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Effect of IPTG Induction on Production of β-Galactosidase-PreS2 Fusion Protein in Recombinant *Escherichia coli*

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Effects of IPTG induction on cell growth and production of β-galactosidase-preS2 fusion protein (βgal-preS2) were studied in a defined medium using a recombinant *Escherichia coli* JM109/pCMHB30. IPTG was added (0.2 mM) to induce the cloned-gene expression in the early-, mid-, and late-log growth phases. The most serious decreases in growth rate and plasmid stability were observed for the induction in the early-log growth phase. The expression level of βgal-preS2 attained by the induction in the mid-log phase was about 0.51 mg fusion protein/mg total cellular protein, which was 2- and 5-fold improvement over the levels obtained with the inductions in the early- and late-log phases. Formation of acidic byproducts including acetate and pyruvate showed different profiles during the fermentation period for each cases of induction; pyruvate was the major byproduct for the induction in the early-log phase while acetate production became more significant for the cases of inductions in the mid- and late-log phases.

With the advanecment of recombinant DNA technology, overproduction of foreign proteins in microorganisms including *Escherichia coli* has been a subject of keen interest to many biotechnologists. When a recombinant fermentation process is to be considered on an industrial scale, one of the most important problems is to maintain the stability of recombinant plasmids during process operation. Genetic approaches made to overcome plasmid instability in both structural and segregational cases, include construction of recombinant plasmids containing *par* (18) or *cer* (20) loci, and/or a switch on-off regulation systems for the control of plasmid copy number (replication control) (14, 21) and of cloned-gene expression (transcription and translation control).

Many cases of cloned-gene expression have been reported to be controlled by temperature shift (2, 19, 22) or chemical inducers (3, 15) including isopropyl-β-D-thiogalactoside (IPTG). In a case of temperature-induced synthesis of *Eco*RI restriction enzymes from a recombinant *E. coli* (2), the early induction, in which the cultivation temperature was shifted from 30℃ to 42℃ in the logarithmic growth phase, showed a higher product yield than the late induction in which temperature-shift was made in the stationary phase. For the production of β-galactosidase (19) and T4 DNA ligase (22) in recombinant *E. coli* containing a temperature-inducible expression system the induction of plasmid genes (temperature shift) was commonly achieved in the early- or mid-log phases of the cell growth. In case of chemical induction systems, it was found that, at high concentrations of an inducer, cell growth and recombinant protein production were significantly inhibited, indicating the existence of optimal concentration of an inducer maximizing the recombinant cell growth and/or the gene product formation (4, 13). Moreover, Seressiotis and Bailey (17) reported the simulation results that plasmid stability and product formation were greatly influenced by the timings of gene expression and plasmid amplification. In a recombinant cell fermentation system, therefore, the determination of induction time as well as the optimal inducer concentration are considered very important operating parameters which determine the overal process productivity.

In the present study, an *E. coli* strain containing the recombinant plasmid pCMHB30, which encodes β-galactosidse-preS2 fusion protein with *tac* promoter, was

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employed to optimize the induction time of plasmid gene expression in a bench-top fermentor. PreS2 peptide is known as an immunodominant surface peptide of hepatitis B virus (HBV) (11). Inducer (IPTG) was added in the various growth phases, to investigate the effects of induction time on recombinant cell growth and product formation in batch fermentations using a chemically-defined medium. Plasmid stability and formation of organic acids including acetate, lactate, pyruvate, and succinate were also monitored after IPTG induction.

A significant improvement in the expression level and product yield of recombinant proteins was obtained by optimizing the induction time and the result is reported this article.

MATERIALS AND METHODS

Microorganism and Plasmid

The plasmid-harboring strain, *Eschericia coli* JM109 /pCMHB30, was kindly provided by DR. M.H., Yu, Genetic Engineering Research Institute, KIST, Taejeon, Korea. The plasmid pCMHB30, as described elsewhere (12), has *tac* promoter, the *lacZ* sequence (encoding 293 N-terminal amino acid residues of β-galactosidase),. the *preS2* gene (encoding 55 amino acids of preS2 pepide), and the repressor gene (*lacI*) of the *lac* operon. The plasmid also contains the ampicillin resistance gene and its size is 7.86 kb. The expression of *lacZ-preS2* fused gene was induced by IPTG, and β-galactosidase-preS2 fusion protein (βgal-preS2) was produced as an inclusion body in the cytoplasm. The recombinant strain was stocked at −20℃ in 20% glycerol. All experiments were started from the frozen cells.

Culture Media

LB medium was used for the inoculum preparation, first preculture, and plasmid stability test. LB medium consisted of (g/*l*); 10.0 bacto-tryptone (Difco), 5.0 yeast extract (Difco), 5.0 NaCl. For the second preculture, minimal A medium was used and its composition was (g/*l*); 2.0 glucose, 1.0 $(NH_4)_2SO_4$, 3.0 $Na_2HPO_4 \cdot 12H_2O$, 0.5 trisodium citrate$\cdot 2H_2O$, and 0.2 $MgSO_4 \cdot H_2O$.

A chemically-defined minimal medium was used in all batch fermentations and it consisted of (g/*l*); 15.0 glucose, 6.0 $(NH_4)_2SO_4$, 1.0 yeast extract, 1.0 KH_2PO_4, 3.0 $Na_2HPO_4 \cdot 12H_2O$, 0.5 trisodium citrate$\cdot 2H_2O$, 0.2 $MgSO_4 \cdot 7H_2O$, and 1.0 m*l* trace element solution (Mo, B, Co, Cu, Mn, Zn, and Fe).

Cultivation Conditions

A single colony isolated on an LB agar plate containing ampicillin was inoculated into LB medium and then incubated for 6 h at 30℃ on a rotary shaker. Cells grown in LB medium were transferred (1%) into the second preculture medium. After incubating for 13 h at 30℃ on a shaker, the whole content of the second preculture was inoculated (3.3%) into the fermentor. The preculture media were supplemented with ampicillin at a concentration of 100 μg/m*l*.

2.5-*l* fermentors (Korea Fermentor Co.) with working volumes of 1.5 *l* were used in this study. The pH, temperature, and aeration rate were controlled at 7.0, 37℃, and 1 vvm, respectively. Dissolved oxygen tension (DOT) was continuously monitored using a galvanic oxygen electrode, and maintained above 20% of air saturation by adjustment of the agitation speed. All batch fermentations in this work were conducted in the absence of the selective pressure, ampicillin.

Cell Grwoth

Cell growth was monitored by measuring the optical density at 540 nm. One absorbance unit (1.0 OD_{540}) corresponded to 0.47 dry wt/*l*. The culture broth samples were diluted appropriately so that OD_{540} readings were made in the range of 0.05 to 0.5.

Plasmid Stability

Samples of the culture broth were aseptically diluted to concentration of about $10^{-6} \sim 10^{-8}$ and then spread on LB agar plates. After incubation, over 100 colonies were transferred by toothpicking to an LB plate containing ampicillin of 100 μg/m*l*, and the phenotypic expression of Amp^R gene was examined. The plasmid stability was defined as the ratio of the colony number exhibiting Amp^R character to the total number of colonies toothpicked.

Glucose and Protein

Residual glucose was determined as a reducing sugar by the 3,5-dinitrosalicylic acid method (10). Concentrations of protein were measured by Lowry's method (9) using bovine serum albumin as a standard.

Organic Acids

The concentration of organic acids, i.e., acetate, lactate, pyruvate, and succinate, was estimated with a reverse-phase HPLC (Tosoh Co.) by isocratic elution (0.8 m*l* /min) of 5 mM $(NH_4)_2HPO_4$ (pH 2.5) at 210 nm. Before analysing the samples, high molecular weight components dissolved in the culture broth were removed by centrifugation (12000 rpm, 5 min) and filtration (0.45 μm pore size filter) after incubating at 80℃ for 20 min. Acetate measurements were confirmed by gas chromatography on arbitrarily chosen samples by and a good correlation with the HPLC analysis was obtained. Analyses of pyuvate and lactate were also confirmed with the enzymatic test kits from Sigma (No. 726-UV and No. 826-UV).

Measurement of β-Galactosidase-PreS2 Fusion Protein

The amount of βgal-preS2 produced was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

as described by Laemmli (8). In this work a 14% gel was found to be adequate for resolving βgal-preS2 (MW 44,000 daltons) from the other cellular proteins. The Coomassie blue stained protein bands were scanned with a densitometer (Ultrascan XL, Pharmacia LKB). The quantification of βgal-preS2 was achieved through multiplying the integrated area of the βgal-preS2 protein band by the protein concentration loaded on the gel.

RESULTS

Fermentation without Induction

Batch fermentations with *E. coli* JM109 harboring a recombinant plasmid, pCMH30, were conducted without addition of the inducer (IPTG) so that the expression of the plasmid gene was repressed. As typically shown in Fig. 1, the recombinant cells fully grew within 15 hrs and finally reached a OD_{540} value of about 12. βgal-preS2 was continuously produced at a very low concentration (<40 mg/l). Even in the absence of ampicillin selection, the recombinant cells were extremely stable probably due to the minimal metabolic burden on the host cell.

From the cell growth curve, three distinctive growth phases were defined; early-, mid-, and late-log growth phases were represented by the cultivation times of 4~6 h, 8~10, and 12~14 h, respectively. Repeated cultivations exhibited similar patterns of cell growth, and these phases were employed for chemical inductions of gene expression in the present study.

Fermentations with Induction

Early induction: When IPTG was added in the early-log phase (OD_{540}=1.0, 5 h after inoculation) to a final concentration of 0.2 mM, it was observed that the cell growth was significantly inhibited (Fig. 2). A successive cultivation for 20 hrs resulted in subsequent cell growth to a final OD_{540} value of 8.3. As can be noted from the plasmid stability data, the subsequent cell growth seemed to be due to the overgrowth of plasmid-free cells (ampicillin sensitive cells), and after 40 h the plasmid free cells became dominant. βgal-preS2 was produced at a concentration of about 0.5 g/l, which corresponded to about 25% of total cellular protein and 15% of dry biomass. The maximum specific production rate of βgal-preS2, 28 mg/g cell·h, was achieved at 20 h after the induction.

The synthesis of βgal-preS2 as a fuction of cultivation time is shown in Fig. 3. The SDS-PAGE band of βgal-preS2 (MW 44,000 daltons) was clearly resolved from the other celllular proteins and illustrated at the same position of ovalbumin (MW 45,000 daltons) as a standard size marker protein.

A similar experiment of the early induction was conducted in which the complex LB medium was supplemented with 20 g/l glucose. The results are summarized in Table 1. It was noted that the expression level and yield of βgal-preS2, 30% of total cellular protein and 17% of dry biomass, were not improved significantly by using the complex medium.

Middle induction: A fermentation time course when IPTG induction was introduced during the mid- logarithmic growth phase (OD_{540}=2.0, 8 h after inoculation), is shown in Fig. 4. Cells grew gradually to their concentration having an OD_{540} value of 12. The specific growth rate (μ) after induction was determined to be 0.216 h^{-1}, which was about 70% of that without induction.

As shown in Fig. 4, before the induction, the recombinant plasmid was maintained in a quite stable state, and plasmid-free cells were not detected. However, at 8 hrs after induction, plasmid-free cells were appeared and

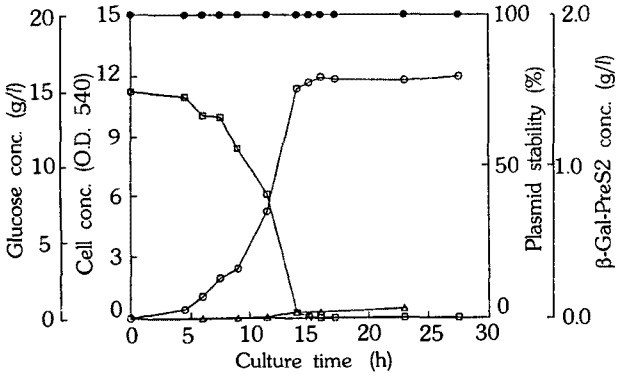

Fig. 1. A typical batch fermentation of *E. coli* JM109 /pCMHB30 when inducer (IPTG) was not added.
(●) plasmid stability (%); (○) cell concentration (OD_{540}); (□) glucose concentration (g/l); (△) β-galactosidase-preS2 concentration (g/l).

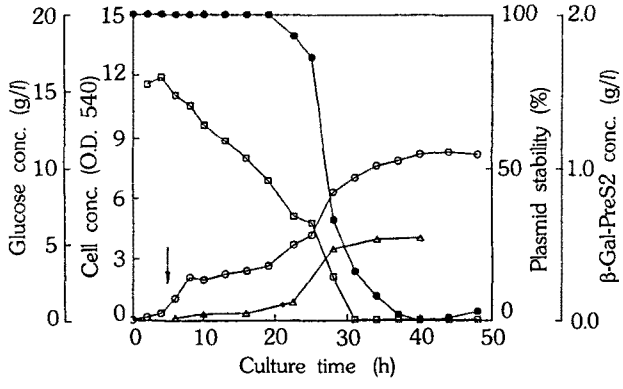

Fig. 2. A time course of batch fermentation of *E. coli* JM109/pCMHB30 when 0.2 mM IPTG was added in the early-log growth phase (early induction).
The symbols are the same as in Fig. 1. The arrow indicates the time of IPTG addition (5 h).

Table 1. Effects of IPTG induction on cell growth and βgal-preS2 production

Inductions	Overall growth yield (g cell/g glucose)	Specific growth yeild rate[a] (h^{-1})	Expression level of βgal-preS2 (mg/mg protein)	βgal-preS2 yield (mg/g cell)
1) Using defined medium				
Host[b]	0.409	0.334	–	–
Non[c]	0.374	0.320	0.015	8.7
Early	0.259	NG[d]	0.249	150.1
Middle	0.367	0.216	0.507	327.6
Late	0.358	0.106	0.101	48.2
2) Using complex medium				
Early	0.343	0.490	0.303	170.5

[a]Maximum specific growth rates for the cases of host cell and recombinant cell without induction (non-induction), and specific growth rate after IPTG addition for the induction experiments. [b]Plasmid-free host cell. [c]No induction with plasmid-containing cell. [d]No growth was observed for 20 h after induction.

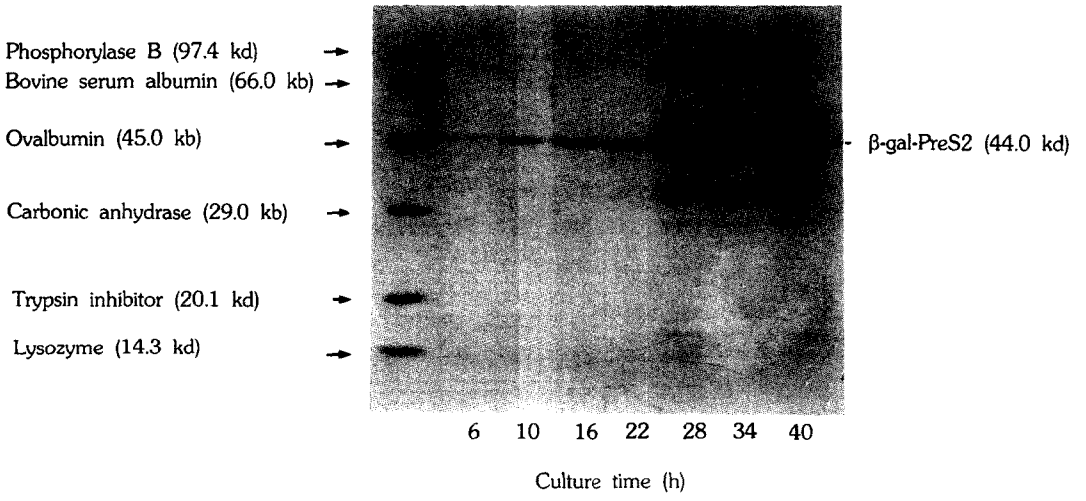

Fig. 3. Induced synthesis of β-galactosidase-preS2 fusion protein as a function of cultivation time in the early induction. Molecular weight standards (Bio-Rad) and insoluble fraction of sonicated cells were loaded on 14% SDS-polyacrylamide gel.

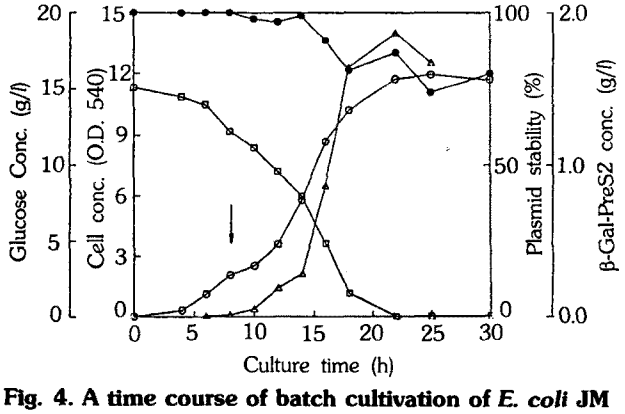

Fig. 4. A time course of batch cultivation of *E. coli* JM 109/pCMHB30 when 0.2 mM IPTG was added in the mid-log growth phase (middle induction).
The symbols are the same as in Fig. 1. Arrow indictes the time of IPTG addition (8 h).

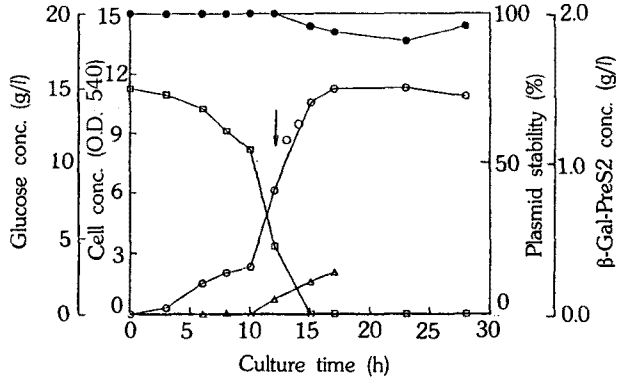

Fig. 5. A time course of batch cultivation of *E. coli* JM 109/pCMHB30 when 0.2 mM IPTG was added in the late-log growth phase (late induction).
The symbols are the same as in Fig. 1. Arrow indicates the time of IPTG addition (12 h).

they increased to more than 20% of the total cells at the end of the cultivation.

Production of βgal-preS2 increased remarkably and its maximum concentration reached 1.9 g/l, which corresponded to about 51% of total cellular protein and 33% of dry biomass. The maximum specific production rate of βgal-preS2 reached 90 mg fusion protein/g cell·hr at 8 hrs after the induction.

Late induction: Fig. 5 shows the cultivation results for the late induction in which IPTG was added into the medium during the late-log phase (OD_{540}=6, 12 h after inoculation). The cell growth continued at a reduced rate for 5 h after induction and the final cell concentration reached an OD_{540} value of 11.3. The specific growth rate after the induction was calculated to be 0.106 h^{-1}, which was just one-third of the specific growth rate obtained in the case without induction.

The concentration of βgal-preS2 reached 0.27 g/l at 5 h after induction, which corresponded to 10% of total cellular protein and 5% of dry biomass. The maximum specific production rate of βgal-preS2 achieved was about 22 mg fusion protein/g cell·h at 1.5 h after the induction. Plasmid-free segregants appeared after induction, but occupied no more than 10% of the total cell population.

Formation of Acidic Byproducts

In an effort to elucidate the physiological behaviour of the recombinant cells after induction, formation of acidic byproducts was monitored. In the experiments, acetate and pyruvate are found to be the major byproducts, and their production patterns after induction were illustrated in Figs. 6 and 7, respectively. The acidic byproducts were also measured for the fermentations of the host cells and recombinant cells without induction; the maximum concentrations of acetate and pyruvate, 5 g/l and 0.8 g/l, respectively, were obtained in the early stationary phase (data not shown).

For the early induction in the present study, acetate was not produced during the fermentation period, whereas the production of pyruvate was most significant. For the middle and late inductions, the highest concentrations of acetate were obtained at the time of glucose exhaustion, and then followed by a steady consumption of acetate. However, the formation and consumption profiles of pyruvate were very different from those of acetate, especially in the early induction. It is interesting to note that the highest concentration of pyruvate is attained coincidently with the maximum specific production rate of the fusion protein.

DISCUSSION

The fermentation results of the host and recombinant cells with and without IPTG induction are summarized in Table 1. Slightly higher overall growth yield and specific growth rate were observed for the host cells (plasmid-free cells) than the plasmid-containing cells in the absence of IPTG induction (non-induction). This suggests that the host cells are free from the metabolic burden rendered by the recombinant plasmid. In the other recombinant *E. coli* cells, it has been observed that the host cell had higher growth yield and specific growth rate than the recombinant cell (16). As compared to the growth rates and yields after induction, it can be noticed that the growth of recombinant *E. coli* cell was significantly influenced by the time or growth phase at which IPTG inducer is introduced into the medium.

The expression level of βgal-preS2 showed 2- and 5-fold higher values for the middle induction than for early and late inductions, respectively. The high expres-

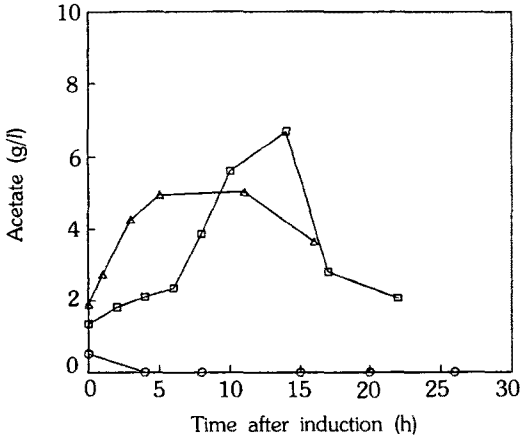

Fig. 6. Effect of IPTG induction time on acetate formation.
(○) early induction; (□) middle induction; (▲) late induction.

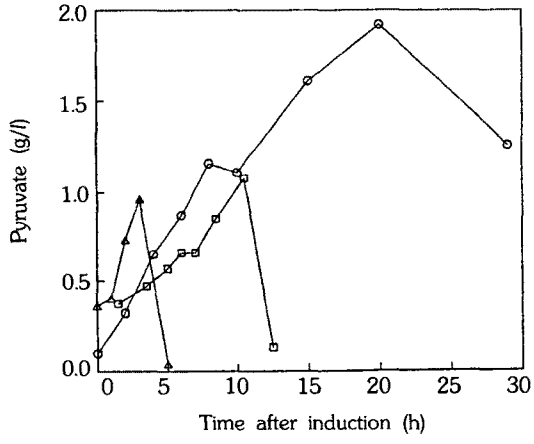

Fig. 7. Effect of IPTG induction time on pyruvate formation.
The symbols are the same as in Fig. 6.

sion level of βgal-preS2 can be explained by the efficiency of protein synthesis system (PSS) within cells. It is known that the efficiency of PSS is closely related to the number of active ribosomes, and the status of the ribosome is drastically changed from the inactive forms into the active ones with a preferable growth condition (6). These facts may, therefore, allow the cells in mid-log phase to possess a sufficient number of active ribosomes to synthesize the recombinant protein of βgal-preS2.

It was apparent that the accumulation or formation of acetate was closely related to the growth rate: i.e., at low growth rates acetate formation was negligible. In the other *E. coli* strains, it was also reported that acidic products did not accumulate up to a certain specific growth rate (5, 7). It is interesting to note that pyruvate produced was immediately consumed as soon as glucose in the culture broth was exhausted, while acetate consumption was delayed even after the glucose exhaustion. It can also be noted that the concentrations of acidic byproducts start decreasing as cell growth entered the stationary phase. Formation and utilization of pyruvate are, therefore, presumably related to the protein synthetic activity induced by IPTG addition. Andersen and Meyenburg (1) reported that *E. coli* cells under an unbalanced state between the capacity of glucose catabolism and the rate of respiration (oxidative phosphorylation) will produce and excrete byproducts via the fermentative energy metabolism (substrate level phosphorylation). In recombinant *E. coli* cell cultivations, it is most likely that the high concentration of acidic byproducts after IPTG induction may have resulted from unbalanced-growth state caused by the overproduction of βgal-preS2. It is, however, not yet fully understood how the glucose metabolism of the host cell is affected by the increased activity of the recombinant protein syntheses.

Although more experimental data should be collected to permit any generalization, it can be concluded that the production of cloned-gene products is strongly dependent on the induction timing of the gene expression, and induction in the mid-logarithmic growth phase is most appropriate for the production of the present recombinant protein.

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