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Phylogenetic Identification of Chitinase Secreting Soil Bacteria Antagonistic to Fungus *Aspergillus*

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ABSTRACT

Economic losses arising from crop diseases caused by phytopathogenic fungi are principally associated with yield reductions. One alternative to fungicides is biocontrol with beneficial bacteria. Next to *Pseudomonas*, the endospore-forming genus *Bacillus* is probably the most widely researched and commercialized biocontrol agent. It is generally recognized that biological control agents are safer and more environmentally sound than is reliance on the use of high volumes of pesticides. The primary objective of current work was to isolate the specific bacterial strain that possesses the potential to act as an antagonist against the fungal pathogen *Aspergillus* which is thought to secrete aflatoxin, the most potent mycotoxin. Many bacterial cells were isolated randomly from the rhizospheric region of groundnut crop by serial dilution method, which were further screened for their antagonistic effect against *Aspergillus*. Screening results show that few of the isolates exhibit significant antagonism on dual culture media. One of the bacterial isolates SJ 609 exhibiting the maximum antagonistic effect was further selected after optimization for further studies. The mechanism of action of SJ 609 was studied *in vitro* for the production of hydrolytic enzymes particularly chitinase which hydrolyses chitin, which is the major component of fungal cell wall. Phylogenetic analysis using 16S rDNA amplification was carried out to establish its identity.

INTRODUCTION

With the advent of agriculture, plant diseases became a problem. Thousands of insects, fungi, viruses, bacteria, nematodes and other living forms are potential hazards to agricultural crops. Fungal diseases like rust and smut in wheat were reported in the Bible as *curses for disobedience of the commandments*. The culprit behind the great Irish Famine of 1840s was a fungus, which attacked potatoes and caused them to rot. As a result, one million people died and two million people migrated to other countries. In 1960s, the fungal infection on cotton crop in Mexico when became tolerant to organophosphate fungicides, triggered the process of rethinking about the use of chemicals for its control (Abramson 1997, Kerry 2000). Various parts of India face the problem not only for cotton but also for legumes, particularly peanuts. One of the most common fungus of legumes and peanuts is *Aspergillus flavus* which is of great importance because of the property of the fungi to produce "aflatoxin" while growing on peanut seeds and its products. Contamination of peanut seeds by aflatoxin is a serious problem in most countries where the groundnut is a major cash crop. Economic losses arising from crop diseases caused by phytopathogenic fungi are principally associated with yield reductions. One alternative to fungicides is biocontrol with beneficial bacteria. Next to *Pseudomonas*, the endospore-forming genus *Bacillus* is probably the most widely researched and commercialized biocontrol agent (Backman et al. 1994). Research in the field of biological control has revealed a variety of natural products that can be exploited for the development of suitable alternative against chemical control measures (Boland & Kuykendall 1998).

MATERIALS AND METHODS

Chemicals: All the chemicals used in order to achieve the proposed objectives were of AR grade or GR grade purchased from Sigma Aldrich (St. Louis, MO, USA), SRL (India) and Sd Fine Pvt. Ltd. (India). Media used for the culture of microorganisms (such as Potato Dextrose Agar, Luria Bertini media, Nutrient Broth, Nutrient Agar, Czapek Agar, etc.) and antibiotic discs to be used as marker in formulation studies were purchased from HI-MEDIA, Mumbai (India). To carry out the molecular biology work such as genomic DNA isolation, PCR amplification and gel electrophoresis, the chemicals were of molecular biology grade and purchased from Bangalore Genei Pvt. Ltd.

Isolation of antagonistic microbes from the rhizospheric soil: Bacterial strains were collected di-

rectly from the soil of the selected fields and the rhizosphere of groundnut crop. The soil was light and sandy loam soil with moderately low amount of organic matter. Organic matter of soil was maintained at the level of 1 to 2% to improve water holding capacity of soil and supply plant nutrients.

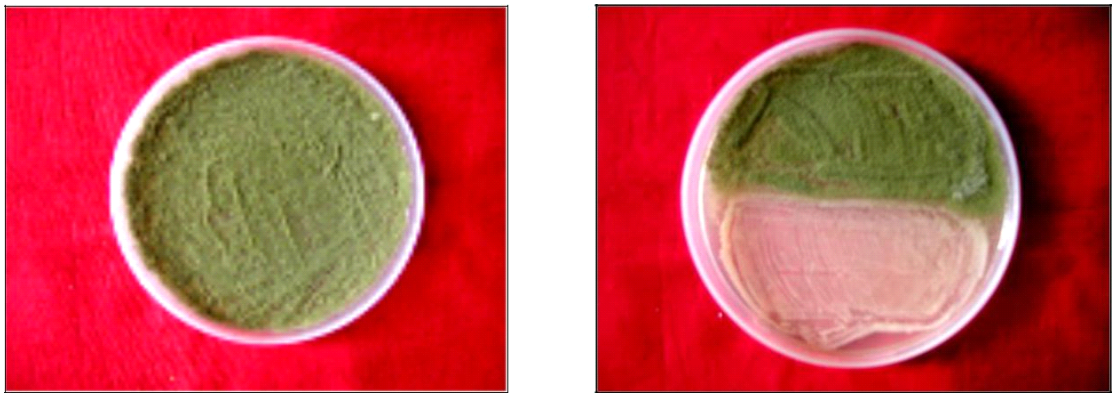
Screening of bacteria for antagonism towards pathogenic fungi *A. flavus*: Randomly chosen strains on the basis of morphology, texture, colony sizes and colour were selected and then subcultured separately and tested for their antifungal activity against pathogenic fungi *Aspergillus* by using dual culture method. Briefly, individual bacterial colonies were cultured on media like LB (Luria Bertini) or nutrient broth (Hi-Media, Mumbai) overnight. Number of bacterial colonies was counted through serial dilution method and a concentration of 10^8 Cfu mL^{-1} was made for each bacterial isolates. Also, spores of *A. flavus* were counted through haemocytometer (Smith et al. 1992) and a suspension of 10^6 spores/mL was used for the study. All the randomly isolated bacterial colonies were tested for their antagonistic effect on dual culture plate (PDA) which was prepared using freshly peeled potatoes [200 g for one litre media followed by addition of dextrose and bacto agar (20 g/L)]. One half of the PDA plate was spread with fungus (freshly grown) while another half was spread with bacterial isolates (10^8 cells mL^{-1}) followed by incubation at 30°C for 5-7 days; plates were observed for antagonism after proper incubation. Control plate was prepared by spreading the fungal spore suspension on to Potato Dextrose Agar (PDA) media. Inhibition or suppression of fungal growth was identified visually (Montealegre et al. 2003).

Screening for the production of chitinase enzyme: Selected bacterial isolates were tested to produce chitinase on chitin agar plate, which was prepared by adding chitin as carbon source with minimal salts that supports the growth of bacteria [colloidal chitin was prepared by the method of Hsu & Lockwood (1975)]. Freshly grown bacterial culture was spot inoculated at five equidistant points on single colloidal chitin agar plate (one plate for each bacteria) followed by incubation at 30°C for 3 days, plates were then observed for zone of hydrolysis around the inoculated area. The bacterial isolate MAK 609 of *Bacillus thuringiensis* produced the clear zone around inoculated area confirming the secretion of chitin by them. Fig. 2 shows the clear area of hydrolysis around the inoculated bacterial strain at five equidistant points. This area of hydrolysis is due to the secretion of chitinase which readily acts upon colloidal chitin which is the sole carbon source in the defined media. This causes the area around the inoculated area to be hydrolysed which confirms the secretion of chitinase by the bacterial isolates MAK 609.

16S rRNA gene amplification: Unique primers P6 (F) 5'GTAACCTGCCTGTAAGACTG3' and P9 (R) 5'GCCC TTTGTTCTGTCCATTG3' specific for 16S rRNA were used for amplification of approx. 1000 bp region of the 16S rRNA gene on T-Cy (Creacon technology, Netherlands) thermal cycler. A 20 µL reaction mixture included 5-10 ng of bacterial DNA as template, 1 µL of each primer (containing 100 ng primer concentration), 1 U of *Taq* DNA polymerase (Bangalore Genei, India) and 100 µm dNTP. The reaction conditions were: initial denaturation of 5 min at 94°C followed by 35 cycles of denaturation of 1 min at 94°C, annealing of 1 min at 58°C and extension of 1 min at 72°C.

The PCR products were analysed by electrophoresis on 1% agarose gel in 1X TAE (40 mM Tris-acetate, 1 mM EDTA pH 8.0 and glacial acetic acid) stained with 10 microgram of ethidium bromide per mL. The same condition for amplification was carried out using Real Time PCR (LC 480; Roche Diagnostics GmbH, Roche Applied Science, Germany) was carried out at Central Instrumentation Facility. The reaction mixture prepared consists of SYBR green master mix (consisting of SYBR green dye, dNTPs, buffer and polymerase) along with target DNA and primers, this mixture was then loaded into the RT-PCR plate having 96 wells, mixture was then mixed properly using a centrifuge having plate rotor. The temperature profile of the cycles was as follows: 1 cycle for denaturation at 95°C for 5 min, 35 cycle of denaturation at 95°C 30 sec., annealing at 60°C for 30 sec and final extension at 72°C for 1 min. PCR product was visualized on 1% agarose gel using ethidium bromide staining and photographed on UV transilluminator chamber. The products obtained after amplification were purified using the spin gel purification kit (Bangalore Genie I). Along with the amplification, melting curve analysis was also done in order to assess the Tm of the amplified DNA.

Sequencing and phylogenetic analysis: The purified amplified product for rRNA gene was sequenced using ABI PRISM 3130 XL and a single primer at a time was used to sequence the amplified product. Sequencing reaction consisting of 20-50 ng of purified amplified product and single primer at the concentration of 100 pg. The obtained sequence was compared with those available in the Genbank at NCBI using BLAST searches to obtain the phylogenetic relationship of the selected antagonistic bacterial isolates. Phylogenetic tree displaying the relationship with the nearest neighbour was constructed using BLAST tree widget available at NCBI.



(A) (B)

Fig.1: Interaction of selected bacterial isolates (from soil) and fungus *Aspergillus flavus* on dual culture plate (PDA) by dual culture method. (A) Control; (B) SJ 609.

RESULTS AND DISCUSSION

Screening of randomly selected bacterial isolates against *Aspergillus* by dual culture assay: Randomly selected visible bacteria isolated from soil collected from the field of groundnut crop, were screened against pathogenic fungus *Aspergillus flavus* by dual culture assay on PDA plate as well as in potato dextrose broth. Fig. 1 shows the interaction between the fungal pathogen *Aspergillus flavus* and selected bacterial isolates (randomly isolated from rhizospheric soil of groundnut crop). One half of PDA plate was spread with suspension of freshly grown fungal spores while other half was spread with freshly prepared bacterial cells. Significant inhibition as compared to control was observed after proper incubation.

Screening for chitinase enzyme: Selected bacterial isolates were tested to produce chitinase on chitin agar plate which was prepared by adding chitin as carbon source with minimal salts that supports the growth of bacteria [colloidal chitin was prepared by the method of Hsu & Lockwood (1975)]. Freshly grown bacterial culture was spot inoculated at five equidistant points on single colloidal chitin agar plate followed by incubation at 30°C for 3 days, plates were then observed for zone of hydrolysis around the inoculated area. The bacterial isolates SJ 609 produced the clear zone around inoculated area confirming the secretion of chitin by them. Fig. 2A and 2B show the clear area of hydrolysis around the inoculated bacterial strain at five equidistant points. This area of hydrolysis is due to the secretion of chitinase which readily acts upon colloidal chitin which is the sole carbon source in the defined media. This causes the area around the inoculated area to be hydrolysed which confirms the secretion of chitinase by bacterial isolate strain SJ 609 of *Bacillus thuringiensis*.

Production profile of chitinase: Enzymatic activity of chitinase producing isolate SJ 609 was analysed under *in vitro* conditions using colloidal chitin as substrate. Production profile for chitinase was checked by observing the chitinase activity at regular interval of 24 hrs. The chitinolytic strains showed the maximum production of chitinase at 48-72 hrs. Five percent of colloidal chitin was added to the defined media followed by subsequent addition of freshly prepared culture of selected bacterial isolates and incubation at 30±2°C in shaker incubator (120 rpm). Enzymatic activity was calculated at subsequent time-interval of 24 hrs by collecting the culture at different time interval. Hydrolysis of chitin in presence of chitinase secreted by bacteria releases the N-acetyl D glucosamine (repeating units of chitin), estimation of released NAG was then carried out by DNS (Dinitrosalicylic acid) test. Corresponding OD was extrapolated on to the standard plot of purified NAG (N-acetyl D glucosamine) (SRL Pvt. Ltd.). Fig. 3 shows the production profile of chitinase at subsequent time-interval of 24 hrs by bacterial isolate SJ 609 which shows the maximum secretion of chitinase at 72 hrs.

Sequencing and phylogenetic analysis: The amplified product from the DNA sample of bacterial isolates after complete run by Agarose gel electrophoresis were observed in the range of approximately 1 Kb as compared to the marker of 1 Kb run along with the sample on the same gel. The amplified and purified product for rRNA gene was sequenced using ABI PRISM 3130 XL (carried at the center for genomics application, New Delhi) and a partial sequence of about 684 bases was obtained using forward primer P^f

(A) (B)

Fig. 2: The secretion of chitinase as observed by hydrolyzed area around inoculated bacterial strains on the colloidal chitin agar plate prepared using colloidal chitin as the sole carbon source in the defined media. (A) *Serratia marcens* used as standard. (B) SJ 609. Clear area around bacterial cell culture inoculation is due to the hydrolysis of colloidal chitin which explains the probable secretion of chitinase by three of the bacterial isolates.

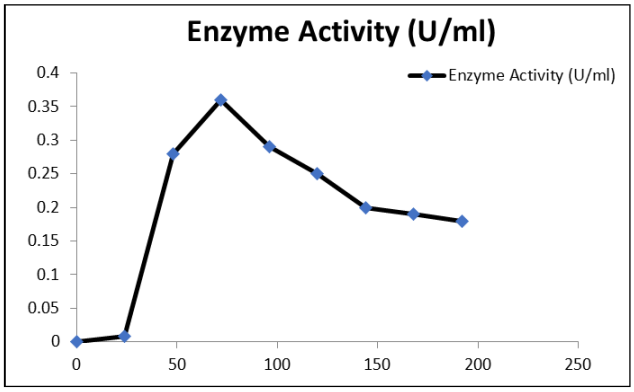


Fig. 3: Graph showing production of chitinase by the bacterial isolate SJ 609. Enzyme activity was calculated after subsequent interval of 24 hours to assess the production profile of chitinase with progression of time.X-Axis represents increase in Time Interval while Y axis represents Enzyme activity (U/ml).

(GTAACCTGCCTGTAAGACTG) in case of SJ 609 (Fig. 4). The sequences obtained were compared with those available in the Genbank using BLAST searches to obtain the phylogenetic relationship of the selected bacteria antagonistic to fungal pathogen. The bacterial isolates sequenced, and analysed by BLAST searches showed homology with *Bacillus thuringiensis* (Fig. 5).

TCCCGGTGTTTGCCAAGGCTCATAAGGGGCATGATGA
TTTGACGTCATCCCCACCTTCCTCCGGTTTGTCA
CCGGCCGTCAAAAGGAGAGTGCCCAACTAAATGATG
GCAACTAAGATCATTGGGTTGCGCTCGTTGCGGGA
CTTAACCCAACATCTCACGACACGAGCTGACGACAA
CCATGCACCACCTGTCACTCTGCTCCCGAAGGAGA
AGCCCTATCTCTAGGGTTGTCAGAGGATGTCAAGAC
CTGGTAAGGTTCTTCGCGTTGCTTCGAATTAAACC
ACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTC
CTTTGAGTTTCAGCCTTGCGGCCGTACTCCCCAGG
CGGAGTGCTTAATGCGTTAACTTCAGCACTAAAGGG
CGGAAACCCTCTAACACTTAGCACTCATCGTTTAC
GGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCC
CCACGCTTTCGCGCCTCAGTGTCAGTTACAGACC
AGAAAGTCGCCTTCGCCACTGGTGTTCCTCCATATC
TCTACGCATTTCACCCGCTACACATGGAATTCCAC
TTTCCTCTTCTGCACTCAAGTCTCCCAGTTTCCAATG
ACCCTCCACGGTTGAGCCGTGGGCTTTTCACATC
AGACTTAAGAAACCACCTGCGCCGCGCTTTACGCC
CAATAATTCC (Accession No. EU 520482)

Fig. 4: Partial sequence of Bacterial Isolate SJ609.

DISCUSSION

Contamination of food and feed stuffs by *Aspergillus* species and their toxic metabolites is a serious problem as they have adverse effects on animal and human health and cause economic problems for international trade in particular of developing countries (Salunkhe et al. 1987, Nielsen & Rios 2000, Batista et al. 2003, Gonzalez et al. 2005). Use of synthetic pesticides is quite harmful owing to their side effects. Of the many research approaches being used to reduce and ultimately, eliminate *A. flavus* contamination, biological control is considered as one of the most promising techniques, particularly for the near-term (Norner 2004). *Bacillus thuringiensis* is a Gram-positive soil bacterium that is known to be a bacterial biopesticide that produces insecticidal proteins called crystal proteins (Cry). Cry toxins are pore-forming toxins that induce cell death by forming ionic pores on epithelial cell membranes following insertion into the membranes (Bravo et al. 2004). Without any negative consequences of synthetic chemicals, the alternate approach sought was to use naturally available antagonistic microorganisms. These microorganisms are present in the

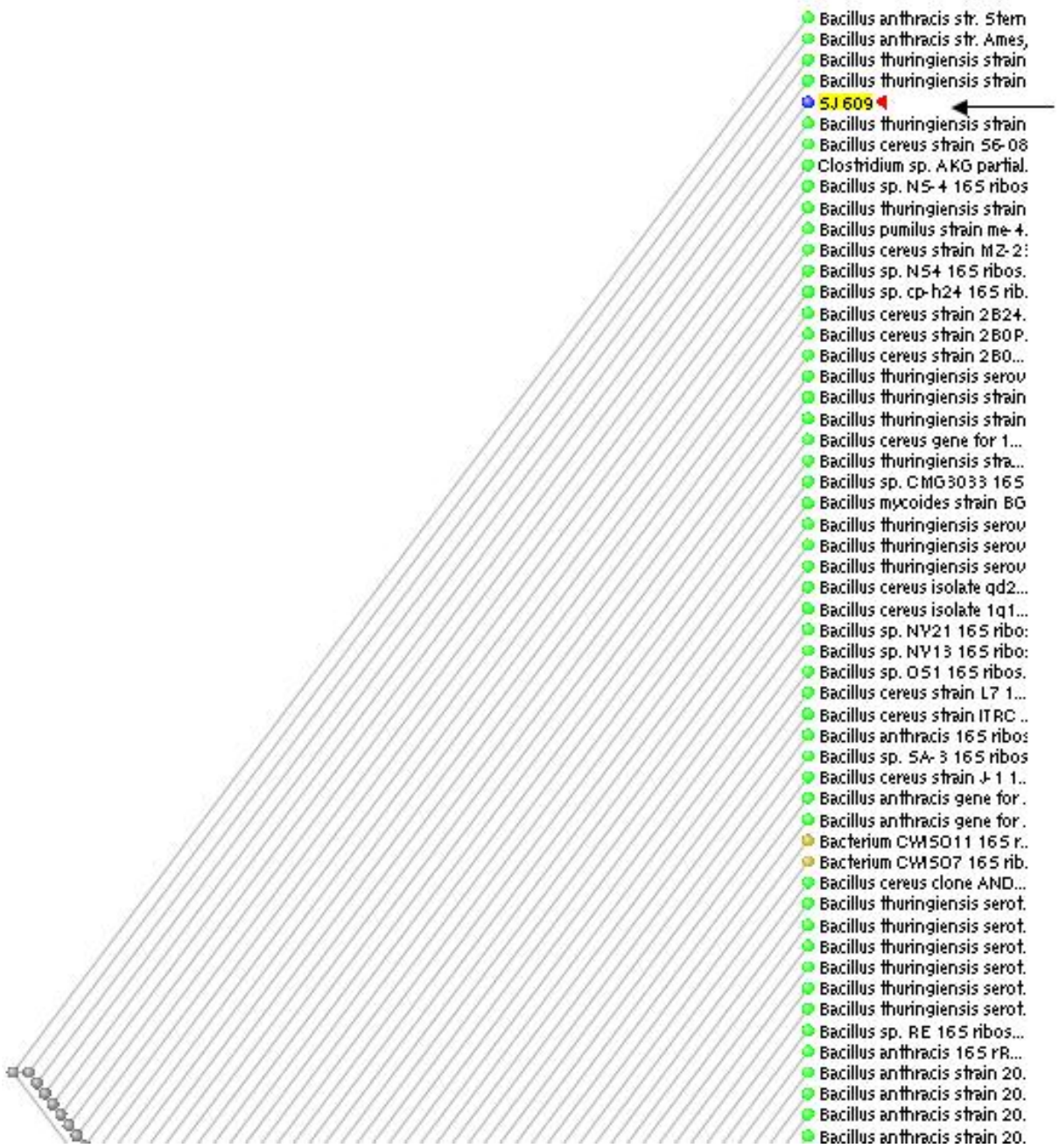


Fig. 5: The phylogenetic tree displaying the nearest neighbour of bacterial strain SJ 609 (shown by arrow) with *Bacillus thuringiensis*.

rhizospheric region of the plant. These microorganisms control the population of fungal pathogens by acting directly or indirectly on them. Therefore, this work was carried out with the primary task to isolate potential antagonistic soil bacteria to control the mycotoxigenic fungi. Earlier, a number of scientists have reported the study of antagonistic fungus against fungus and antagonistic bacteria against fungus by carrying out the dual culture assay on PDA plate with one half of the plate being spread with bacterial cells (freshly grown) while other half with fungal spores (freshly grown) (Cuero et al. 1987). One of the major mechanisms involved in the biological control of fungal pathogen is parasitism via degradation of cell wall. Chitin, the unbranched homopolymer of N-acetyl D glucosamine in β, 1-4 linkage is a structural component of cell walls in most of the fungi. Chitinase, which hydrolyses this polymer is

produced by various organisms and have been implicated in the biocontrol process. The bacterial isolate SJ 609, was found secreting chitinase enzyme that hydrolyses chitin. Chitin is one of the most abundant polymers in nature and occurs as a structural polymer in fungi. Chitinase, a chitin-degrading enzyme is, therefore, of great significance for its role in biological control as chitin can easily be accessible to them. The use of these bacterial strains as biocontrol agents can be quite helpful in control of fungal infection under field conditions. Analysis of 16S rRNA homology was also carried out for bacterial identification. Gene coding for 16S rRNA has many features, which makes it a valuable tool for phylogenetic studies, the rRNA sequence contains highly conserved regions suitable for species identification sequencing of purified PCR product was carried out to obtain the partial sequence of 16 S rRNA. The analysis of obtained sequence by carrying out BLAST on to the database available on NCBI which confirm the identity of the bacterial isolates by providing the homology against the sequences available in the database. Maximum homology for the partial sequence of SJ 609 (684bp) was found to be with *Bacilli thuringiensis* which provides a fruitful perspective to develop a suitable formulation against fungus *Aspergillus*.

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