

Hexadecane-Degradation by *Teskumurella* and *Stenotrophomonas* Strains Isolated From Hydrocarbon Contaminated Soils

Hamid Tebyanian¹, Mehdi Hassanshahian^{2,*}, Ashraf Kariminik¹

¹Department of Microbiology, Science and Research Branch, Islamic Azad University, Kerman, IR Iran

²Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, IR Iran

³Department of Microbiology, Islamic Azad University, Kerman, IR Iran

*Corresponding author: Mehdi Hassanshahian, Department of Biology, Faculty of Sciences, Shahid Bahonar University of Kerman, Kerman, IR Iran. Tel: +98-9132906971, Fax: +98-3413202032, E-mail: mshahi@uk.ac.ir

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Background: Petroleum hydrocarbons are mix compounds and divided into four groups: Saturates, Aromatics, Resins and Asphalten. Among various phases of crude-oil, the alkanes with medium length chain are favorable substrates that rapidly degraded, although short-chain alkanes are very toxic and long-chain alkanes have low solubility in water that reduce its bioavailability and make resistant to biodegradation.

Objectives: The main goal of this study is the isolation, molecular identification and degradation properties of hexadecane degrading bacteria from contaminated soils.

Materials and Methods: In this research to isolate aliphatic degrading bacteria, sampling from hydrocarbon contaminated soil with of petroleum reservoirs regions, Tehran were performed. Alkane degrading bacteria were isolated by enrichment in Bushnel-Hass medium with hexadecane as sole source of carbon and energy. The isolated strains were identified by amplification of 16S rDNA gene and sequencing. Alkane hydroxylase gene (alk-B) was identified in all strains by PCR with specific primers.

Results: Among 8 strains three strains with high growth rate on hexadecane selected for further study. These three selected strains identified as *Stenotrophomonas maltophilia* strain M2, *S. maltophilia* strain Q2 and *Teskumurella tyrosinosolvans* strain Q3. In comparison to the other bacteria, these bacterial strains can degrade hexadecane 2 times more; with a high emulsification activity.

Conclusions: The 2.5% concentration of hexadecane was the best concentration that supports the growth of these strains. Among these strain *T. tyrosinosolvans* strain Q3 was the best strain for biodegradation of hexadecane. Alk-B gene was identified in all strains.

Keywords: Biodegradation; Hexadecane; Pollution; *Stenotrophomonas*

1. Background

Petroleum hydrocarbons are important energy resources used by industry and in our everyday lives. Together petroleum is a major pollutant of the environment. The petroleum containing thousands of individual hydrocarbons and related compounds (1). Aliphatic and aromatic hydrocarbons which are the most common pollutants are the main contaminants in oil-polluted environments. Oil pollution may arise either accidentally or operationally during oil production, transportation, storing, proceeding or using at sea or on land (2-4). The traditional methods to cope with oil spills are confined to physical containment. Biological methods have the advantage over physicochemical treatment regimes in removing spills as they offer institute biodegradation of oil fraction by microorganisms (5, 6). Many of these remediation strategies employ microorganisms which can degrade

the hydrocarbons.

Bioremediation of contaminated sites relies on either the activity of microorganisms which are presented at the site or the addition of selected microorganisms with desired catabolic features and bio-augmentation systems (7, 8). The rate of biodegradation depends on the oil concentration, alkanes length, biosurfactant and microorganism strains (9). It has been reported that the saturated components of crude oil are mostly the alkanes with intermediate length (C10 - C20) that are more readily to biodegrad (10). The rate of uptake and mineralization of various organic compounds depends on the compound concentration, high concentrations of hydrocarbon cause inhibition of biodegradation by inducing nutrient or oxygen limitation or through toxic effects of volatile hydrocarbons (11-13).

Alkane hydroxylase is a key enzyme in alkane degra-

Implication for health policy/practice/research/medical education

The environmental remediation of hazardous materials is important for global health. Oil hydrocarbons are the most environmental polluting factor. Bio degradation of these pollutants was studied in this research.

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dation. This enzyme which carries the oxygen atom of molecular oxygen into the alkane substrate, plays an important role in crude oil bioremediation. Phylogenetic analyses have revealed that the alkane hydroxylase genes classified into three clusters. The alkane hydroxylase group (I) encoding *alk-B* gene catalyzes medium chain length (C6 - C12) n-alkanes. The genes classified group (II) encoding *alk-M* catalyzes long chain alkanes > C12 this gene is mostly contained in *Acinetobacter* genomes. The gene classified group (III) encoding *alk-B* is unknown for substrate specificity, alkane oxidation pathway and oxidation system (14-16).

2. Objectives

The main goal of this study was the isolation, molecular identification of hexadecane degrading bacteria isolated from contaminated soils.

3. Materials and Methods

3.1. Sampling

For isolation of alkane-degrading bacteria, contaminated soils were collected from 10 different sites of Tehran petroleum reservoirs regions. The samples were taken from 1 - 12 cm of the subsoil using a sterile scalpel. Collected samples were transported on ice to the laboratory for isolation (17).

3.2. Isolation and Selection of Alkane Degrading Bacteria

A synthetic Bushnell Hass Mineral Salts medium (BHMS) was used for isolation. BHMS medium contain (g.L-1) KH₂PO₄, 1; K₂HPO₄, 0.2; MgSO₄.7H₂O, 0.2; CaCl₂, 0.02; NH₄NO₃, 1; and 2 droplet of FeCl₃ 60%. The pH adjusted to 7. BHMS medium was supplemented with 1% (v/v) hexadecane as sole carbon source of energy. Portion of soil (1 g) samples were added to 250 mL Erlenmeyer flasks containing 100 mL BHMS medium and the flask incubated for 10 days at 30° and 180 rpm C on rotary shaker (INFORS AG, Switzerland). Then 5 mL aliquots were remove to fresh BHMS medium. After a series of four further passages, inoculums from the flask were streaked out and different colonies phenotypically purified on BHMS agar medium. colonies grown on the plates were transferred to fresh BHMS medium with and without hexadecane to eliminate the autotrophs and agar-utilizing bacteria. The procedure was repeated and isolates only showed a pronounced growth on hexadecane were stored for further characterization (18).

3.3. Identification of Isolates

3.3.1. Biochemical Characterization

To identify and characterize the bacteria isolates, bio-

chemical tests such as Gram staining, oxidation/ fermentation, production of acid from carbohydrates, hydrolysis of gelatin and citrate carried out according to the Bergey's manual of Systematic Bacteriology (19).

3.3.2. Molecular Identification

Analysis of 16S rDNA was performed to characterize the isolated strains taxonomically. Total DNA extraction was performed using the CTAB method (20). The bacterial 16S rDNA loci were amplified using the forward domain-specific 16S primer, Bac27_F (5'-AGAGTTTGATCCTGGCTCAG-16 3) and universal reverse primer Uni_1492R (5'-TACGYTACCTGTTACGACTT-173). The amplification reaction was performed in a total volume of 50 µL containing, 1X solution Q (Qiagen, Hilden, Germany), 1X Qiagen reaction buffer, 1 µM of each forward and reverse primer, 10 µM dNTPs (Gobco, Invitrogen Co, Carlsbad, CA), and 2 U of Qiagen Taq polymerase (Qiagen). Amplification for 35 cycles was performed in a thermocycler GeneAmp 5700 (PEApplied Biosystem, Foster City, CA, USA).

The temperature profile for PCR was set at 95°C for 5 min (1 cycle); 94 °C for 1 min annealing at 55°C for 30 s and 72°C for 1 min (35 cycles), followed by 72°C for 10 min at the end of final cycle (21). The 16S amplified (1400 bp) was sequenced with a Big Dye terminator V3.1 cycle sequencing kit on an automated capillary sequencer (model 3100 Avant Genetic 1 Analyzer, Applied Biosystems). The similarity rank from the Ribosomal Database Project RDP (22) and FASTA Nucleotide Database Query were used to determine partial 16S rDNA sequences to estimate the degree of similarity to other 16S rDNA gene sequences. Analysis and phylogenetic affiliates of sequences was performed as described previously (23).

3.4. Growth and Hexadecane Removal Assay

The isolates growth curves were indirectly assessed by turbidity measurement (O.D 600 nm) by a UV-visible spectrophotometer (Shimadzu UV-160, Japan) in culture of bacteria in BHMS + hexadecane medium. The hexadecane removal assay was carried out by Gas chromatography analysis (18).

3.5. Emulsification Activity and Bacterial Adherence to Hydrocarbons (BATH) Test

The emulsification activity (E24) was determined by addition of hexadecane, to the same volume of cell free culture broth, vortex mixed for 2 minutes and left to stand for 24 h. The emulsification activity was determined as the percentage of height of the emulsified layer (mm) divided by the total height of the liquid column (mm) (17). Bacterial adhesion assessment to hydrocarbon was carried out according to Cappello et al. 2012 (16).

3.6. Detection of Alkane Hydroxylase Gene (*alk-B*) in Bacterial Isolates

Total DNA extraction of bacterial strain was performed using CTAB method (20). The purified DNA extracts were subsequently screened by PCR to detect catabolic *alkB* gene that encodes enzymes involve in alkane degradation pathways. Alkane hydroxylase gene was detected by *alk-3F* (5'-TCGAGCACATCCGCGGCCACCA-3') and *alk-3R* (5'-CCG TAGTGCTCGACGTAGT-3') primers (14, 24). A total volume of 25 μ L PCR mixture containing 2 mM MgCl₂, 10X strength PCR reaction buffer (200 mM Tris, 500 mM KCl), 1 mM each dNTP, 0.15 mM each primer and 1 Unit taq DNA polymerase was added to each tube.

Target DNA was amplified in a thermal cycler (Eppendorf AG 22331, Hamburg, Germany) with initial denaturation of the target DNA at 94°C for 2 min and then 30 repeated cycles comprised of three-step PCR amplification consisting of denaturation at 94°C for 1 min, primer annealing at 54 °C for 30 s and primer extension at 72°C for 30 s. The samples were incubated at 72°C for 5 min at the end of amplification cycles to complete the extension reaction. PCR products (330 bp) were visualized by gel electrophoresis using a horizontal 2 % agarose gel (Sigma, St. Louis, MO) with 1X TBE buffer. Gels were stained in a solution of ethidium bromide and visualized under a UVP UV transilluminator (UVP Inc., San Gabriel, CA) (14, 25).

4. Results

4.1. Isolation and Identification of Bacteria

Among 8 strains that isolated from enrichment cultures which established at 30°C for one week, three bacterial strains were selected for further studies. These strains were isolated from soil contaminated hydrocarbons. These strains were first differentiated by classical biochemical tests. Molecular identification of isolates was performed by amplification and sequencing the 16SrDNA gene sequencing and matching it to the database of known 16SrDNA sequences. The results of identification were presented in Table 1. As shown in this Table, these three strains belonged to *Stenotrophomonas maltophilia* and *Tsukamurella tyrosinosolvens* genus. The phylogenetic trees of these three isolated were illustrated in Figure 1. Full sequences of three bacteria were submitted to EMBL database. Accession numbers of these strains in EMBL are: HE862282, HE862285 and HE86227 respectively.

4.2. Growth Rate and Hexadecane Removal by Strains

All bacterial strains were grown in the presence of 1 % hexadecane for one week while shaking. After one week microbial growth, hexadecane biodegradation were analyzed by spectrometry and GC method respectively. The results of growth and degradation were presented in Table 2. As reported in this Table, *T. tyrosinosolvens* strain Q3 has demonstrated a high hexadecane biodegradation and growth rate. On the other hand, the *S. maltophilia* strain Q1 has the lowest percentage of hexadecane removal (68 percent) and growth rate among all isolates.

4.3. The Effect of Various Hexadecane Concentrations on Bacterial Growth

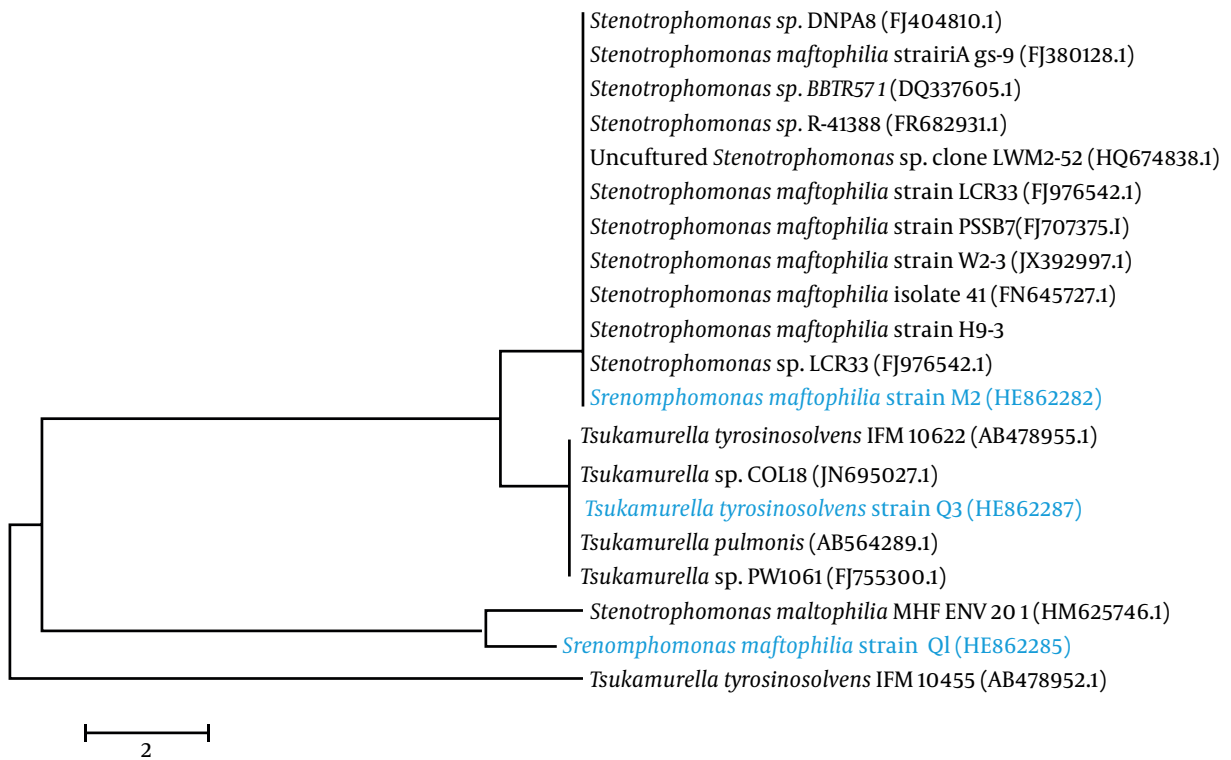
For determine the effect of various hexadecane concentrations on bacterial growth, selected bacterial isolates were grown at different hexadecane concentrations (1, 2.5, 4, 5.5 and 7 percent) after one week-incubation at 30°C, the optical density was read at 600 nm. The result of this experiment was presented in Figure 2. As shown in this Figure when the hexadecane concentrations of increased the growth rate of degradative bacteria were decrease. For example *S. maltophilia* strain M2 has an optical density of 1.5 at 1% hexadecane concentration 1% while this amount decreases to 0.3 in 7% hexadecane concentration. The optimum hexadecane concentration that leads to the high bacterial growth was 2.5%.

4.4. Adherence to Organic Phase and Emulsification Activity by Isolates

Cell surface hydrophobicity and emulsification activity of the isolates were studied by emulsification (E24%) and bacterial adhesion to hydrocarbon tests (BATH). The results were presented in Table 2. These results indicated that there is a direct relationship between cell surface hydrophobicity and emulsification activity with alkane biodegradation. These results demonstrate that when a bacterial strain has high cell surface hydrophobicity it can produce more emulsifiers that enhance the alkane biodegradation.

Table 1. Closest Relatives of 16rDNA Gene Sequences of Isolated Bacteria in This Study

Isolate	Closest hit	Accession Number	ID, %
M2	<i>Stenotrophomonas maltophilia</i> strain MHF ENV 20	HE862282	98
Q1	<i>S.maltophilia</i> strain Ags-9	HE862285	98
Q3	<i>Tsukamurella tyrosinosolvens</i> IFM 10623	HE862287	100

Figure 1. Phylogenetic Tree of 16S rDNA Sequences of the Bacterial Isolates Obtained From Petroleum Reservoirs Waste Water

The tree was constructed using sequences of comparable 16S rDNA gene sequences available in public databases. Neighbour-joining analysis using 1,000 bootstrap replicates was used to infer tree topology. The bar represents 0.1% sequence divergence. Sequenced data show the location of selected isolated strain.

Table 2. Growth Rate, Hexadecane Removal, Measurement of Emulsification Activity (E24 %) and Cell Surface Hydrophobicity (BATH %) in Isolated Strain

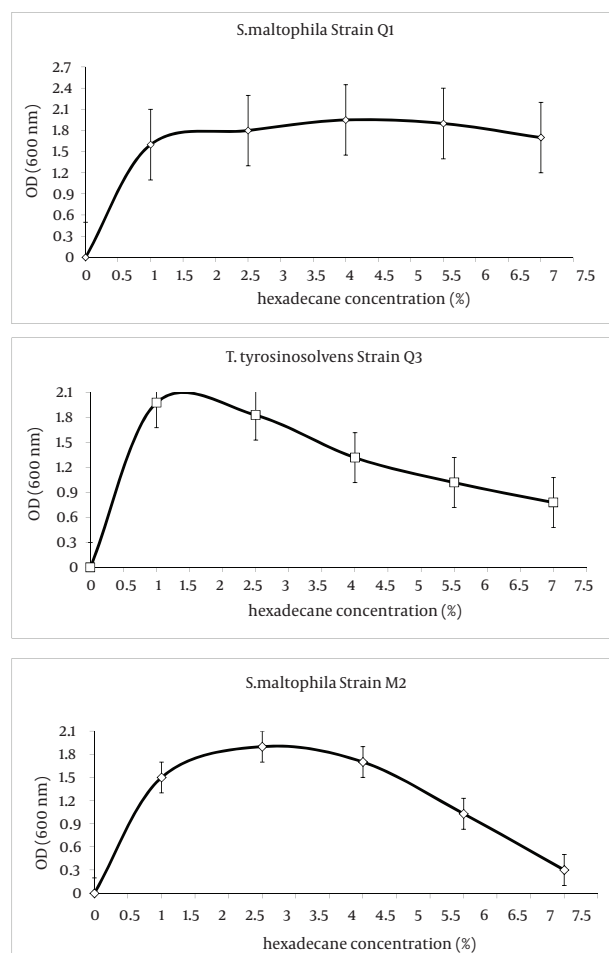
Isolate	Growth Rate, OD600nm	Percent of hexadecane Removal	Emulsification activity, E24, %	Cell Surface Hydrophobicity, BATH, %
<i>Stenotrophomonas maltophilia</i> strain M2	1.32	74	29	6
<i>S.maltophilia</i> strain Q1	1.4	68	6.12	24
<i>Tsukamurella tyrosinosolvens</i> strain Q3	1.12	76	7.14	29

4.5. Detection of Catabolic Gene by PCR

The presence of catabolic gene encoding alkane hydroxylases enzymes (alkB gene) of isolated strains was determined by PCR using specific primers of this functional

gene. Alk-B primers successfully amplified PCR products of expected size from all isolated strains. Figure 3 show the results of PCR analysis which verified alkane hydroxylase gene existed in all isolated strains.

Figure 2. Effect of Various Concentrations of Hexadecane (1- 7%) on Bacterial Growth (as Optical Density (O.D.) at 600 nm) of Three Bacterial Strains



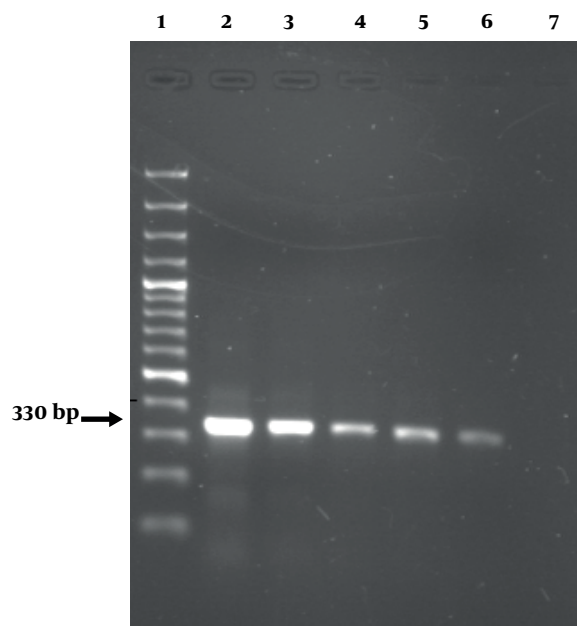
All strains were grown for 7 day at 30°C.

5. Discussion

Tsukamurella spp. primarily are known as environmental saprophytes of soil, water and foam (thick stable scum on aeration vessels and sedimentation tanks) of activated sludge. *Tsukamurella* are represented in HPC populations in drinking-water. *Tsukamurella* spp. appears to be transmitted through devices such as catheters or lesions (26, 27). *S. maltophilia* is ubiquitous species that exists in a wide variety of environments and geographical regions, and different strains of this organism were isolated from soil as well as various nosocomial and clinical specimens. Characteristic of *S. maltophilia* are unusual lipopolysaccharides containing relatively large amount of the uncommon fatty acids (28, 29).

Sudarat et al. isolated these bacteria from soil sample of a contaminated site located in Warracknabeal (30).

Figure 3. Amplification of alk-B Gene (Alkane Hydroxylase) by PCR Analysis of DNA Extracts From Bacterial Isolates



lane 1, 100 bp DNA size marker Ladder; lane 2, positive control (*Acinetobacter* sp.2769A); lane 3, isolate M2; lane 4, isolate Q1; lanes 5, 6 isolate Q3; lane 7, negative control.

Nicole et al. isolated these bacteria from soil of a bioremediation system at Rositz, a village in Thuringia, Germany. The contamination consisted of benzene, toluene, ethylbenzene and xylene (BTEX), polyaromatic compounds, as well as phenols (31). The key difference between our study and other reported ones is the isolation of these bacteria from petroleum reservoirs waste water and also hydrocarbon-contaminated soils in Iran (Tehran). Many reports about the isolation of aromatic hydrocarbon degrading bacteria from contaminated soil, sediment, sludge and water are available but no report on the isolation of hexadecane degrading bacteria from petroleum reservoirs waste water has been published yet.

In this study three bacterial strains with hexadecane degradation ability were isolated and identified by 16SrDNA sequence analysis. These strains belonged to the genus *Stenotrophomonas*, and *Tsukamurella*. Jurelevicius et al. has been defined the alkane degrading bacteria in soils from King George Island in Maritime Antarctic (32). They found that there is a high diversity of alkane degrading bacteria in this environment. Quatrini et al. isolated some gram positive n-alkane degraders from a hydrocarbon contaminated Mediterranean shoreline (33). Hence it is believed that bacteria must have certain mechanisms to uptake and use this hydrophobic substrate. In this study the effect of increased concentration of hexadecane from 1% to 7% on isolated bacterial growth was studied.

We found that when the hexadecane concentration

increased the growth rate of bacteria will decreased, however this reduction is not the identical in all strains. The optimum hexadecane concentration for growth was found 2.5%. The water solubility of hexadecane is less than 0.9×10^{-6} mg/L (18). Vomberg et al. isolated 45 alkane degrading bacteria belong to 37 bacterial species from soil contaminated to crude-oil in Germany. They have studied the distribution of *alk-B* among isolates by PCR-hybridization method and have concluded that the group (III) alkane hydroxylase is predominant among isolates (34). To date, the microorganisms carrying the *alkB* gene have been found in various environments, such as Alaska sediments, contaminated soil, cold ecosystems, oil-contaminated site, a shallow aquifer, bulk soil, a land treatment unit, Arctic and Antarctic soil and Sea water (35).

The gene *alkB* could possibly be used as a potential prediction marker to predict the of microorganism in different environments with oil degradation capability (35). Heissblanquet et al. showed that there are strong differences in the predominance of the two *alk-B* genotypes of freshwater and soil microcosms. However both types of *alk-B* gene prevalence increased in the most polluted soils (15). In this study distribution of *alk-B* is almost the same among 3 bacterial isolates. This result indicated that alkane hydroxylase enzyme encoded by *alk-B* gene is essential for hydrocarbon biodegradation.

The described results of this paper show that hexadecane degrading bacteria have more diversity and biodegradation ability of petroleum reservoirs waste water of Iran. the new isolates capability to degrade PAHs could be also investigated in further studies.

Authors' Contribution

None declared.

Financial Disclosure

None declared.

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