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Conjugational Transformation of Wild Type *Bacillus halodurans* CM1 by Methylated Recombinant Plasmid Harbouring a Gene Encoding for Alkaline Protease and the Protease Activity Assay of the Transformant

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ABSTRACT

Protease contributes significantly to various industrial sectors, as shown by its increasing demand. An indigenous previously isolated bacteria, *Bacillus halodurans* CM1, is a wild-type bacterium whose ability to produce alkalotermophilic protease. In this study, the transformation of methylated pBBRE194 plasmid containing alkaline protease gene from this strain (PBBRE194-prot-CM1) into itself using a conjugation approach was conducted, and the measurement of the alkaline protease activity of the recombinant bacterium was carried out. The recombinant *B. halodurans* CM1 has been verified to carry the methylated pBBRE194 prot-CM1 by examining its ability to degrade protein in media containing skim milk and tetracycline, but also by amplifying tetracycline resistance gene sequence with 1,024 bp length of plasmid pBBRE194 prot-CM1 by PCR method from the recombinant bacterium. The results confirmed that recombinant *B. halodurans* CM1 was positively harboring plasmid pBBRE194 prot-CM1. Alkaline protease produced by recombinant CM1 reached higher activity than wild type between 18-36 h of cultivation. The alkalotermophilic wild type *B. halodurans* could accept the recombinant plasmid into their cells via conjugational transformation is firstly reported to our further knowledge.

1. Introduction

Proteases are the most popular and applicable enzymes in various industries (Naveed *et al.* 2021), including food (Tucker and Woods 1995), pharmaceutical (Drag and Salvesen 2010; Rao *et al.* 1998), textile (Haki and Rakshit 2003), and detergent industry (Rekik *et al.* 2019). The enzymes share a significantly large percentage (about 65%) of the total commercial industrial enzymes (Rao *et al.* 1998). The annual growth rate of the overall global market for protease was expected to increase by approximately 7% over the period from 2015 to 2020 (Thakur *et al.* 2018). The high demand for proteases in the industrial field could increase production efficiency, reduce toxic waste, work specifically, and be environmentally friendly (Razzaq *et al.* 2019).

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Nowadays, most commercial proteases originally come from *Bacillus* (Razzaq *et al.* 2019). To meet industrial requirements, a high level of protease production is needed. Genetic engineering, including gene cloning, is one of the solutions to improve the strain so that *Bacillus* can produce more significant amounts of proteases (Banerjee and Ray 2017; Razzaq *et al.* 2019). Rekik *et al.* (2019) successfully cloned the sapRH gene encoding detergent-applicable serine alkaline protease (SAPRH) from *B. safeness* RH12 to the plasmid pDESTTM17 and expressed it in *E. coli* BL21. Ariyaei *et al.* (2019) successfully cloned an alkaline protease gene (as animal feed) from *B. thuringiensis* C405 into plasmid pET28a and was transformed into *E. coli* BL21. However, the transformations of undomesticated wild-type *Bacillus* are still limited.

B. halodurans CM1 is a wild-type bacterial strain that has not been domesticated yet. It is an alkalothermophilic bacterium isolated from

Indonesia's local habitat and has the potential as an amylase, xylanase, gelatinase, and protease producer (Ulfah *et al.* 2011). Therefore, this indigenous alkalotermophilic protease-producing bacterium has the opportunity for further strain improvements by genetic engineering.

Previously, an alkalotermophilic protease gene from *B. halodurans* CM1 was isolated (Helianti *et al.* 2018). A shuttle *E. coli-Bacillus* plasmid vector pBBRE194 containing this protease gene has been constructed and successfully inserted in *B. subtilis* DB104 (Rahmawati *et al.* 2019); however, the introduction of the recombinant plasmid into wild type *B. halodurans* CM1 has not been successful yet. According to Wallace and Breaker 2011, methylated plasmid recombinant could be protected from restriction enzyme activity, and then efficiency transformation could be improved (Wallace and Breaker 2011).

This study transformed previously *in vivo* methylated pBBRE194 prot-CM1 plasmid (Priyanka *et al.* 2020) into *B. halodurans* CM1 using conjugation method. We also observed the protease activity of recombinant *B. halodurans* CM1 containing this recombinant pBBRE194 prot-CM1.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions

The plasmid used in this work was methylated pBBRE194 prot-CM1 plasmid. In contrast, the bacterial strains were *E. coli* S17-1 as a donor bacterium and *B. halodurans* CM1 as a recipient bacterium isolated from Cimanggu Hot Spring, Bandung, West Java, a collection of National Agency of Research and Innovation (BRIN)-Culture Collection, LAPTIAB-Puspiptek-Serpong. *E. coli* S17-1 was grown on an LB plate, and *B. halodurans* CM1 was grown on Horikoshi medium plate without antibiotics (Horikoshi 1971).

2.2. Conjugational Transformation of Methylated Plasmid to *E. coli* S17-1

Conjugational shuttle vector plasmid pBBRE194 containing alkaline protease gene from *B. halodurans* CM1 (pBBRE194 prot-CM1) has been described previously (Rachmawati *et al.* 2019). The *in vivo* methylation of this plasmid was also reported elsewhere (Priyanka *et al.* 2020). Preparation of *E. coli* S17-1 competent cells was conducted according to Hanahan (1983) and Inoue *et al.* (1990). Five µl (0.15 µg/µl) of methylated pBBRE194 prot-CM1 were mixed in 50 µl *E. coli* S17-1 competent cells and then homogenized. The plasmids were transformed into *E. coli* S17-1 competent cells by heat-shock method (Hanahan 1983). The *E. coli* S17-1 transformants were inoculated into LB plates containing 12.5 µg/ml tetracycline at 37°C for 17 hours. A single positive colony of *E. coli* S17-1 transformant was used as a donor bacterium.

The conjugation protocol was conducted based on Richardt *et al.* (2010) protocol. The donor strain was *E. coli* S17-1 harboring methylated pBBRE194 prot-CM1, and the recipient strain was *B. halodurans* CM1. The CM1 bacterial strain was grown in 5 ml of Horikoshi pH 8, and *E. coli* S17-1 harboring methylated pBBRE194 prot-CM1 was cultivated in 5 ml of LB containing 12.5 µg/ml tetracycline and ten µg/ml chloramphenicol. Cultivations were performed at 37°C for 17 hours. An Erlenmeyer flask with 30 ml Horikoshi medium pH 8.5 was subsequently inoculated with 3 ml of the overnight *B. halodurans* CM1 culture, then grown at 37°C until an OD_{600} = 0.7. Another Erlenmeyer flask with a 30 ml LB medium containing 12.5 µg/ml tetracycline and ten µg/ml chloramphenicol was subsequently inoculated with 3 ml of the overnight *E. coli* S17-1 harboring methylated pBBRE194 prot-CM1 culture and grown at 37°C until an OD_{600} = 0.7. Cells were collected by centrifugation at 4°C and 3,000 rpm for 15 min, and the pellet was washed twice in 15 ml holding buffer (12.5 mM K_2HPO_4, 12.5 mM KH_2PO_4, one mM $MgSO_4$, pH 8), repelleted by centrifugation at 4°C and 3,000 rpm for 15 min. Both pellets were mixed and resuspended in 30 ml of holding buffer. The mixture was compressed on a sterile nitrocellulose filter with a pore size of 0.45 µm. The filter was then placed on a sporulation medium pH 8 plate (Schaeffer *et al.* 1965), the cells forming the top layer, then incubated at 30°C for 48 h.

Grown cells were subsequently suspended in a 2 ml holding buffer and incubated at 80°C for 20 min. Two hundred fifty µl of culture were spread in dilution 10^{-7} on Horikoshi pH 8.5 plate (containing 2% (w/v) skim milk and 12.5 µg/ml tetracycline) and incubated at 37°C for 48 h. A single colony that exhibits a clear zone determined as recombinant *B. halodurans* CM1 was harboring methylated pBBRE194 prot-CM1.

2.3. Verification of Recombinant *B. halodurans* CM1 Containing Recombinant Plasmid

A single colony of wild-type *B. halodurans* CM1 and recombinant one was suspended in 10 µl ddH_2O, respectively. The suspensions were heated in the microwave for 1 min and incubated in ice for 5 min, repeated three times, then ready to be used as the template for polymerase chain reaction (PCR). The PCR reactions contained 5 µl GoTaq Green Master Mix [Promega] 2X, 0.5 µl primer forward (10 µM), 0.5 µl primer reverse (10 µM), 3.5 µl ddH_2O, and 0.5 µl template. The primers are Tet-F (5'-ATGAAATCTAACAATGCGCTCATCGTC-3') and Tet-R (5'-AGCCGCGAGCGATCCTTGAAG-3'). PCR reaction was started with an initial denaturation at 95°C for 2 min, continued with denaturation at 95°C for 30 sec, annealing at 60°C for 1 min, extension at 72°C for 1 min, repeated 30 cycles, and final extension at 72°C for 10 min, then hold at 4°C. The templates used were recombinant *B. halodurans* CM1, plasmid pBBRE194 prot-CM1 (as positive control), and wild-type *B. halodurans* CM1 (negative control). PCR results were visualized by electrophoresis using 0.8% agarose (w/v) and carried out at 100 V for 25 min.

2.4. Protease Production

A single colony of recombinant *B. halodurans* CM1 harboring pBBRE194 prot-CM1 was cultured in 5 ml of Horikoshi pH 9 containing 12.5 µg/ml tetracycline. The culture was incubated overnight at 37°C for bacterial growth. One point five ml of the overnight culture was reinoculated into 15 ml of the same medium as mentioned before. *B. halodurans* CM1 wild-type were also cultured similarly to recombinant one. Both cultures were grown in vigorous shaking at 37°C until OD_{600} = 0.8. Then, the culture could be used as a starter for producing the crude enzyme. Ten ml of each starter cultures were re-inoculated into 100 ml of Mamo medium (0.5% (w/v) peptone, 0.1% (w/v) KH_2PO_4, 0.2% (w/v) NaCl, 0.01% (w/v) $MgSO_4$, 0.01% (w/v) $CaCl_2$, dan 1% (w/v) Na_2CO_3) pH 9 (Mamo *et al.* 2006) containing 4.37% (w/v) corncobs, respectively. Cultures were incubated in a shaking incubator at 150 rpm and 37°C for 36 hours. Five hundred µl of each culture was sampled every 6 h for enumerating bacteria population (Total Plate Count), protease activity assay, and protein concentration. The crude enzyme was collected by centrifugation at 9,000 rpm at 4 C for 10 min, and the supernatant was stored at 4°C.

2.5. Bacterial Growth Observation

The diluent solution was composed of 0.85 g of NaCl in 100 ml of water which was then sterilized in an autoclave at 121°C for 15 min. The wild-type of *B. halodurans* CM1 culture was grown for 6, 12, 18, 24, 30, and 36th hours were dissolved into a sterile diluent solution to obtain 10^{-6}, 10^{-8}, 10^{-8}, 10^{-11}, 10^{-14}, and 10^{-17}, respectively. Whereas the recombinant *B. halodurans* CM1 culture was grown for 6, 12, 18, 24, 30, and 36th h, were dissolved into a sterile diluent solution to obtain 10^{-5}, 10^{-7}, 10^{-7}, 10^{-10}, 10^{-13}, and 10^{-14}, respectively. The dilution reaction is taken from each microtube using a micropipette of 250 µl and then inoculated into a sterilized Horikoshi pH nine plate. Each dilution is done in duplicate. The plates are incubated in an incubator for 24 hours at 37°C. The grown colonies in the plate are calculated in a formula:

$$\frac{\text{number of colonies}}{\text{volume x factor of dilution}} \quad \text{CFU/ml}$$

2.6. Protein Concentration and Enzyme Assay

Protein estimation was measured as described by Bradford (1976). Quantitative measurements of protease activity were performed as described by Folin and Ciocalteu (1938) and Anson (1938) and modified by the Amano K protease assay (Amano 2007) . Tyrosine is an amino acid used as a result of protein hydrolysis. One unit of enzyme activity was defined as the number of enzymes that produce one µg tyrosine per minute. A comparison of protease activity between recombinant and wild-type bacterium was measured at 50°C and pH 12.

3. Results

3.1. Verification of the Presence of Methylated Plasmids in *B. halodurans* CM1

A clear zone from recombinant *B. halodurans* CM1 grew in Horikoshi Medium pH 8.5 containing 2% skim milk, and 12.5 µg/ml tetracycline after 48 hours at 37°C was found (Figure 1). The suspected recombinant *B. halodurans* CM1 colony was verified using the PCR method. The result showed a DNA band of 1,024 bp of the tetracycline resistance gene in pBBRE194 prot-CM1 (Figure 2).

3.2. Bacterial Growth, Protein Concentration, and Protein Activity

The growth of the wild-type *B. halodurans* CM1 population was higher than that of the recombinant strain within 36 h. The total population of wild-type and recombinant within 36 hours increased to 3.1 x 10^{19} CFU/ml and 4.04 x 10^{15} CFU/ml, respectively (Figure 3).

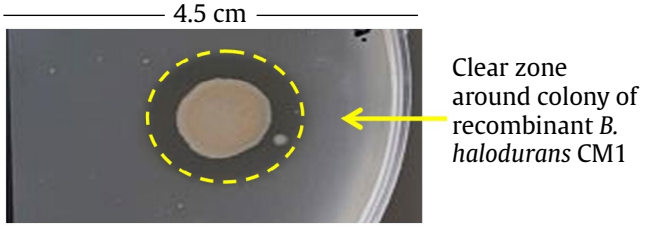

Figure 1. Clear zone from recombinant *B. halodurans* CM1 that grown in Horikoshi Medium pH 8.5 containing 2% skim milk and 12.5 µg/ml tetracycline after 48 hours at 37°C

3.3. Protease Activity

The protease activity of recombinant CM1 reached a higher value significantly than that of wild-type cultivation between 18-36 h. The protease activity of recombinant CM1 after 24 h reached above 1,000 U/ml, whereas the wild type was still less than that (Figure 3).

4. Discussion

In nature, lateral gene transfer is considered to prompt the evolution of bacteria, including natural transformation, bacterial conjugation, and transduction (von Wintersdorff *et al.* 2016). Via bacterial conjugation, DNA can move from one bacterium to another, although the bacteria are phylogenetically distant (Burton and Dubnau 2010; Chen *et al.* 2005). Bacterial conjugation was first discovered in *E. coli*. DNA can be seceded

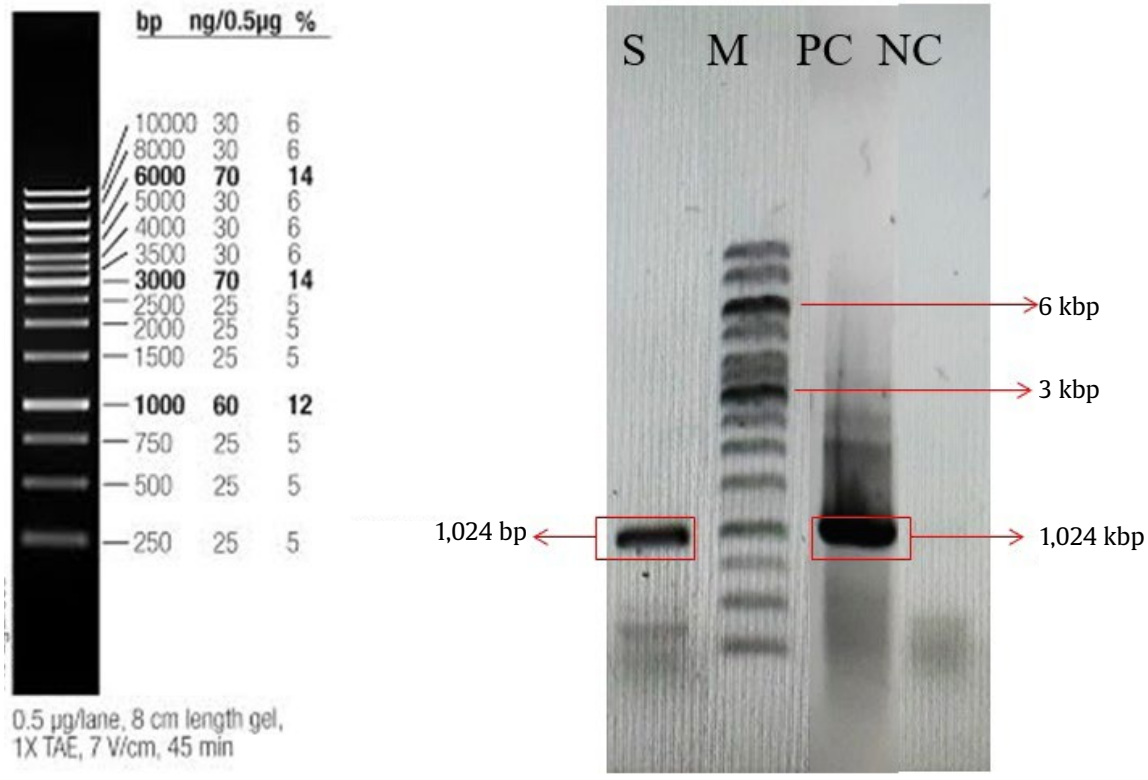

Figure 2. Amplification result of tetracycline resistance gene from pBBRE194 prot-CM1 in recombinant *B. halodurans* CM1 colony. S: sample (recombinant *B. halodurans* CM1 colony), M: DNA ladder 1 kb [Thermo Scientific], PC: positive control (PBBRE194 prot-CM1 plasmid), NC: negative control (wild type *B. halodurans* CM1 colony)

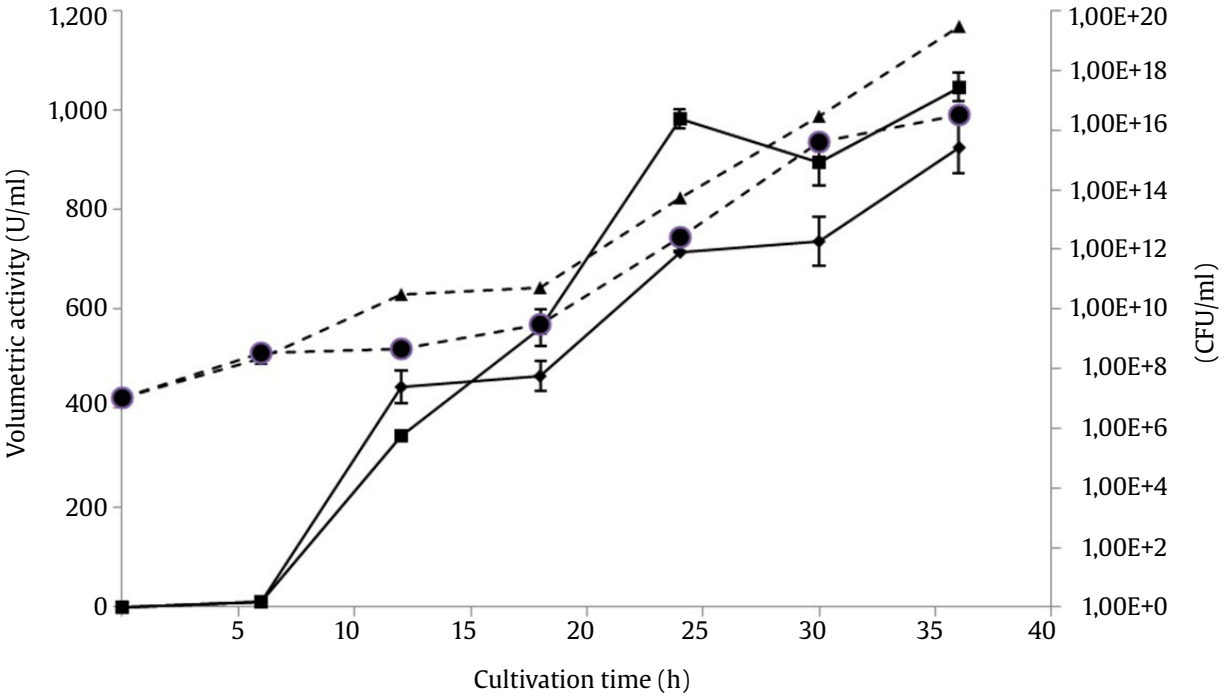

Figure 3. The growth curve and the protease activity of wild type and recombinant *Bacillus halodurans* CM1. -▲- : The growth of wild type *B. halodurans* CM1, ●: the growth of recombinant *B. halodurans* CM1,◆: protease activity of wild type *B. halodurans* CM1, ■: protease activity of recombinant *B. halodurans* CM1

from a donor cell and moved into a recipient cell through intense contact with bacterial cells during matings (Lederberg and Tatum 1946). This bacterial conjugation inspired the gene cloning technology in transforming naturally incompetent wild type bacterial strains to be able to receive exogenous genes; hence, it could be a helpful tool for introducing the genetic modifications into various biotechnologically relevant but challenging to transform bacteria such as wild type Lactobacilli (Samperio *et al.* 2021). The problematic DNA transformation was also found in *B. halodurans*, and the study of this species was often hindered. However, genetic manipulation of *Escherichia coli* or *B. subtilis* is routine (Wallace and Breaker 2011). As a source of potential industrial enzymes, our indigenous *B. halodurans* CM1 (Ulfah *et al.* 2011) must be transformed to improve the strain. Thus, in this study, the conjugational transformation of *in vivo* methylated plasmid pBBRE194 containing alkaline protease gene into wild type *B. halodurans* CM1 was carried out.

The cell densities of recipient and donor bacterium have to reach the mid-exponential OD. The intense contact occurs by compressing both recipient and donor on the surface of a sterile nitrocellulose filter and allowing matings to proceed for 24 hours. In this study, *E. coli* S17-1 was transformed with the plasmid pBBRE194 prot-CM1 and afterward served as the donor bacterium in matings with *B. halodurans* CM1 based on Richhardt *et al.* (2010) that used *E. coli* S17-1 as donor strain to transform the pJR1 plasmid into *B. megaterium* DSM 319 using conjugation protocol. A similar protocol was also performed for transforming the cellulase gene into *B. megaterium* (Helianti *et al.* 2014).

The successfully obtained *B. halodurans* CM1 harbored pBBRE194 prot-CM1, as proven by the resistance against tetracycline and PCR colony using specific tetracycline-resistant genes. Previously, several attempts to transform recombinant shuttle vectors containing protease gene or xylanase gene into wild type *B. halodurans* CM1 via protoplasts have been conducted (data was not shown); however, the transformation has not been successful (Rachmawati 2019). Introducing recombinant plasmids into target bacteria can be hampered by restriction-modification (RM) in bacteria cells (Priyanka *et al.* 2020). Therefore, to attain stable recombinant clones, the recombinant plasmid containing protease gene has been *in vivo* methylated before the genetic transformation. Using the methylated recombinant plasmid, positive recombinant colonies have been obtained. However, only one transformant showed plasmid presence stability. The *in vivo* methylation of plasmid also increased the rate of transformation of other strains of *B. halodurans* (Wallace and Beaker

2011), alkaliphilic *Bacillus* sp. (Gao *et al.* 2011), and *Methylomonas* sp. DH-1 (Ren *et al.* 2020).

In this study, recombinant *B. halodurans* CM1 showed a slower growth rate than wild-type within 36 hours of cultivation. The slower growth of recombinant bacteria might be due to cells using the metabolic energy for plasmid maintenance. The plasmid-free strain, with time, will outcompete the plasmid-containing strain (Ashby and Stacey 1984).

However, even though the bacterial growth was slower, the protease activity of recombinant CM1 that had two alkaline protease genes in their cells (in chromosomal DNA and plasmid) was higher. At optimal conditions, the highest protease activity could be produced within 24-30 hours (896.61±18.21 U/mg) by recombinant *B. halodurans* CM1 instead of 30 hours (814.32±48.72 U/mg) by wild-type. The ability of recombinants to produce higher protease activity proved that the protease gene in plasmid pBBRE194 prot-CM1 could be appropriately expressed in recombinant *B. halodurans* CM1.

In conclusion, the protease gene in methylated pBBRE194 prot-CM1 was successfully transformed by conjugation into *B. halodurans* CM1 verified by a clear zone in selective media and detecting tetracycline resistance gene by PCR. Recombinant protease produced by recombinant *B. halodurans* CM1 exhibited higher activity than wild-type, and the highest recombinant protease activity was at 24th-hour cultivation with conditions 50°C and pH 12. Therefore, it was considered alkalothermophilic protease. This result is the first report that the alkalotermophilic wild type *B. halodurans* could accept the recombinant plasmid into their cells via conjugational transformation.

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