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Isolation and characterization of *Dietzia maris* from midgut of *Aedes albopictus*: A suitable candidate for paratransgenesis

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Abstract
The genus *Dietzia* is remarkably similar to genus *Rhodococcus.* Bacterial species *Rhodococcus rhodnii* of genus *Rhodococcus* was used for genetically modification for expression of potent antimicrobial molecules which shows strong deleterious effect against *Trypanosoma cruzi*. Hence, *Dietzia maris* can be a suitable candidate for genetic modification for expression of effector molecules against the parasite like dengue and chikungunya in the mosquitoe's midgut as was possible in *R. rhodnii.* In this study the *D. maris* was isolated for the first time from mosquitoes *Aedes albopictus* collected from Arunachal Pradesh, North East India. After purification bacteria was first morphologically and biochemically characterized which showed high salt tolerance and exhibited slow growth rate appearing on agar plate after 48-72hr. The isolated bacterial species was finally conformed as a *D. maris* on the basis of results obtained from MALDI-TOF MS and 16S rRNA gene based analysis.

Keywords: *Dietzia maris*, midgut, *Aedes albopictus*, parasites, paratransgenesis

1. Introduction
The genus *Dietzia* was first proposed by Rainey *et al.,* in 1995, to accommodate the actinomycetes previously named as *Rhodococcus maris* [1, 2]. Initially, it was known as '*Flavobacterium maris'* [3] but later classified as *R. maris* [2]. *D. maris* are very similar to *Rhodococcus equi,* in morphology and clinical appearance and commonly encounters by microbiologists in their daily practice. *Dietzia* spp. might be misidentified as a *Rhodococcus* spp. due to high degree of similarity and absence of simple, appropriate and specific methods for their identification [4].

D. maris (family Dietziaceae, order Actinomycetales) was first isolated by Harrison, (1929) in halibut and subsequently in 1982, by Nesterenko *et al.,* from the skin and intestinal tract of carp (*Cyprinus carpio*) [2, 3]. According to data of Gharibzahedi *et al.* (2014), till 2014 a total of thirteen *Dietzia* spp. have been reported from various sources viz: *D. maris*, *D. natronolimnaea, D. psychralcaliphila*, *D. cinnamea*, *D. kunjamensis*, *D. papillomatosis*, *D. cercidiphylli*, *D. schimae, D. lutea*, *D. timorensis*, *D. aerolata*, *D. alimentaria* and *D. aurantiaca* [1, 5-16].

D. maris was isolated from various resources but to the best of our knowledge, it was not reported from any mosquitoes and we for the first time isolated it from *Aedes albopictus* collected from the foot hills of Himalaya at Bhalukpong, Arunachal Pradesh, India. *Ae. albopictus* and *Ae. aegypti* are responsible for transmission of dengue and chikungunya virus world wide, mainly in tropical and sub-tropical regions, mostly in urban and semi-urban areas. In the recent decades, the incidence of dengue has increased dramatically. A recent report estimated that, in 128 countries about 3900 million persons are at risk of dengue infection [17] and every year about 500000 people with severe dengue infection has been reported with about 2.5% casualty. Hence, there is an urgent need to develop an effective control method for disease transmission [18].

The bacterial species, *Rhodococcus rhodnii* of genus *Rhodococcus* have been used for development of paratransgenesis techniques against *Trypanosoma cruzi*, a causative agent of Chagas disease transmitted by reduviid bug, *Rhodnius prolixus* [19, 20]. Due to high similarity of *Dietzia* spp. to *Rhodococcus* spp., the paratransgenesis technique may also be developed using *D. maris* against invading pathogen inside mosquito's midgut which causes severe disease. With this objective, we isolated and characterized a bacterial strain *D. maris* from the midgut of

Ae. albopictus from Arunachal Pradesh, India. Thus, a novel, cheap and eco-friendly technique might be developed for management of diseases transmitted by mosquitoes.

2. Materials and Methods

2.1 Isolation of bacterial isolate

Bacterial strain Bab3-8 was isolated from the midgut of female *Ae. albopictus* which emerged from larvae collected from Bhalukpong, Arunachal Pradesh, North East India which is located at the foothills of the Himalayas. (27.01° N, 92.65° E). The samples were collected during post monsoon season when the breeding of mosquitoes were at peak. Midgut of female *Ae. albopictus* was dissected out and the homogenate was prepared in Phosphate buffered saline (PBS) and transferred to nutrient agar for isolation of bacterial strain as described earlier [21]. Isolated bacteria were purified by streaking on agar plate.

2.2 Characterization of isolates

2.2.1 Phenotypic Characterization

The colony morphology like shape, size, colour, margin, opacity, elevation of purified bacterial colonies was studied microscopically. Hanging drop method was used to study the motility. Gram staining kit (HiMedia, India) was used for determination of gram staining reaction according to the manufacturer's instruction. Salt tolerance of bacterial isolate was studied on various salt concentrations from 1 to 20%.

Various biochemical tests such as, nitrate reduction, urease activity, citrate utilization, H_2S production, Voges Proskauer (VP), Methyl Red (MR), β– galactosidase activity, ornithine and lysine utilization activity and carbon source utilization profile were performed using biochemical kit following manufacturer's instruction (HiMedia, India).

2.2.2 Scanning Electron Microscopy (SEM)

Fresh overnight broth culture of Bab3-8 was used in Scanning Electron Microscope analysis. The sample was prepared as following described protocol. Bacterial pellet was washed two times with Phosphate-buffered saline (PBS) followed by addition of 2.5% Glutaraldehyde in PBS for fixation of the pellet, which was mixed properly and incubated at 4ºC for 6hrs. After fixation, the pellet was again washed three times with Phosphate-buffered saline (PBS). For dehydration of bacterial specimen ethanol was used with gradual increase of concentration (30%, 50%, 80% and 100%). During washing with 100% ethanol, samples were mixed with ethanol and was directly transferred to presterilized cover slip and allowed to air dry. Small piece of sample was cut and platinum coated and examined under (SEM) JEOL-JSM-6390LV Scanning Electron Microscope.

2.2.3 Matrix-assisted laser desorption / ionization time-of-flight mass spectrometry (MALDI-TOF MS)

The MALDI-TOFMS identification system was used for the identification of bacterial strain in which the mass spectrum acquired from unknown organism was compared to the reference Biotyper database [22, 23]. The fresh bacterial culture was directly deposited on a MALDI-TOF MTP 96 target plate (Bruker Daltonik GmbH) and inactivated with 96% formic acid (1µl) followed by overlaying 1 µl of matrix solution (α-cyano-4-hydroxycinnamic acid in 50% acetonitrile, and 2.5% trifluoroacetic acid). Crystal formation of matrix-sample was allowed by air-drying at room temperature. Microflex III mass spectrometer (Bruker Daltonik) with a 337 nm nitrogen laser was used for performing the measurement and the spectra was recorded. The obtained raw spectra were matched with mass spectra of different bacterial species as a reference from Biotyper database using Bio Typer software, ver. 3.0 [24].

2.2.4 Genomic DNA isolation and PCR amplification of 16S rRNA

For 16S rRNA gene amplification, the genomic DNA was isolated from overnight fresh bacterial culture according to protocol described earlier [21]. Two sets of primer, forward 16S1 (5'-GAGTTTGATCCTGGCTCA-3') and reverse 16S2 (5'-CGGCTACCTTGTTACGACTT-3') were used for amplification of 1.5 kb 16S rRNA gene according to Alam *et al.*, (2006) [25]. Prior to sequencing the PCR product was purified according to the Chromous PCR Cleanup kit and sequencing was done on the ABI 3500xL Genetic Analyzer (Applied Biosystems Inc. Foster City, CA) at Chromous Biotech, Banglore, India. After forward and reverse sequence alignment, the obtained sequence of 16S rRNA gene was submitted to GenBank under the accession number (KT380950).

2.3 Phylogenetic Analyses

The BLAST (http://www.ncbi.nlm.nih.gov/BLAST) and EzTaxon server (http://147.47.212.35:8080) was used for searching the homologous sequences in the Genbank data base and the closest related sequences were retrieved for performing the multiple sequences alignment using CLUSTAL W program. End trimming of unaligned sequences and phylogenetic relatedness was performed using neighbor joining and Kimura 2 distances parameter method in MEGA 6.0 package [26]. One thousand bootstrap replicates were generated for examination of the robustness of the trees and the consensus tree was used for analysis.

3. Results

The Bab3-8 bacteria strain was isolated from the midgut of female *Ae. albopictus*, collected from Bhalukpong, Arunachal Pradesh, North East India, a hot biodiversity zone of India. Initially, the morphological characteristics of the bacterial isolate was studied and found to be gram positive, aerobic, circular in shape, deep orange red in colour, convex or elevated, opaque with a smooth margin (Fig. 1). The bacterial colony exhibited very slow growth, appeared after 48-72 hours on agar plate and high salt tolerance, grows up to 10% (w/v) NaCl concentration (Fig. 2).

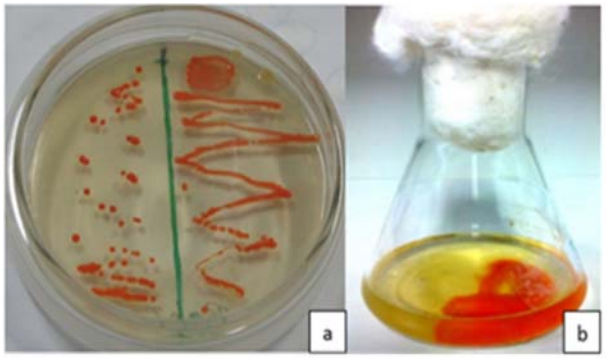

Fig 1: Purified culture of isolate a. on nutrient agar and b. in nutrient broth

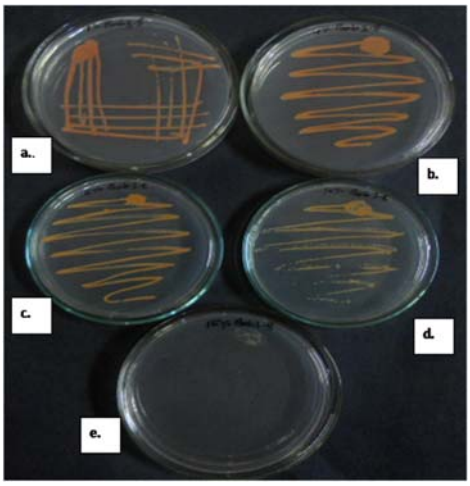

Fig 2: Salt tolerance of bacterial strain- a. at 1%, b. at 4%, c. at 8%, d. at 10% and e. at 15%

Biochemical reactions like Nitrate reduction, H_2S production, urease activity, Voges–Proskauer (VP) and methyl red (MR) tests are some important reaction used for determination and discrimination of *Dietzia* strains [27]. In our result, the bacterial strain Bab3-8 was able to hydrolyze urea and reduces nitrates to nitrogen. It was negative for production of H_2S, Voges Proskauer's and Methyl Red. In carbon utilization profiling, the Bab3-8 showed positive result only for glucose utilization (Table 1).

Table 1: Biochemical characteristics of bacterial Isolate Bab3-8

S. No.	Test	Result
1	ONPG	-
2	PYR	-
3	Arginine utilization	+
4	Lysine utilization	±
5	Ornithine utilization	±
6	Urease	+
7	Phenylalanine deamination	V
8	Nitrate reduction	+
9	H_2S production	-
10	Citrate Utilization	+
11	Voges Proskauer's	-
12	Methyl Red	-
13	Indole	-
14	Malonate utilization	-
15	Esculin hydrolysis	-
16	Sucrose	-
17	Sorbitol	-
18	Mannitol	-
19	Arabinose	-
20	Xylose	±
21	Adonitol	-
22	Rhamnose	-
23	Cellobiose	-
24	Melibiose	-
25	Saccharose	-
26	Raffinose	-
27	Trehalose	-
28	Glucose	±
29	Lactose	-
30	Oxidase	-

+ = Positive (more than 90%), - = Negative (More than 90%), V =11-89% positive

The bacterial sample Bab3-8 was also analyzed through Scanning Electron Microscopy (SEM) at 5000X magnification. In Scanning Electron Microscopy image the bacterial cell is seen as single and also in clustered form (Fig. 3).

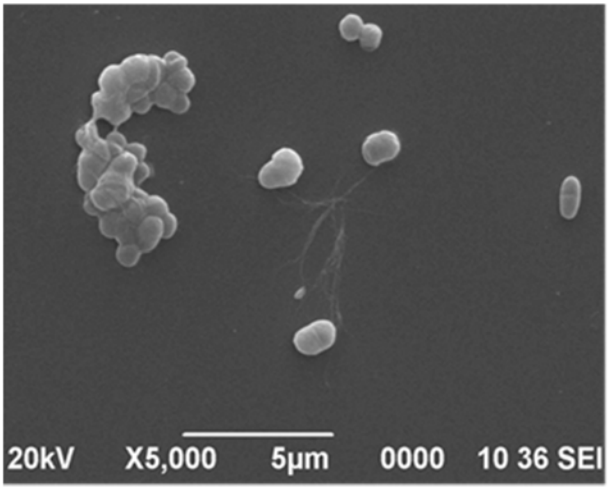

Fig 3: Scanning Electron Microscopy (SEM) image of Bacterial sample at 5000X magnification

The obtained result was confirmed by using MALDI-TOF mass spectrum analysis. MALDI-TOF mass spectrum analysis is an easy and discriminatory tool for bacterial species identification [28]. On the basis of obtained score value (1.8) from the MALDI-TOF mass spectrum analysis and matching to Biotyper database, the bacterial strain Bab3-8 was identified as *Dietzia maris* (Fig. 4).

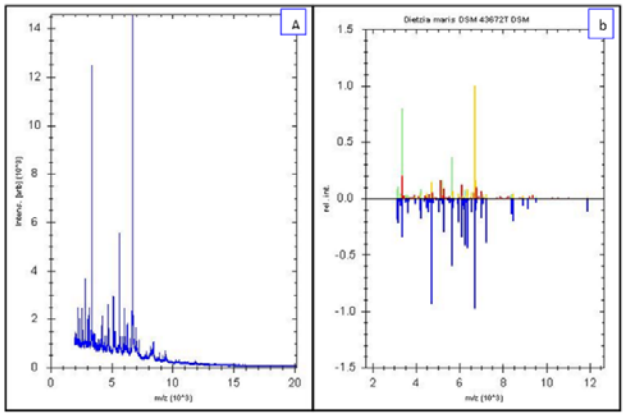

Fig 4: (a) Spectra generated by Microflex III mass spectrometer (b) Identified by matching with Biotyper database

The result obtained from MALDI-TOF mass spectrum analysis was finally confirmed to the result of 16S rRNA gene sequence analysis. The sequence of bacterial isolates Bab3-8 was aligned with Genbank database and shows highest similarity to the *D. maris* in the EzTaxon server (99.86%) (http://147.47.212.35:8080) and NCBI data base (99.0%). The homologous sequences were downloaded from database for construction of phylogenetic tree in which *Staphylococcus epidermidis* was used as an out group (Fig. 5). The phylogenetic tree was divided in two mega clusters- one of genus *Rhodococcus* and second of genus *Dietzia* (Fig. 5).

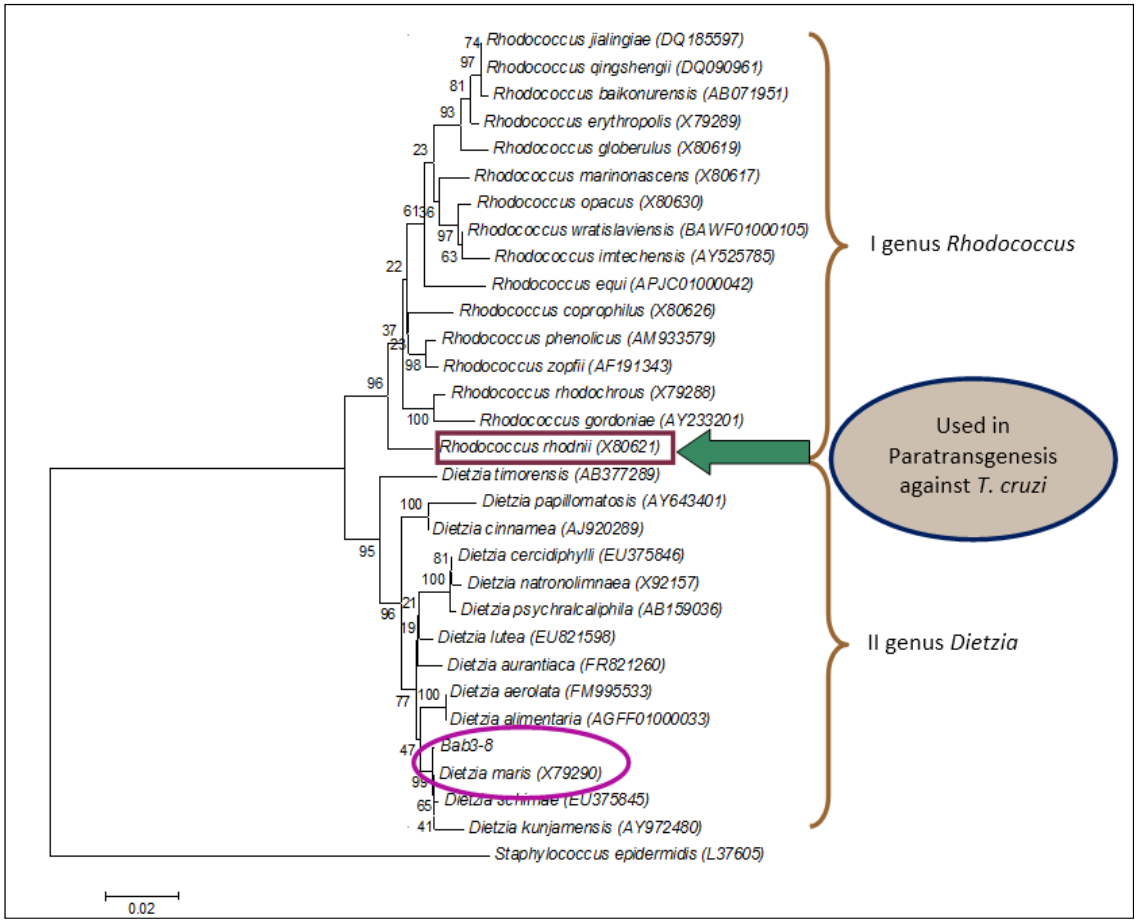

Fig 5: Dendrogram based on 16S rRNA gene sequencing constructed by neighbor joining method using Kimura 2 distances parameter

4. Discussion

In our study, the isolated bacterial strain was initially morphologically and biochemically identified, and was similar to previous reports which supported our findings [4, 27]. This bacterial species showed high salt tolerance and growth upto 10% (w/v) NaCl concentration and the optimum growth temperature was found to be 30-37 ^{o}C [11]. Thus, we observed that the bacterial strain Bab3-8, isolated from *Ae. albopictus* collected from Bhalukpong, North East India, has close proximity with *D. maris.* The bacterial strain was identified by MALDI TOFF MS based analysis and with 16S rRNA gene sequencing which confirms that, the isolated strain was *D. maris.* In the phylogenetic analysis, tree has been divided in two clades- one of genus *Dietzia* and another of genus *Rhodococcus* which are highly close to each other as described previously (Fig. 4) [4]. In the clade I of genus *Rhodococcus,* the bacterial species *R. rhodnii* have been used in paratransgenesis approach against *T. cruzi.* The Clade I is closely associated to clade II and it has been previously described that genus *Rhodococcus* is highly similar to genus *Dietzia* [4]. If the *R. rhodnii* have been used in development of paratransgenesis techniques, then there is a great possibility of using *D. maris* in this approach.

D. maris was isolated from various environmental resources but limited report shows its isolation from insects. In 2006, *D. maris* was isolated from the gut of an insect's pupae *Wohlfahrtia magnifica* (Order: Dipetra), an obligate parasitic fly, causing myiasis in domestic animals [29], and later on from larvae gut of *Trypoxylus dichotomus* (Order: Coleoptera), Japanese Horned Beetle [30]. As an endosymbiont, it has been also reported from the dinoflagellate *Pyrodinium bahamense* [31].

The genus *Rhodococcus* was used for development of paratransgenesis techniques against *T. cruzi*, a causative agent of Chagas disease transmitted by reduviid bug, *R. prolixus* [19, 20]. The bacterial species of genus *Rhodococcus, R. rhodnii* is situated in the lumen of *R. prolixus,* with close proximity to *T. cruzi* and was genetically modified for expression of a highly potent antimicrobial active molecule, Cecropin A, which causes cell lysis and death [32, 33, 34]. Cecropin A shows paltrier effects on genetically manipulated bacteria *R. rhodnii* and *R. prolixus* gut tissue, but exhibited strong deleterious effect against *T. cruzi* [19].

As previously described, in morphology, *Dietzia* are very similar to *Rhodococcus equi*. Due to high similarity and absence of simple, appropriate and specific methods for their identification, it might be misidentified as a *Rhodococcus* spp. [4]. To the best of our knowledge, *D. maris* was isolated for the first time from the midgut of mosquito, *Ae. albopictus*. Hence, there is a great potentiality to apply biotechnological tools for development of paratransgenesis approach using *D. maris*. The genetic manipulation of *D. maris* could be possible for the expression of anti-parasite molecules in the midgut of mosquitoes to prevent the development of parasites causing dengue, chikungunya, malaria, etc. The advantage of using *D. maris* in the vector management is that, this bacterial species can survive in a broad range of temperature and pH and has high salt tolerance capability [4, 11]. Due to this property of

bacterial species, it could be stable in the midgut of any mosquitoes like *Culex, Anopheles* or *Aedes* which survives in broad range of environmental condition. Using biotechnological tools, *D. maris* could be used for the development of a novel, stable, eco-friendly and highly effective defense mechanism for prevention of disease transmission.

5. Conclusion

In our study a midgut bacteria *D. maris* has been isolated from lumen of *Ae. albopictus*, collected from foothill of Himalaya, Arunachal Pradesh, North East India. Various techniques like MALDI-TOFF, 16S rRNA, SEM were used for characterization of the isolated bacterial strain. In previous studies, it has been proved that, the bacterial genera *Dietzia* exhibited highly similar characteristic to *Rhodococcus* genera and was under it but later on it was established as a separate genus. Using *R. rhodnii,* paratransgenesis techniques have been already developed against *T. cruzi*, a causative agent of Chagas disease transmitted by reduviid bug, *R. prolixus.* Hence, there is a great possibility for development of paratransgenesis approach using *D. maris* for expression of anti-parasite molecules to prevent parasite development in mosquito's midgut. The knowledge obtained from this study will provide valuable information for development of novel, stable, eco-friendly and highly effective defense mechanism against various disease like, dengue, chikungunya, malaria, etc.

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