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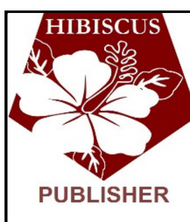

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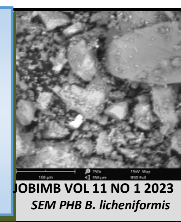

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Characterizing Atrazine Degradation by Molybdenum-reducing *Pseudomonas* sp.

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ABSTRACT

The most feasible and economical technique for removal of toxic compounds in the polluted environment is bioremediation. This technique surpasses other physicochemical methods in recent time for being effective particularly at a lower concentration of the toxicant. In this study, seven (7) previously isolated molybdenum-reducing bacteria were screened for their potential to degrade atrazine herbicide as sole carbon source for growth. Bacterial colony count on mineral salt medium supplemented with atrazine was used for the screening, while the effects of incubation time, concentration, temperature, pH, inoculum size and heavy metals on atrazine biodegradation was used in characterizing the candidate isolate. Of the seven isolates, an isolate identified as *Pseudomonas* sp. that grew best with a count of 195 CFU/mL was chosen. The optimum conditions supporting atrazine degradation by *Pseudomonas* sp. were found to be temperature 35 °C, pH 7.0, incubation time 48 hours and 400 µL inoculum. The use of atrazine as carbon and electron donor source for molybdenum reduction, poorly support molybdenum blue (Mo-blue) production. At a concentration (2 ppm), heavy metals such as lead and copper did not significantly ($p>0.05$) affect atrazine biodegradation relative to control, iron and silver shows a relative stimulatory effect to the process, while mercury and zinc showed significant ($p<0.05$) inhibitory effect when compared to control. The ability of the isolate to degrade atrazine makes it an important instrument for bioremediation of this herbicide.

INTRODUCTION

Bioremediation of toxic compound in polluted environment is currently considered as the most economical and eco-friendly approach, particularly at a lower concentration of the toxicant, where other physicochemical techniques are ineffective [1]. World-wide, herbicides have been widely utilized for controlling noxious or undesirable plant growth generally referred to as weeds in crop and non-crop areas and this has contributed greatly to modern agriculture [1,2]. The use of herbicides in weed control has grown significantly in Nigeria in recent years [3]. This increase has been linked to the ease of application, and the effectiveness of the herbicides in controlling the common weeds. These are in addition to the growing difficulty in hiring labor to carry out the traditional method of manually cutting off the weeds. Most of the applications of the herbicides are, however, carried out by illiterate farmers who apply the herbicides indiscriminately, mostly because they had not received any training on the judicious application of the herbicides [4]. This poses a significant threat to the environment, crop yield and on human health, as studies have indicated that such indiscriminate use of herbicides can leach into the environment, threatening non target organisms, harm soil microorganisms, thus affecting soil fertility and can accumulate in harvested crops, possibly causing direct harm to humans, upon the consumption of the harvested crops [5,6].

One of the most widely used herbicides in agriculture is atrazine (2-chloro-4-ethylamino-6-isopropylamino-1,3,5-triazine) [7-9]. It is primarily applied as a selective herbicide for controlling broad-leaf and some grassy weeds in crop production such as corn, maize, pineapple, sorghum and sugar cane [8-11] and as a non-selective herbicide for non-cropped fallow lands and industrial lands [12]. Nevertheless, the long-term and excessive

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application of atrazine could lead to its high concentration presence and persistence in the soil which could be dissipated through leaching into ground water and drinking water as well as washed (as run-off) into surface water, if applied prior to irrigation or heavy rainfall [13]. In a USEPA report in 1988, the maximum atrazine contaminant level in drinking water is 3.0 gL^{-1}. However, the type of soil determines the mobility and environmental fate of atrazine through absorption to soil particles. Atrazine is more readily adsorbed on muck or clay soils than on soils of low clay and organic matter content, therefore, adsorption to certain soil constituents significantly limits the downward movement or leaching [14,15]. Atrazine is persistent in water and together with its metabolites (which are often more hazardous than the parent product) can accumulate in drinking water sources downstream from farms. As of 2001, atrazine is the most commonly detected pesticide contaminating drinking water in the US with 75% of surface water and 84% of all groundwater in agricultural areas tested by the US Geological Survey contaminated with atrazine [16].

Atrazine is a non-polar, non-volatile and low soluble compound [8]. It is also a chlorotriazine and has in its structure a hexameric aromatic ring, symmetrical, composed by three carbon atoms and three nitrogen atoms in alternated positions (**Fig.** 1).

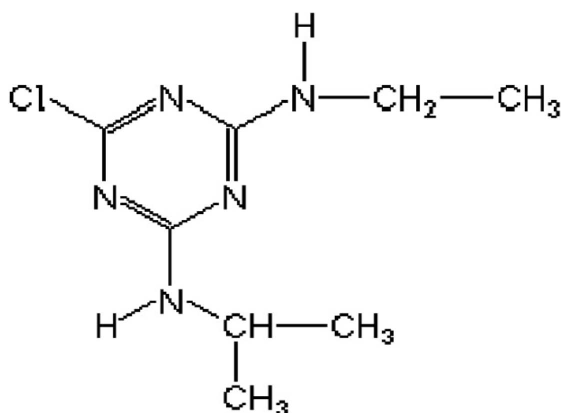

Fig. 1. Chemical structure of s-triazine herbicide atrazine.

The degradation of atrazine occurred predominantly by biological process, and microbial activity has been deemed as the most influential that significantly facilitate removal of atrazine [17]. Many microorganisms with potential to degrade atrazine have been isolated and characterized. These include strain of Pseudomonas [18], Acinetobacter [19], Rhodococcus [20], Arthrobacter [21], Bacillus [22], Variovorax etc. [17]. Environmental atrazine removal involves either biotic transformation processes mediated by microorganisms, or abiotic processes such as chemical and photochemical reactions [23]. Until present, report on microbial degradation of atrazine from Northern Nigeria is scarce or even lacking despite the high Agricultural practices in this region, Similarly, the continuous indiscriminate use of this noxious herbicide on the farmlands that may eventually wash-off and contaminate the environment necessitate the search for a microbe with potential to biodegrade the herbicide. This research, characterized the atrazine-degrading potential of previously isolated molybdenum-reducing *Pseudomonas* sp.

MATERIALS AND METHODS

Chemicals and equipment

The materials used were autoclave (RAV-530D, Stockholm, Sweden), Weighing machine (SPO 401, China), Biosafety cabinet (AC2-4E8, Singapore), Incubator (063105, England), Spectrophotometer (6705, United Kingdom). All chemical reagents and media ingredients utilized in this research were of analytical grade, obtained from Biochemistry Lab Bayero University Kano. The equipment used were washed with 10% (v/v) nitric acid, thoroughly rinsed and sterilized by autoclaving at 121 °C, 115 Kpa for 15 minutes. Atrazine (ultrazine; active ingredient atrazine 80% WP) was manufactured by Zhejiang Zhongshan Chemical Industry Group Co., Ltd. Zhongshan, Xiaopu, Changxing, Zhejiang, China. All the experiments were done on triplicate to minimize experimental Error. Likewise, all inoculations were done on a bio-safety cabinet with laminar air-flow to avoid contamination with other microorganisms.

Media and Media Preparation

Mineral Salt Medium

The medium was prepared by dissolving (g/L), NaCl: 0.5 g, $(NH_4)_2SO_4$: 2 g, $MgSO_4.7H_2O$: 0.2 g, CaCl: 0.01 g, $FeSO_4.7H_2O$: 0.001 g, Na_2HPO_4: 0.6 g, KH_2PO_4: 1.5 g into a liter of distilled water and the pH was adjusted to 7.0 prior to sterilization by autoclaving at 121 °C,115 Kpa for 15 minutes. Atrazine was also autoclaved separately and was added to the medium afterwards.

Low Phosphate-Molybdenum Medium (LPM)

This medium was prepared according to the method of [24] with slight modification. Briefly, into a liter of deionized water, $(NH_4)_2SO_4$: 3 g, $MgSO_4.7H_2O$: 0.5 g, NaCl: 5 g, $Na_2MoO_4.2H_2O$: 2.42 g, Na_2HPO_4: 0.71 g, yeast extract: 0.5 g and glucose: 10 g were dissolved, and the medium was adjusted to pH 7.5 prior to autoclaving at 121°C, 115 kPa for 15 minutes. Glucose was separately autoclaved and added to the medium afterwards.

Isolates Screening for Atrazine Biodegradation

To the mineral salt medium, 3 g of Agar-agar was added prior to sterilization by autoclaving at 121 °C, 115 kPa for 15 minutes. Atrazine was also autoclaved separately and added to the medium afterwards. The molten medium was allowed to cool about 50 °C before pouring into sterilized and disposable Petri dish (the agar-agar helps the medium to solidify on the Petri dish). 200 µl of the different bacterial suspension were inoculated into the Petri dishes and kept at 30 °C in an incubator, and were checked for bacterial growth every 24 hours.

Effect of Incubation Time on Atrazine Biodegradation

Onto the mineral salt medium (pH 7.0), 200 µl of the bacterial suspension was inoculated and incubated at 30 °C for the period of 72 hours in order to determine the optimum incubation time for atrazine biodegradation by *Pseudomonas* sp. Optical density was checked at 600 nm starting from 0 hour every 24 hours.

Effect of Concentration Atrazine Biodegradation

The effect of atrazine concentration on growth of *Pseudomonas* sp. was ascertained by varying its concentration from 0.5 – 10 g/L (0.5, 1, 2, 4, 8 and 10 g/L) in mineral salt medium pH 7.0, inoculated and incubated at 30 °C for the period of 72 hours Optical density was checked at 600 nm starting from 0 hour every 24 hours.

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Effect of Temperature on Atrazine Biodegradation

The effect of different temperature on the biodegradation of atrazine was studied in a temperature-controlled water bath to determine the optimum temperature. The temperature ranges were between 25 – 45 °C (25, 30, 35, 40 and 45 °C) and the optical density was checked as before.

Effect Initial pH on Atrazine Biodegradation

The initial pH of the mineral salt medium was adjusted from 5.5 – 8.0 (pH 5.5, 6.0, 6.5, 7.0, 7.5 8.0 and 8.5) prior to autoclaving at 121 °C, 115Kpa for 15 mins. Optical density was checked as before.

Effect of Inoculums Size on Atrazine Biodegradation

Various amount (50, 100, 200, 400, 600, 800 and 1000 µL) of the bacterial suspension was used in the mineral salt medium. Optical density was checked as before.

Effect of Atrazine as Carbon Source of Molybdenum Bioremediation

Low phosphate-molybdenum media prepared according to [24] containing glucose (5 g/L) was used as control and in test sample, glucose was replaced with 4 g/L atrazine to determine if it can be used as carbon source for molybdenum bioreduction. Optical density was checked at 865 nm for molybdenum-blue production.

Effect of Heavy Metals on Atrazine Biodegradation

Different heavy metals (mercury, zinc, copper, iron, lead and silver) were used at 2 ppm on this study in order to determine their interaction with atrazine biodegradation. Optical density was checked at 600 nm.

RESULTS

Screening for Atrazine Biodegrading Isolates

Table 1 shows the colony count for the screening of isolates with potential to grow on atrazine supplemented media. From the table, isolate G have the highest number of colonies compared to others qualifying it as a better candidate for atrazine biodegradation.

Table 1. Screening for atrazine degrading bacterial isolate.

Isolate	Colony count (cfu/mL)
A	180
B	105
C	68
D	98
E	123
F	65
G	195

Effect of Incubation Time

The effect of incubation time on atrazine biodegradation by *Pseudomonas* sp. was determined over a period of 72 hours. From the result obtained in **Fig. 1**, the optimum incubation time for atrazine degradation was 48 hours. A significant decrease ($p<0.05$) in growth was observed beyond 48 hours.

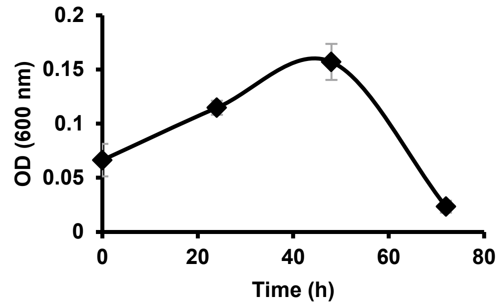

Fig 1. Effect of incubation time on atrazine biodegradation by *Pseudomonas* sp. after 72 hours of incubation. Data represent mean ± standard deviation of triplicate.

Effect of Atrazine Concentration

The effect of concentration on atrazine biodegradation by *Pseudomonas* sp. presented in **Fig. 2**. The result shows that growth of this bacterium was optimum at a concentration of 4 g/L. Growth of this isolate significantly reduced ($p<0.05$) at concentrations below and above the optimum.

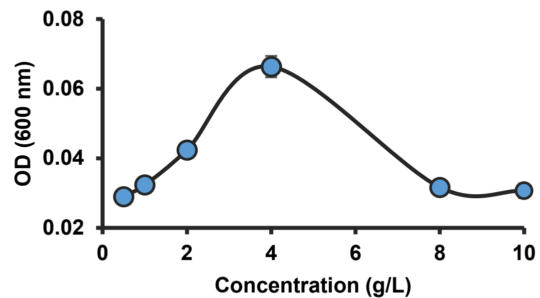

Fig 2. Effect of concentration on atrazine biodegradation by *Pseudomonas* sp. Data represent mean ± standard deviation of triplicate.

Effect of Temperature

Various temperature ranges (25 °C - 45 °C) were used in this study, the result shown in **Fig. 3** indicates that 35 °C as the optimum temperature supporting atrazine biodegradation by *Pseudomonas* sp. was 35 °C. Analysis of variance (ANOVA) shows a significant decrease ($p<0.05$) in growth at temperatures below and above the optimum except for 40 °C that show the difference to be not significant ($p>0.05$).

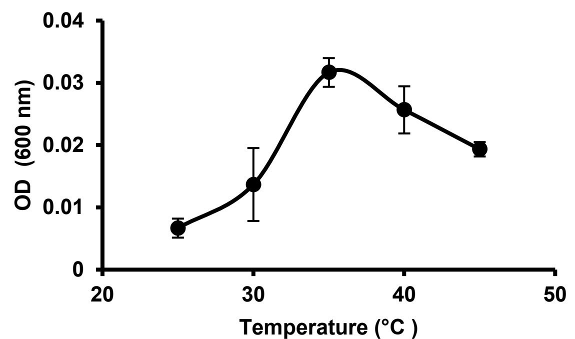

Fig 3. Effect of different temperature on atrazine biodegradation by *Pseudomonas* sp. after 24 hours of incubation. Data represent mean ± standard deviation of triplicate.

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Effect of initial pH

Various pH ranges 5.5-8.5 were used in this study, the result in **Fig. 4** shows that the optimum pH was 7.0, with significant ($p<0.05$) growth reduction at pH beyond and below the optimum pH.

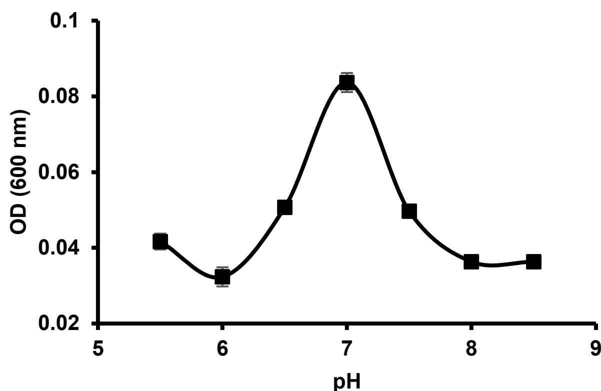

Fig 4. Effect of optimum pH on atrazine biodegradation by *Pseudomonas* sp. Data represent mean ± standard deviation of triplicates.

Effect of Inoculums Size

In this study, various inoculums sizes were used ranging from 50-1000 µL. From the result obtained (**Fig**. 50, 400 µl was found to be the optimum inoculum. Analysis of variance (ANOVA) shows a significant decrease ($p<0.05$) above the optimal inoculum.

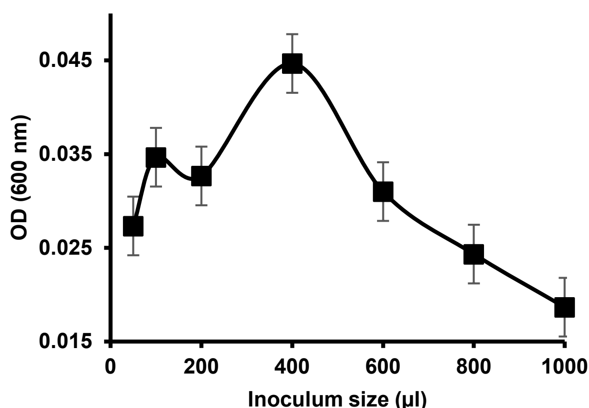

Fig 5. Effect of optimum inoculums size on atrazine biodegradation by *Pseudomonas* sp. Data represent mean ± standard deviation of triplicates.

Atrazine of as Carbon Source of Molybdenum reduction

In study, glucose in the low phosphate medium was replaced with atrazine (**Fig**. 6) to determine if can be used as a source of carbon for molybdenum reduction by *Pseudomonas* sp. The result shows no significant difference ($p>0.05$) in Mo-blue produced after 24 hours of incubation. However, further incubation above 24 hours reveals significant difference ($p<0.05$) in Mo-blue produced in the low phosphate medium supplemented with glucose and that supplemented with atrazine as carbon sources.

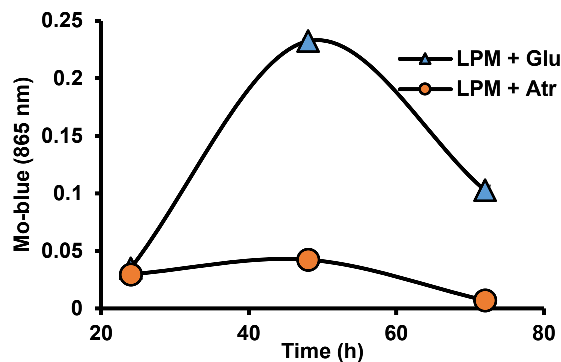

Fig 6. Effect atrazine as carbon source of molybdenum-blue production by *Pseudomonas* sp. Data represent mean ± standard deviation of triplicate.

Effect of Heavy Metals Interaction

Various heavy metals viz; copper, iron, lead, mercury, silver and zinc were used in this study to determine their inhibitory or stimulatory effects on atrazine biodegradation by *Pseudomonas* sp. (**Fig**. 7). The result shows that lead and copper did not significantly ($p>0.05$) affect the process relative to control, iron and silver shows a relative stimulatory effect, while mercury and zinc showed significant ($p<0.05$) inhibitory effect when compared to control.

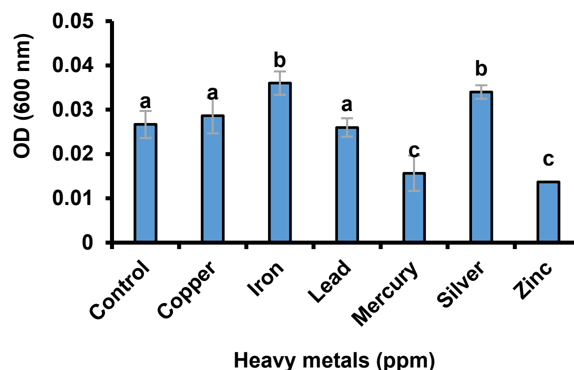

Fig 7. Effect of heavy metals interactions on the biodegradation of atrazine by *Pseudomonas* sp. Data represent mean ± standard deviation of triplicates.

DISCUSSION

The fate of commonly used herbicides such as atrazine in natural environments has been thoroughly investigated in the past 40 years [25]. The biodegradation of atrazine and other herbicides in the environment is a complex process. Microorganisms such as bacteria and fungi are the key agents of bioremediation, with bacteria assuming the dominant role and fungi becoming more significant. However, factors influencing the degradation, such as the content and concentration of the atrazine, the physico-chemical environmental conditions and the composition of the microbial consortia, decide the rate of the overall microbial degradation process.

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Most of the reports on atrazine biodegradation use *Pseudomonas* sp. as their isolates of degradation [26,27]. However, other isolates have been used such as *Bacillus, Pseudomonas, Klebsiella, Actinomycetes, Nocardia, Streptomyces, Actinomycetes, Escherichia coli*, *Azotabacter* and *Alcaligenes* [28].

Perhaps, the significant increase in the bacterial population observed as the incubation period progresses from 0 to 48 hours (**Fig**. 1) was due to changes from lag phase to the stationary phase through exponential phase utilizing atrazine as sole carbon source. Similarly, the decrease observed after 48 hours was due to bacterial cell's death that occurs as a consequence of substrate (atrazine) depletion. The decrease in growth observed at lower concentration of atrazine is due to the bacteria gearing-up to acclimatized and degrade atrazine as the only source of carbon for growth, while at high concentrations, decease in the growth is attributable to the inhibitory effect of atrazine to its degradation (**Fig**. **2**). This was similarly reported in the biodegradation most xenobiotics [1,17,18,25-27].

Temperature is an important environmental variable in bioremediation technologies. Most microorganisms have temperature optimum at which they grow and produce enzymes that affect catalysis. These enzymes are thermo-unstable. Hence, temperature below or above the optimum can result in total loss of their catalytic function. Therefore, the significant difference observed was probably due to the bacteria been cooled as a result of the low temperature as such the enzymes may not likely to degrade the atrazine faster at 25 °C than it does at the optimum. Many reports recorded an optimum temperature of around 30 – 40 °C. The report of [26] and [29] has an optimum temperature of 35 °C and 37 °C respectively which interestingly are in line with this finding. However, [27] and [30] reported an optimum of 30 °C each. The optimum temperature recorded (**Fig**. **3**) may be due to the fact that the bacterium (*Pseudomonas* sp.) was isolated from the temperate region.

A pH is the measure of acidity, neutrality and alkalinity of a given medium, most bacteria produce enzymes that affect catalysis at a particular pH range and are liable to irreversible denaturation when the pH is altered. Their ability to survive in higher or lower pH depends on their capacity to regulate the pH of the intracellular and extracellular environment. Thus, it is necessary to determine the pH range for optimum growth. To date, nearly all the optimum pH reported was around 7.0 [31-33], however, a recent work [34] reported an optimum pH of 9.0. Similarly, another report [29] indicates an optimum pH between 5.0-8.0.

This is the first report on the use of molybdenum-reducing bacteria for atrazine degradation from Nigerian soil. Interestingly, finding an isolate with a capacity to treat multiple xenobiotics is advantageous to bioremediation process [1]. Determination of carbon source to be used in a bacterial media is an important factor in biodegradation process because it gives an idea of the carbon source preferable to the bacteria, as bacteria like all other organism, need a source of nutrient. We report for the first time the use of atrazine herbicide as carbon and electron donor source for molybdenum reduction. Although, when tested, little Mo-blue was produced, indicating that atrazine could be utilized as carbon source in molybdenum bioremediation, but not as the preferred electron donor source, as seen when glucose was used as control (**Fig**. **6**).

The possible heavy metals interaction has also been studied, the presence of these metals in the bacterial media will either inhibits or stimulates the biodegradation process by *Pseudomonas* sp. There is scarcity of report on heavy metals interaction with atrazine biodegradation, only of recent that [34] reported that copper inhibits the process, while zinc facilitates the bioremediation of atrazine by *Klebsiella variicola* strain FH-1.

CONCLUSION

The screening of previously isolated molybdenum-reducing bacteria for atrazine degradation, confirms isolate G identified as *Pseudomonas* sp. as best atrazine degrader by virtue of its highest colony counts. The characterization *Pseudomonas* sp. for atrazine degradation revealed optimum conditions as incubation time of 48 hours, concentration at 4 g/L, temperature at 35 °C, pH of 7.0 and inoculum size of 400 µL with iron and silver facilitating the degradation, while zinc and mercury as inhibitors to atrazine degradation. Thus, this bacterium is a suitable candidate that can utilize atrazine contaminant for molybdenum bioremediation. Research is ongoing to explore the potentials of this isolate to degrade other xenobiotics.

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