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Consequences of *cps* mutation of *Klebsiella pneumoniae* on 1,3-propanediol fermentation

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Abstract The filtration in 1,3-propanediol (1,3-PD) downstream process is influenced by the large amounts of capsular polysaccharides (CPS) produced by *Klebsiella pneumoniae* CGMCC 1.6366. The morphological and fermentation properties were investigated with the CPS-deficient mutant *K. pneumoniae* CGMCC 1.6366 CPS. Similar biomass was obtained with CGMCC 1.6366, and the mutant strain in batch cultures indicating the cell growth was slightly inhibited by CPS defection. The viscosity of fermentation broth by mutant strain decreased by 27.45%. The flux with ceramic membrane filter was enhanced from 168.12 to 303.6 l h^{-1} m^{-2}, exhibiting the great importance for downstream processing of 1,3-PD fermentation. The products spectrum of mutant isolate changed remarkably regarding to the concentration of fermentation products. The synthesis of important 1,3-PD and 2,3-butanediol was enhanced from 9.73 and 4.06 g l^{-1} to 10.37 and 4.77 g l^{-1} in batch cultures. The noncapsuled *K. pneumoniae* provided higher 1,3-PD yield of 0.54 mol mol^{-1} than that of encapsuled wild parent in batch cultures. The fed-batch fermentation of mutant strain resulted in 1,3-PD concentration, yield, and productivity of 78.13 g l^{-1}, 0.53 mol mol^{-1}, and 1.95 g l^{-1} h^{-1}, respectively.

Keywords Capsule · Downstream process · Glycerol · *Klebsiella pneumoniae* · 1,3-Propanediol

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Introduction

The increasing demand caused by a growing world population and the rapid industrial development in many countries have stimulated the discussion on possible alternative raw material sources for fuel and chemical production (Schrader et al. 2009). 1,3-Propanediol (1,3-PD) is one of the building blocks in the chemical industry and can be synthesized either from petrochemical or renewable resources (Zeng and Biebl 2002). The value of 1,3-PD lies predominantly in its use in polymers prepared from 1,3-PD and terephthalic acid, demand of which is estimated to be 1 to 2 billion lb $year^{-1}$ in 10 years (Nakamura and Whited 2003). The microbial approach to 1,3-PD production becomes an emerging area of intellectual endeavor and industrial practice with great promise in recent years (Emptage et al. 2003; Gonzalez-Pajuelo et al. 2005; Xiu and Zeng 2008; Zheng et al. 2009). Glycerol conversion to 1,3-PD by several microorganisms including *Bacillus*, *Clostridia*, *Citrobator*, *Lactobacillus*, and also *Klebsiella* was well documented (Biebl and Marten 1995; Reimann et

al. 1998). *K. pneumoniae* is one of the most competent 1,3-PD producing strains. 1,3-PD of 102.06 g l^{-1} was produced with lactate deficient *K. pneumoniae* HR 526 in our previous work (Xu et al. 2009). In the absence of an external oxidant, glycerol is fermented by a dismutation process involving two pathways, one molecule serving for glycerol oxidation and the other for the consumption of the reducing equivalents generated.

The downstream separation of 1,3-PD plays an important role in the industrial commercialization, which accounts for up to 50–70% of the total production cost (Xiu and Zeng 2008). The generic processing of 1,3-PD includes biomass removal, by-product removal and 1,3-PD distillation. The flocculation, membrane filtration, electrodialysis, extraction, distillation, and chromatograph were employed for 1,3-PD separation and purification (Adkesson et al. 2005; Gong et al. 2004; Hao et al. 2005; Hermann and Patel 2007; Hilaly and Binder 2002; Xiu et al. 2007). In our practices with *K. pneumoniae*, the combined route with nanofiltration, electrodialysis, condensation, and distillation was developed for the large scale 1,3-PD production. As reported previously, however, *K. pneumoniae* produced large amounts of capsular polysaccharides (CPS) as reflected by the formation of glistening mucoid colonies with viscid consistency (Lai et al. 2003). Consequently, the CPS caused filtration blocking of 1,3-PD broth is liable to occur. Although the acid hydrolyzation pretreatment could enhance the filtration flux (Liu et al. 2006), the subsequent desalination and condensation was influenced by addition of H_2SO_4.

To this end, the construction of CPS-deficient mutant *K. pneumoniae*, the time profiles of fermentation, and morphological change will be studied in this work to facilitate the separation of 1,3-PD.

Materials and methods

Bacterial strains and media K. pneumoniae CGMCC 1.6366 was stored in China General Microbiological Culture Collection Center (CGMCC, Beijing, China). The culture was cryo-conserved at −70°C to enhance stability. The basic culture media for 5-l fermentation contained (gram per liter): glycerol—20~25, K_2HPO_4—0.69, KH_2PO_4—0.25, $(NH_4)_2SO_4$—4, $MgSO_4{\cdot}7H_2O$—0.2, yeast extract—1.5, and 1 ml of trace element solution. The composition of the trace element solution was (milligram per liter): $MnSO_4{\cdot}4H_2O$—100, $ZnCl_2$—70, $Na_2MoO_4{\cdot}2H_2O$—35, H_3BO_3—60, $CoCl_2{\cdot}6H_2O$—200, $CuSO4{\cdot}5H_2O$—29.28, $NiCl_2{\cdot}6H_2O$—25, and 0.9 ml HCl (37%).

Culture conditions Erlenmeyer flasks (250 ml) containing 50 ml of medium were inoculated and incubated at 37°C in a rotatory shaker at 140 rpm for 10 h to prepare the inoculums. A 1.5% (*v*/*v*) inoculum was added aseptically. The fermentations were conducted in a 5-l B. Braun B Plus fermenter (B. Braun, Germany) with a 4-l working volume at a temperature, pH, stirring rate, and air flow rate of 37°C, pH 6.5, 320 rpm, and 0.65 vvm, respectively. A solution of 10 M NaOH was used to maintain the pH value of the broth. The feeding with pure glycerol in fed-batch cultures was started when the glycerol concentration in broth was below 5 g l^{-1}. The glycerol level was controlled at 5–10 g l^{-1} in subsequent cultures.

Analytical methods Dry cell mass was computed from the optical density calibration curved determined at 650 nm (OD_{650}). Glycerol, 1,3-PD, 2,3-butanediol (2,3-BD), lactate, succinate, acetate, and ethanol were determined by a Shimadzu 10AVP HPLC system using an Aminex HPX-87H column (300×7.8 mm; Bio-Rad, Palo AHO, Ca, USA) as described (Zheng et al. 2008a). 3-HPA was determined using the colorimetric method described by Circle et al. (1945). The viscosity measurements were carried out in Ubbelohde viscometer as described (Nouh et al. 2009). The ceramic microfilter MFS-25 (Ever-shine, Hunan, China) with pore size of 50 nm was employed to detect the flux.

Construction of gene-specific mutants A 1,000-bp segment of truncated *cps* gene ORF3 of *K. pneumoniae* was polymerase chain reaction (PCR)-amplified with oligonucleotides primer1 (5′-ACTCCCAATTGTGACCGAAATCC-3′) and primer 2 (reverse 5′-GAGCCACTGGTTCCAGAACTTCA-3′). The segment was cloned into the pMD18-T simple vector (Takara, Japan) to be amplified and sequenced. A terminator sequence (5′-AGTCCGGCCATTTGGCCGGACTTTTTTTT-3′) was designed from the *K. pneumonia cps* gene. The terminator sequence was added to the 1,000-bp segment by PCR-amplification for two times with the primer 3 (forward 5′-TTGGCCGGACTTTTTTTTGCTGTACAATAGTTCCTGTTC-3′), the primer 4 (forward 5′-GAATTCAGTCCGGCCATTTGGCCG-GACTTTTTTTTG-3′), and the primer 5 (reverse 5′-GAATTCGAGCCACTGGTTCCAGAACTT-3′). The segment was cloned into pGEM-T easy vector (Promega, USA) and then transferred to suicide vector pGPCm after being digested with *Eco*RI, resulting in vector pCPS. The pCPS vector was then transformed into *Escherichia coli* SM10 (Miller and Mekalanos 1988), and the resulting strain was used as donor in conjugation with *K. pneumoniae*. Transconjugants were selected for both chloromycetin resistance from pGPCm inserting and ampicillin resistance because the *K. pneumoniae* strain is resistive to ampicillin while the *E. coli* SM10 is sensitive.

Assays of the insertion mutants PCR analysis were used to confirm that the transconjugants were the correct insertion

mutants. Oligonucleotides primer 6 (forward 5′-CATG CGCTCCATCAAGAAGA-3′) and primer 7 (reverse 5′-GTGGGTCTCGCGGTATCATT-3′) were designed to amplify a 1,160-bp segment of pGPCm. Total DNA from *K. pneumoniae* and the four transconjugants were used as template, and the pGPCm plasmid DNA was used as positive control.

Transmission electron microscopy analysis Morphology and size of *K. pneumoniae* were examined by transmission electron microscopy (TEM) analysis (JEOL 120 CX; JEOL, Tokyo, Japan). The stained (methanolic uranyl acetate and lead citrate) slices of the cell of wild and mutant strains of *K. pneumoniae* were obtained.

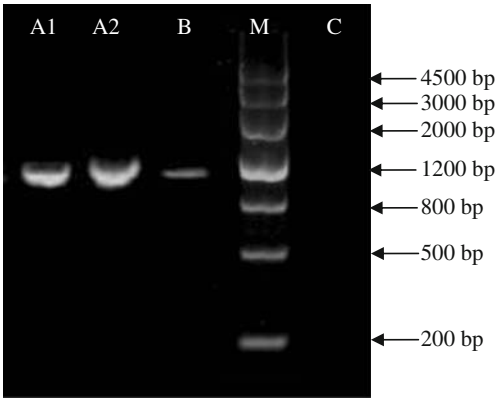

Fig. 1 Electrophoretic analysis of the amplified products from the *K. pneumoniae* CGMCC 1.6366 wild-type strain (*lane C*), the plasmid pCPS (*lane B*), and the mutant strain CGMCC 1.6366 CPS (*lane A1* and *A2*)

Results

Construction of a capsule-defective mutant The genomic organization of *K. pneumoniae* isolate *cps* region responsible for capsular polysaccharide synthesis has been determined previously (Arakawa et al. 1995). A total of 15 open reading frames organized into two transcriptional units have been identified as indispensable, 13 of them (ORF3 to ORF15) being part of a same transcriptional unit. A 1,000-bp DNA fragment from the capsule-encoding region of *K. pneumoniae* CGMCC 1.6366 was amplified by PCR with primers complementary to regions of ORF3 terminator. The resulting sequence was cloned into the suicide vector, pLD55, to construct pCPS harboring chloromycetin and ampicillin resistance. pCPS was then introduced by conjugational transfer into *K. pneumoniae* CGMCC 1.6366, giving rise to chloromycetin-resistant isolate.

The occurrence of an insertion event was checked by PCR as mentioned in "Materials and Methods". As shown in Fig. 1, the 1,160-bp DNA fragment was observed when genomic DNA from the *K. pneumoniae* CGMCC 1.6366 (CPS), and plasmid pCPS was used as a template (Fig. 1, lane A1, A2, and lane B) whereas no fragment was amplified with genomic DNA from the wild *K. pneumoniae* CGMCC 1.6366 (lane C). The fragments were subjected to DNA sequencing, confirming that the sequence data of CGMCC 1.6366 CPS was identical with that of pCPS. This result indicated that the pCPS vector had integrated into the chromosome of the transconjugant strains. The occurrence of the insertion event was further confirmed by Southern hybridization experiments with genomic DNA from the mutant and DNA probes specific for the ORF3 of *cps* region (data not shown).

Characterization of CPS-defective mutants K. pneumoniae strains produce copious exopolysaccharide in the form of various capsule (Clegg and Sebghati 2001). *K. pneumoniae* mutant on Luria–Bertani agar plates were quite different from the ones obtained with wild-type *K. pneumoniae* CGMCC 1.6366; they were smaller and did not exhibit the smooth phenotype characteristic of surface polysaccharide producing colonies. Since capsule synthesis is known to be dependent on the physiological state status, such as carbon excess and growth (Favre-Bonte et al. 1999), TEM was performed with both the wild-type and mutant strains at exponential growth phase to observe the capsule formation (shown in Fig. 2). The wild-type strain was surrounded by the lighter material while the noncapsuled mutant strain cell was uniform dark, indicating that this mutant was in fact defective in the synthesis of capsular polysaccharides. The reduction of mucoidy with the value of 27.45% was also evident when the bacterial cultures were subjected to the viscosity analysis (Table 1). What should be mentioned was that the filtration flux was enhanced from 168.12 to 303.6 l h^{-1} m^{-2}, exhibiting the great importance for downstream processing of 1,3-PD fermentation.

1,3-PD fermentation of capsule-defective mutant Batch cultures were performed at an initial concentration of 23 g l^{-1} glycerol, 0.5 vvm air flow, and 320 rpm (Table 2). In the absence of oxygen or if the rate of carbon consumption is greater than the capacity to deoxidize the reduced equivalents (NADH) generated, the microbial resorts to metabolite overflow mechanism to maintain the intracellular stability (Zheng et al. 2008b). Together with 2,3-butanediol, lactate, acetate, succinate, and ethanol, 1,3-PD was secreted into the culture broth during fermentation. It was noticed that similar biomass (3.09 and 3.01 g l^{-1}) was obtained with CGMCC 1.6366 and the mutant strain in batch cultures, indicating the microbial growth was inhibited by CPS defection. However, the product spectrum of mutant isolate changed remarkably regarding the concentration of 2,3-

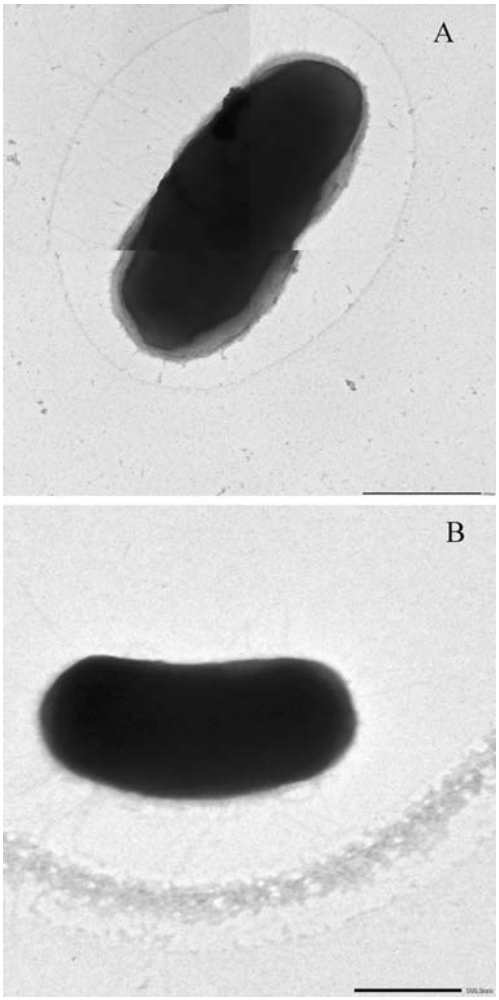

Fig. 2 Aspects of the colonies of *K. pneumoniae* CGMCC 1.6366 (**a**) and its capsule-defective mutant strain CGMCC 1.6366 CPS (**b**)

butanediol, lactate, acetate, succinate, ethanol, and 1,3-PD. The synthesis of important 1,3-PD and 2,3-butanediol was enhanced from 9.73 and 4.06 g l^{-1} to 10.37 and 4.77 g l^{-1}, respectively, whereas the variation of organic acids formation was negligible. The fermentation with noncapsuled *K. pneumoniae* provided higher 1,3-PD yield of 0.54 mol mol^{-1} than that of encapsuled wild parent. The time profiles of fed-batch fermentation by *K. pneumoniae* CGMCC 1.6366 wild-type strain and the mutant strain are indicated in Figs. 3 and 4, respectively. The fermentation efficiency for 1,3-PD was improved obviously by CGMCC 1.6366 CPS under these conditions. 1,3-PD concentration, yield, and productivity of mutant strain were 78.13 g l^{-1}, 0.53 mol mol^{-1}, and 1.95 g l^{-1} h^{-1}, respectively, while higher than that of 1,3-PD concentration, yield, and productivity of wild strain were 67.05 g l^{-1}, 0.52 mol mol^{-1}, and 1.68 g l^{-1} h^{-1}, respectively. Taken together, these results indicated that the CPS mutation leads to the redistribution of carbon flux.

Discussion

With increasing oil prices and demand from emerging economies worldwide, the production of low-grade glycerol will increase, resulting from ever spread of biodiesel. The glycerol-based 1,3-PD is more attractive than fossil-based 1,3-PD because of less nonrenewable resource consumption and greenhouse gas emissions (Urban and Bakshi 2009). In fermentation with *Klebsiella*, metabolic engineering expands the range of chemical process solutions and enables improved cost effectiveness of options based on natural biological routes. The metabolism in 1,3-PD fed-batch fermentation by a D-lactate-deficient mutant of *K. pneumoniae* was investigated in our work (Xu et al. 2009). The final lactate concentration decreased dramatically from more than 40 g l^{-1} to less than 3 g l^{-1}. The diol (1,3-PD and 2,3-BD) yield increased from 0.55 mol mol^{-1} to a maximum of 0.65 mol mol^{-1}. A tetracycline resistance marker was inserted to inactivated *aldA* gene encoding ALDH in *K. pneumoniae* YMU2 to block ethanol synthesis (Zhang et al. 2006). The final titer, the productivity of 1,3-PD, and the yield of 1,3-PD relative to glycerol of the mutant strain reached 927.6 mmol l^{-1}, 14.05 mmol l^{-1} h^{-1}, and 0.699 mol mol^{-1}, respectively. Recently, the glycerol oxidative pathway was inactivated to eliminate the by-product during 1,3-PD fermentation with *K. pneumoniae* (Seo et al. 2009). The 1,3-PD production yield was higher in the recombinant strain (0.57 mol mol^{-1}) than the wild-type Cu strain (0.47 mol mol^{-1}).

This work aimed at reducing CPS synthesis via inactivating *cps* gene in *K. pneumoniae* CGMCC 1.6366 to facilitate downstream processing of 1,3-PD production. Transmission electronic microscopy of *cps* mutant has shown that the no noticeable morphological variation of

Table 1 Mucoidy and filtration flux comparison of wild-type and mutant strains

Strain	CGMCC 1.6366	CGMCC 1.6366 CPS
Viscosity[a] (mPa s)	1.78	1.29
Filtration flux[b] (l h^{-1} m^{-2})	168.12	303.6

[a] Viscosity was detected with Ubbelohde viscometer which has an internal diameter of 1.2 mm

[b] Filtration flux was detected with a ceramic filter (pore size of 50 nm) at 80°C and 0.3 MPa

Table 2 Batch fermentation by *Klebsiella pneumoniae* CGMCC 1.6366

Parameters	CGMCC 1.6366	CGMCC 1.6366 CPS
Glycerol consumed (g l^{-1})	22.95	22.8
Biomass (g l^{-1})	3.09	3.01
1,3-Propanediol (g l^{-1})	9.73	10.37
2,3-Butanediol (g l^{-1})	4.06	4.77
Succinate (g l^{-1})	0.27	0.34
Acetate (g l^{-1})	0.23	0.19
Lactate (g l^{-1})	0.09	0.08
Ethanol (g l^{-1})	1.19	1.51
1,3-Propanediol yield (mol mol^{-1})	0.55	0.58
Carbon recovery[a] (%)	85.1	94.94

[a] Exclusive of carbon dioxide

cell occurred, and CPS formation decreased significantly. *K. pneumoniae* is a Bio-safety Level 2 microorganism, causing suppurative infection, pneumonia, urinary tract infection, and septicemia in humans (Boddicker et al. 2006). The pathogenicity-related factors of *Klebsiella* include capsule, lipopolysaccharide, siderophores, and adhensins, etc. The noncapsulated variants of Gram negative bacteria were isolated by many research groups previously via the genetic manipulation (Dean et al. 2005; Regueiro et al. 2006; Scorpio et al. 2008; Wikraiphat et al. 2009). These works mainly focused on the mechanism of the interaction between bacterium and human cell, immune response to bacterial infection and human neutrophil-killing ability. ORF3, designed *wzi* which is located in the *cps* conserved region of 2,872–4,305 nt, is responsible for the surface assembly of the capsule. The results in this study indicated PCR-based mutagenic and conjugation protocol were efficient to obtain *wzi*-deficient isolate.

The metabolic flux exhibited the sequential evolution profile in fermentation by *K. pneumoniae* CGMCC 1.6366. The exponential phase was the most vigorous period for 1,3-PD formation except for lactate (Zheng et al. 2008b). 1,3-PD is the growth-associated metabolite, playing a key role in NADH consumption to regulate the intracellular-reducing equivalent balance of *K. pneumoniae* (Zeng et al. 1993). It was revealed that the growth of *cps* mutant isolate was not influenced by genetic modification. The similar growth pattern could be noticed in fed-batch fermentations by the wild and the mutant strains (Figs. 3 and 4). 1,3-PD synthesis was enhanced slightly with the *cps* deficient strain. Another important chemical 2,3-butanediol formation reached the high level of about 15 g l^{-1} before 20 h in fed-batch cultures with the *cps*-deficient strain. The plausible explanation could be that more carbon flux was directed to the central carbon pathway (EMP and TCA) instead of HMP pathway due to blocked capsule synthesis.

This work is the first try to apply the noncapsuled *K. pneumoniae* via inactivating *cps* gene to facilitate the downstream process of the biotechnology. The ceramic membrane filtration flux of *cps* mutant strain broth

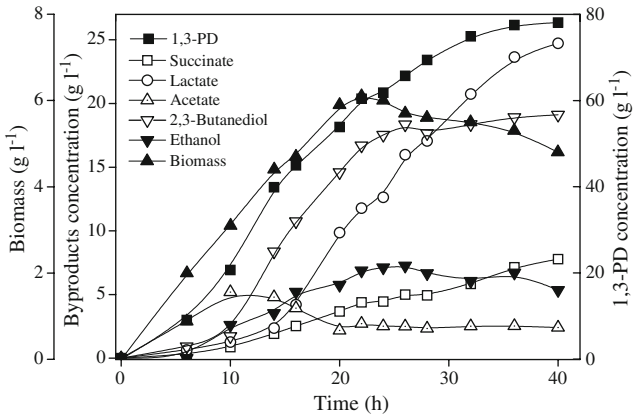

Fig. 3 Time profile of capsule-defective mutant strain *K. pneumoniae* CGMCC 1.6366 in fed-batch fermentation at 37°C, 0.65 vvm, and 320 rpm, respectively. The addition of pure glycerol into the fed-batch cultures was started when the glycerol concentration in the broth was below 5 g l^{-1}. The glycerol level in subsequent cultures was controlled at approximately 5–10 g l^{-1}

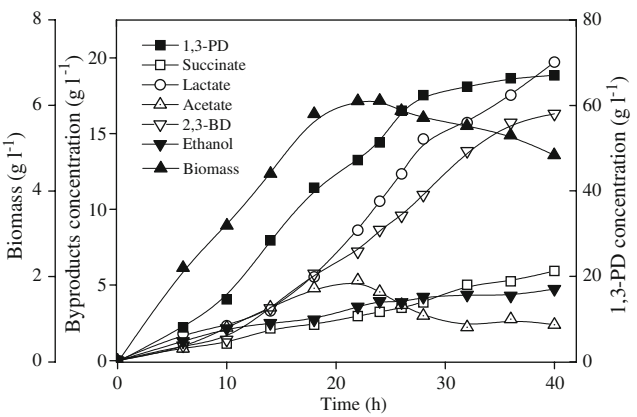

Fig. 4 Time profile of *K. pneumoniae* CGMCC 1.6366 in fed-batch fermentation at 37°C, 0.65 vvm, and 320 rpm, respectively. The addition of pure glycerol into the fed-batch cultures was started when the glycerol concentration in the broth was below 5 g l^{-1}. The glycerol level in subsequent cultures was controlled at approximately 5–10 g l^{-1}

increased by 80.59%, confirming CPS was the significant factor influencing cell removal for 1,3-PD purification. Thus, the acid hydrolyzation pretreatment could be eliminated in our processes (Liu et al. 2006). Moreover, the organic content (BOD_5) in 1,3-PD downstream wastewater decreased. The energy consumption in the aerobic-activated sludge pond could be saved. Aside from the significant increase in the final 1,3-PD concentration, the inactivation of CPS formation demonstrated the potential value in view of 1,3-PD commercialization and biosafety.

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