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Effect of Curing of *Anoxybacillus rupiensis* Strain Ir3 (JQ912241) Plasmid (s) in their Ability for Biodegradation of Carbazole

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Authors' contributions

This work was carried out in collaboration between all authors. Authors MHAJ and AMAF designed the study and wrote the protocol. Author MSM wrote the first draft of the manuscript and managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: To study the effect of curing agents on *A. rupiensis* Ir3 (JQ912241) plasmid(s) and the plasmid(s) role in utilization of carbazole in this bacterium.
Study Design: Experimental study.
Place and Duration of Study: Department of Biotechnology, College of Science, Al-Nahrain University. Baghdad, Iraq, between October 2012 and February 2013.
Methodology: *Anoxybacillus rupiensis* strain Ir3 (JQ912241) a newly thermophilic bacterium capable to utilize aromatic hydrocarbons, was used. Plasmid profile of this bacterium was determined. This bacterium was treated with two curing agent's in order to cure their plasmids.
Results: Plasmid profile of *A. rupiensis* strain Ir3 (JQ912241) showed that this bacterium contains large and small plasmid DNA bands. In order to determine the role of plasmid in utilization carbazole, many attempts were made to cure plasmid (s) of this bacterium using Sodium Dodecyl Sulfate (SDS) and Ethidium Bromide (Et.Br). Results indicated that no cured colonies (lost their ability to utilize carbazole at 70℃) were obtained. P lasmid isolated from some of these colonies

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being treated with Et.Br, indicated that these colonies are still harboring the large plasmid.
Conclusion: It was difficult to cure the large plasmid, and the utilization trait might be located on it or on the chromosome.

Keywords: Anoxybacillus rupiensis; carbazole; curing agent; plasmid; biodegradation.

ABBREVIATIONS

CAR: Carbazole, CDM: Chemically defined medium, Et.Br: Ethidium bromide, MIC: Minimum inhibitory concentration, LB: Luria-Broth and SDS: Sodium dodecyl sulfate.

1. INTRODUCTION

The genus *Anoxybacillus* belongs to the order bacillales under the firmicutes phylum in the domain bacteria [1]. The genus with the type species *Anoxybacillus pushchinoensis* was separated from the genus *Bacillus* [2]. Euzeby mentioned that there are 16 species and 2 subspecies described in the genus *Anoxybacillus* [3]. It has been proven that 5 of 16 species were aerobes. Among them, *A. kamchatkensis* [4], *A. flavithermus* sp. [2], *A. contaminans* [5], *A. amylolyticus* [6] and *A. rupiensis* [7]. It is appeared that all the members of this genus show thermophilic features. The species that belongs to the genus *Anoxybacillus* show 97% or more 16S rRNA sequence similarity, while they have proven to be different species by DNA-DNA hybridization. Hence, the 16S rRNA sequences are applicable tools for classifying the genus *Anoxybacillus* in genus level, and inappropriate for species level [8]. In the study of Inan et al, the applicability of rpoB gene was investigated, which encodes the β subunit of RNA polymerase to be used as an alternative to 16S rRNA gene sequence similarity analysis in the thermophilic genus *Anoxybacillus*. The *rpoB* gene was found to provide a better resolution for *Anoxybacillus* species, with lower interspecies sequence similarities. The *rpoB* sequence similarity analysis permitted a more accurate discrimination of the species within the *Anoxybacillus* genus than the more commonly used 16S rRNA gene. They also reported that *Anoxybacillus* species are widely distributed and readily isolated from geothermal heated environments, with a continually increasing industry interest in their thermostable gene product. Therefore isolating the new strains of this novel bacterial genus is not a taxonomical concern, but also a necessity in order to exploit its biotechnological potential completely [9].

Anoxybacillus rupiensis sp. nov. isolated from hydrocarbon contaminated soil in Iraq [10] it was an efficient strain for utilizing aromatic compounds and the identity of the carbazole-degrading culture. It was investigated using biochemical tests, microscopic observation, and a determination of its 16S rRNA gene. The culture is a facultative Gram positive (or gram variable) long rod that form, medium sized, smooth, round colonies with cream color, regular and complete margins on LB agar plate. Cell of this strain is appeared as motile, strictly aerobic, thermophile. Most cells occur in exponential growth phase singly or in chains. Terminal endospores are observed. Obligate thermophilic growing between 40 and 70℃ optimum 55-65℃ and in the pH range from 5.0-9.0 (optimum 7.0), the insole is not produced, the Voges-Proskauer reaction is negative, catalase and oxidase reaction are positive and methyl red test is negative. The 16S rRNA gene sequence of this bacterium compared with a database of NCBI with BLAST program has 97% similarity to *Anoxybacillsus rupiensis* (HQ 696615.1). These data indicated that this carbazole-degrading bacterium can be identified to the genus and species level as *Anoxybacillus rupiensis* (JQ 912241) [11]. According to the biochemical and molecular properties, the isolate may represent a new CAR-degrading bacterium. This strain was able to utilize naphthalene as a carbon and energy source and carbazole, nitrobenzene and ρ-nitrophenole as a carbon, nitrogen and energy source.

Carbazole is one of the most predominant polyaromatic N-heterocyclic compounds in oil [12], Naphthalene belongs to poly aromatic hydrocarbons, and Nitrobenzene and ρ-nitrophenole belong to nitroaromatic compounds whose reduction alters petroleum quality. Therefore, further research on this novel species may develop bioremediation strategies for polluted environments, and improvements of biorefining processes. For several decades, thermophilic bacteria have attracted the interest of many scientists due to their biotechnological

potential [13]. In particular, phenotypic and genotypic characterization of thermophilic bacteria has been done for many geothermal areas in different parts of the world, including Turkey [14], Italy [15], Bulgaria [16], China [17], India [18]. According to that mentioned above and due to the limited studies about the genetic of such bacteria, this work aimed to study the role of *A. rupiensis* Ir3 (JQ912241) plasmid(s) in utilization of carbazole.

2. MATERIALS AND METHODS

2.1 *A. Rupiensis* Strain Ir3 (JQ912241)

The bacterium used in this study (*Anoxybacillus rupeinsis* Ir3 (JQ912241)) is a novel strain able to utilize aromatic compounds. It was isolated from hydrocarbon-contaminated soil [10].

2.2 Plasmid Extraction of *A. rupiensis* Strain Ir3 (JQ912241)

The plasmid DNA was isolated by using the following methods:

2.2.1 Salting out method [19]

Culture of bacteria grown in 20 ml of LB broth was pelleted by centrifugation at 6000 rpm for 15 min. The pellet washed with 3 ml of SET buffer, and the cells were resuspend with 1.6 ml of SET buffer, and then freshly prepared lysozyme (final concentration 1 mg/ml) was added and incubated at 37℃ for 30 min. One ml of 10% SDS was added and mixed by inversion, then incubated at room temperature for 30 min. Two ml of 5 M NaCl was added and mixed by inversion at room temperature. An equal volume of chloroform was added, mixed by inversion for 15 min., then centrifuged (6000 rpm at 4℃) for 20 min. The aqueous phase was transferred to another sterile tube, and 0.6 volume of isopropanol was added and mixed by inversion. It was kept at room temperature for 5 min. Then, it was centrifuged at 13000 rpm for 15 min at 4℃. The isopropanol was discarded and the precipitated DNA dissolved in 100 µl TE buffer and stored at -20℃.

2.2.2 Gene JET^{TM} plasmid miniprep kit (Fermentas)

The pelleted cells were resuspended in 250 µl of resuspension solution. Lysis solution (250 µl) was added and mixed thoroughly by inverting the tube 4-6 times until the solution becomes viscous and slightly clear. Neutralization solution (350 µl) was added and mixed immediately and thoroughly by inverting the tube 4-6 times. Centrifugation for 5 min to pellet debris and chromosomal DNA. The supernatant was transferred to the Gene JET^{TM} spin column by decanting. Centrifuge for 1 min and discard the flow-through. Five hundred microliter of the wash solution was added to the Gene JET^{TM} spin column, centrifuge for 30-60 Sec and discarded the flow through. This step was repeated. The Gene JET^{TM} spin column was transferred into new 1.5 microcentrifuge tube. Fifty microliter of the elution buffer was added to the center of the Gene JET^{TM} spin column membrane, incubate for 2 min at room temperature and centrifuge for 2 min, to elute the plasmid DNA. The purified plasmid was stored at -20℃.

2.2.3 Pure Yield^{TM} plasmid miniprep kit (Promega)

Six hundred microliter of bacterial culture grown in LB medium was transferred to a 1.5 ml microcentrifuge tube. Cell lysis buffer (100 µl) was added and mixed by inverting the tube six times. Of the cold (4-8℃) neutralizing solution (350 µl) was added and mixed thoroughly by inverting the tube. Centrifugation at maximum speed in a microcentrifuge for 3 min. The supernatant was transferred to a pure yield^{TM} minicolumn. The minicolumn was placed into a pure yield^{TM} collection tube, and centrifuged at maximum speed for 15 seconds. The flow through was discarded, and the minicolumn was placed into the same pure yield^{TM} collection tube. Two hundred microliter of endotoxin removal wash was added to the minicolumn, centrifuged at maximum speed for 15 Sec. Four hundred microliter of the column wash solution was added to the minicolumn, centrifuged at maximum speed for 30 Sec. The minicolumn was transferred to a clean 1.5 ml microcentrifuge tube, and then 30 µl of the elution buffer was added to the minicolumn matrix and let stand for 1 min at room temperature. Centrifugation at maximum Speed for 15 Sec to elute the plasmid DNA. The eluted plasmid DNA was stored at -20℃.

2.3 Curing of Plasmid DNA

Curing experiments were performed on the bacterium *A. rupiensis* strain Ir3 (JQ912241) by using two types of curing agents (SDS and Et.Br) according to Trevors [20]. The bacterium was grown in 5 ml of LB broth to mid log phase, then

0.1 ml (300×10^6) inoculums of fresh culture (18 hrs) were inoculated in a series of 5 ml fresh LB broth containing various concentrations of SDS (0%, 0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, 5%) or Et.Br (0, 70, 80, 90, 100, 125, 150, 175, 200, 225, 250 µg/ml). All tubes were incubated with shaking (150 rpm) at 55℃ for 24-48 hrs. The growth density of different tubes was observed by naked eye and compared with the control to determine the effect of SDS and Et.Br on bacterial growth. The lowest concentration of each curing agent that inhibited the growth of bacterial isolate considered as the minimal inhibitory concentration (MIC). Samples were taken from tubes containing the highest concentration of either SDS or Et.Br that still allowed bacterial growth, and diluted appropriately, then 0.1 ml from proper dilutions were spread on LB agar plates and incubated overnight at 55℃ to score the survived colonies. These colonies (100 colonies) were replica plated (using a sterile toothpick) on LB agar plate (master plate) and on mineral agar plate (CDM) [21] containing 1 mM carbazole [11]. If the colonies were able to grow on the master plate, but not on the selective medium containing carbazole, it means that the cells of this colony are cured cells that lost the ability to grow on carbazole as a sole source of carbon, nitrogen and energy. The DNA has been isolated from these colonies, and gel electrophoresis was performed in order to compare between them and wild type.

2.4 Gel-electrophoresis [22]

Agarose gels (0.7% and 1%) were run horizontally in Tris-borate-EDTA (TBE 1X). A sample of DNA was mixed with 1/10 volume of the loading buffer and added to the wells of the gel. Generally, gel was run for (50 min., 1 hrs. 1.5 hrs. and 2 hrs.) at 5V/cm and the gel buffer added up to the level of horizontal gel surface. Agarose gel was stained with ethidium bromide by immersing them in distilled water containing the dye at a final concentration of 0.5 µg/ml for 30-45 min. DNA bands were visualized by UV illumination at 302 nm on a UV transilluminator. Gels were distained in distilled water for 30-60 min. to get rid of the background before photographs were taken.

3. RESULTS AND DISCUSSION

3.1 Plasmid Isolation

In order to determine the plasmid profile of *A. rupiensis* strain Ir3 (JQ912241), salting out method [19] and two types of plasmid isolation miniprep kits Promega and Fermentas were used.

The results showed that this strain harbored small and large plasmid DNA bands. Small plasmid DNA bands were seen when salting out method was used. Whereas large plasmid DNA bands have been seen when plasmid miniprep kits were used as shown in Figs. 1, 2, and 3 respectively.

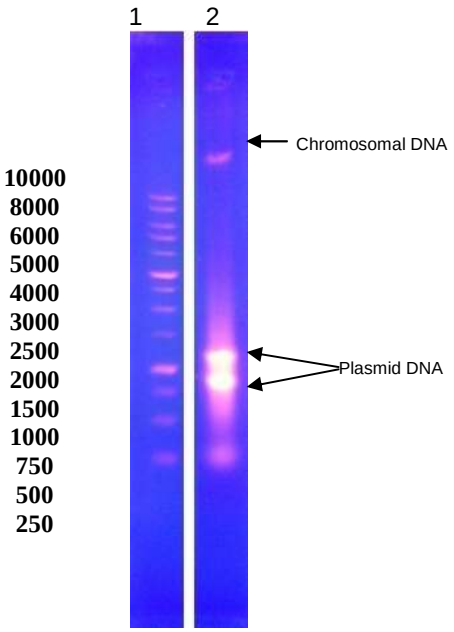

Fig. 1. Gel electrophoresis for plasmid isolation from *A. rupiensis* strain Ir3 (JQ912241) by salting out method. Electrophoresis was performed on (0.7%) agarose gel with 5V/cm for 2 hours

1: markers, DNA ladder 10Kb RTU 2: A. rupiensis

It was reported that microbial biodegradation pathways are often, either fully or partially, encoded on mobile genetic elements, including catabolic plasmids. As these plasmids frequently contain beside catabolic genes/operons, the full set of determinants necessary for conjugative transfer, they are relatively large from approximately 50 kb up to more than 1 Mb, for example Pseudomonas putida EST1020 harbored pEST1026 (109 Kb) responsible for phenol degradation and Arthrobacter keyseri 12B harbored pREI (130 Kb) for phthalate degradation [23].

Previous studies showed that biodegradation of some oil derivatives depends on plasmids.

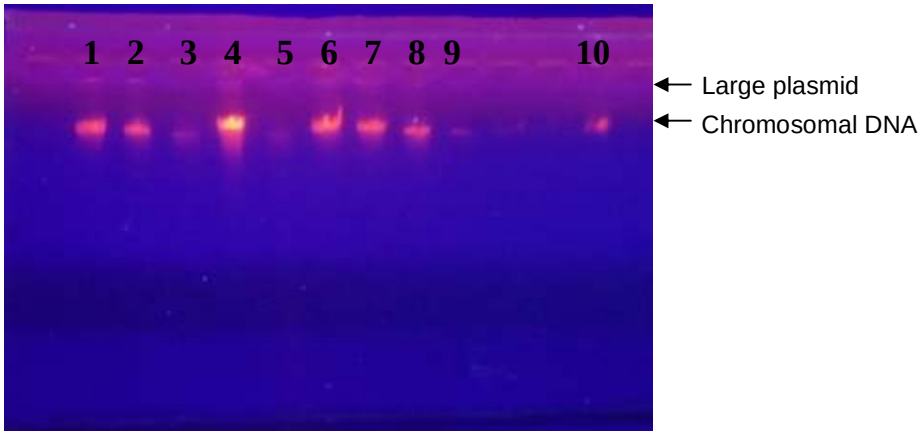

Fig. 2. Gel electrophoresis for plasmid isolation from *A. rupiensis* strain Ir3 (JQ912241) and the other nine thermophilic bacterial isolates by using the Promega plasmid miniprep kit. Electrophoresis was performed on (0.7%) agarose gel and run with 5V/cm for 50 minutes

1: 9SM, 2: 2G, 3: 3A, 4: Ir3, 5: 12SM, 6: 6A, 7: 13SM, 8: 14SM, 9: 21SM, 10: 34SM

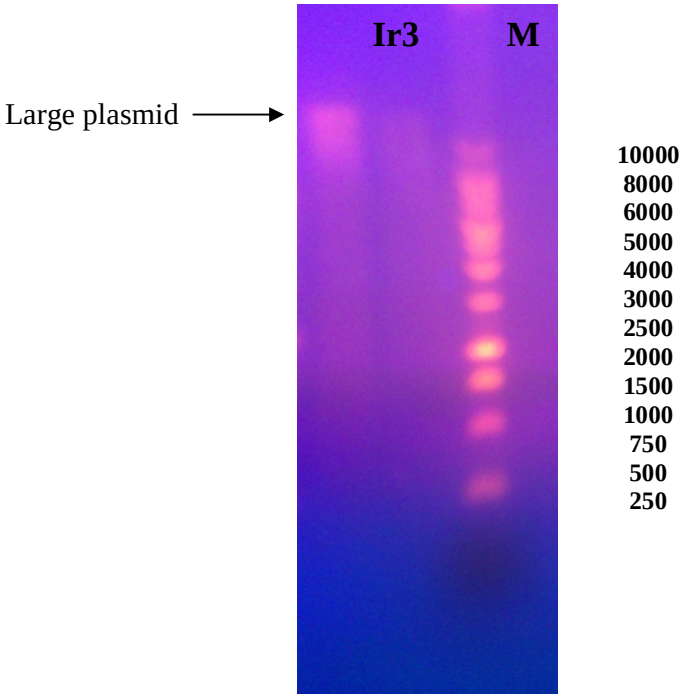

Fig. 3. Gel electrophoresis for plasmid isolation from *Anoxybacillus rupiensis* Ir3 (JQ912241) by using Fermentas miniprep kit. Electrophoresis was performed on (1%) agarose gel and run with 5V/cm for 1.5hours

M: markers, DNA ladder 10Kb RTU.
Ir3: Anoxybacillus rupienses Ir3 (JQ912241).

Plasmids pWWO, pTOM, pNAH, and pOCT are involved in the degradation of benzene, toluene, naphthalene and alkane [24].

It was found that certain plasmids play an important role in adaptation of natural microbial populations to oil and other hydrocarbons. Some of the microbial catabolic pathways responsible for the degradation, including the alk (C5 to C12 n-alkanes), nah (naphthalene) and xyl (toluene) pathways have been extensively characterized and are generally located on large catabolic plasmids [25].

It was elucidated that the carbazole degradative car-/gene cluster (carAalBalBblClACl) of *Sphingomonas* sp. strain KA1 is located on the 254 kb circular plasmid pCAR3. Carbazole conversion to anthranilate is catalyzed by carbazole 1, 9a-dioxigenase (CARDO;carAalAcl), meta- cleavage enzyme (carBalBbl), and hydrolase (carCl) [26]. Also, it was stated that the carbazole-catabolic plasmid pCAR1 isolated from *Pseudomonas resinovorans* strain CA10 was sequenced in its entirety to elucidate the mechanism by which the car operon may have been assembled and distributed in nature. pCAR1 is a 199,035-bp circular plasmid, and carries 190 open reading frames, and it was found that pCAR1 carries the class II transposon Tn4676 containing carbazole-degradative genes, also a new plasmid designated pCAR2 was isolated from *P. putida* strain HSO1 [27].

3.2 The role of Plasmid in Carbazole Degradation

In order to study the role of plasmid of *A. rupiensis* strain Ir3 (JQ912241) in carbazole degradation, curing experiments were done.

3.2.1 Curing

Plasmid curing of *A. rupiensis* strain Ir3 (JQ912241) was used to determine whether the genes responsible for hydrocarbon degradation are located on the plasmid or not. For this purpose, many attempts were made in order to cure *A. rupiensis* strain Ir3 (JQ912241) plasmid(s) using SDS and Et.Br. No colonies appeared after treatment with different concentrations of SDS (0.5%, 0.6%, 0.7%, 0.8%, 0.9%, 1%, 2%, 3%, 4%, and 5%), which indicated that this bacterium is sensitive for SDS. The results in Table (1) indicated that the highest concentration of Et.Br that still allows the bacterial growth was 225 µg/ml. From this treatment, an appropriate dilutions were made and spread on LB agar plates, (incubated at 70℃, in order to determine the role of plasmid in temperature resistance), and on selective media, mineral salt agar (CDM) containing carbazole (1 mM), in order to determine the cured colonies that lost their ability to utilize carbazole.

The result indicated that all the tested colonies of *A. rupiensis* strain Ir3 (JQ912241) (100 colonies were selected randomly) were still able to grow in the presence of carbazole at 70℃.

Plasmid isolation from a number of these colonies showed that they are still harboring plasmid, Fig. (4) showed one of these colonies. No cured colony from *A. rupiensis* strain Ir3 (JQ912241) was obtained, and this may be related to large plasmid that cannot be cured easily. Therefore, other molecular techniques to confirm the role of plasmid may be needed or the utilization trait may be located on the chromosome.

Catabolic pathways, which encode degradative routes of different aromatic and aliphatic hydrocarbon, are frequently located on plasmids, although degradative genes can be located on either chromosome or plasmid [28].

It was noticed that *Arthrobacter protophormia* strain RkJ100 is capable of utilizing ρ-nitrophenol as the sole source of carbon, nitrogen, and energy. A large plasmid of approximate 65 kb was found to be responsible for harboring genes for ρ-nitrophenol degradation in this strain. This was based on the fact that ρ-nitrophenol (-) derivative was devoid of plasmid and had simultaneously lost their capability to grow on this nitroaromatic compound [29]. Also the plasmid mediation of ability to degrade phenanthrene was demonstrated [28].

Shimizu et al. found that many aromatic degradation genes are known to be encoded on plasmid DNAs [30]. In particular, several *Rhodococcus* strains harbor aromatic degradation genes on large linear plasmids, for example *Rhodococcus erythropolis* strain BD2 harbors a linear 210 Kb plasmid pBD2 carrying the ipb genes for isopropylbenzene degradation [31].

Table 1. Effect of Et.Br on the growth of *A. rupiensis* strain Ir3 (JQ912241)

Et.Br concentration (µg/ml)	70	80	90	100	125	150	175	200	225	250
Bacterial growth	+++	+++	+	+	+	±	±	±	±	-

(+++): Very good growth; (-): No growth; (++): Good growth
(±): Slight growth; (+): Moderate growth

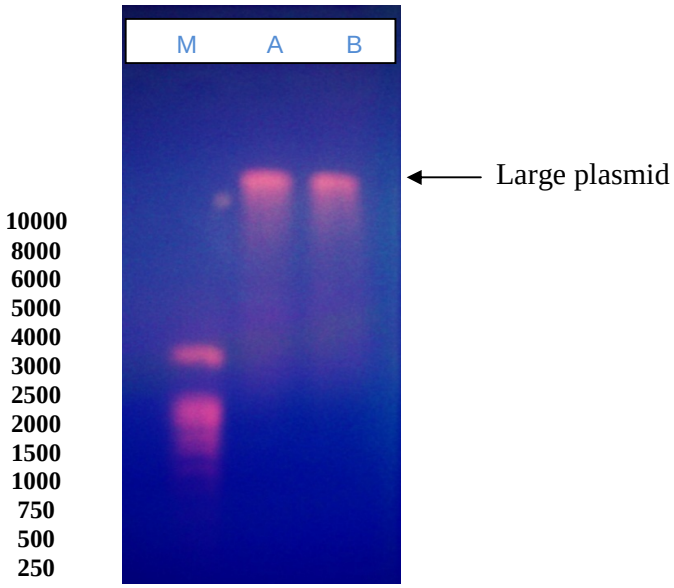

Fig. 4. Gel electrophoresis of the plasmid content of A. rupiensis strain Ir3 (JQ912241). Electrophoresis was performed on (1%) agarose gel and run with 5V/cm for 1.5 hours

M: markers, DNA ladder 10Kb RTU
A: plasmid content of the A. rupiensis strain Ir3 (JQ912241).
B: plasmid content of the A. rupiensis strain Ir3 (JQ912241) (after treatment with Et.Br)

4. CONCLUSION

It was difficult to cure large plasmid of *A. rupiensis* strain Ir3 (JQ912241), and this may be related to its large molecular size that cannot be cured easily. Therefore, other molecular techniques may be needed such as using other curing agents and/or transform the plasmids to strains free of plasmids to confirm the role of the plasmid in biodegradation of carbazole. The biodegradation trait in this bacterium may be located on the large plasmid or on the chromosome.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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