**Background**

Microbial cells could synthesize extracellular polymeric substances (EPS) to build biofilm communities with enhanced survival and metabolic capacities [1]. In natural settings, biofilms are commonly formed by cells attached to surfaces or interfaces, like the surfaces of water pipes, stones in a river, and indwelling devices in hospital patients. In laboratory, biofilms are usually attached to the inside wall of incubators, grown on agar plates or static liquid surfaces [2], and their formation could often be facilitated by solid carriers submerged in culture media, such as cotton fibre, plastic, stainless steel, glass or clay brick [3, 4]. Some nutritional factors were shown to be important for biofilm formation by some species. For some prokaryotes like *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *P. fluorescens*, iron limitation repressed biofilm formation while high iron rescued biofilm formation [5, 6]. In *Escherichia coli*, *Salmonella* sp. and anaerobic sludge communities, low-nutrient media (e.g.,
Clostridium acetobutylicum biofilm of solventogenic biomanufacturing [10]. One canonical example is the biofilm of solventogenic Clostridium acetobutylicum which is an important industrial platform capable of producing a range of biofuels and bulk chemicals [11]. It was shown that butanol tolerance of C. acetobutylicum cells in biofilm was three orders of magnitude higher than that of planktonic cells under certain conditions [12]. Operated in a continuous mode, Clostridium biofilms increased the productivity by almost 50-fold [4, 13]. Enhanced metabolism of pentose as well as hexose and enhanced solvent biosynthesis were also extensively demonstrated for C. acetobutylicum biofilm [14–16].

In general, superior phenotypes (such as the improved tolerance and metabolic activities) of EPS-encased biofilm cells could be attributed to two aspects: genetic regulation and EPS architecture. Living together in biofilms, cells tend to exhibit a different pattern of gene expression. Some genes are repressed or activated, thus cellular structure and functions are modulated [17, 18]. On the other hand, highly hydrated EPS matrix can be a protective barrier and provides cells with a favourable microenvironment. EPS plays an important role in exclusion of toxic substances [17, 19]. It keeps cells in close proximity and enables the development of high-density cell communities with intense cell–cell communication and cooperation [19, 20]. EPS matrix also provides excellent conditions for retention of extracellular proteins, functioning as an enzyme reservoir for external processes [1].

However, so far little has been known about C. acetobutylicum biofilm. Despite the observation of C. acetobutylicum biofilm under various conditions, the underlying molecular basis and regulatory processes remain to be explored [21]. Deciphering the EPS matrix and cell physiology in the biofilm will be important for optimization and control of biofilm-based processes. Recently we reported the first transcriptomic study of C. acetobutylicum biofilm [22] and revealed that heterogeneity of C. acetobutylicum EPS conferred improved resistance to harsh environments [23]. In this study, we will further shed light on C. acetobutylicum biofilm by investigating the cell physiology and EPS composition in the biofilm.

Methods

Culture and medium

Cultures of C. acetobutylicum B3 (CGMCC 5234) were grown in modified P2 medium containing 10 g/L glucose as the sole carbohydrate source for seed culture. Fermentation experiments were performed anaerobically in 2-L stainless steel columns containing 1.5 L of P2 medium (glucose 60 g/L; K2HPO4 0.5 g/L; KH2PO4 0.5 g/L; CH3COONH4 2.2 g/L; MgSO4·7H2O 0.2 g/L; MnSO4·H2O 0.01 g/L; NaCl 0.01 g/L; FeSO4·7H2O 0.01 g/L; p-aminobenzoic acid 1 mg/L; thiamine 1 mg/L; biotin 0.01 mg/L) at 37°C with initial inoculum 10%(v/v). Cotton towel was used to facilitate the formation of biofilm and continuous culture was performed with broth replacement every 12 h, see our previous work for details [12, 22].

Quantification of biofilm formation

Each piece of cotton towel (2 cm × 3 cm) with attached biofilm was taken from fermenters at predetermined time, immersed in 20 mL of 0.1 M NaOH (the mass of NaOH solute was calculated as W1) and vortexed to completely dissolve the biofilm. Then, the piece was removed and rinsed twice with a total of 40 mL of pure water. All the volumes were mixed together and the total volume was determined. Then, 3 mL of the mixture was dried and weighed to deduce the total dry weight (W2) of the mixture. Biofilm formation was quantified as (W2 − W1)/(2 cm × 3 cm).

Transcriptomic analysis

To collect biofilm cells for transcriptomic analysis, pieces of cotton towel were harvested from the fermenter typically at 6 h after each broth replacement and rinsed twice with PBS buffer (137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4 and 2 mM KH2PO4, pH 7.4, 4 °C) to remove contaminating planktonic cells. Then, the cotton towel was submerged in 15-mL PBS buffer and the biofilm was scraped off the cotton towel. The resulting suspension was centrifuged at 8000×g for 6 min at 4 °C to pellet the biofilm cells. All the cells were frozen immediately using liquid nitrogen and then stored at −80 °C prior to RNA extraction. RNA extraction and transcription analysis were performed as previously described [22]. Resulting microarray data were uploaded to the Gene Expression Omnibus (GEO) database under Accession Number GSE72765. Hierarchical clustering was performed using R-software and clusters were visualized with Tree-view [22].

Microscopy

Light microscopy was used for morphological observation. Each piece of cotton towel (approximately
2 cm × 3 cm) with attached biofilm was taken from fermenters at predetermined time points, immersed in 0.1 M NaOH at 4 °C for 10 min and vortexed to completely disperse cells within the biofilm. Then, 50 ul of suspension was transferred to a microscope slide and air-dried. Safranin O was used as a fluorescence dye that can be excited with green light [24]. It is well known for nuclear staining and was also reported to stain mucus, cartilage, starch and plant tissues [25]. In this study, it also differentially stained cells and endospores under light microscopy. Cells were stained with 0.5% safranin O for 30 s, washed gently with water and then air-dried for light microscopy. As the real production system with cotton towel as biofilm carrier was not suitable for detailed observation of the biofilm, fluorescence microscopy using microscope slides as biofilm carriers was used to observe the biofilm in detail. Microscope slides were immersed in 100-µL NaOH at 4 °C for 10 min and vortexed to completely disperse cells within the biofilm. Then, 50 ul of suspension was transferred to a microscope slide and air-dried. Safranin O as confirmed by microscopy. Also, control experiments solely with an equal volume of C. acetobutylicum cells going through the same procedures did not yield apparent sediments.

Isolation of polysaccharides
An aliquot of 0.5 g wet polysaccharide extract was dissolved in 35 mL of 0.1 M NaOH. The polysaccharide solution was filtered through a 0.45-µm membrane filter (Fisher Scientific). Polysaccharides were isolated with the Q-Sepharose fast flow (QFF) chromatography column (AKTA, GE Healthcare, USA), eluted with a step gradient of NaCl (dissolved in 0.1 M NaOH) in 0, 0.3, 0.4, 0.6, and 0.8 M steps, at a flow rate of 1.5 mL/min. Eluent was monitored at 280 nm and carbohydrate content of each fraction (200 µL) was determined according to the phenol–sulfuric acid method [27].

Monosaccharide composition analysis
The 1-phenyl-3-methyl-5-pyrazolone (PMP) derivatization method [28] was used to analyze monosaccharide composition. Each polysaccharide (2 mg) was hydrolyzed with 2 M trifluoroacetic acid (TFA) at 105 °C for 3 h. TFA was evaporated by adding methanol under reduced pressure. The hydrolysis product was derivatized with PMP in 0.3 M NaOH for 1 h at 70 °C, and then neutralized with HCl. The derivatives were analyzed using a Thermo C18 column (250 mm × 4.6 mm) coupled to an Agilent HPLC–DAD at 245 nm, at a flow rate of 0.8 mL/min of mobile phase: phosphate buffer (0.1 mol/L, pH 7.0)/CH3CN = 83/17 (v/v). Monosaccharide composition and the molar ratio analysis were carried out by comparing the retention times and peak areas with those of monosaccharide standards.

Mass spectrometric analysis of extracellular proteins
Proteins precipitated after the pH adjustment were sent to Shanghai Boyuan Biological Technology Co., LTD (Shanghai, China) for LC–MS/MS analysis. Proteins isolated by two-dimensional (2D) SDS-PAGE were analyzed using an ABI 4800 Plus MALDI TOF/TOF system (Life Technologies). Protein identification was performed using MASCOT 2.3 (http://www.matrixscience.com/, Matrix Science, UK) against the NCBI-Clostridium acetobutylicum database using a significance threshold of p < 0.05.

Results
Sporulation and morphological changes of biofilm cells
Biofilm formation by C. acetobutylicum in fermenters during continuous cultivation was quantified. Accumulation of biofilm was most apparent during day 3 and day 4. The biofilm could be built up with a maximum dry weight....
of 28 mg/cm² on the surface of cotton towel (Fig. 1). It was found that during continuous cultivation of *C. acetobutylicum* biofilm, the cells eventually eliminated sporulation and displayed a vegetative growth. As shown in Fig. 2, swollen clostridial-form cells first appeared at 18 h. These cells are to be the mother cells for spores. With the sporulation, fore spores were formed at 30 h. The final mature spores were released and peaked at 42 h, after which they disappeared rapidly. By 102 h, spores could hardly be observed, leaving almost exclusively vegetative cells. At the same time, the vegetative cells underwent significant morphological changes: from short single cells to long-chain cells. The long chains of cells were observed from 66 h, apparent at 102 h, and predominantly present in the biofilm after 150 h with a length around 100 µm.

**Decreased expression of sporulation genes in biofilm cells**

Inspired by the elimination of sporulation in the biofilm cells during long-term cultivation, expression of sporulation-related genes was investigated. In general, expression of the genes responsible for spore formation was apparently down-regulated in the biofilm over time (Fig. 3). Of the sporulation regulators in *C. acetobutylicum*, the gene encoding σK (*sigK, CA_C1689*) was downregulated over time by eightfold. The most down-regulated genes were those involved in spore coat synthesis (*CA_C0613-0614, CA_C1335, CA_C1337-1338, CA_C2808-2910, CA_C3317*), which decreased over time by 6- to 24-fold. An operon *CA_C2086-2093* related to stage III sporulation was down-regulated 6- to 12-fold. The small, acid-soluble proteins (SASP) that are used to coat DNA in spores (encoded by *CA_C1487*...
and CA_C1522) were also significantly down-regulated by 48-fold ($p<0.01$; Student t test), and the CA_C2365 was down-regulated by 200-fold. Overall, the decreased expression of sporulation-related genes over time was consistent with the elimination of sporulation in the biofilm.

**EPS and wire-like structures in C. acetobutylicum biofilm**

EPS and wire-like structures of *C. acetobutylicum* biofilm were observed. As shown in Fig. 4, at the early stage of biofilm development (4 h), cells were all buried in a gel-like extracellular matrix (as indicated by the blurred area in the image) which was typically excreted by the cells to help adhere onto surfaces. Then it developed into a three-dimensional, high-density cell colony (16 h). At the edges of the colonies, wire-like structures could be clearly observed. The wires were surprisingly long (could be more than 50 µm) and could be cross-connected. With the development, the wires were eventually imbedded in cells aggregates (28 h). At the late stage (40 h), EPS pellicles were also shown. In recent years, a kind of “nanowire” structure has been reported for some bacterial biofilms and was supposed to function in extracellular electron transport [29]. Whether the wire structures observed here are similar to the nanowires and what their roles are in *C. acetobutylicum* biofilm remain to be investigated.
After polysaccharides and proteins in C. acetobutylicum biofilm were extracted, the polysaccharides were further isolated by anion exchange chromatography (Fig. 5). Three polysaccharides were obtained which were designated SM1, SM2, and SM3 according to their elution order. A peak at 280 nm occurred concurrently with each of the polysaccharide peaks, indicating possible presence of polysaccharide-associated proteins. The SM1 comprised the largest fraction (53%, w/w) of the polysaccharides, followed by SM2 (26%, w/w) and SM3 (21%, w/w). SM1 was also the most purified polysaccharide as indicated by a much smaller peak at 280 nm (Fig. 5).

Analysis of monosaccharide composition showed that all the three polysaccharides were heteropolysaccharides with glucose as the primary component (Table 1). SM2 and SM3 were very similar in both of monosaccharide type and molar ratio, consisting of glucose (47–53%, molar ratio, the same hereinafter), mannose (13–15%), rhamnose (10–16%), galactose (9–10%), aminoglucose (7–9%), and a little ribose (4–5%). Compared to SM2 and SM3, the SM1 polysaccharide consisted of more glucose (58%), mannose (21%), and aminoglucose (13%), but much less rhamnose (0.8%), galactose (0.8%) and ribose (0.4%). SM1 also consisted of unique galacturonic acid (5.5%). The presence of uronic acid might explain why the C. acetobutylicum biofilm matrix was alkali soluble.

**Characterization of C. acetobutylicum biofilm polysaccharides**

Proteins extracted from the C. acetobutylicum biofilm were identified by LC-MS/MS. The proteins were next ranked according to their emPAI (exponentially modified protein abundance index) which reflects their relative abundance [30]. Table 2 lists the Top 30 abundant proteins of C. acetobutylicum biofilm. Strikingly, most of the proteins are commonly known as physiological process related proteins, especially the molecular chaperones and stress proteins. The three most abundant proteins were GroEL, surface layer (S-layer) protein and rubrerythrin, which typically functions as molecular chaperone, structure protein and oxidative stress protein, respectively. Surprisingly, many of the proteins are typically known as intracellular proteins such as the enzymes normally functioning in central metabolism, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), triose phosphate isomerase, pyruvate: ferredoxin oxidoreductase, electron transfer flavoprotein and alcohol dehydrogenase. Meanwhile, the biofilm proteins were isolated by 2D gel electrophoresis and major protein spots were identified by MALDI TOF/TOF mass spectrometry. The major proteins identified on 2D gel were well included in the Top 30 abundant proteins identified by LC-MS/MS, and
the spots of the three most abundant proteins, GroEL, S-layer protein and rubrerythrin were indeed the most distinct protein spots on 2D gel (Fig. 6).

In fact, most of the \textit{C. acetobutylicum} biofilm proteins identified here have been recognized as a kind of non-classically secreted proteins that do not contain known sequence motifs for secretion or anchoring onto the cell surface [31, 32]. A great number of these proteins have been found to be “moonlight proteins” that have a canonical biochemical function inside the cell and perform a second biochemical function on the cell surface or extracellularly [33, 34]. Table 3 lists the proteins (from the Top 30 abundant proteins list) that have been reported as non-classically secreted proteins or moonlighting proteins. Obviously, many proteins with canonical function in central metabolism, chaperone activity, or

---

**Table 1** The molar ratio of each monosaccharide in \textit{C. acetobutylicum} biofilm polysaccharides

|    | Glc | Man | GlcN | GalA | Rha | Gal | Rib |
|----|-----|-----|------|------|-----|-----|-----|
| SM1 | 100 | 36  | 23   | 9.4  | 1.5 | 14  | 0.7 |
| SM2 | 100 | 25  | 17   | –    | 18  | 20  | 9.0 |
| SM3 | 100 | 32  | 16   | –    | 33  | 20  | 9.6 |

SM1, SM2 and SM3 are three isolated polysaccharides

\textit{Glc} glucose, \textit{Man} mannose, \textit{GlcN} aminoglucose, \textit{GalA} galacturonic acid, \textit{Rha} rhamnose, \textit{Gal} galactose; \textit{Rib} ribose
protein synthesis and nucleic acid stability could moonlight as bacterial adhesins and interact with the environment. While these proteins were abundant in the biofilm, no apparent regulation of their gene transcription was observed (Additional file 2: Sheet 3).

**Discussion**

*C. acetobutylicum* has attracted considerable interest due to its unique capability of biosynthesizing a range of liquid fuels and bulk chemicals that are fundamentally important to human society. It has been long accepted that sporulating clostridial form of *C. acetobutylicum* cells is the solvent-forming phenotype, that is, solventogenesis is coupled to sporulation. However, Tracy and his co-workers observed a stronger correlation between solvent production and the vegetative cell type than the clostridial-form type based on flow cytometry assisted cell-sorting techniques. They also demonstrated that a *sigF* mutant blocked sporulation but still produced comparable solvent in batch fermentation [71, 72]. Despite this, the view that solventogenesis is coupled to sporulation is still prevailing in the field [73, 74]. Here, our results clearly showed that *C. acetobutylicum* could eliminate sporulation and display vegetative growth in biofilm over time. In this way, instead of being impaired, the solvent production was greatly improved [12, 22].

### Table 2 Top 30 extracellular proteins in *C. acetobutylicum* biofilm identified by LC–MS/MS

| Gene locus | Score | Mass | Matches | Sequences | emPAI | Description                                                                 |
|------------|-------|------|---------|-----------|-------|----------------------------------------------------------------------------|
| 1 CA_C2703 | 10,961 | 58,166 | 408     | 29        | 12.3  | Molecular chaperone GroEL (Hsp60)                                          |
| 2 CEA_G3563 | 3556  | 47,277 | 139     | 17        | 10.3  | Putative S-layer protein                                                   |
| 3 CA_C3597 | 8141  | 20,493 | 187     | 10        | 10.3  | Rubrerythrin                                                               |
| 4 CA_C2710 | 2299  | 28,089 | 71      | 14        | 9.5   | Electron transfer flavoprotein beta-subunit                               |
| 5 CA_C0709 | 1619  | 35,999 | 72      | 15        | 8.1   | Glyceraldehyde-3-phosphate dehydrogenase                                  |
| 6 CA_C2452 | 572   | 15,611 | 24      | 7         | 7.7   | Flavodoxin                                                                 |
| 7 CA_C1555 | 2446  | 29,503 | 80      | 10        | 7.5   | Flagellin                                                                  |
| 8 CA_C1747 | 556   | 8602   | 21      | 3         | 6.9   | Acyl carrier protein, ACP                                                  |
| 9 CA_C3136 | 5404  | 43,482 | 207     | 18        | 6.8   | Elongation Factor Tu (Ef-Tu)                                              |
| 10 CA_C2990 | 396   | 7307   | 23      | 2         | 6.2   | Cold shock protein                                                         |
| 11 CA_C1834 | 448   | 9203   | 19      | 4         | 5.9   | Host factor I protein, Hfq                                                 |
| 12 CA_C3125 | 299   | 7908   | 7       | 3         | 5.4   | Ribosomal protein L29                                                      |
| 13 CA_C2712 | 571   | 28,400 | 28      | 10        | 4.9   | Crotonase                                                                  |
| 14 CA_C1807 | 182   | 10,251 | 7       | 5         | 4.8   | Ribosomal Protein S15                                                      |
| 15 CA_C3211 | 111   | 10,341 | 6       | 4         | 4.7   | DNA binding protein HU                                                     |
| 16 CA_P0164 | 1098  | 23,666 | 33      | 8         | 4.6   | Acetoacetyl-CoA:acetate/butyrate CoA-transferase subunit B                 |
| 17 CA_C2704 | 745   | 10,420 | 27      | 5         | 4.6   | Molecular chaperone groES (Hsp10, Hsp60 cofactor)                          |
| 18 CA_C3145 | 553   | 12,670 | 24      | 5         | 4.4   | Ribosomal protein L7/L12                                                   |
| 19 CA_C3076 | 627   | 32,321 | 30      | 12        | 4.3   | Phosphate butyryltransferase                                               |
| 20 CA_C1281 | 757   | 17,734 | 29      | 7         | 3.8   | Heat shock protein grpE (hsp20, Hsp70 cofactor)                            |
| 21 CA_C1282 | 2280  | 65,723 | 77      | 24        | 3.8   | Molecular chaperone DnaK (Hsp70)                                           |
| 22 CA_C2229 | 4951  | 129,740| 191     | 43        | 3.7   | Pyruvate ferredoxin oxidoreductase                                         |
| 23 CA_C2641 | 782   | 49,565 | 37      | 19        | 3.7   | FKBP-type peptidyl-prolyl cis-transisomerase (trigger factor)              |
| 24 CA_C3075 | 744   | 39,146 | 33      | 15        | 3.7   | Butyrate kinase, BUK                                                      |
| 25 CA_C2873 | 1247  | 41,443 | 53      | 16        | 3.7   | Acetyl coenzyme A acetyltransferase (thiolase)                             |
| 26 CA_P0165 | 725   | 23,797 | 22      | 8         | 3.3   | Acetoacetate decarboxylase                                                 |
| 27 CA_P0162 | 3768  | 95,774 | 175     | 32        | 3.2   | Alcohol dehydrogenase E                                                   |
| 28 CA_C0711 | 452   | 26,698 | 17      | 11        | 3.1   | Triosephosphate isomerase                                                  |
| 29 CA_C2597 | 461   | 17,599 | 19      | 5         | 3.1   | Hypothetical protein                                                       |
| 30 CA_C3558 | 1128  | 48,599 | 51      | 10        | 3.0   | Probable S-layer protein                                                   |

*a* All scores were statistically significant (*p* < 0.05; Student t test). Higher score means higher probability

*b* Theoretical molecular mass

*c* The number of peptides that matched the identified protein with *p* < 0.05

*d* The number of distinct (nonredundant) peptides that matched the identified protein with *p* < 0.05 (Student t test)

*e* Exponentially modified Protein Abundance Index
Therefore, it is plausible that sporulation and solventogenesis can be uncoupled in \textit{C. acetobutylicum}. This is of particular importance, because it would encourage researchers to develop long-term continuous cultivation processes. Besides elimination of sporulation, \textit{C. acetobutylicum} biofilm cells also exhibited significant morphological changes. The prolonged chain-like morphology observed for \textit{C. acetobutylicum} biofilm cells was also observed for \textit{Bacillus subtilis} biofilm cells. In \textit{B. subtilis}, a transcriptional regulator SinR represses the genes responsible for EPS production and promotes cell separation and motility. During biofilm development, SinR activity is antagonized. Low SinR activity results in EPS production and loss of cell motility. Thus, motile single cells switch to long chains of nonmotile cells [75]. Considering the presence of SinR in \textit{C. acetobutylicum}, this is probably also the case in \textit{C. acetobutylicum}.

Despite the fact that \textit{C. acetobutylicum} biofilm has been extensively exploited for producing industrial products [4, 12, 16, 21], the biosynthetic process and molecular composition of \textit{C. acetobutylicum} biofilm remain completely unknown. Here, for the first time isolated polysaccharides and proteins from \textit{C. acetobutylicum} biofilm were reported. \textit{C. acetobutylicum} biofilm contained three polysaccharides which were all heteropolysaccharides. Recently, a polysaccharide separated from \textit{C. acetobutylicum} culture supernatant was reported [76]. Consistent with our results, the supernatant polysaccharide was also a heteropolysaccharide and its monosaccharide composition seemed similar to those of the SM2 and SM3. While the supernatant polysaccharide was characterized with glucose (34\%, molar ratio), rhamnose (40\%), mannose (13\%) and galactose (10\%) as its primary monosaccharides, the SM2 and SM3 characterized here also consisted of glucose (47–53\%), rhamnose (10–16\%), mannose (13–21\%) and galactose (9–10\%) as their primary monosaccharides (Table 1), although the monosaccharide ratio differed. However, the polysaccharide SM1 that represented the major polysaccharide in \textit{C. acetobutylicum} biofilm had a more distinct composition: it contained predominantly glucose (58\%), mannose (21\%), and aminoglucose (13\%). Altogether, it seemed that C.
acetobutylicum liked to produce a variety of heteropolysaccharides varying in monosaccharide composition. In addition, the biofilm polysaccharides, especially the SM1, proved hard to re-dissolve after lyophilization. Also, they were possibly associated with some non-carbohydrate substances. Despite our try of various protein removal methods, they still defied $^1\mathrm{H}$-NMR analysis.

Strikingly, a great variety of proteins were found abundantly present in C. acetobutylicum biofilm. One of the most abundant proteins was a protein annotated as putative S-layer protein (Table 2; Fig. 6). The gene encoding this protein is designated CEA_G3563 in C. acetobutylicum EA2018 and SMB_G3598 in C. acetobutylicum DSM 1731. In both strains, it is located in an operon together with and downstream of an S-layer protein encoding gene [77]. It shows 81% sequence identity to a S-layer protein from C. felsineum DSM 794 (Sequence ID: WP_077894211), but both have been poorly studied. S-layers are crystalline monomolecular assemblies of protein or glycoprotein, which represent one of the most common cell envelope structures in bacteria [78]. In Clostridium difficile, S-layer proteins were demonstrated essential for biofilm formation perhaps due to the fact that S-layer is essential for anchoring cell wall associated proteins that are required for adhesion during biofilm formation [79]. Studies also showed that S-layer was required for normal growth in C. difficile [80, 81], while a non-matured S-layer protein induced the apparition of a bigger biofilm [82]. Except the putative S-layer protein, most of the C. acetobutylicum proteins were typically intracellular proteins (Tables 2, 3). It has previously been reported that a variety of Gram-positive bacteria, such as S. aureus and B. subtilis, release intracellular proteins into the external environment during stationary phase [83, 84]. These proteins are considered to be secreted in a non-classical pathway and some of them (e.g., the GroEL) have been extensively found to moonlight as adhesins and contribute to the biofilm formation [32, 33, 84]. For instance, it was shown that deletion of GroEL-phosphorylating PrkC in Bacillus anthracis abrogated biofilm formation, while overexpression of GroEL led to increased biofilm formation [85, 86]. Another intracellular protein abundant in the biofilm is a rubrerythrin encoded by rbr3B (CA_C3597). In this rubrerythrin, the order of the two functional domains is reversed compared to normal rubrerythrins [87]. Although this rubrerythrin has been demonstrated to be involved in H$_2$O$_2$ and O$_2$ detoxification, its role in biofilm remains to be studied.

### Table 3 Major C. acetobutylicum biofilm proteins that have been reported as non-classically secreted proteins with potential moonlighting functions

| Intracellular function | Moonlighting function |
|------------------------|-----------------------|
| **Chaperones**         |                       |
| Molecular chaperone groel | Adhesin [33, 35–37]; bind mucin, invertase and fibronectin [38, 39] |
| Molecular chaperone dnak | Bind plasminogen and invertase [40–42] |
| Heat shock protein grppe | Not characterized [32, 39, 43] |
| Molecular chaperone groes | Not characterized [32, 43] |
| Cold shock protein | Not characterized [44, 45] |
| **Protein synthesis and nucleic acid stability** |                       |
| Elongation factor Tu (EF-Tu) | Attatch to human cells, bind fibronectin and plasminogen [46–49] |
| Trigger factor | Not characterized [43, 44, 50, 51] |
| Ribosomal protein L29 | Not characterized [43, 45, 51] |
| Ribosomal protein S15 | Not characterized [52, 53] |
| Ribosomal protein L7/L12 | Not characterized [52, 54] |
| **Central metabolism** |                       |
| Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) | Adhesin [55–57]; bind plasminogen, collagen, fibronectin and RNA [34, 58, 59] |
| Triosephosphate isomerase | Adhesin [33, 60]; bind plasminogen, laminin and fibronectin [61, 62] |
| Alcohol dehydrogenase | Bind plasminogen [63, 64] |
| Pyruvate: ferredoxin oxidoreductase | Adhesin [65, 66] |
| Electron transfer flavoprotein beta-subunit | Not characterized [44, 50, 54] |
| Acetyl coenzyme A acetyltransferase (thiolase) | Not characterized [44, 67] |
| Rubrerythrin | Not characterized [52, 68] |
| Acyl carrier protein, ACP | Not characterized [69, 70] |
cells inside is likely lysed due to various mechanisms [88]. The biofilm matrix could act as a recycling center by keeping the components of lysed cells available [19, 88]. A biofilm-forming mechanism was recently proposed using *Staphylococcus aureus* or *Pseudomonas aeruginosa* biofilm as a model [83, 89]. In these models, cytoplasmic proteins that were released from cells or cell lysate proteins could associate with the cell surface in response to decreasing pH during biofilm formation. Considering the presence of abundant cytoplasmic proteins in the *C. acetobutylicum* biofilm as well as a low pH level (usually around pH 4.2) during *C. acetobutylicum* fermentation, this mechanism also be plausible for *C. acetobutylicum* biofilm.

**Conclusions**

*Clostridium acetobutylicum* biofilm cells eliminated sporulation and performed vegetative growth over time, indicating that vegetative *C. acetobutylicum* cells rather than the spore-forming cells were the solvent-forming cells. EPS and wire-like structures were observed. The biofilm contained three heteropolysaccharides. The major fraction consisted of predominantly glucose, mannose and aminoglucose. A variety of proteins including non-classically secreted proteins were present in the biofilm, with GroEL, a S-layer protein and ruberythrin being the most abundant. Of these proteins, many proteins such as GroEL, EF-Tu and glyceraldehyde-3-phosphate dehydrogenase could moonlight as adhesins which might contribute to the biofilm formation. This study provides important insights into *C. acetobutylicum* biofilm. Future studies should genetically manipulate the main components to elucidate their specific roles in *C. acetobutylicum* biofilm.

**Additional files**

Additional file 1. Evaluation of different extraction methods and 1H-NMR spectra of polysaccharides.

Additional file 2. Full list of the biofilm proteins and relevant gene expression data.

**Abbreviations**

2D: two-dimensional; EPS: extracellular polymeric substances; LC–MS/MS: liquid chromatography coupled with tandem mass spectrometry; MALDI-TOF/TOF: matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometer; PMP: 1-phenyl-3-methyl-5-pyrazolone; QFF: q-sepharose fast flow chromatography column; SDS-PAGE: sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

**Authors’ contributions**

DL and HY designed experiments. DL and ZY performed experiments. WZ, YC, HN, and JW contributed materials and sample analysis. DL and ZY analyzed data. DL and HY wrote the manuscript. All authors read and approved the final manuscript.

**Author details**

1. State Key Laboratory of Materials-Oriented Chemical Engineering, College of Biotechnology and Pharmaceutical Engineering, Nanjing Tech University, No. 30, Puzhu South Road, Nanjing 211800, China. 2. Jiangsu National Synergetic Innovation Center for Advance Material (SICAM), No. 30, Puzhu South Road, Nanjing 211800, China.

**Acknowledgements**

We thank Dr. Xia Zhao from Qingdao Haiyang University (Shandong, China) for the help with analysis of polysaccharides.

**Competing interests**

The authors declare that they have no competing interests.

**Availability of data and materials**

The raw transcriptomic data were uploaded to the Gene Expression Omnibus (GEO) database under Accession Number GSE72765. All other datasets supporting the conclusions of this article are included within the article.

**Consent for publication**

Not applicable.

**Ethics approval and consent to participate**

Not applicable.

**Funding**

This work was supported by the Jiangsu Provincial Natural Science Foundation of China (Grant No.: BK20150938), the National Nature Science Foundation of China (Grant No.: 21706123); the Major Research Plan of the National Natural Science Foundation of China (21390204); the key program of the National Natural Science Foundation of China (21636003); the Program for Changjiang Scholars and Innovative Research Team in University (IRT_14R28); the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD), and the Jiangsu Synergetic Innovation Center for Advanced Bio-Manufacture.

**Publisher’s Note**

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

**Received:** 21 July 2018  **Accepted:** 13 November 2018

**Published online:** 20 November 2018

**References**

1. Flemming H-C, Wingender J, Szewzyk U, Steinberg P, Kjelleberg S. Biofilms: an emergent form of bacterial life. Nat Rev Microbiol. 2016;14:563.

2. Monikawa M. Beneficial biofilm formation by industrial bacteria Bacillus subtilis and related species. J Biosci Bioeng. 2006;101:1–8.

3. Dolejš I, Rebroš M, Rosenberg M. Immobilisation of *Clostridium* sp. for production of solvents and organic acids. Chem Pap. 2014;68:1–14.

4. Qureshi N, Schripsema J, Lienhardt J, Blaschek H. Continuous solvent production by *Clostridium beijerinckii* BA101 immobilized by adsorption onto brick. World J Microbiol Biotechnol. 2000;16:377–82.

5. Pratt LA, Kolter R. Genetic analyses of bacterial biofilm formation. Curr Opin Microbiol. 1999;2:598–603.

6. Weinberg E. Suppression of bacterial biofilm formation by iron limitation. Med Hypotheses. 2004;63:863–5.

7. Dewanti R, Wong AC. Influence of culture conditions on biofilm formation by *Escherichia coli* O157: H7. Int J Food Microbiol. 1995;26:147–64.

8. Speranza B, Corbo MR, Sinigaglia M. Effects of nutritional and environmental conditions on *Salmonella* sp. biofilm formation. J Food Sci. 2011;76:M12–6.

9. Miqueleto A, Dolosic C, Pozzi E, Foresti E, Zaia M. Influence of carbon sources and C/N ratio on EPS production in anaerobic sequencing...
batch biofilm reactors for wastewater treatment. Bioreour Technol. 2010;101:1324–30.

10. Berlanga M, Guerrero R. Living together in biofilms: the microbial cell factory and its biotechnological implications. Microb Cell Fact. 2016;15:165.

11. Liu D, Yang Z, Wang P, Niu H, Zhuang W, Chen Y, Wu J, Zhu C, Ying H, Ouyang P. Towards acetone-uncoupled biofuels production in solven-
togenic Clostridium through reducing power conservation. Metab Eng. 2018;47:102–12.

12. Liu D, Chen Y, Li A, Ding F, Zhou T, He Y, Li B, Niu H, Lin X, Xie J. Enhanced butanol production by modulation of electron flow in Clostridium acetobutylicum B3 immobilized by surface adsorption. Bioreour Technol. 2013;129:321–8.

13. Survae SA, van Heiningen A, Granstrom T. Continuous bio-catalytic conversion of sugar mixture to acetone–butanol–ethanol by immobili-
cized Clostridium acetobutylicum DSM 792. Appl Microbiol Biotechnol. 2012;93:2309–16.

14. Chen Y, Zhou T, Liu D, Li A, Xu S, Liu Q, Li B, Ying H. Production of butanol from glucose and xylose with immobilized cells of Clostridium acetobutylicum. Biotechnol Bioprocess Eng. 2013;18:234–41.

15. Liu D, Chen Y, Ding F-Y, Zhao T, Wu J-L, Guo T, Ren H-F, Li B-B, Niu H-Q, Cao Z. Biobutanol production in a Clostridium acetobutylicum biofilm reactor integrated with simultaneous product recovery by adsorption. Biotechnol. Lett. 2014;36:75.

16. Huang W-C, Ramey DE, Yang S-T. Continuous production of butanol from MR-1 nanowires are outer membrane and periplasmic extensions of Shewanella oneidensis. 2014;111:12883–8.

17. Maht-F, O’toole GA. Mechanisms of biofilm resistance to antimicro-
bial agents. Trends Microbiol. 2001;9:34–9.

18. Harrison JJ, Ceri H, Turner RJ. Multimetal resistance and tolerance in biofilm induced tolerance to acetic acid and butanol. RSC Adv. 2016;15:165–169.

19. Wampler JL, Kim K-P, Jaradat Z, Bhunia AK. Heat shock protein 60 acts as a receptor for the Lipidase adherence protein in Caco-2 cells. Infect Immun. 2004;72:931–6.

20. Aral T, Ochiai K, Senpuku H. Actinomyces naeslundii GroEL-dependent initial attachment and biofilm formation in a flow cell system. J Microbiol Methods. 2015;109:160–6.

21. Bergonzelli GE, Granato D, Pridmore RD, Marvin-Guy LF, Donnica D, Cortés-Thualiez IE. GroEL of Lactobacillus johnsonii La1 (NCC 533) is cell surface associated: potential role in interactions with the host and the gut microbiota. J Biotechnol. 2014;175.

22. Filmann H-C, Wingender J. The biofilm matrix. Nat Rev Microbiol. 2007;5:928.

23. Fleeming S, Jendrossek D. Physical features of intracellular proteins that moonlight in extracellular proteomes. Proteomics. 2017;7:3332–41.

24. Beck HC, Madsen SM, Grinting J, Petersen J, Israelsen H, Nørreløkke MR, Antonsson M, Hansen AM. Proteomic analysis of cell surface-associated proteins from probiotic Lactobacillus plantarum. FEMS Microbiol Lett. 2009;297:61–6.

25. Gohar M, Gilois N, Graveline R, Garreau C, Sanchis V, Lereclus D. A comparative study of Bacillus cereus, Bacillus thuringiensis and Bacillus anthracis extracellular proteomes. Proteomics. 2010;10:5966–111.

26. Xoo XX, Han MJ, Lee SY, Yoo JS. Comparative study of the extracellular proteomes of Escherichia coli B and K-12 strains during high cell density cultivation. Proteomics. 2008;8:10089–103.

27. Zhang Y, Xiang X, Lu Q, Zhang L, Ma F, Wang L. Adhesions of extracellular biofilm–layer–associated proteins in Lactobacillus MS-L and Q8-L. J Dairy Sci. 2016;99:1011–8.

28. Granato D, Bergonzelli GE, Pridmore RD, Marvin L, Rouvet M, Cortés-Thualiez IE. Cell surface-associated elongation factor Tu mediates the attachment of Lactobacillus johnsonii NCC353 (La1) to human intestinal cells and mucins. Infect Immun. 2004;72:2160–9.

29. Widjaja M, Harvey KL, Hagemann L, Gründel A, Steele JR, Padula MP. Elongation factor Tu is a surface-exposed human plasminogen receptor upregulated in response to bile salts. Microbiology. 2010;156:1609–18.

30. Gohar M, Hidalgo N, Graveline R, Garreau C, Sanchis V, Lereclus D. Comparative study of Bacillus cereus, Bacillus thuringiensis and Bacillus anthracis extracellular proteomes. Proteomics. 2010;10:5966–111.

31. Xoo XX, Han MJ, Lee SY, Yoo JS. Comparative study of the extracellular proteomes of Escherichia coli B and K-12 strains during high cell density cultivation. Proteomics. 2008;8:10089–103.

32. Zhang Y, Xiang X, Lu Q, Zhang L, Ma F, Wang L. Adhesions of extracellular biofilm–layer–associated proteins in Lactobacillus MS-L and Q8-L. J Dairy Sci. 2016;99:1011–8.

33. Granato D, Bergonzelli GE, Pridmore RD, Marvin L, Rouvet M, Cortés-Thualiez IE. Cell surface-associated elongation factor Tu mediates the attachment of Lactobacillus johnsonii NCC353 (La1) to human intestinal cells and mucins. Infect Immun. 2004;72:2160–9.

34. Widjaja M, Harvey KL, Hagemann L, Berry IJ, Jarocki VM, Raymond BBA, Tacchi JL, Gründel A, Steele JR, Padula MP. Elongation factor Tu is a multifunctional and processed moonlighting enzyme. Scientific reports. 2017;7:11227.

35. Kunert A, Losse J, Greusen C, Hühn M, Kaendler K, Mikkat S, Volke D, Hammerschmidt S, Brigidi P. DnaJ of Bidobacterium animalis subsp. lacticis is surface displayed on the cell surface cytosolic proteins that recognize yeast mannoproteins. Appl Microbiol Biotechnol. 2010;86:319–26.

36. Hagemann L, Grundel A, Jacobs E, Dumke R. The surface-displayed chaperones GroEL and DnaK of Mycoplasma pneumoniae interact with human plasminogen and components of the extracellular matrix. Pathog Dis. 2017;75:1. https://doi.org/10.1093/femsdp/fvx017.

37. Xolalpa W, Vallecillo AL, Lara M, Mendoza-Hernandez G, Comini M, Spallek R, Singh M, Espitia C. Identification of novel bacterial plasminogen-binding proteins in the human pathogen Mycobacterium tuberculosis. Proteomics. 2007;7:3332–41.

38. Beck HC, Madsen SM, Grinting J, Petersen J, Israelsen H, Nørreløkke MR, Antonsson M, Hansen AM. Proteomic analysis of cell surface-associated proteins from probiotic Lactobacillus plantarum. FEMS Microbiol Lett. 2009;297:61–6.

39. Gohar M, Gilois N, Graveline R, Garreau C, Sanchis V, Lereclus D. Comparative study of Bacillus cereus, Bacillus thuringiensis and Bacillus anthracis extracellular proteomes. Proteomics. 2010;10:5966–111.

40. Xoo XX, Han MJ, Lee SY, Yoo JS. Comparative study of the extracellular proteomes of Escherichia coli B and K-12 strains during high cell density cultivation. Proteomics. 2008;8:10089–103.

41. Zhang Y, Xiang X, Lu Q, Zhang L, Ma F, Wang L. Adhesions of extracellular biofilm–layer–associated proteins in Lactobacillus MS-L and Q8-L. J Dairy Sci. 2016;99:1011–8.

42. Granato D, Bergonzelli GE, Pridmore RD, Marvin L, Rouvet M, Cortés-Thualiez IE. Cell surface-associated elongation factor Tu mediates the attachment of Lactobacillus johnsonii NCC353 (La1) to human intestinal cells and mucins. Infect Immun. 2004;72:2160–9.

43. Widjaja M, Harvey KL, Hagemann L, Berry IJ, Jarocki VM, Raymond BBA, Tacchi JL, Gründel A, Steele JR, Padula MP. Elongation factor Tu is a multifunctional and processed moonlighting enzyme. Scientific reports. 2017;7:11227.

44. Kunert A, Losse J, Greusen C, Hühn M, Kaendler K, Mikkat S, Volke D, Hammerschmidt S, Brigidi P. DnaJ of Bidobacterium animalis subsp. lacticis is surface displayed on the cell surface cytosolic proteins that recognize yeast mannoproteins. Appl Microbiol Biotechnol. 2010;86:319–26.

45. Hagemann L, Grundel A, Jacobs E, Dumke R. The surface-displayed chaperones GroEL and DnaK of Mycoplasma pneumoniae interact with human plasminogen and components of the extracellular matrix. Pathog Dis. 2017;75:1. https://doi.org/10.1093/femsdp/fvx017.

46. Xolalpa W, Vallecillo AL, Lara M, Mendoza-Hernandez G, Comini M, Spallek R, Singh M, Espitia C. Identification of novel bacterial plasminogen-binding proteins in the human pathogen Mycobacterium tuberculosis. Proteomics. 2007;7:3332–41.

47. Beck HC, Madsen SM, Grinting J, Petersen J, Israelsen H, Nørreløkke MR, Antonsson M, Hansen AM. Proteomic analysis of cell surface-associated proteins from probiotic Lactobacillus plantarum. FEMS Microbiol Lett. 2009;297:61–6.
proteome of Bacillus licheniformis grown in different media and under different nutrient starvation conditions. Proteomics. 2006;6:268–81.

52. Mao S, Luo Y, Bao G, Zhang Y, Li Y, Ma Y. Comparative analysis on the membrane proteome of Clostridium acetobutylicum wild type strain and its butanol-tolerant mutant. Mol BioSyst. 2011;7:1660–77.

53. Thein M, Sauer G, Paramasivam N, Grn L, Linke D. Efficient subfractionation of gram-negative bacteria for proteomics studies. J Proteome Res. 2010;9:6135–47.

54. Bittel M, Gastiger S, Amin B, Hofmann J, Burkovski A. Surface and extracellular proteome of the emerging pathogen Corynebacterium ulcerans. Proteomes. 2018;6:18.

55. Tunio SA, Oldfield NJ, Ala’Aldeen DA, Wooldridge KG, Turner DP. The role of glyceraldehyde 3-phosphate dehydrogenase (GapA-1) in Neisseria meningitidis adherence to human cells. BMC Microbiol. 2010;10:280.

56. Bergmann S, Rohde M, Hammerschmidt S. Glyceraldehyde-3-phosphate dehydrogenase of Streptococcus pneumoniae is a surface-displayed plasminogen-binding protein. Infect Immun. 2004;72:2416–9.

57. Zhu W, Zhang Q, Li J, Wei Y, Cai C, Liu X, Zin M. Glyceraldehyde-3-phosphate dehydrogenase acts as an adhesin in Erysipelothrix rhusiopathiae adhesion to porcine endothelial cells and as a receptor in recruitment of host fibroconnectin and plasminogen. Vet Res. 2017;48:16.

58. Barbosa MS, Bão SN, Andreotti PF, de Faria FP, Felipe MSS, dos Santos Feitosa L, Mendes-Giannini MJS, De Almeida Soares CM. Glyceraldehyde-3-phosphate dehydrogenase of Paracoccidioides brasiliensis is a cell surface protein involved in fungal adhesion to extracellular matrix proteins and interaction with cells. Infect Immun. 2006;74:382–9.

59. Garcia ED. GAPDH as a model non-canonical AU-rich RNA binding protein. Seminars in cell and developmental biology. New York: Elsevier; 2018.

60. Pereira LA, Bão SN, Barbosa MS, Da Silva JLM, Felipe MSS, De Santana JM, Mendes-Giannini MJS, De Almeida Soares CM. Analysis of the Paracoccidioides brasiliensis triosephosphate isomerase suggests the potential for adhesin function. FEMS Yeast Res. 2007;7:1381–8.

61. Miranda-Ozuna JF, Hernández-García MS, Brueba LG, Benítez-Cardoza CG, Ortega-López J, Gonzalez-Robles A, Arroyo R. The glycolytic enzyme triosephosphate isomerase of Trichosporon vaginalis (TnTIM) is a surface-associated protein induced by glucose that functions as a laminin-and fibronectin-binding protein. Infect Immun. 2016;84:00538.

62. Kinney B, Booth NA, Svensser G. Plasminogen binding by oral streptococci from dental plaque and inflammatory lesions. Microbiology. 2008;154:924–31.

63. Crowe JD, Sievwright IK, Auld GC, Moore NR, Gow NA, Booth NA. Candida albicans binds human plasminogen: identification of eight plasminogen-binding proteins. Mol Microbiol. 2003;47:1637–51.

64. Jagaabedan B, Koo OK, Kim K-P, Burkholder KM, Mishka KK, Arcoonual A, Bhunia AK. LAP, an alcohol acetaldehyde dehydrogenase enzyme in Listeria, promotes bacterial adhesion to enterocyte-like Caco-2 cells only in pathogenic species. Microbiology. 2010;156:2782–99.

65. Meza-Cervantez P, Gonzalez-Robles A, Cárdenas-Guerra RE, Ortega-Lopez J, Saavedra E, Pineda E, Arroyo R. Pyruvate: ferredoxin oxidoreductase in pathogenic species. Microbiology. 2010;156:2782–95.

66. Arora G, Sajid A, Virmani R, Singhal A, Kumar C, Dhasmana N, Khanna T, Minton NP, Serruto D, Unnikrishnan M. Multiple factors modulate biofilm formation by the anaerobic pathogen Clostridium difficile. J Bacteriol. 2012;194:01980.

67. Dembek M, Barquist L, Boinet CJ, Akin CA, Mayho M, Lawley TD, Fairweather NF, Fagan RP. Fagan RP. High-throughput analysis of gene essentiality and sporation in Clostridium difficile. MBio. 2015;6:e02383.

68. Kirk JA, Gebhart B, Buckley AM, Lok S, Scholl D, Douce GR, Govoni GR, Fagan RP. New class of precision antimicrobials redefines role of Clostridium difficile S-layer in virulence and viability. Sci Transl Med. 2017;9:eaah6813.

69. Pantaleon V, Soavelomandrosa AP, Bouttier S, Brandet R, Roxas B, Chu M, Collignon A, Janior C, Vedantam G, Candela T. The Clostridium difficile protease Cwp84 modulates both biofilm formation and cell surface properties. PLoS ONE. 2015;10:e0124971.

70. Foulston L, Eltolsh AK, DeFrancesco AS, Losick R. The extracellular matrix of Staphylococcus aureus biofilms comprises cytoplasmic proteins that associate with the cell surface in response to decreasing pH. Microbiology. 2014;160:01667.

71. Yang C-K, Ewis HE, Zhang X, Lu C-D, Hu H-J, Pan Y, Abdelat AT, Tai PC. Classical protein secretion of Bacillus subtilis in the stationary phase is not due to cell lysis. J Bacteriol. 2011;193:005897.

72. Aoraga S, Said S, Virmani R, Singhal A, Kumar C, Dhasmana N, Khanna T, Maja M, Misra R, Molle V. Ser/Thr protein kinase PrkC-mediated regulation of GroEL is critical for biofilm formation in Bacillus anthracis. NPJ Biofilms Microbiomes. 2017;3:7.

73. Jain S, Smyth D, O’Hagan BM, Heap JT, McMullan G, Minton NP, Ternan NG. Inactivation of the dnaK gene in Clostridium difficile 630 Δ erm yields a temperature-sensitive phenotype and increases biofilm-forming ability. J Bacteriol. 2011;193:2429–40.

74. Sleytr UB, Sára M. Bacterial and archaeal S-layer proteins: structure-function relationships and their biotechnological applications. Trends Biotechnol. 1997;15:20–6.

75. Wallenius J, Maehmeo H, Eerikäinen T. Carbon 13-metabolic flux analysis derived constraint-based metabolic modelling of Clostridium acetobutylicum in stressed chemostat conditions. Biotechnol Biofuels. 2016;219:378–86.

76. Hu S, Zheng H, Gu Y, Zhao J, Zhang W, Yang W, Wang Z, Zhao G, Yang S, Jiang W. Comparative genomic and transcriptomic analysis revealed genetic changes of bacteria related to solvent production and xylene utilization in Clostridium acetobutylicum EA 2018. BMC Genom. 2011;12:93.

77. Hu S, Zheng H, Gu Y, Zhao J, Zhang W, Yang W, Wang Z, Zhao G, Yang S, Jiang W. Comparative genomic and transcriptomic analysis revealed genetic changes of bacteria related to solvent production and xylene utilization in Clostridium acetobutylicum EA 2018. BMC Genom. 2011;12:93.

78. Sleytr UB, Sára M. Bacterial and archaeal S-layer proteins: structure-function relationships and their biotechnological applications. Trends Biotechnol. 1997;15:20–6.