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ISOLATION AND IDENTIFICATION OF RHIZOSPHERIC BACTERIA ASSOCIATED WITH LEMONGRASS FOR POTENTIAL BIOREMEDIATION

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Abstract

Contamination of soil and groundwater pollution is a severe problem, has been attracting considerable public attention over the last decades. With the demand for green and cleaner technology for remediation process, there is an increased interest in moving away from conventional technologies towards bioremediation technologies. Rhizospheric zone is a suitable place for harboring bacteria that are capable to utilize chemical compounds which will be used either to facilitate growth of bacteria or the host plants. Identification of the specific microbial members should allow for better strategies to enhance biodegradation. This study aimed to isolate and identify the rhizospheric associated microbes of lemongrass (*Cymbopogon citratus*), a plant that commonly available in South East Asia, which could be used in future research on degradation studies of dibenzofuran. This probably is due to their ability to harbor large numbers of bacteria on their highly branched root systems. A total of 68 strains of dibenzofuran (DF)- degrading bacteria isolated from the rhizospheric soil of lemon grass from 2 different unpolluted sites were characterized. The isolates showed the ability to utilize dibenzofuran as the sole carbon and energy source up to 40 ppm. Identification of the isolates based on 16S rRNA gene sequence assigned them as members of the phyla Proteobacteria and Firmicutes, among which those of the genera, Proteobacteria were most abundant. The presented results indicated the potential of these bacterial isolates in bioremediation of dibenzofuran-contaminated soil.

Keywords: Rhizospheric, lemongrass, dibenzofuran-utilizing bacteria, bioremediation, *16S rRNA* gene

1.0 INTRODUCTION

Dioxin is a group of chloroaromatic compounds which includes Polychlorinated dibenzo-p-dioxin (PCDDs) and Polychlorinated dibenzofuran (PCDFs) or generally known as dioxins and furans respectively.

Contamination of dioxins, poses one of the most challenging problems in environmental science and technology because of their toxicity, persistence, and mutagenic properties [1] as they can strongly attach to solid surfaces and not easily leach from soil or sediment [2]. In short, their extreme persistence in the environment and bioaccumulation in the food chain makes them great environmental and human health risks that require remedial action [3]. The increased level of this toxic group has been linked with many incidents of human health risks which include cancer [4].

In recent years a number of studies have focused on biodegradation of dioxin and related-compounds, including biphenyl, dibenzofuran, and dibenzo-*p*-dioxin using microorganisms, which is a subject of major concern in environmental microbiology in connection with bioremediation of polluted environment. A wide variety of microorganisms have shown to possess the ability to degrade some highly chlorinated dibenzofuran (DFs) and dibenzo-*p*-dioxin (DDs).

Several bacteria that utilize DF as a sole source of carbon and energy have been isolated, including Gram-negative (*Burkholderia xenovorans* strain LB400 [5], [6], *Pseudomonas resinovorans* strain CA10 [7], *Rhodococcus sp.* strain YK2 [8], *Sphingomonas wittichii*

strain RW1T [9], [10], [11], [12] *Klebsiella sp.* [13] and some *Terrabacter* strains [14], [15], [16], [17], [18] now classified as members of the genus *Janibacter* [19] or Gram-positive such as *Staphylococcus* sp. [20].

In terms of their vitality, adaptability to various surroundings and the relative ease of modifying functions through molecular biological techniques, bacteria may be more efficient than other organisms to counteract environmental dioxin pollution [21].

According to Glick, 2010 [22], some rhizosphere microorganisms can act directly on organic pollutants using their own degradative capabilities (phytostimulation or rhizodegradation). Highly branched and widely distributed roots of grasses such as lemon grass (*Cymbopogon citratus*) enable them to cover a large surface area per unit volume of soils and enable to harbor large numbers of bacteria thus increase the potential of rhizoremediation process. The screening of bacterial strains that inhabit the rhizospheric zone of lemon grass is important in order to identify the potential bacteria that possess the capability to degrade xenobiotic compounds.

Furthermore, it is necessary to discover or construct novel bacteria for a successful bioremediation in nature. In this experimental study, DF was used as substrate in which the bacteria that capable of degrading dibenzofuran (DF) as the sole source of carbon and energy were isolated and characterized.

2.0 EXPERIMENTAL

2.1 Soil Sampling

The soil sample used in this study was collected from the rhizospheric zone (1-3 mm) of lemon grass with no history of previous dioxin pollution at two different sites, Permatang Badak, Kuantan and IIUM Kuantan Campus. The plant residues and soil fauna were removed. Prior to the collection of samples, the pH of the soil was monitored. Ten grams of rhizospheric soil were mixed with sterile distilled water and spread on plates containing Luria Bertani (LB) medium. The sample was incubated at 30 °C. The population density of the target bacteria was described as CFU/µL.

2.2 Screening of DF-degrading Bacteria

Dibenzofuran (DF) with CAS number 132-64-9 was used in screening studies. Bacterial isolates were cultured using fresh M9 minimal medium supplemented with dibenzofuran as sole carbon and energy sources. Bacterial growth was measured in different concentration of dibenzofuran; 2.5 ppm, 10 ppm, and 40 ppm. Cultures were incubated at 30 °C for 48 hours in an incubator shaker at 200 rpm. Bacterial growth was monitored by measuring the optical density of the culture at 600 nm (OD_{600}).

2.3 Extraction and Amplification of Bacterial DNA

The extraction of total DNA were performed using QIAamp DNA Mini Kit directly according to protocol provided by manufacturer. Polymerase Chain Reaction (PCR) were performed to amplify the *16S rRNA* gene from the genomic DNA of bacteria selected from the rhizospheric soil of lemongrass. PCR amplification was performed using Mastercycler® gradient thermocycler (Eppendorf). The PCR mixture used contained 10 µl of the extracted DNA, 5 µl of each primer, 2.5 µl of dNTPs, 1.5 µl *Taq* DNA polymerase, 12.5 µl PCR buffer, 8.5 µl $MgCl_2$ and 55 µl sterile dH_2O to a final volume of 100 µl. *16S rRNA* gene were amplified using primers 27F (5'-AGAGTTTGATCMTGGCTCAG- 3' and 1492R 5'-GGTTACCTTGTTACGACTT-3' (purchased from 1st BASE Sdn Bhd, Malaysia). These primers were specifically amplified the *16S rRNA* (~1.5 kb) sequences of the DNA template. The PCR amplification were performed according to the following temperature profile: Initial denaturation at 95°C for 5 min, 30 cycles of denaturation (95 °C for 1 min); annealing (55 °C for 1 min); extension (72 °C for 1 min) and final extension at 72 °C for 10 min. PCR amplicons were analyzed by agarose gel electrophoresis. NucleoSpin® Gel and PCR Clean-up (*Macherey-Nagel*) were used for purification process. The procedure was carried out according to manufacturer's protocol. The samples were sent for further sequencing service at 1st BASE Sdn Bhd, Malaysia.

2.4 Sequence Analysis and Phylogenetic Tree Construction

Basic Local Alignment Search Tool (BLAST) were used for similarity searches between sequences obtained. Sequences that show high similarity with the sample were chosen from the NCBI database for phylogenetic analysis and *Sulfolobus acidocaldarius* were used as an outgroup. The evolutionary distances were computed using the Kimura 2-parameter method [23] and are in the units of the number of base substitutions per site. Phylogenetic analysis were done using software MEGA version 6.0 [24]. Neighbour-joining method were used to show the relatedness and a distance matrix of all samples.

3.0 RESULTS AND DISCUSSION

3.1 Isolation and Characterization of DF-Utilizing Strain

In the present study, the isolation and characterization of rhizospheric bacteria from lemon grass, which could utilize dibenzofuran as the sole source of carbon and energy, were investigated. It was found that total 68 bacterial strains were isolated from the rhizospheric soil of lemon grass with no history of previous dioxins pollution at two different sites, Permatang Badak, Kuantan and IIUM Kuantan Campus that were able to grow in M9 minimal media containing dibenzofuran as substrate through enrichment culture. Various researchers have also reported a highly variable potential of different strains of bacteria that capable to degrade dibenzofuran as carbon and energy sources [25], [26], [27], [28], [29], [30]. Hence, all the isolates have been shown to possess the ability to utilize dibenzofuran (DF) as the sole carbon and energy sources. DF-degrading isolates were identified phylogenetically based on 16S rRNA gene sequence analysis.

Most of the isolates achieved their highest cell number within 12 hours of incubation with optimum growth temperature at 30 °C. The results showed the growth of all strains, expressed as the increased turbidity (OD_{600}). According to previous results observed by Simarro *et al.* [30], the addition of substrate with different concentration in the culture had significant influenced in biodegradation. In essence, assessment of the optimal conditions would make the bioremediation process more effective. These findings suggested that all of the isolates showed the highest growth rate when supplemented with 40 ppm dibenzofuran compared to 10 ppm and 2.5 ppm. It was further noted that, the level of degradation was concentration dependent. The concentration of the compound of interest, thus important for the optimal growth of bacterial cells increases the efficiency of the degradation process. This study has shown that at 2.5 ppm and 10 ppm concentration, the cell growth decreased after 48 hours. This result indicated that there is high tendency of insufficient substrate in the culture which is to provide a carbon source for the bacteria. Hence, if the concentration is too low, bacteria would not be able to multiply as they use the available carbon source to generate energy for cell activity that is vital to sustain their survival. However, if the concentration is too high, it could be toxic to the cell or it could lead to accumulation of metabolites to the level that is toxic to the cells. Therefore, the optimum concentration was at 40 ppm as most strains were able to achieve the highest cell number when being incubated at 30 °C with the initial OD_{600} of 0.05.

The following conclusion can be drawn from the present study where all isolated bacteria from rhizosphere zone able to utilize dibenzofuran (DF) as the sole sources of carbon and energy and have potential to be used in degradation studies in order to degrade contaminated of dibenzofuran.

3.2 16S rRNA Gene Based Identification and Phylogenetic Analysis Using Neighbour-Joining Method

Phylogenetic analysis allows the identification of bacteria genus, species and also subspecies level through both distance-based and character based method. Commonly, the Neighbour-Joining tree plot is a distance based method where the evolutionary distance between the unknown bacteria and their most possible bacteria identity was studied.

The evolutionary history was inferred using the Neighbor-Joining method [31]. The optimal tree with the sum of branch length = 6.56354003 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches [32]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The analysis involved 98 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 573 positions in the final dataset.

Neighbour-Joining method compares the result, evolutionary distance of each sequence with database sequence. From the above result, it showed that isolate, JH-1, JH-2, JH-3, JH-5 and JH-47 is most similar to *Pseudomonas fulva* with distances of 0.000. Likewise, JH-16, it was closely related to *Burkholderia sp.* with distance of 0.002. For sample NA-21, it is most similar to *Pseudomonas sp.* with distances of 0.000. NA-4 is most similar to *Chromobacterium sp.* by 0.000 distances. On the other hand, NA-2, NA-8, NA-9, NA-12, NA-15, NA-17, NA-18, NA-20, and JH-28 were closely related to *Stenotrophomonas maltophilia* with 2.679 base substitutions per site. Based on Figure 1, the phylogenetic trees show that NA-2, NA-8, NA-9, NA-12, NA-15, NA-17, NA-18, NA-20, and JH-28, are in the same clade. While for isolate NA-10, it is most similar to *Staphylococcus saprophyticus* with a distance of 0.000. Another isolate of NA-14 and NA-16, it was closely related to *Staphylococcus succinus* with a distance of 0.000. Besides, NA-11 is most similar to *Aeromonas sobria* with distances of 0.004. Isolate JH-12, JH-35 and JH-38 with a distance of 0.000 most similar to *Klebsiella sp.*. Next, isolate JH-17, JH-18, JH-19, JH-20, JH-21, and JH-22 with 0.025 base substitutions per site represent *Citrobacter werkmanii*. Whereas, the isolate of NA-1, NA-3, NA-5, NA-6, NA-7, NA-13, NA-19 is very likely represented *Enterobacter mori* with distance of 0.000. Lastly, isolates of JH-4, JH-6, JH-7, JH-8, JH-9, JH-11, JH-13, JH-15, JH-23, JH-24, JH-25, JH-26, JH-27, JH-30, JH-33, JH-34, JH-36, JH-37, JH-39, JH-40, JH-41, JH-43, JH-44, JH-45 was closely related to *Pantoea agglomerans*, by 0.000 base substitutions per site.

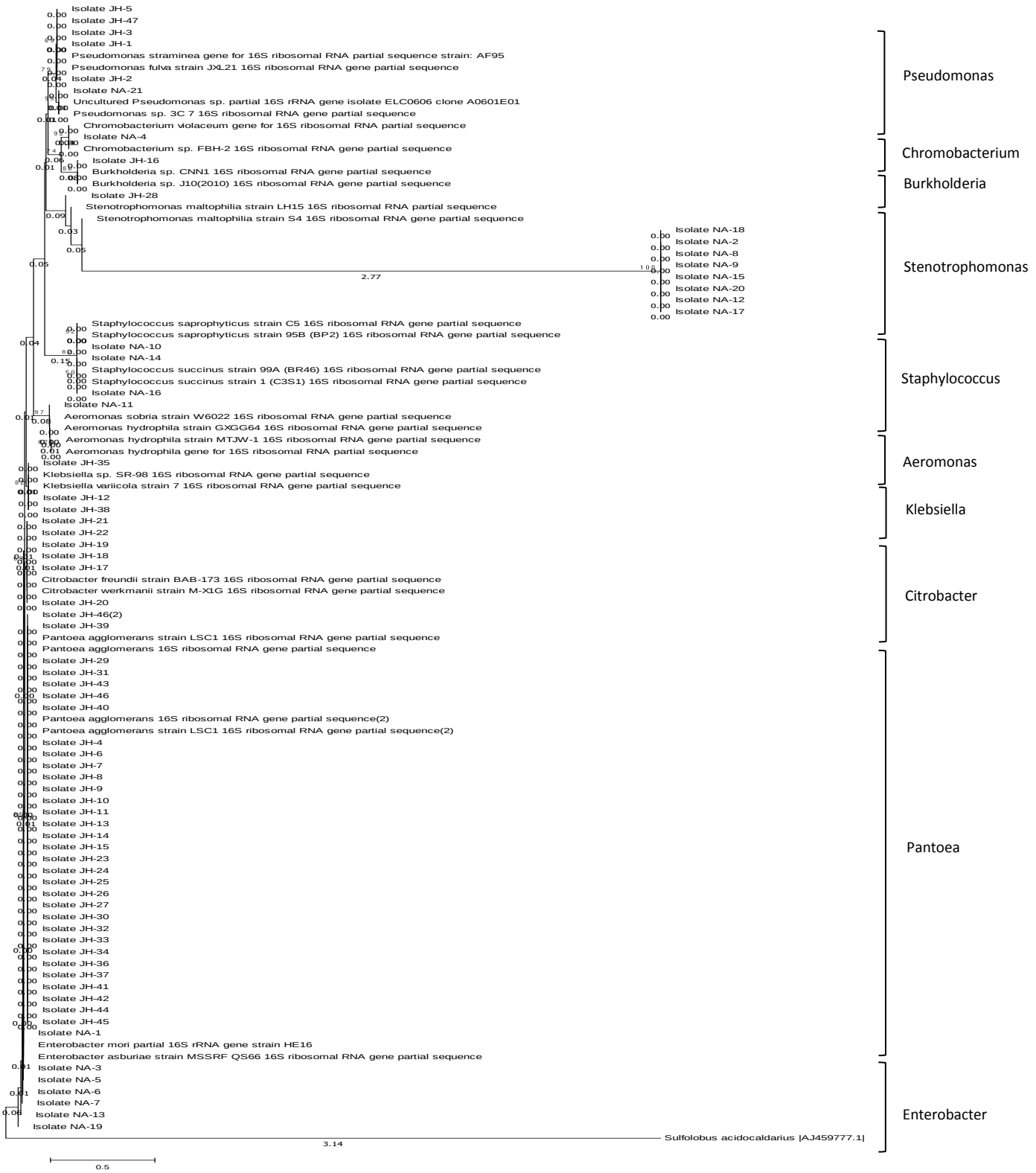

Figure 1 Phylogenetic analysis of dibenzofuran-utilizing bacteria. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the distance of isolated strains with the nearest species of the genus *Bacillus*. *Sulfolobus Acidocaldarius* AJ459777.1 was used as an out group. Bootstrap percentage values as obtained from 1,000 resamplings of the date set are given at the nodes of the tree. Bar, 0.5 substitutions per nucleotide position

Table 1 The Highest Sequence Similarity and Most Possible Bacterial Related to Bacterial Samples Based on Blast Sequence Alignment

Isolates	Possible Organisms	Sequence Length (bp)	Max score/Total score	E-Value	Max. Identity (%)	Gene bank accession number
NA-1, NA-3, NA-5, NA-6, NA-7, NA-13, NA-19	*Enterobacter mori* partial 16S rRNA gene, strain HE16	1406	2471/2471	0.0	98	LN624803.2
NA-10	*Staphylococcus saprophyticus* strain C5 16S ribosomal RNA gene, partial sequence	1400	2582/2582	0.0	100	KM016945.1
NA-11	*Aeromonas sobria* strain W6022 16S ribosomal RNA gene, partial sequence	1435	2603/2603	0.0	99	JX987063.1
NA-4	*Chromobacterium sp.* FBH-2 16S ribosomal RNA gene, partial sequence	1417	2494/2494	0.0	99	KJ619639.1
NA-14, NA-16	*Staphylococcus succinus* strain 99A (BR46) 16S ribosomal RNA gene, partial sequence	1387	2337/2337	0.0	97	KF254629.1
NA-21	Uncultured *Pseudomonas sp.* partial 16S rRNA gene, isolate ELC0606, clone A0601E01	1403	2547/2547	0.0	99	HE575562.1
JH-1, JH-2, JH-3, JH-5 , JH-47	*Pseudomonas fulva* strain JXL21 16S ribosomal RNA gene, partial sequence	627	1155/1155	0.0	100	KP980573.1
JH-16	*Burkholderia sp.* CNN1 16S ribosomal RNA gene, partial sequence	1400	2481/2481	0.0	99	HQ231922.1
JH-17, JH-18, JH-19, JH-20, JH-21, JH-22	*Citrobacter werkmanii* strain M-X1G 16S ribosomal RNA gene, partial sequence	1404	2564/2564	0.0	99	KJ806341.1
NA-2, NA-8, NA-9, NA-12, NA-15, NA-17, NA-18, NA-20, JH-28	*Stenotrophomonas maltophilia* strain LH15 16S ribosomal RNA, partial sequence	1407	2599/2599	0.0	100	KM893074.1
JH-12, JH-35, JH-38	*Klebsiella sp*. SR-98 16S ribosomal RNA gene, partial sequence	1410	2604/2604	0.0	100	KC455423.1
JH-4, JH-6, JH-7, JH-8, JH-9, JH-11, JH-13, JH-15, JH-23, JH-24, JH-25, JH-26, JH-27, JH-30, JH-33, JH-34, JH-36, JH-37, JH-39, JH-40, JH-41, JH-43, JH-44, JH-45	*Pantoea agglomerans* 16S ribosomal RNA gene, partial sequence	1394	2564/2564	0.0	99	EU879089.1

Table 1 shows the highest sequence similarity between isolates and database sequence. *Staphylococcus saprophyticus, Pseudomonas fulva, Stenotrophomonas maltophilia, and Klebsiella sp.* have high percentage similarity of 100% with isolate (NA-10), (JH-1, JH-2, JH-3, JH-5, JH-47), (NA-2, NA-8, NA-9, NA-12, NA-15, NA-17, NA-18, NA-20, JH-28) and (JH-12, JH-35, JH-38), respectively. This proves that the isolate have a 100% chance to be *Staphylococcus saprophyticus, Pseudomonas fulva, Stenotrophomonas maltophilia, and Klebsiella sp.*. For isolates NA-1, NA-3, NA-5, NA-6, NA-7, NA-13, NA-19, it is most similar to *Enterobacter mori* with 98%. NA-11 and NA-4 have 99% of similar sequence of *Aeromonas sobria* and *Chromobacterium sp.* respectively. According to the result, NA-14, NA-16, isolates are most similar to *Staphylococcus succinus* by 97% of identity. Pseudomonas sp. and *Burkholderia sp.* are the species which are most similar to isolates NA-21 and JH-16 with percentage of 99%.

Phylogenetic analysis of the selected bacterial strains assigned them to three major groups. The first group was Gamma proteobacteria comprised of different isolates of *Pseudomonas, Citrobacter, Pantoea, Enterobacter, Klebsiella, Aeromonas* and *Stenotrophomonas*. The second group was Betaproteobacteria comprised of different isolates of *Burkholderia* and *Chromobacterium*. The third group comprised of a single isolate of Staphylococcus was Bacilli.

4.0 CONCLUSION

DF-utilizing bacteria which can utilize DF as a sole source of carbon and energy were isolated. This ability makes the strains attractive for field bioremediation applications. Hence, a future work will focus on biodegradation analysis of dioxin by isolates is therefore suggested.

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