932_2000} promoter is stronger than the other two. Overexpression of D-sorbitol dehydrogenase (*sldh*) by P_{B932_2000} in *G. oxydans* WSH-003 enhanced the titer and productivity of L-sorbose synthesis from D-sorbitol by 12.0 % and 33.3 %, respectively. These results showed that proteomics analysis is an efficient way to identify strong promoters. The isolated promoter P_{B932_2000} could further facilitate the metabolic engineering of *G. oxydans*.

Keywords L-ascorbic acid · Acetic acid bacteria · Constitutive promoter · Metabolic engineering · D-sorbitol

Introduction

Gluconobacter oxydans is an obligatory aerobic Gram-negative bacterium, belonging to the family of acetic acid bacteria [12]. This bacterium is well adapted to high concentrations of sugar and alcoholic environment; thus, it is often found in habitats such as fruits, flowers, and alcoholic drinks [2].The organism is known for its efficient incomplete oxidation of various sugars, sugar acids, and sugar alcohols [27] via effective membrane-bound dehydrogenases and oxidoreductases [7, 20]. Owing to its unique oxidizing activities, over recent decades, *G. oxydans* has been successfully used for the industrial production of food-related products, pharmaceuticals, and cosmetics, e.g., 2-keto-gluconic acid (vitamin C precursor) [9, 46], 6-amino-L-sorbose (precursor of the antidiabetic drug miglitol) [45], and dihydroxyacetone [11].

Because *G. oxydans* is widely used in industry, many investigations aiming at strain improvement including genetic engineering have been performed [15, 19, 31]. One basic, but important approach is to find a series of powerful promoters that are crucial to gene expression [24]. Thus far, little progress in identifying *G. oxydans* promoters has been made. In 1997, Saito et al. [30] found that the promoter $P_{E.\ coli\_tufB}$ was more effective compared with two other *E. coli* promoters, P_{tac} and P_L, in homologous expression of the *sndh/sdh* gene in *G. oxydans* G24. In addition, the promoter activities of $P_{G.\ oxydans\_tufB}$ and $P_{G.}$

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$_{oxydans\_gdh}$ were compared and eventually it was shown that the former was better when they were applied to improve the gene expression level of membrane-bound *ga5dh*, which catalyzes the conversion of glucose to 5-keto-D-gluconic acid [21]. Recently, a new promoter called $P_{ghp0169}$ was isolated from the chromosome of *G. oxydans* DSM 2003 and characterized by Shi et al. [34]. Compared with the strong $P_{G.\ oxydans\_tufB}$ promoter, $P_{ghp0169}$ exhibited even stronger promoter activity when it was evaluated with a GFP protein and a membrane-bound type II NADH dehydrogenase [32].

Most previous studies have mainly focused on promoters of known genes with high transcription levels, such as *tufB* coding for elongation factor Tu and *gdh* for glucose dehydrogenase [18, 21], which largely limited broad screening for new promoters. More comprehensive techniques, such as proteomics analysis, would make it easier to achieve this goal [1]. A protein is translated from a certain gene and the protein profile is highly correlated to the gene transcription level [29]. Furthermore, the proteome is the expressed protein complement of a genome and proteomics is functional genomics at the protein level [3]. Therefore, it should be possible to find a new strong promoter of a highly expressed gene through proteomics analysis.

In this study, a new promoter, designated as promoter P_{B932_2000}, was identified based on proteomics analysis and the promoter was shown to be useful for the expression of homologous and heterologous genes in *G. oxydans* WSH-003. A protein with a high expression level called hypothetical protein B932_2000 was found through two-dimensional gel electrophoresis (2-DE) experiments. The corresponding promoter region was characterized including the −10 and −35 motif sequences using the bioinformatics tool BPROM with the help of the genome sequence information. Enhanced green fluorescent protein (*egfp*) was used as the reporter gene to compare the new promoter with the strong $P_{E.\ coli\ tufB}$ and $P_{G.\ oxydans\_tufB}$ promoters. Finally, the strong promoter P_{B932_2000} was successfully used to overexpress *sldh* in *G. oxydans* WSH-003 to enhance L-sorbose production. The results showed that proteomics analysis is an efficient way to identify strong promoters. The strong promoter can further facilitate the metabolic engineering of *G. oxydans*.

Materials and methods

Strains and plasmids

Strains and plasmids used in this study are listed in Table 1. *G. oxydans* WSH-003 was obtained from Jiangsu Jiangshan Pharmaceutical Co., Ltd., and had been sequenced in our previous study [10]. *G. oxydans* ATCC 621H (DSM 2343) was purchased from the American Type Culture Collection (ATCC) [27]. *Escherichia coli* JM109 used as the host for plasmid construction was purchased from Novagen (Darmstadt, Germany).

Culture conditions

Gluconobacter oxydans strains were cultivated in D-sorbitol medium (D-sorbitol 150 g/L, yeast extract 10 g/L), at 30 °C, 200 rpm. As *G. oxydans* possesses a natural resistance toward cefoxitin, cultivation was performed using 25 μg/mL cefoxitin. All *E. coli* strains were cultivated at 37 °C, 200 rpm, on Luria–Bertani medium (yeast extract 5 g/L, tryptone 10 g/L, NaCl 10 g/L) with appropriate antibiotics when needed.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Sources
Strain		
E. coli JM109	*recA1, endA1, gyrA96, thi, hsdR17, supE44, relA1, Δ(lac-proAB)/F'[traD36,proAB+, lacIq, lacZΔM15]*	Novagen
G. oxydans WSH-003	Wild type, cef^R	This laboratory
G. oxydans 621H	Wild type, cef^R	Prust et al. [27]
Plasmid		
pNCS-egfp	*Amp^r, pUC ori, P_{T7},gfp*	This laboratory
pBBR1MCS-2	*rep*, $Kana^R$, *lacZ*	Kovach et al. [17]
pBBR1-$P_{E.\ coli\_tufB}$-*egfp*	pBBR1MCS-2 expressing *egfp* with $P_{E.\ coli\_tufB}$	This study
pBBR1-$P_{G.\ oxydans\_tufB}$-*egfp*	pBBR1MCS-2 expressing *egfp* with $P_{G.\ oxydans\_tufB}$	This study
G. oxydans-P_{B932_2000}-*egfp*	pBBR1MCS-2 expressing *egfp* with P_{B932_2000}	This study
pBBR1-$P_{G.\ oxydans\_tufB}$-*sldh*	pBBR1MCS-2 expressing *sldh* with $P_{G.\ oxydans\_tufB}$	This study
pBBR1-P_{B932_2000}-*sldh*	pBBR1MCS-2 expressing *sldh* with P_{B932_2000}	This study

Protein extraction and analysis

Intracellular protein extracts were prepared for analysis by 2-DE based on the method of He et al. [13]. For *G. oxydans* WSH-003, 1 g of wet cell pellet was resuspended in 2 mL extraction buffer (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), 10 mg/mL dithiothreitol). A protease inhibitor cocktail (Sangon Biotech, Shanghai, China) was added to the cell suspension, which was sonicated to disrupt the cells and break down the genomic DNA. The cell extract was centrifuged and the supernatant was collected. Membrane proteins were obtained by using a FOCUS™ Global Fractionation kit (G-Biosciences, St. Louis, MO). The protein extract was then purified using a 2-D Clean-Up Kit (GE Healthcare, Milwaukee, WI), and the purified protein sample was dissolved in rehydration solution (7 M urea, 2 M thiourea, 4 % (w/v) CHAPS, 0.002 % (w/v) bromophenol blue) supplemented with 2 % (v/v) Bio-Lyte 3-10 (Bio-Rad, Hercules, CA) and 10 mg/mL dithiothreitol. Total protein concentration was determined using the Non-Interference Protein Assay Kit (Sangon Biotech, China).

For the first-dimension isoelectric focusing (IEF), 800 μg of protein (450 μL) was loaded on a 24-cm Immobiline Dry-Strip pH 3–10 (Bio-Rad) (Immobiline Dry-Strip pH 4–7 for membrane proteins). IEF was performed with an Ettan™ IPGphor™ 3 system (GE Healthcare, USA). Before the second dimension (SDS-PAGE), the IPG strips were equilibrated. Second-dimension electrophoresis was performed on the Ettan DALTsix Electrophoresis System (GE Healthcare) with 12.5 % SDS-PAGE gels. Proteins were visualized using Coomassie staining as described by Neuhoff et al. [22]. Then the stained gel images were captured using Image Master LabScan (GE Healthcare). Image data were analyzed using the PD-Quest 8.0.1 software package (Bio-Rad, USA). Spots of interest were excised and in-gel digestion with trypsin performed as described by He et al. [13]. Peptide mass spectra were obtained using the Bruker UltraFlex III MALDI-TOF/TOF MS (Bruker Daltonics, Karlsruhe, Germany).

Bioinformatics analysis of the promoter sequence

The peptide fragment ion data acquired from MALDI-TOF/TOF MS were used to search for protein candidates in the NCBInr database through Mascot (Matrix Science) integrated in BioTools (Bruker Daltonics) (http://www.matrixscience.com/). Then, according to the protein research results, the corresponding gene information was obtained based on the genome sequence of *G. oxydans* WSH-003. The promoter sequence was predicted using a professional online tool, BPROM, which is a bacterial promoter recognition program with about 80 % accuracy and specificity [39].

Construction of recombinant plasmids

Based on the available sequence information of *G. oxydans* WSH-003 and *G. oxydans* 621H [10, 27], the promoters $P_{E.\ coli\_tufB}$, $P_{G.\ oxydans\_tufB}$, and P_{B932_2000} and the genes *egfp*, *sldh* were amplified by PCR with the templates and primers listed in Tables 1 and 2. The amplified promoter fragments $P_{E.\ coli\_tufB}$, $P_{G.\ oxydans\_tufB}$, and P_{B932_2000} were ligated with *egfp* and *sldh*, respectively, using overlap extension PCR methods [4]. The resulting fusion DNA fragments were digested and inserted into the *BamH*I/*Kpn*I site of pBBR1MCS-2, resulting in pBBR1-$P_{E.\ coli\_tufB}$-*egfp*, pBBR1-$P_{G.\ oxydans\_tufB}$-*egfp*, pBBR1-P_{B932_2000}-*egfp*, pBBR1-$P_{G.\ oxydans\_tufB}$-*sldh*, and pBBR1-P_{B932_2000}-*sldh*, respectively. The vectors were sequenced by the Sanger method for correction and then introduced into *G. oxydans* WSH-003 by electroporation. Transformants were selected with both 25 μg/mL of cefoxitin and 20 μg/mL of kanamycin.

RNA isolation and quantitative real-time PCR (qRT-PCR)

The recombinant strain *G. oxydans* WSH-003 pBBR1-$P_{E.\ coli\_tufB/G.\ oxydans\ 621H\_tufB/B932\_2000}$-*egfp* cells were harvested at the beginning of the stationary phase (at 24 h). Then, total RNA was isolated using the Qiagen RNeasy mini purification kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The cDNA was synthesized from the total RNA using a PrimeScript RT Reagent Kit (Perfect Real Time) (Takara, Dalian, China) according to the manufacturer's protocol. Expression levels of different *egfp* genes were measured with the 16S rRNA gene as the internal standard. Primer pair sets for the genes are listed in Table 2. qRT-PCR analysis was performed in 96-well plates on a LightCycler 480 II instrument (Roche, Mannheim, Germany) using double-stranded DNA-specific fluorochrome SYBR Green I. Amplification was carried out in a 20-μL (final volume) mixture containing 100 ng of cDNA sample, 0.2 μM forward primer, 0.2 μM reverse primer, and 10 μL of SYBR Premix ExTaq (Takara, Japan). The amplification procedure involved incubation at 95 °C for 40 s for the initial denaturation, followed by 40 cycles consisting of denaturation at 95 °C for 5 s, annealing/extension at 55 °C for 30 s, and cooling at 50 °C for 30 s. The threshold cycle (CT) values were determined with LightCycler software (version 3.3) [47].

Measurement of fluorescence intensity

The recombinant strains for *egfp* expression were grown in D-sorbitol medium at 30 °C, 200 rpm, and fluorescence and OD_{600} were determined by a BioTek Synergy H4 Multi-Mode Reader every 4 h. The whole cell fluorescence

Table 2 Primers used in this study

Primer	Sequence (5′-3′)[a]	Notes
sldh-R	GGGGTACCTCAGTGCTTGATGGCATCAG	*Kpn*I
egfp-R	GGGGTACCGGCCGCAAATTAAAGCCTTC	*Kpn*I
E. coli_tufB-F	CGGGATCCCAGGAGCGCATTGTTGAGCA	*BamH*I
E. coli_tufB(*egfp*)-R	AGTTCTTCTCCCTTACCCATCGATTGTCCCTCTAAGACACGGA	
egfp(*E. coli_tufB*)-F	GTGTCTTAGAGGGACAATCGATGGGTAAGGGAGAAGAACTTTTCAC	
G. oxydans_tufB-F	CGGGATCCGTACGATGGTAAGAAATCCACTG	*BamH*I
G. oxydans _tufB(*egfp*)-R	AGTTCTTCTCCCTTACCCATCGTCTTTCTCCAAAACCCC	
egfp(*G. oxydans_tufB*)-F	GGGGTTTTGGAGAAAGACGATGGGTAAGGGAGAAGAACTTTTCAC	
B932_2000-F	CGGGATCCGAGGTATTTGGAATGAGTCGCC	*BamH*I
B932_2000(*egfp*)-R	AGTTCTTCTCCCTTACCCATCGTCTATCTCCAAAACCCTGC	
egfp(*B932_2000*)-F	CAGGGTTTTGGAGATAGACGATGGGTAAGGGAGAAGAACTTTTCAC	
G. oxydans_tufB(*sldh*)-R	TGGAAATATTTCACAATCACCGTCTTTCTCCAAAACCCC	
sldh(*G. oxydans_tufB*)-F	GGGGTTTTGGAGAAAGACGGTGATTGTGAAATATTTCCAAAAT	
B932_2000(*sldh*)-R	TGGAAATATTTCACAATCACCGTCTATCTCCAAAACCCTGC	
sldh(*B932_2000*)-F	CAGGGTTTTGGAGATAGACGGTGATTGTGAAATATTTCCAAAAT	
RT*egfp*-F	ACTTGTCACTACTCTTACTTATGG	
RT*egfp*-R	TCCTTGAAGAAGATGGTCCTC	
16S rRNA-F	GCGGTTGTTACAGTCAGATG	
16S rRNA-R	GCCTCAGCGTCAGTATCG	

[a] Restriction sites and reverse complementary sequences for fusion PCR used for cloning are underlined

intensity (RFU/OD_{600}, the relative fluorescence unit divided by the corresponding cell density) was measured according to Xu et al. [43], using Gen5 Data Analysis Software (BioTek, USA) with an excitation of 488 nm and emission at 509 nm upon measuring. Cells were harvested by centrifugation and washed twice with phosphate-buffered saline (PBS) buffer (pH 7.4). Finally, the cells with maximum fluorescence intensity were photographed using a Nikon Eclipse 80i microscope (Nikon, Tokyo, Japan).

L-sorbose and D-sorbitol analysis

D-sorbitol and L-sorbose were evaluated by HPLC (Agilent 1100 series, Santa Clara, CA), with an Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad, Hercules, CA) at 35 °C with a flow rate of 0.6 mL/min and 5 mM H_2SO_4 as the eluent [48]. The results are reported as the averages of three biological replicates.

Results

Identification of the protein with the highest expression level in *G. oxydans*

As promoter strength is often highly correlated to gene expression level, the 2-DE technique was firstly used to obtain the protein with the highest expression in the whole proteome. The protein was extracted from the wild strain cells at three different growth phases, 8 h (early logarithmic phase), 20 h (late logarithmic phase), and 36 h (middle stationary phase), to comprehensively study the protein expression levels spanning the strain growth curve. As shown in Fig. 1, the whole protein expression levels were represented at three different time points by 2-DE. Among the gel spots, one distinct big spot was present at all three growth phases, which revealed the existence of a corresponding potential high-expression gene such as a housekeeping gene. Finally, Mascot search results demonstrated that the protein of interest was the hypothetical protein B932_2000 (GenBank ID: 414342978) in *G. oxydans* WSH-003.

Bioinformatics analysis of the P_{B932_2000} promoter region

Referring to the genome information of *G. oxydans* WSH-003, gene *B932_2000* codes for an open reading frame (ORF) of 396 amino acids with 1188 nucleotides. The regions immediately upstream from gene *B932_2000* ORF were analyzed, using the specialized promoter prediction tool BPROM. The sequence between *B932_2000* and the gene ahead of it was chosen to be analyzed in case there was a promoter far away from the initiation codon. The results showed that the core motif sequence at −35 box is CTGAAG and that of −10 box is

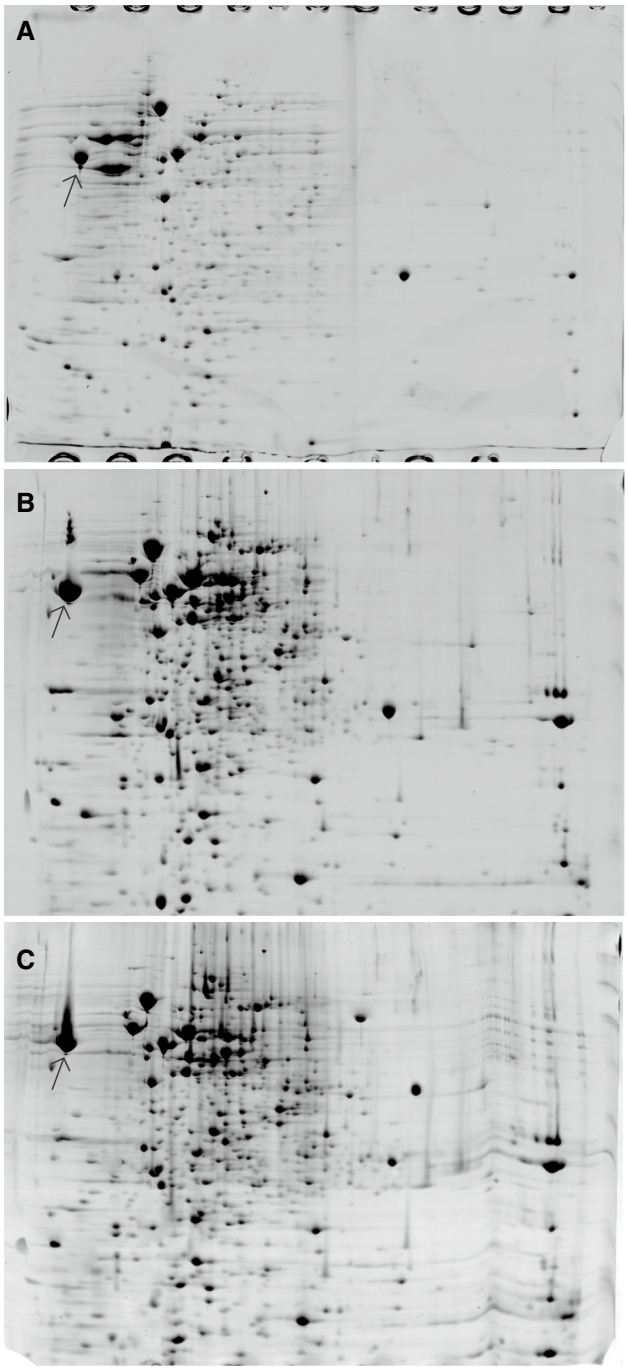

Fig. 1 Two-dimensional electrophoresis gels of the whole protein fraction of *G. oxydans* WSH-003. Proteins were extracted from cells at different growth phases. **a** Cells harvested at 8 h; **b** cells harvested at 20 h; **c** cells harvested at 36 h. *Arrows* point to the high-profile protein B932_2000

AGTCACTAT, which are at the positions of 147 nucleotides and 127 nucleotides upstream from the gene ORF, respectively. The core sequence positions and spacing length between these two were similar to those of most bacterial promoters.

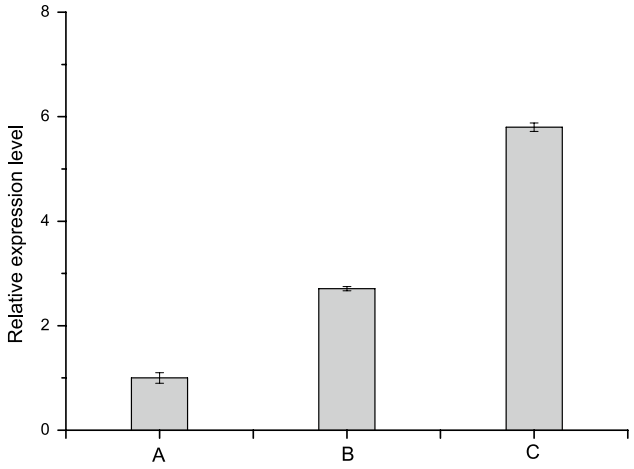

Fig. 2 Transcriptional levels of *egfp* with different promoters. **a** *G. oxydans* WSH-003 pBBR1-$P_{E.\ coli\_tufB}$-*egfp*; **b** *G. oxydans* WSH-003 pBBR1-$P_{G.\ oxydans\_tufB}$–*egfp*; **c** *G. oxydans* WSH-003 pBBR1-P_{B932_2000}–*egfp*. The 16S rRNA gene was used as the internal control gene to normalize the results

qRT-PCR and fluorescence analysis of the expression of *egfp* with P_{B932_2000}

To evaluate the relative promoter strength of P_{B932_2000}, the *egfp* gene was used as a reporter in *G. oxydans*, while the promoters $P_{E.\ coli\_tufB}$ and $P_{G.\ oxydans\_tufB}$ were used as controls. After plasmids construction, three recombinant strains were obtained, carrying pBBR1-$P_{E.\ coli\_tufB}$-*egfp*, pBBR1-$P_{G.\ oxydans\_tufB}$-*egfp*, and pBBR1-P_{B932_2000}-*egfp*, respectively. The promoter strengths were firstly determined by *egfp* transcription level using qRT-PCR. The 16S rRNA gene was used as the internal standard, and the *egfp* gene expression data obtained were normalized to the expression level in the control strain *G. oxydans* WSH-003 pBBR1-$P_{E.\ coli\_tufB}$-*egfp* (Fig. 2). From the results, *G. oxydans* WSH-003 pBBR1-P_{B932_2000}-*egfp* achieved the highest expression levels, which were about 5.7- and 2.4-fold higher than pBBR1-$P_{E.\ coli\_tufB}$-*egfp* and pBBR1-$P_{G.\ oxydans\_tufB}$-*egfp*, respectively. This result showed that mRNA transcription of the reporter *egfp* gene with promoter P_{B932_2000} was improved compared with the existing two strong promoters in *G. oxydans*.

In addition, the promoter activity was indirectly determined by measuring RFU/OD_{600}. As shown in Fig. 3, the RFU values of the three recombinant strains largely increased linearly along with the growth of *G. oxydans* until the stationary phase (24 h), and RFU/OD_{600} reached a maximum value at the late logarithmic phase. The strain with promoter P_{B932_2000} showed remarkably higher values of both maximum RFU and RFU/OD_{600} compared to the other two strains. Meanwhile, the whole cells of the three strains with the highest fluorescence intensities were photographed, using a Nikon Eclipse fluorescence microscope. Again,

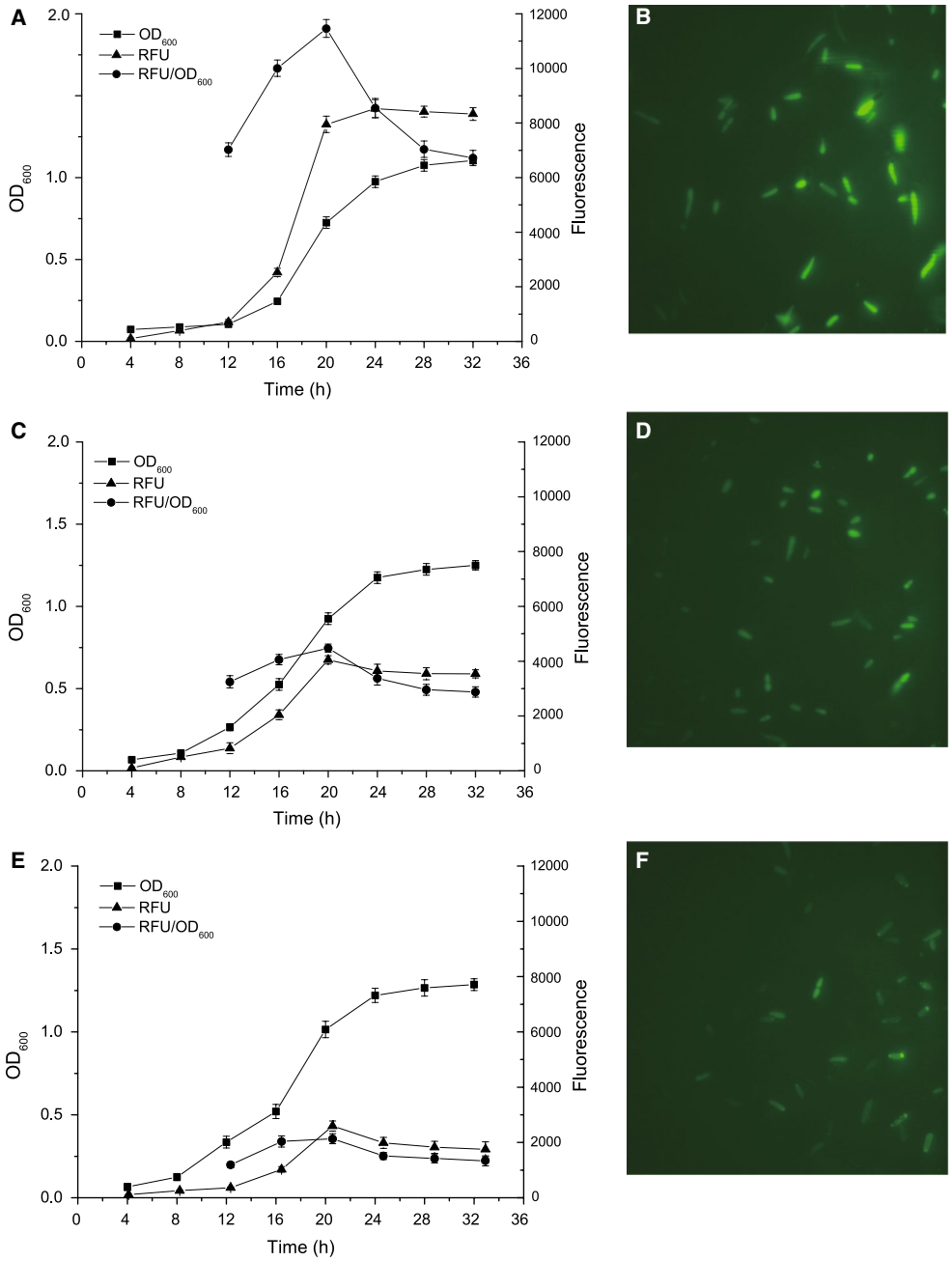

Fig. 3 Expression of green fluorescent protein in *G. oxydans* WSH-003 under the control of different promoters. The whole cell fluorescence intensities of strains with P_{B932_2000} (**a**), $P_{G.\ oxydans\_tufB}$ (**c**) and $P_{E.\ coli\_tufB}$ (**e**) were measured using a BioTek Synergy H4 Multi-Mode Reader. Images of cells with the highest RFU/OD_{600} are shown in **b** (strain with P_{B932_2000}), **d** (strain with $P_{G.\ oxydans\_tufB}$), and **f** (strain with $P_{E.\ coli\_tufB}$)

cells expressing EGFP protein under regulation of P_{B932_2000} exhibited noticeably stronger fluorescence (Fig. 3).

Enhanced expression of D-sorbitol dehydrogenase in *G. oxydans* by P_{B932_2000}

To apply the newly identified strong promoter P_{B932_2000} to enhance gene expression in *G. oxydans* WSH-003, engineered strains *G. oxydans* pBBR1-P_{B932_2000}-*sldh*, *G. oxydans* pBBR1-$P_{G.\ oxydans\_tufB}$-*sldh*, and *G. oxydans* pBBR1MCS-2 were constructed. Then, detailed studies of the time course of D-sorbitol to L-sorbose conversion by the *sldh* overexpression strains and the control strain were carried out in shake-flask experiments. Overall, the L-sorbose yield and fermentation time from *G. oxydans* pBBR1-P_{B932_2000}-*sldh* were both superior to the other two

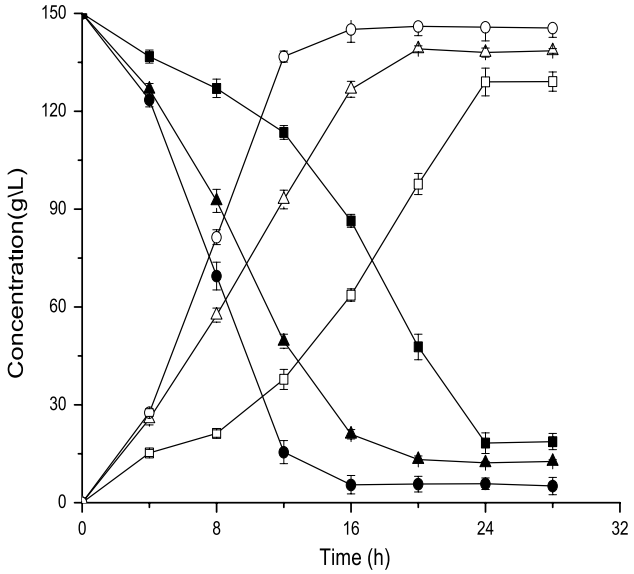

Fig. 4 Overexpression of *sldh* with different promoters in *G. oxydans* WSH-003. The time courses of the oxidative fermentations of D-sorbitol (*solid markers*) and L-sorbose (*open markers*) by control strain *G. oxydans* pBBR1MCS-2 (*squares*) and the over-producing strains *G. oxydans* pBBR1-$P_{G.\ oxydans\_tufB}$-*sldh* (*triangles*) and *G. oxydans* pBBR1-P_{B932_2000}-*sldh* (*circles*) were determined. *Error bars* represent standard deviation from the mean for the three batches

control strains, with a yield of 144.5 g/L and 16-h fermentation time (Fig. 4). In detail, the yields obtained by *sldh* overexpression regulated by P_{B932_2000} achieved 5.0 and 12.0 % increases compared with *G. oxydans* pBBR1-$P_{G.\ oxydans\_tufB}$-*sldh* and *G. oxydans* pBBR1MCS-2, respectively. More importantly, the fermentation was shortened to 16 h, in comparison to 20 and 24 h with the control stains, respectively.

Discussion

Gluconobacter oxydans has been successfully used for the industrial production of food-related products, pharmaceuticals, and cosmetics. Metabolic engineering of the bacterium is essential to further improve strain performance [11, 21, 26]. Here, a new promoter was identified in *G. oxydans* based on proteomics analysis, which can enable researchers to obtain available genetic tools for metabolic engineering. P_{B932_2000} was identified from 2-DE analysis due to the high protein expression driven by this promoter and further characterized by the −10 and −35 sequence motifs using bioinformatics tool. Strong promoter activity was demonstrated using the *egfp* reporter gene and was applied to enhance *sldh* gene expression in *G. oxydans* WSH-003.

There are several ways to find new promoters. Among them, a traditional way to identify promoters is a promoter trap system aimed at genome scale, which consists of a plasmid containing a multiple cloning site at the front of a promoter-less marker gene [6, 44]. However, this method often requires a lot of work and seems less efficient. Besides, high-throughput DNA sequencing technology has largely accelerated sequencing of many bacterial genomes and the prediction of promoters [40]. However, it is not yet possible to accurately predict promoters, largely because of the complex expression behavior of genes [28]. Recently, the prevalent high-throughput sequencing technology of transcriptomes (RNA-Seq) has been used in many studies including promoter analysis [33, 35]. RNA-Seq enables the identification of promoters and determination of strong promoters in a genome-wide manner [42]. This transcriptomics-based method, while comprehensive, is a little time-consuming and quite expensive to carry out for most researchers. Compared with the method above, a new strategy based on proteomics analysis is not only easy but also efficient for obtaining the desired strong promoters of high expression genes.

A core promoter is a DNA sequence with two conservative sequences known as −10 and −35 elements, which specify the binding site for an RNA polymerase holoenzyme and the transcriptional starting point [29]. According to the function and type of a gene, a promoter can be classified as a housekeeping or stress-inducible one [38]. In this experiment, the protein isolated by 2-DE was similar to a housekeeping protein, as the gene expression level was extremely high compared to other levels and, also, the protein was constitutively expressed during different strain growth phases. Usually, strong constitutive promoters are used for the construction of expression plasmid vectors as well as for replacing the native promoters of selected genes in engineered strains [8, 14, 21].

To gain comprehensive knowledge of the promoter, the core motif sequence of the promoter was analyzed by BPROM, which is a bioinformatics tool for bacterial promoter prediction that has been used in many promoter-related studies [16, 37, 41]. To analyze promoters, reporter genes are convenient tools for screening promoters and measuring promoter activities. The commonly used reporter genes include green fluorescent protein (*gfp*), chloramphenicol acetyl transferase (*cat*), and β-galactosidase (*lacZ*) [23, 36], among which *gfp* is the most widely used reporter system with many advantages such as lack of host dependency and direct visualization [5, 38]. In our study, a modified *gfp* named *egfp* was used, which is a derivative of the original that displays advantages such as enhanced stability and stronger fluorescence intensity [25]. Then with the help of the *egfp* reporter gene, the promoter assessment was determined at both transcription and translation levels and compared with the commonly used strong promoters $P_{E.\ coli\_tufB}$ and $P_{G.\ oxydans\_tufB}$ [21, 30, 31].

In summary, a pipeline for screening of strong promoters by proteomics analysis was established here. Based on the procedure, a new promoter was isolated. The −10 and −35 core motifs were further characterized using a bioinformatics tool. The promoter strength was then determined using the *egfp* gene with two control promoters, and the new entity eventually proved to be a strong promoter. Finally, the new promoter was applied to enhance *sldh* gene expression in *G. oxydans* WSH-003, and the yield was improved with shorter fermentation time. Based on the procedure and results presented here, it can be concluded that proteomics analysis is an efficient way to identify strong promoters. Moreover, a strong promoter can further facilitate the metabolic engineering of *G. oxydans*.

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