



Molecular identification of isolates from local microorganisms as potential biofertilizer

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ABSTRACT

Local microorganisms (MOL) are liquid fertilizers commonly used by farmers to help increase crop production. Beneficial microbes in MOL need to characterize their interactions and ability to produce growth drive compounds. The purpose of this research is to identify the superior microbial isolates from MOL made by farmers from Cibodas Lembang Bandung, Indonesia that can produce phytohormones as biofertilizers. The results of the microbial selection of MOL derived from three best microbes are 1A-2 NFB, 4A-1 NFB, and 4B-1 NFB with the ability to produce auxin, i.e., 19.41 ppm, 17.18 ppm, and 10.59 ppm, respectively. The compatibility test between the three isolates showed negative results so that it was possible to apply three microbes as a consortium. The results of a molecular identification with a 16S rRNA analysis indicate strain microbe 1A-2 NFB: *Bacillus cereus* (99.88% homology), 4A-1 NFB: *Bacillus cereus* (99.76% homology), and 4B-1 NFB: *Lysinibacillus* sp. (99.88% homology).

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1. INTRODUCTION

The use of biological fertilizer can be a solution to increasing the efficiency of using chemical fertilizer. Biofertilizer is an active biological product consisting of a single microbe or a consortium that can improve fertilization efficiency, soil fertility, and soil health. Biofertilizers are live microbes that are given into the soil as inoculants to facilitate or provide certain nutrients for plants. Soil microorganisms such as *Rhizobium*, *Azospirillum*, *Bacillus* sp., *Burkholderia* sp., *Azotobacter*, phosphate-solubilizing bacteria, N₂-fixed bacteria, *Mycorrhiza*, *Tricoderma* sp., and several types of other microbes used as biofertilizers that are already widely available in the market, such as *Bion-Up* biological liquid fertilizer that is produced by Pupuk Kujang Cikampek Company and *Provibio* production of the Indonesian Association of Seed Banks and Technology (AB2TI) Bogor.

One of the current constraints is the lack of knowledge about the use of bioagents both the type of microbes and their interaction with plants (Hadi et al., 2019). Stated that to achieve the goal of improving productivity, efficiency, and

sustainability of farming, the principle of providing accurate nutrients according to plants need (balanced fertilization). The main requirement in the process of making biofertilizer is to find superior microorganisms, for example, can come from bamboo Apus roots (*Gigantochola apus*) that contain the bacteria *Pseudomonas fluorescens* and could increase the solubility of P in the soil (Yulistiana et al., 2020), and *Bacillus cereus* 11UJ isolated from the rhizospheric zone of sugarcane is widely used for antifungal activity tests (Suryadi et al., 2015). Later, these microorganisms can pass through various tests, including the pathogens test, phytohormonal test, and compatibility test. These tests become the standard quality of biological fertilizers that can have a positive impact when used by farmers (Walida et al., 2019). The utilization of biofertilizers is based on a positive response to the effectiveness and efficiency of fertilization so that it can save the cost of fertilizer and labor; of course, it can be achieved if the ability of isolates contained in biological fertilizer (Moelyohadi et al., 2012).

Potential sources of microorganisms can be explored from various ecosystems, both in the extreme ecosystem and nearby ecosystem of farming. The adaptability and survival of the microbes in the field conditions are the basis for choosing microbes as biofertilizers (Pratiwi et al., 2020). One of the potential sources of microbes can originate from local microorganisms (MOL). Local microorganisms can be sourced from various local materials, such as cow urine, banana stem, gamal leaves, fruit waste, rice stale, household waste, bamboo shoots, and elephant grass and can play a role in the process of livestock waste management, both solid wastes to be used as compost and livestock liquid waste.

In this research, the selection and identification of the superior microorganisms originating from MOL Farmer Village production Cibodas Lembang Bandung, Indonesia that can potentially become biofertilizer were performed. It is important to know the potential microbes of MOL origin used by some Cibodas village farmers. If there is already an isolate in isolation is expected next Cibodas farmers do not need to make MOL again but simply multiply the microbe isolation results and then used them on agricultural land.

2. MATERIALS AND METHODS

The results of the pathogens test in the *blood agar* medium and tobacco leaves indicated 33 isolates later in the application on the seed germination of peppers (Sodiq et al., 2019) from test application on the efficiency of select 10 isolates with the best sprout growth power to be followed by testing the ability to produce an auxiliary phytohormone (IAA).

2.1. Growth hormone production test

A total of 10 samples were grown at a temperature of 28°C for 24 h on an NB medium by Merck to screen isolates. A hundred microliters of culture were added to 10 ml of the selective medium consisting of 0.136% KH_2PO_4 , 0.213% Na_2HPO_4 , 0.02% MgSO_4 , 4.7 H_2O , and 10 ml trace element (700 mg $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 300 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 40 mg $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 20 mg $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 3 mg H_3BO_3 , 7 mg $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 4 mg Na_2MoO_4 , H_2O , and 1 ml H_2SO_4 for a total volume of 1 liter) that has been added with 1 ml of L-tryptophan (10% glucose, 1% L-tryptophan, and 0.1% yeast extract filtered using a 0.2 m millipore filter). Next, the isolated culture was incubated at 28 °C for 48 h. Once, it is done centrifugation culture at a speed of $6,000 \times g$, at a temperature of 4 °C for 10 min to separate the bacterial cells with its supernates. One milliliter of the supernatant of the culture was taken and coupled with 2 ml reagents (1 ml 8.12% $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 50 ml HClO_4 35% in the dark vial) and incubated at 28 °C for 25 min. The absorption reading was at a length of 530 nm using a spectrophotometer. Auxin concentration (IAA) in each sample was compared with the standard IAA curve.

The absorbance was done in triplicate for each type of bacteria followed by Gupta et al., 2012. The analysis of gibberellin and cytokines (Zeatin & Kinetin) was performed by modification of methods (Linskens & Jackson, 2012) with a working step, take 5 ml of the sample and add 20 ml of

MeOH 65% solvent extraction in homogenizing by using the vortex for 2–3 minutes then in the at 4,000 rpm for 30 minutes and filtered using Whatman 42 next take the supernatant and in strain using millipore and samples in the Injection 5–10 μl to HPLC with wavelength 254 Ω using a UV-Visible detector C-18 Phase motion Methanol 65% and a column temperature of 40°C.

2.2. Compatibility test

Compatibility testing is needed to create a consortium culture. A microbial consortium is a group of microbes that live together and interact with both the fellow and the plant. The method used refers to Hidayati et al. (2014). Compatibility test grown on an NA medium in the petri dish and then incubated for 24–72 hours at room temperature. Furthermore, there is or is not clear zone/inhibition (positive results) that formed in the intersection growth of three types of isolates. When a clear zone is formed, it signifies the nature of antagonism between bacteria so that it cannot be used in a consortium; if it does not form a clear zone, the bacteria are compatible with one another.

2.3. Identify bacteria using rDNA 16s sequencing

Identification of molecular bacteria began with the inoculation of bacteria in NB media as much as 10 mL for 3 days. Deoxyribonucleic acid (DNA) isolation of bacterial genome was performed using the Sodium Dodecyl Sulfate (SDS) method. The culture was planted from the solution by being centrifuged at a speed of 8,000 rpm for 10 minutes. The pellets were washed by using a buffer of tris-HCl ethylenediaminetetraacetic acid (EDTA)-TE as much as 1 mL until it forms a suspension and then centrifuged again. Supernatant discarded and pellet plus 200 μl of TE buffer and added SDS 10% as much as 40 μl and incubated in a waterbath at 65°C for 90 minutes. Suspension cooled at room temperature then added proteinase-K as much as 10 mg/mL. The DNA suspension is stored in incubators at 37 °C for 4 hours, and phenol and chloroform were then added as much as 120 μl to emulsion form. DNA solutions were homogenized using the reverse of the 2 mL Eppendorf tube containing DNA and then centrifuged at a speed of 8,000 rpm for 10 minutes. The suspension containing DNA and removed into the 2 mL Eppendorf tubes a new and presipitation using ethanol 100 μl . Pellet DNA results in precipitation coupled with 40 μl sterile aqua-bidest and used as stock DNA. Total DNA checking is performed through agarose gel electrophoresis. Gel used is 0.8 g agarose dissolved in 100 mL tris acetic NaEDTA (TAE) 0.5 times. Electrophoresis was carried out by filling tank electrophoresis with a buffer TAE 0.5 times. Printed agarose gels are inserted into the tank that contains the TAE buffer until the agarose gel is flooded. Total DNA used as much as 2 μl mixed with 3 μl of a loading buffer. The DNA mixture with buffer loading is inserted into the agarose gel well and electrophoresis. Electrophoresis result gel is colored by being soaked in the Ethidium ipratropium bromide (EtBr) solution for 10 minutes and rinsed using aquadest for 5 minutes. The undocumented agarose gel uses a digital camera on an ultraviolet illumination (UV) transilluminator.

The polymerase chain reaction (PCR) process began with making PCR composition with a total volume of 75 µl consisting of 7.5 µl Buffer PCR, 2.25 µl MM Deoksinukleosida triphosphate (DNTP), 1.5 µl 10 MM MgSO₄ 10 µl Primary 165f, 10 µl Primer 165r, 1 µl of DNA templates, 1 µl Taq DNA polymerase, and 60.25 µl of sterile aqua-bidest. PCR stages performed as many as three cycles consisting of (1) pre-denaturation at 94 °C for 30 seconds, (2) annealing at 50 °C for 30 seconds, (3) the polymerization (extension) at 70 °C for 2 minutes, (4) the final cycle of polymerization (post extension) for 7 minutes, and (5) cooling at a temperature of 4 °C. PCR results were visualized using a 1% agarose gel electrophoresis in a solution 0.5 Buffer TAE. PCR products performed sequencing analysis by using a DNA sequencer tool. Template DNA sequence uses the sequence kit method by Roche®. The sequence result is compared to data from the GenBank National Center for Biotechnology Information through the stage of the Local Alignment Search Tool (BLAST) on the <http://www.ncbi.nlm.nih.gov/BLAST> site to achieve the highest level of homology in the nucleotide sequence. A total of 15 strands of DNA sequences from GenBank including the DNA sequences of isolate samples are compiled with the FASTA3 format for the performance of similarity analysis using the ClustalW program from the www.ebi.ac.uk/clustalW site. The results obtained were stored on the TreeConW software, which was subsequently used to create a phylogenetic tree in the Phylip format using the program with bootstrap 100× replication. The type and number of access DNA sequences are used for the manufacture of phylogenetic trees.

3. RESULTS

3.1. Growth hormone production test results

Based on Table 1, the result IAA indicated by isolates 4a-1 NFB, 1A-2 NFB, and 4B-1 NFB shows only three isolates were continued to measure gibberellin and cytokinin (zeatin & kinetin). The results of the measurement of gibberellin hormones, zeatin, and kinetin are the best results shown by the isolates of 1A-2 NFB; they indicate that the isolates of 1A-2 NFB derived from the bamboo root MOL have the ability to produce the best phytohormones. The results of the test measurement phytohormone 10 isolates selected can be seen in Table 1.

3.2. Compatibility test results

The results of the compatibility of three isolates selected the growth hormone production test results in showing no inhibitory zones formed in all isolates tested. This indicates that the bacteria are compatible with one another and can be used in a consortium. As isolates 1a-2 and 4B-1 in Figure 1 do not form a clear zone on the petri dish that has been overgrown isolate 4A-1. Figure 2 indicates that the isolate of 1A-2 and 4A-1 does not form a clear zone on the petri dish that has been overgrown with an isolate of 4B-1.

Figure 3 indicates that isolates 4a-1 and 4B-1 do not form a clear zone; there is a petri dish that has been overgrown with isolate 1a-2. These results can support the possibility of the consortium's use of isolates. The use of microbes is expected to provide a more optimal impact on the

application media. The effectiveness of microbial utilization determined its ability to adapt to the environment.

3.3. Molecular identification results

Three isolates (1A-2, 4A-1, and 4B-1) on molecular identification results with 16S rRNA analysis are 1A-2 NFB as *Bacillus cereus* (99.88% homology), 4A-1 NFB as *Bacillus cereus* (99.76% homology), and 4B-1 NFB as *Lysinibacillus* sp. (99.88% homology). Subsequent sequences of nucleotides and phylogenetic trees are presented in Table 2 and Figure 4. Table 2 indicates that the result of three isolates that are identified with codes 1A-2, 4A-1, and 4B-1 are bacteria.

4. DISCUSSION

The best three isolates selected to produce phytohormones are identified as *Bacillus cereus* (99.88% homology) from MOL bamboo root, *Bacillus cereus* (99.76%) from MOL rabbit manure, and *Lysinibacillus* sp. from rabbit feces MOL (99.88%) (Table 2 and Figure 4). There is still one more condition so that the microbes that have been identified can be used as a biofertilizer by farmers, namely the test of the ability to dissolve nutrients (PTM biofertilizer no. 01 of 2019) but with a sample of bacteria that is proven to be able to produce phytohormones, three isolates can be a strong basis for use as a biological fertilizer where it is widely known to benefit phytohormones and applications simultaneously (consortium) of microbes if it has been proven not to give each other negative impacts (Figures 1, 2, and 3).

In this study, the ability to produce the auxiliary phytohormone (IAA) (Table 1) became the basis for choosing the best isolates as a potential biological fertilizer. Microbial ability to produce IAA is expected to be able to have a positive impact when applied to plants. The selected microbes are also intended to allow for pre-selected isolates to be applied in a consortium; a consortium of microbes is expected to provide more optimal results in multi-site and multi-variety experiments.

Table 1. Phytohormone observation results

No.	Isolate Code	Concentration (ppm)			
		IAA	Gibberellin	Zeatin	Kinetin
1.	1A-2 NFB**	14.09	3.149	0.584	0.352
2.	2A-3 NFB	1.88	-	-	-
3.	3A-3 NFB	2.39	-	-	-
4.	3B-1 NFB	1.56	-	-	-
5.	3B-2 LG	2.39	-	-	-
6.	4A-1 NFB*	19.21	2.993	0.536	0.252
7.	4A-3 NFB	1.21	-	-	-
8.	4B-1 NFB***	9.68	3.080	0.475	0.209
9.	5A-1 NFB	0.062	-	-	-
10.	5B-2 NFB	3.20	-	-	-

Remarks: The isolate code indicates that the first number is the MOL making number, the next letter is a repetition of the creation of MOL, the next number is the isolate sequence that is isolated from the MOL number and the string of letters at the end is the media code in the initial isolate selection.

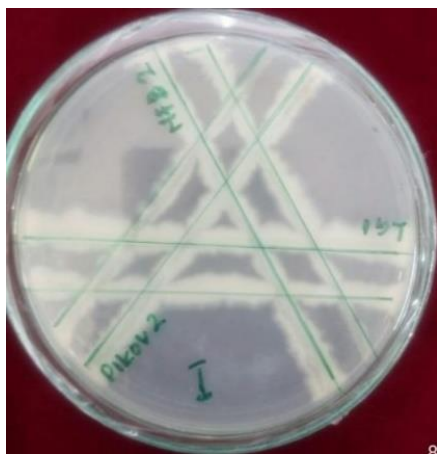


Figure 1. Isolates 4A-1 media test and overgrown by isolates 1A-2 and 4B-1



Figure 2. Isolates 4B-1 media test and overgrown by isolates 1A-2 and 4A-1

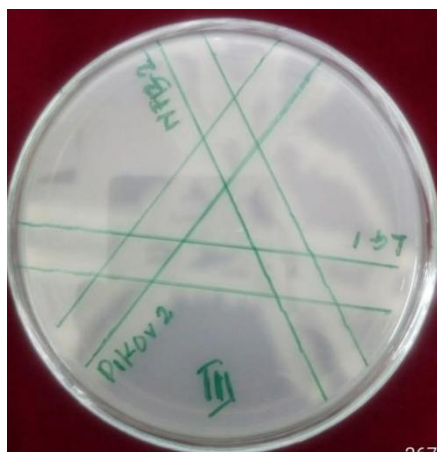


Figure 3. Isolates 1A-2 media test and overgrown by isolates 4a-1 and 4B-1

It is supported by the opinion (Iswati, 2012) that the bamboo roots that have been weathered are suspected to contain bacteria that can produce cellulase enzymes. Microbe-secreted hormones can stimulate enzymatic processes, which biologically increase seed germination and accelerate the synthesis of organic nitrogen compounds at the roots.

Phytohormones play an important role in the growth and development settings of plants. Phytohormones are important regulators for the growth of plants that control various processes such as cell division, cell differentiation,

organogenesis, and morphogenesis (Bielach et al., 2012). The auxin phytohormone is synthesized by all high-level crops. IAA serves to stimulate root growth such as initiation and development of lateral roots, gravitropism, and root extensions. Phytohormones are widely found in seed embryos and active meristematic tissues such as crop shoots and root end. In this study, the three best auxins (IAA) phytohormone producers were continued to test their ability to produce other phytohormones.

The biological approach of utilizing a consortium of phytosphere microbial and microbial of rhizosphere that has the ability to inhibit N, dissolve P, and secrete the plant growth hormone is one step of reducing the use of inorganic fertilizer and improving the quality of the environment (Pas et al., 2015). This formulation used to make bioorganic fertilizer is usually enriched with microbes using various types of microbes as a consortium (Sukmadewi et al., 2017). The ability of enzyme work from each different microbial type A consortium of microbes tends to provide more optimal results compared to the use of single isolates (Nafiah & Prasetya, 2019). These constraints can be answered by utilizing microbes in the form of a consortium. Microbial applications in the form of a consortium can reduce the risk of failure to use microbial in the field (Pas et al., 2015). Currently, it is difficult to find biofertilizers that only use a single isolate; almost all biofertilizers that have been circulating in the market are biofertilizers with a consortium of microbes.

Molecular identification tests are conducted to determine the type of microbes, it is important to know the specific types and capabilities of these microbes. So, in the next experiment, the ability of a biofertilizer and a biological fungicide can be better explored. The result of the microbial identification test from MOL is known as *Bacillus cereus* and *Lysinibacillus* sp. (Table 2). Known bacteria have a high potential as fertilizers. One of the isolates that can be used as a bioinsecticide and bio fungicide is isolates of 1A-2 *Bacillus thuringiensis* (homology 99.65%). To reduce the attack of the *Ostrinia furnacalis*. The *B. thuringiensis* and *B. subtilis* bacteria groups are reported to have antibacterial power through the production of extracellular enzyme chitinase, with secondary metabolites reducing the length of mycelium and fungal colonies. Also reported as *Bacillus cereus* 11UJ which was isolated from the land of the rhizosphere of sugarcane plants in Jember area can be attributed to chitin in mushroom cell wall (Suryadi et al., 2013). The results of the research related to *Bacillus subtilis* and *Lysinibacillus* sp. reported many such bacteria as potential bioagents that can have a positive impact on crop protection. Some antagonistic microbe can control pathogens with resistance induction mechanisms, such as *Bacillus subtilis* and *Lysinibacillus* sp. (Leiwakabessy et al., 2018; Pieterse et al., 2014).

Table 2. Identification of isolates with codes 4A-1,1A-2, and 4B-1

Isolate Code	Result The sequence of nucleotides (% similarity)
1A_2	<i>Bacillus cereus</i> (99.88% homology)
4A_1	<i>Bacillus cereus</i> (99.76% homology)
4B_1	<i>Lysinibacillus</i> sp. (99.88% homology)

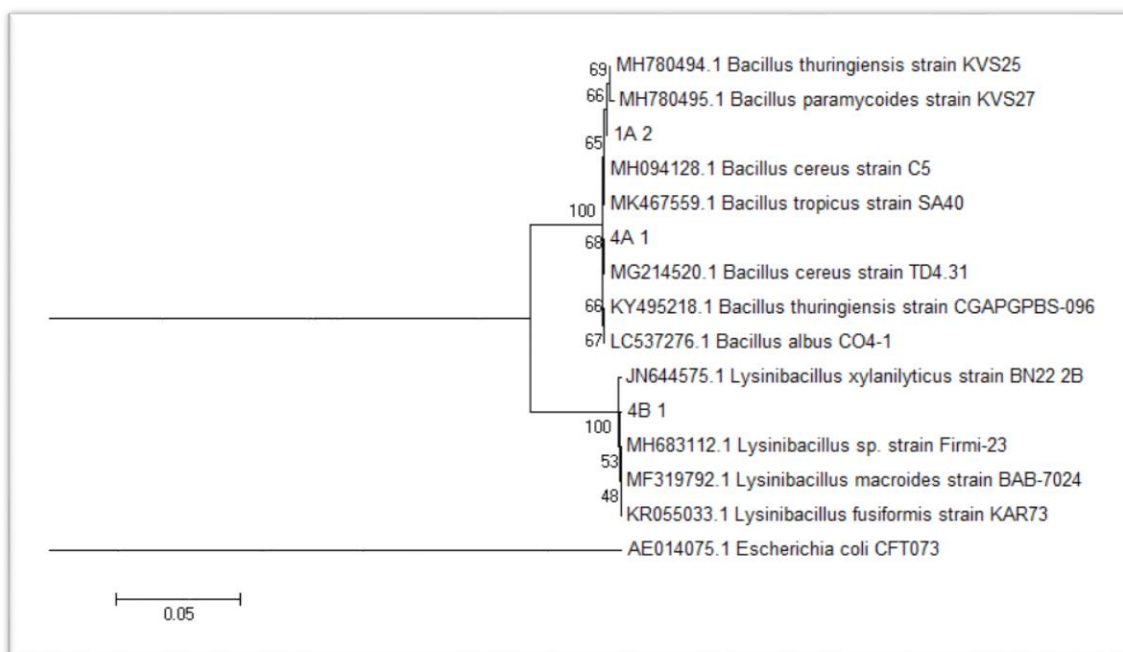


Figure 4. Result of phylogenetic tree of three isolates (1A-2, 4A-1, and 4B-1)

According to [Leiwakabessy et al. \(2018\)](#), *Lysinibacillus* sp. is an endophytic bacterium capable of the systemic induction of resistance to *Xanthomonas oryzae*, the cause of bacterial leaf blight in rice. The ability of an antagonistic microbe to suppress the development of pathogens can be increased by conducting a consortium or merger ([Hersanti et al., 2019](#)). Also reported a mixture of *Bacillus subtilis* and *Lysinibacillus* sp. in nano-silica, as well as carbon fiber, is able to increase the resistance of red onion plants with inhibition of purple spot disease intensity by 71.2% ([Hersanti et al., 2019](#)).

Bacteria *B. subtilis* and *B. cereus* have high antifungal activity against the pathogenic fungus *Fusarium solani*. Bacteria in the *Bacillus* sp. produce antifungal compounds that may result in hypha growth being abnormal (malformations). In addition, the activity of the enzyme chitinase causes the mushroom cell wall to be lysed ([Abidin et al., 2015](#)). The secondary metabolite resulting from the bacteria extract *B. cereus* 11uj can inhibit the growth of mushrooms *R. solani* and *P. oryzae* which is good enough at concentrations of 1,000 ppm, and the antifungal activity produced more effectively inhibits the growth of *P. oryzae* ([Suryadi et al., 2015](#)). One mechanism of emphasis by the endophytic bacteria in the genus *Bacillus* is antibiosis, through the generation of a bulbiformin antibiotic that is toxic to various plant pathogens ([Marsaoli et al., 2019](#)). Bacterial isolates are potential as biofertilizers that produce enzymes; bacteria as a source of enzymes are more beneficial because of their rapid growth. They can grow on the inexpensive substrate, and more easily improved results can be achieved through the setting of growth conditions.

5. CONCLUSION

The best microbes are 1A-2 NFB, 4A-1 NFB, and 4B-1 NFB with the ability to produce Auxin (IAA) of 19.41 ppm, 17.18 ppm, and 10.59 ppm, respectively. Molecular identification results indicate 1A-2 NFB, 4A-1 NFB as *Bacillus cereus*, and

4B-1 NFB as *Lysinibacillus* sp. Further testing should be conducted on the functions of the three isolates that have been identified to meet the biofertilizer distribution permit.

Declaration of Competing Interest

The authors declare no competing financial or personal interests that may appear and influence the work reported in this paper.

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