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Original Research Paper

GC-MS Analysis of Antibacterial Metabolites Extracted from *Rhodococcus rhodochrous* Isolated from Soil Samples

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ABSTRACT

The genus *Rhodococcus* is a Gram positive and non motile bacterium with different capability. Nowadays scientists are using the organism for the production of their antimetabolite products. Therefore, in the present study, the genus was isolated from the soil of different parts around the manganese mine in Qom city, Iran. The isolates were characterized using biochemical and antimicrobial test. Antimicrobial properties were evaluated using pathogenic bacteria, including: *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC8739), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC14028), *Shigella dysenteriae* (PTCC 1188), *Klebsiella pneumophila* (ATCC 700603), *Candida albicans* (ATCC 10231), *Bacillus subtilis* (PTCC1715), *Bacillus cereus* (PTCC 1015), *Listeria monocytogenes* (PTCC1295), *Enterococcus faecalis* (ATCC51299), *Rhodococcus erythropolis* (PTCC1767), *Corynebacterium glutamicum* (PTCC 1532). Furthermore, antibacterial compounds from culture supernatant were extracted using different solvents and the extracts were analysed using GC-MS. The results indicated that 9 of the isolates correctly belonged to the genus *Rhodococcus*. In addition, the results exhibited potential antibacterial activity against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC8739), with average zone inhibition of 8 and 25 mm respectively, but it had no effect on the other organisms. On the other hand the chromatogram showed nine special peaks and the identified compounds were: cyclopenthasiloxane, azulene, 2,7 octadiene, docosane, cyclohexasiloxane, bis (3,5,5 trimethyhexyl, tetraedacane-2 methyl, cycloheptasiloxane and heptadecanol. Phylogenetic analysis showed that the isolated strain from soil around manganese mine in Qom city, Iran formed a distinct subclade within the genus *Rhodococcus* with *R. rhodochrous*.

INTRODUCTION

The genus *Rhodococcus* is Gram positive, non motile and aerobic bacteria, which have been found in different types of environments including soil, water and eukaryotic cells (McLeod et al. 2006). According to 16S rRNA molecular typing, the genus has been assigned to four subclades, including *Rhodococcus equi, Rhodococcus rhodnii, Rhodococcus rhodochrous* and *Rhodococcus erythropolis* (McMinn et al. 2000, Wang et al. 2008). Therefore, at present because of new techniques, the discovery of novel *Rhodococcus* species has been greatly facilitated.

A few secondary metabolites produced by rhodococci have been characterized. The first report of an antimicrobial produced by *Rhodococcus* was in 1999, which was discovered by Chiba and his colleague with antifungal activity against *Candida albicans* and *Cryptococcus neoformans* without any antibacterial activity (Chiba et al. 1999). On the other hand, Treadway et al. (1999) reported that the organism has ability to produce indene, a precursor to the AIDS drug indinavir. Furthermore, the strains are important due to their ability to degrade a broad group of compounds and capability to produce different types of bioactive compounds (Kitagawa & Tamura 2008a). During the last decades, scientists tried to find out and screen microorganisms with new antimicrobial compounds. Indeed, they showed that the genus has capability to synthesize many different biologically active secondary metabolites such as antibiotics, herbicides, pesticides, anti-parasitic and enzymes (Oskay et al. 2004). At the moment, life of humans is threatened by infectious and antibiotic resistant bacteria (Bandow et al. 2003, Westh et al. 2004), the aim of this study was to evaluate the antimicrobial compounds extracted from *Rhodococcus* isolates from soil around manganese mine in Qom city, Iran against some pathogenic bacteria.

MATERIALS AND METHODS

Isolation of *Rhodococcus rhodochrous* from soil samples: Totally, 120 soil samples have been collected from different parts around the manganese mine in Qom city, Iran. The

samples were collected from 10 cm depth of soil using a sterile polyethylene bag and transferred to the laboratory within one hour of sampling. All samples were subjected to detection of *Rhodococcus* immediately upon arrival in the laboratory. Hence, 0.1 mL of serially diluted (10^{-1}-10^{-6}) soil samples were poured on ISP5 agar medium and the plates were kept at 30°C for 48-72 hrs.

Characterization of the isolates: All suspected colonies were characterized using microscopic examination, Gram and acid fast staining, catalase, oxidase and motility tests (De Wever et al. 1997). Then the suspected colonies with different pigments were identified using Api Coryne kit (bioMérieux).

Identification of the isolates using Api Coryne kit: The API Coryne system consists of 20 microtubes containing dehydrated substrates for the demonstration of 11 enzymatic activities (nitrate reduction, pyrazinamidase, pyrrolidonylarylamidase, alkaline phosphatase, glucuronidase, β-galactosidase, α-glucosidase, N-Acetyl-f, glucosaminidase, aesculin, urease and hydrolysis of gelatin) or the fermentation of eight sugars (glucose, ribose, xylose, mannitol, maltose, lactose, sucrose and glycogen). For this purpose the inoculum was prepared in distilled water with turbidity greater than 6 on the McFarland scale measured by comparing it with the turbidity control included in the kit and they were used for enzymatic tests. The strip was incubated at 37°C for 24 hours (Sotto et al. 1994).

Test organisms: The pathogenic bacteria have been prepared from American and Persian type culture collections including *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC8739), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC14028), *Shigella dysenteriae* (PTCC 1188), *Klebsiella pneumophila* (ATCC 700603), *Candida albicans* (ATCC 10231), *Bacillus subtilis* (PTCC1715), *Bacillus cereus* (PTCC 1015), *Listeria monocytogenes* (PTCC1295), *Enterococcus faecalis* (ATCC51299), *Rhodococcus erythropolis* (PTCC1767), *Corynebacterium glutamicum* (PTCC 1532), then they were activated accordingly in a nutrient broth medium.

Antibacterial activity of collected bacterial isolates: Antibacterial activity of the cultivated bacteria was evaluated using the agar well diffusion method against test bacteria. For this purpose 0.1 mL of pathogenic bacteria was spread over the agar surface of the plates. Then, two equally spaced wells of 6 mm diameter were made in the agar with sterilized core borer. The isolates were cultivated in the broth culture medium, then centrifuged at 9000 rpm for 15 min, subsequently the supernatant was poured into the well and the other well was used as control. The plates were kept at 37°C for 24 hrs (Kumar et al. 2009).

Extraction of antibacterial compounds from culture supernatant: The bacterial isolates were cultivated in TSB medium, and the culture broth were then centrifuged at 9000 rpm for 15 min to separate bacterial cells. Then the supernatant were acidified to pH 2.5 with concentrated HCl and added ammonium sulphate (40%) up to saturation to precipitate the antibacterial metabolites. The solvents used were ethanol, methanol, chloroform and ethyl acetate to determine the best solvent for extraction (Augustine et al. 2005). Then the solvents were (1:1 ratio) added to the supernatant. The mixture of solvent and supernatant were agitated for 90 min with homogenizer and the solvent was separated from the supernatant using separating funnel. Then all the solvent extract was assayed for their antibacterial activity by well agar diffusion method (Kumar et al. 2009).

GC-MS analysis of the extracted microbial metabolites: The gas chromatography combined with mass spectrometry detection technique is a qualitative and quantitative analysis of the crude extracts with high sensitivity even with trace the amount of constituents. Identification of the chemical moiety of crude extracts of *Rhodococcus rhodochrous* which showed valuable antibacterial activities against the selected human pathogens was analysed. The GC-MS analysis was done by standard specification by dissolving 10 mg of crude extracts in one millilitre of ethyl acetate. The aliquot of 0.1 µL was injected automatically into 0.25 mm × 30 m column of GC-MS model (Agilent 5973N, HP-5MS), 5% methyl phenyl polysiloxane as stationary phase and ionization energy 70 eV. Helium was used as a carrier gas at 17.69 psi pressure with the flow of 1.5 mL/min at the flow rate of 0.4 m/min. The temperature gradient program was implemented for the evaporation of organic solvent to identify the chemical constituent. The initial temperature was 60°C and gradually accelerated to 246°C at a rate of 3°C per minute. The sample was injected after 18 minutes at 250°C. The maximum peaks representing mass to charge ratio characteristics of the antimicrobial fractions were compared with those in the mass spectrum library of the corresponding organic compounds (Pandey et al. 2010). The concentration of such compound was calculated by the following formula:

$$\text{Compound concentration percentage} = [P1/P2] \times 100$$

Where, P1 is the peak area of the compound and P2 is whole peak areas in the fractioned extracts.

Molecular Identification of the Isolates

Bacterial genomic DNA extraction: DNA was extracted using bacterial DNA extraction kit Cinna Clone CO (Iran), according to the manufacturer's instructions. Bacteria from 5 mL aliquots of a stationary phase broth culture were cultured in LB broth medium containing NaCl, 5 g/L; Bacto

yeast extract, 5 g/L; BactoTryptone, 10 g/L (pH 7.2) and then precipitated by centrifugation at 4000 rpm for 5 min.

***Phylogenic analysis of isolated strain*:** For final assessment of isolated bacteria, a partial sequence of 16SrRNA was amplified by PCR using two sets of primers (Reverse1541: 5'-AAGGAGGTGATCCAGCCGCA-3' and Forward8: 5'-AGAGTTTGATYMTGGCTCAG- 3') (Walter et al. 2001). Reaction mixture for PCR amplification was prepared as follows: 5 µL DNA template, 1 U of Taq DNA polymerase (Fermentas, Russia), 1.5µL of $MgCl_2$, 50 µL of PCR buffer 10 × 1µL of each primers, 10 µL of dNTP mix and 16.8 µL of double distilled water in a final volume of 25 µL. Thermal cycling PCR program was as follows: initial denaturation of DNA at 95°C for 2 min, then denaturation at 95°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 40 s in 30 cycles. Final extension was performed at 72°C for 5 min and then the reaction mixture was held at 10°C till analysis. PCR product was analysed by electrophoresis on 0.8% agarose gel. After purification of PCR product from gel agarose by gel extraction kit (Life Bioscience, England), purified DNA was sequenced by Life Bioscience. Subsequently resulting sequence was compared with the sequence database in NCBI and phylogenic analysis of the isolate was obtained and aligned with the 16SrRNA gene sequence of the *Rhodococcus* species (obtained from GenBank/EMBL/DDBJ) using CLUSTALX (Thompson et al. 1997). Phylogenetic analysis was performed using the software package MEGA 4 (Kumar et al. 2004). Distances were calculated and clustering was performed with the neighbour-joining method (Wang et al. 2008).

RESULTS

Isolation and identification of the isolates: Of the 87 Coryne form organisms studied using the API system, 9 of them were correctly and completely identified within 24 hours to species level and they belonged to the genus *Rhodococcus*.

Antibacterial effect of the microbial metabolite: The results obtained from this study indicated that the metabolite exhibited potential antibacterial activity against *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC8739), with average zone inhibition of 8 and 25 mm respectively, but it had no effect on the other organisms.

GC-MS analysis of the extracted microbial metabolites: Analysis was carried out to identify the chemical compounds present in it. The chromatogram showed nine special peaks and mass spectrum detected the compounds present in the respective peak areas (Fig. 1). The identified compounds were cyclopenthasiloxane, azulene, 2,7 octadiene, docosane, cyclohexasiloxane, Bis (3,5,5 trimethyhexyl), tetraedacane

Table 1: Compounds in *Rhodococcus rhodochrous.*

Pk#	RT	Area%	Library/ID	Ref#	CAS#	Qual
1	15.64	1.18	C:\DATABASE\wiley7n.L			
			Cyclopentasiloxane, decamethyl-	313068	000541-02-6	83
			Cyclopentasiloxane, decamethyl-	313066	000541-02-6	78
			Cyclopentasiloxane, decamethyl-...	313067	000541-02-6	78
2	17.27	12.01	C:\DATABASE\wiley7n.L			
			Azulene (CAS) $$ Cyclopentacycl...	25286	000275-51-4	93
			Azulene	25289	000275-51-4	91
			Azulene	25287	000275-51-4	91
3	19.10	7.40	C:\DATABASE\wiley7n.L			
			2,7-OCTADIENE, 4-METHYL-, CIS/T...	21458	068701-77-9	64
			2,7-Octadiene, 4-methyl-	21571	000000-00-0	64
			Cyclopropane, 1,1-dimethyl-2-(2...	21488	069147-03-1	43
4	21.81	0.80	C:\DATABASE\wiley7n.L			
			Docosane (CAS) $$ n-Docosane $$...	261318	000629-97-0	64
			2-Octyldodecan-1-ol	248021	000000-00-0	39
			Azetidine, 2-methyl- (CAS)	1470	019812-49-8	38
5	22.94	2.66	C:\DATABASE\wiley7n.L			
			Cyclohexasiloxane, dodecamethyl...	352523	000540-97-6	90
			2,4-di(trimethylsiloxy)-6,7-(me...	312619	000000-00-0	50
			Cyclohexasiloxane, dodecamethyl-	352522	000540-97-6	47
6	25.63	0.96	C:\DATABASE\wiley7n.L			
			Bis-(3,5,5-trimethylhexyl) ether	214181	000000-00-0	35
			1-Dodecanol (CAS) $$ n-Dodecano...	95947	000112-53-8	35
			1-Fluorooctane $$ Octane, 1-flu...	28182	000463-11-6	35
7	25.93	1.27	C:\DATABASE\wiley7n.L			
			Tetradecane, 2-methyl- (CAS) $$...	134024	001560-95-8	53
			Heptadecane, 2-methyl- (CAS) $$...	193159	001560-89-0	53
			Octadecane, 2-methyl- (CAS) $$ ...	211469	001560-88-9	53
8	29.87	2.76	C:\DATABASE\wiley7n.L			
			Cycloheptasiloxane, tetradecame...	371831	000107-50-6	87
			TETRADECAMETHYLCYCLOHEPTASILOXA...	371832	000107-50-6	53
			EICOSAMETHYLCYCLODECASILOXANE $...	388915	018772-36-6	16
9	33.41	1.16	C:\DATABASE\wiley7n.L			
			1-Heptadecanol (CAS) $$ n-Hepta...	195647	001454-85-9	50
			Cyclopentane, 1,1,3,4-tetrameth...	23181	053907-60-1	43
			(E)-2-decenal	53223	000000-00-0	38

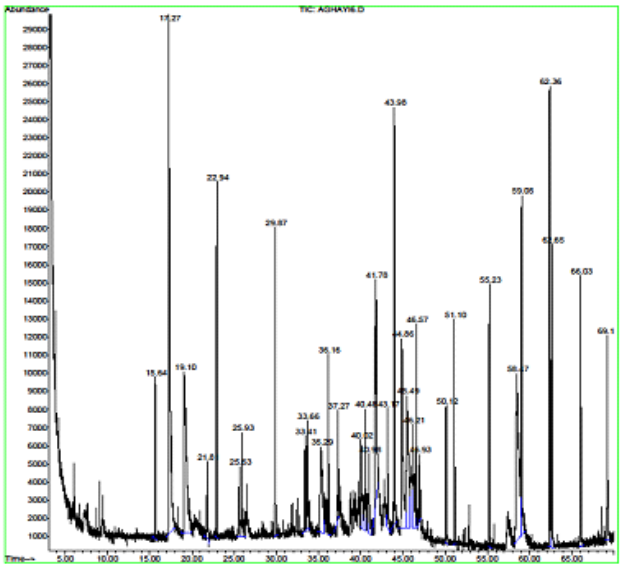

Fig. 1: Chromatogram of crude extract of *Rhodococcus rhodochrous.*

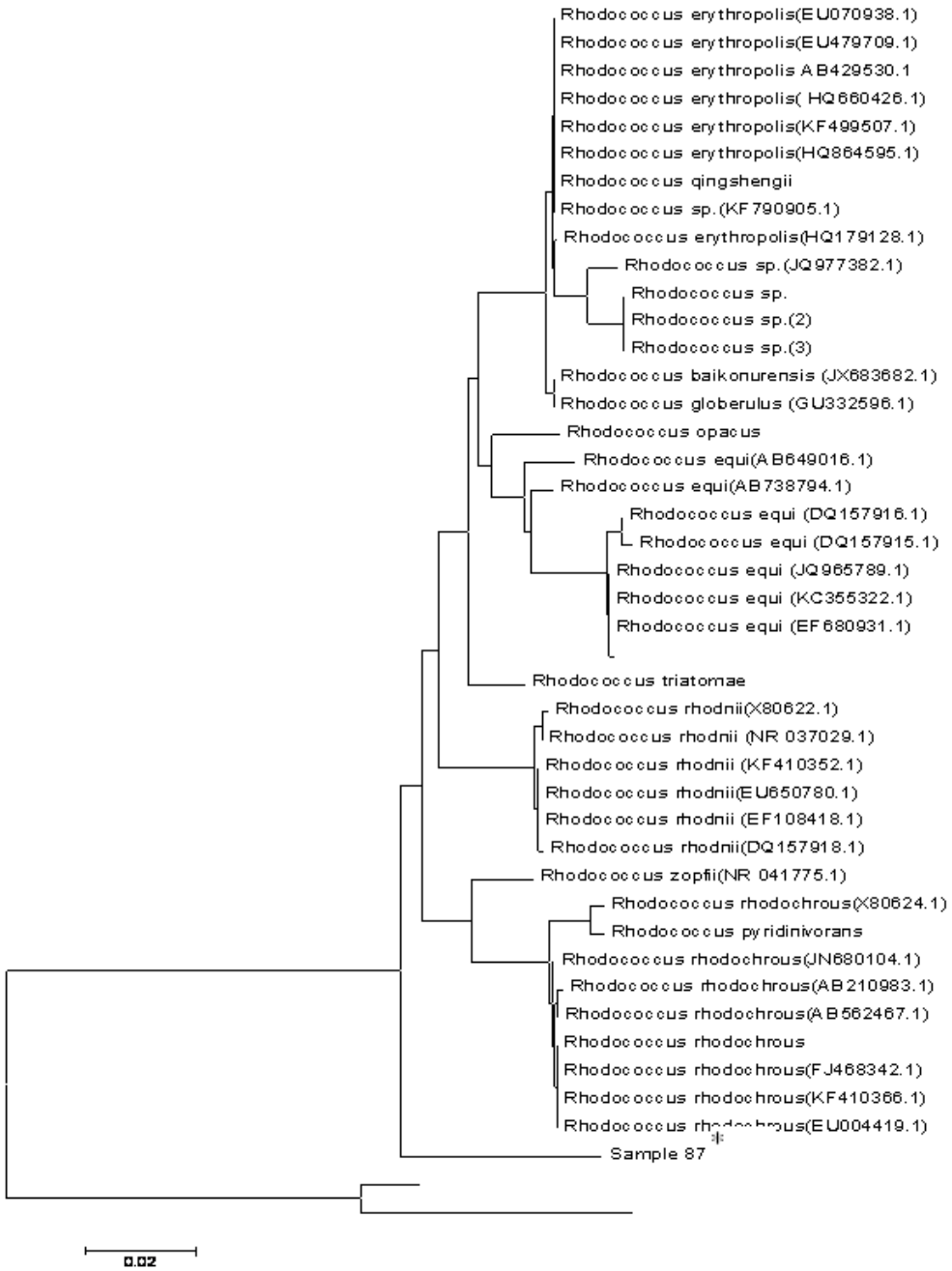

Fig. 2: Phylogenetic tree showing evolutionary relationships of the isolated strain (sample 87) producing bioactive metabolites. The number in parentheses indicates accession number of the other taxa obtained from GenBank database.

-2 methyl, cycloheptasiloxane and heptadecanol (Table 1).

Molecular identification of the isolate: Phylogenetic analysis showed that the isolated strain from soil around manganese mine in Qom city, Iran formed a distinct subclade within the genus *Rhodococcus* with *R. rhodochrous* (Fig. 2). The 16S rRNA gene sequence similarities of the isolated strain to type strains of species of the genus *Rhodococcus* with validly published names were 91%.

DISCUSSION

Rhodococcus is a bacterial genus that could be isolated from different sources, including soil, marine sediments, rocks, groundwater, boreholes, animal dung, insect guts, healthy and diseased plants and animals (Goodfellow 1989a, Ivshina

et al. 1994). Furthermore, the organism has been isolated from high-level nuclear waste plume (Fredrickson et al. 2004) and even a medieval grave (Takeuchi et al. 2002). The organism is a Gram positive, aerobic, non-motile, mycolate containing nocardioform actinomycetes (Goodfellow 1989b) which demonstrate a large metabolic diversity with capability to degrade different types of pollutants. The genus has ability to produce some metabolites including: surfactants, polymers, amides and flocculants (Bell et al. 1998), therefore, these capacities make them useful for biotechnological research (Thomas et al. 2002, Carvalho & da Fonseca 2005, Rzeznicka et al. 2010). Hence the aim of this study was isolation of the *Rhodococcus* from soil with capability to produce antimicrobial metabolite. For this purpose nine coryeneform bacteria were isolated, but only the *Rhodococcus rhodochrous* has the ability to produce antimicrobial compounds. Along with our research, Chiba et al. (1999) isolated the *Rhodococcus* sp. from a soil sample at Mt. Hayachine, Iwate Prefecture, Japan. The rhodopeptins were isolated as either colourless solids or white powders and were soluble in acetic acid, dimethyl sulphoxide, methanol, and slightly soluble in water but insoluble in chloroform and ethyl acetate (Chiba et al. 1999), while, our extracted antimicrobial metabolite were more soluble in ethyl acetate.

Furthermore, Iwatsuki et al. (2006) isolated a strain of *Rhodococcus*, *R. jostii* K01-B0171 from a soil sample in Yunnan, China and discovered that this strain is capable of producing 2 antibacterial compounds lariatin A and B. Both lariatins had antimycobacterial properties against *Mycobacterium smegmatis*; however, lariatin A also inhibited the growth of *Mycobacterium tuberculosis.* The team determined that the lariatins are cyclic peptides with a lasso structure. Lariatins A and B were also soluble in water, methanol, and DMSO while insoluble in chloroform and ethyl acetate (Iwatsuki et al. 2007). Our metabolites were examined against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC8739), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC14028), *Shigella dysenteriae* (PTCC 1188), *Klebsiella pneumophila* (ATCC 700603), *Candida albicans* (ATCC 10231), *Bacillus subtilis* (PTCC 1715), *Bacillus cereus* (PTCC 1015), *Listeria monocytogenes* (PTCC 1295), *Enterococcus faecalis* (ATCC 51299), *Rhodococcus erythropolis* (PTCC 1767), and *Corynebacterium glutamicum* (PTCC 1532). The results obtained indicated that *Esherichia coli* was more sensitive to the extracted metabolite. Research about the new metabolite continued till 2008, Kitagawa and Tamura illustrated that 80 *Rhodococcus* strains which were acquired from Japanese and German culture collections had antibacterial activity and they were classified in 3 groups based on the type of antibiotic they produced (Kitagawa & Tamura 2008a, Kitagawa & Tamura 2008b). At the same time Kurosawa et al. (2008) worked on horizontal gene transfer from *Streptomyces padanus* to *R. facians* and they allowed the new *Rhodococcus* strain independently to begin producing 2 new antimicrobial compounds. In addition, Narayana et al. (2008) isolated *Streptomyces* sp. from soil samples with capability to produce antimicrobial compound. They showed that the GC-MS spectrometry monitored four active fraction against Gram positive, Gram negative and fungi, among which hydorxyquinoline exhibited strong antibacterial and antifungal activity, while in our study 9 fractions have been screened and azuelene were more strong than the others.

In general, different species have been isolated from the soil samples but only one strain had the capability to produce antimicrobial products, hence, it seems that additional studies would be needed to assess the potential of the extracts for introduction of novel antibiotics from this geographical area of investigation.

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