Int J Pharm Bio Sci 2013 Apr; 4(2): (B) 1168 - 1175

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

BIOCHEMICAL AND MOLECULAR TAXONOMY OF CHITINOLYTIC *CITROBACTER FREUNDII* STR. NOV. HARITD11, ISOLATED FROM INDIAN MARINE ENVIRONMENT

HARITHA MERUVU* AND SRI RAMI REDDY DONTHIREDDY

Center for Biotechnology, Department of Chemical Engineering, College of Engineering, Andhra University, Visakhapatnam 530 003, Andhra Pradesh, India

ABSTRACT

A strain of *Citrobacter* with chitinolytic activity was isolated by screening of five marine sediment samples near NTPC area of the Visakhapatnam Coast of Bay of Bengal (India), an arm of the Indian Ocean. The taxonomic position of a chitinolytic marine isolate, strain haritD11 was established using a polyphasic approach. The organism merits species status in the genus *Citrobacter* according to the chemical and phenotypic data. Phylogenetic analysis of the strain based on its 16S rDNA sequence shows that there was 100% similarity and identity without any nucleotide gaps with the species *Citrobacter freundii* strain DSM 30039(T). As the organism was distinguished with significant differences in certain genotypic and phenotypic characteristics, it was proposed as a new strain variety of *Citrobacter freundii* and designated as *Citrobacter freundii* str. nov haritD11 (GenBank accession number of strain D11 is KC344791).

KEYWORDS: ***Citrobacter freundii*, chitinolytic, polyphasic taxonomy, phylogenetic analysis, 16S rDNA sequence**

HARITHA MERUVU
Center for Biotechnology, Department of Chemical Engineering, College of Engineering, Andhra University, Visakhapatnam 530 003, Andhra Pradesh, India

INTRODUCTION

Citrobacter is a genus of Gram-negative, facultative anaerobic, non-spore forming rods belonging to *Enterobacteriaceae* family. As their name indicates, they usually utilize citrate as sole carbon source[1]. At present and according to Bergey's Manual of Systematic Bacteriology, eleven species are reported in the genus including: *C. freundii*, *C. koseri*, *C. amalonaticus*, *C. farmeri*, *C. youngae*, *C. braakii*, *C. werkmanii*, *C. sedlakii*, *C. rodentium*, *C. gillenii* and *C. murliniae* [2]. These bacteria can be found almost everywhere in soil, water, wastewater, etc. They are rarely a source of illnesses, except for infections of the urinary tract, infant meningitis and sepsis [3, 4]. *Citrobacters* are a part of the fecal flora of humans, however they have been isolated from the intestinal tract of dogs, cats, horses, cows, birds and tortoises[5]. *Citrobacters* were also recovered from sea water[5,6], sewage and soil[1]. *Citrobacter amelonaticus* isolated from the intestines of insectivorous bats of Indiana was found to produce chitinase[7]. Similar report stating isolation of *Citrobacter sp*., from the gut microflora of insectivorous bats, with the ability to digest the chitin component in the bat diet was provided by Irulan *et al*., (2011)[8]. *Citrobacter freundii* B1A was also reported to show chitinase activity [9]. However based on literature search there is no report to date on chitinolytic *Citrobacter freundii* strain isolated from Indian marine environment. In this study, we isolated a Gram-negative rod-shaped bacterium from a marine sediment of Bay of Bengal (NTPC area, Visakhapatnam, India). The isolate belongs to the *Citrobacter* genus in the family *Enterobacteriaceae* in the class *Gammaproteobacteria* and is a novel strain with chitinolytic activity. Different molecular, physiological and biochemical tests were carried out to further identify and describe this isolate.

MATERIALS AND METHODS

Collection of samples

A total of five sediment samples from Bay of Bengal from different places (at different distances and depths) at NTPC area (17°31' 51" North latitude and 83°4' 53" East longitude) were collected and screened for chitinolytic bacteria.

Screening and isolation of bacteria

Marine sediment samples were stored at 4˚C until isolation. Bacteria are isolated by plating out the samples in proper dilutions. About 1g of the above sample was taken into a 250 ml conical flask containing 100 ml of sterile water and kept on a rotary shaker for 15 minutes. The suspension was serially diluted upto 10^{-6} level. Isolation was carried out on nutrient agar (supplemented with 75µg/ml cycloheximide to inhibit fungal contamination) plates, which have been seeded with a sediment sample suspension of 1.0 ml each and incubated at 30˚C for 24-48 hours. Single separate colonies on the agar plates which appeared different from one another to the naked eye were selected and streaked on the nutrient agar slants and incubated for 24-48 hours at ± 30˚C. Code names were given to each of the isolates and stored at ± 4˚C and recultured every 4 weeks until the identification process is completed. About 59 pure bacterial colonies in total were isolated from the above samples. The isolated colonies were streaked upon chitin-containing minimal agar plates and checked for growth. The bacterial colonies that could grow on the minimal chitin agar plates were observed for chitinolytic activity on basis of the zone of chitin utilization around the bacterial colonization for upto 3 days. The bacterial isolate, strain haritD11 which produced clear zone of hydrolysis over 0.5 cm[15] was selected as the promising isolate with chitinolytic activity.

Biochemical identification

The strain haritD11 isolate was Gram-stained and tested for motility using hanging drop method. The biochemical tests included in the

system were: growth temperature range, growth pH range, sodium chloride tolerance, starch hydrolysis, casein hydrolysis, citrate utilization, MRVP Test, nitrate reduction test, indole test, catalase test, oxidase test, urea test and carbon source utilization and acid production.

Molecular identification by Genotypic characterization (16s rDNA analysis)

Genomic DNA was prepared by using standard methods [10,11]. Preparation of DNA from lyophilized ampoules consisted of suspending the lyophilized cells in 0.2 ml of 10 mM tris-hydrochloride (pH 8.3)–2.5 mM $MgCl_2$–50 mM KCl. This was added to approximately 0.1 ml of 0.1-mm-diameter acid-washed glass beads, 10 µl of 20% sodium dodecyl sulphate, and approximately 200 µl of phenol, saturated with the above buffer. This sample was shaken in a 500-µl microcentrifuge tube, seated within a 2.0 ml tube, in a Mini-Bead Beater for 2 min. After phase separation in 20 to 50 µl of TE buffer (10mM Tris hydrochloride [8.0], 1Mm EDTA), and between 2 and 5% of this resuspended DNA was put into the PCR amplification. Approximately 1.0 to 3.0 µg of genomic DNA was amplified in a 100 µl reaction by using the Geneamp kit (Perkin-Elmer Cetus). When the lyophilized ampoule DNA was amplified, 1.0 µl was used. Conditions consisted of 25 to 35 cycles of 95˚C (2 minutes), 42˚C (30 seconds) and 72˚C (4 minutes), plus one additional cycle with a final 20-minute chain elongation. All amplifications were performed in Perkin-Elmer temperature controller and purified on centricon 100 columns followed by ethanol precipitation. The GC content of the DNA was determined using the Genetool software with PGEM as the control. The amplified product was sequenced using universal primers (5' to 3') fD2 (ccgaattcgtcgacaacAGAGTTT GATCATGGCTCAG) and rP1(cccgggatcc aagcttACGGTTACCTTGTTACGACTT). The nucleotide sequence was obtained by processing DNA sequencing samples using ABI 3130 (4 capillary) electrophoresis instruments. The resultant 16S rDNA sequence was aligned using Gene Tool Lite version 1.0 software program against corresponding sequences retrieved from the Genbank database. The phylogenetic tree was constructed based on almost complete 16S rDNA sequence showing the relationship between strain D11 and its related taxa by neighbor joining tree method with maximum sequence difference of 0.75 using BLASTN 2.2.27 program[12].

RESULTS AND DISCUSSION

Isolation and microscopic studies of strain haritD11

From the five marine sediment samples screened, the bacterial isolate strain haritD11 produced a clear zone of hydrolysis over 0.5 cm and was selected as the promising isolate with chitinolytic activity (Fig. 1). The isolate D11 showed proficient growth on yeast-malt extract agar, nutrient agar and minimal chitin agar. The isolate D11, when streaked on agar medium appears to grow as smooth, slightly convex, distinct shining white colonies. Microscopic observations (Figures 2 and 3) showed that cells were gram negative, long rod-shaped bacteria typically 1.0 µm in diameter, 1-5 µm in length and were found to be non motile through motility test.

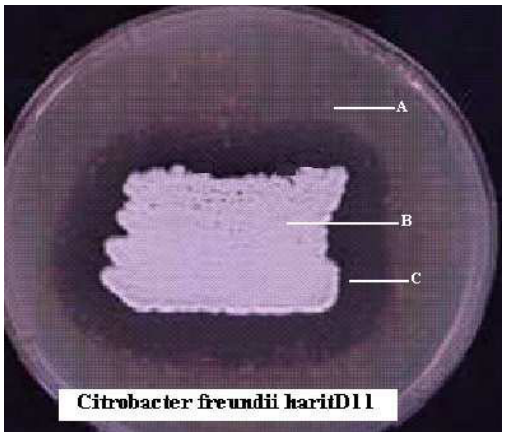

Figure 1

Growth of Citrobacter freundii haritD11 on minimal chitin agar plate showing the zone of clearance caused by hydrolysis of chitin (A-chitin agar, B-Citrobacter and C-zone of clearance).

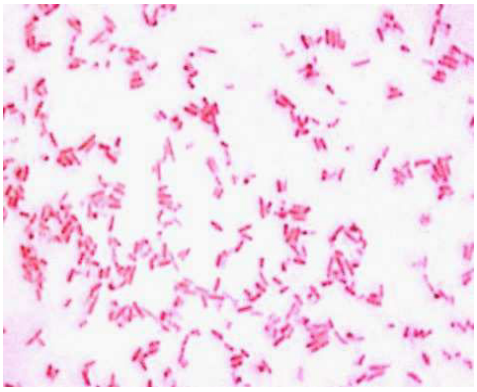

Figure 2

Gram stained Citrobacter freundii haritD11 cells observed under Optical microscope at 100x magnification

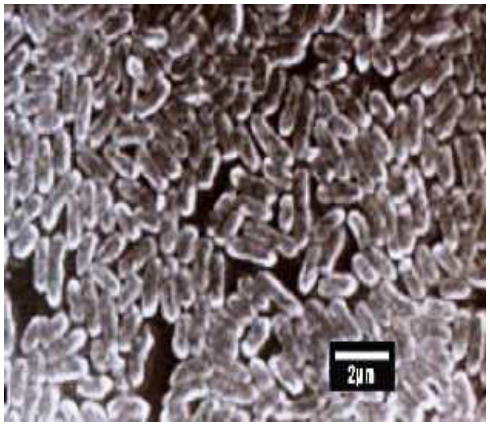

Figure 3

Citrobacter freundii haritD11 cells observed under Scanning Electron microscope at 7000x magnification

Biochemical properties

The haritD11 strain was tested for several biochemical activities and showed a few differences (Table 1). The biochemical tests showed that the strain was Gram-negative, catalase-positive, voges-proskauer negative and reduce nitrate just like other *Citrobacter* bacteria[13]. However, there were biochemical differences between the isolate strain haritD11 and other *Citrobacters* such as, the strain D11 was non motile, negative with urea, MR, oxidase, indole tests and oxidase-positive unlike the others. Another important biochemical property showing uniqueness of *Citrobacter freundii* strain haritD11 is that it could not utilize citrate as sole carbon source unlike most other *Citrobacters.*

Table 1
Phenotypic characters of Citrobacter freundii str. nov. haritD11

Character	Result	
	Strain haritD11	***Citrobacter freundii*** [13]
Gram staining	-	-
Motility	-	+
Growth at 15˚C	-	ND
Growth at 25˚C-37˚C	+	+
Growth at 40˚C	-	ND
Growth at pH 5.2	-	ND
Growth at pH 8.0-10.0	+	ND
Growth on NaCl 2%-10%	+	ND
Starch hydrolysis	+	ND
Casein hydrolysis	-	ND
Citrate utilization	-	+
MR	-	+
VP	-	-
Nitrate reduction	+	+
Indole	-	V
Catalase	+	+
Oxidase	+	-
Urease	-	V
Acid production from		
Arabinose	+	+
Galactose	+	ND
Glucose	+	+
Mannitol	+	+
Raffinose	-	+
Salicin	-	-
Xylose	-	ND
Sucrose	+	+
Rhamnose	+	ND
meso-inositol	-	ND
Fructose	+	ND

+, positive or present; -, negative or absent; V, variable (15 to 85% positive), ND, not done

Molecular identification of strain haritD11

The 16S rDNA sequence was aligned with other 16S rDNA bacterial sequence obtained from GenBank by basic local alignment search tool (BLAST) program[14]. The 16S rRNA gene sequence similarity between the isolate D11 and its nearest neighbor, *Citrobacter freundii* DSM 30039(T) was 100%; this value corresponds to 0 differences out of the 1409 nucleotide positions compared (Table 2).

Table 2
16S rDNA similarity values between strain haritD11 and the representatives of the genus Citrobacter.

Rank	Name	Strain	Accession	Identities	Diff/total nt
1	*Citrobacter freundii*	DSM 30039(T)	AJ233408	1409/1409 100%)	0/1409
2	*Citrobacter brakii*	CDC080-58(T)	AF025368	1405/1409 (99%)	4/1409
3	*Citrobacter murlinae*	CDC2970-59(T)	AF025369	1404/1409 (99%)	5/1409
4	*Citrobacter werkmanii*	CDC0876-58(T)	AF025373	1398/1409 (99%)	11/1405
5	*Citrobacter gillenii*	CDC4693-86(T)	AF025367	1394/1409 (99%)	15/1409
6	*Citrobacter youngae*	ATCC29935(T)	AJ564736	944/948 (99%)	4/948
7	*Citrobacter farmeri*	CDC 2991-81	AF025371	1374/1411 (97%)	37/1411
8	*Citrobacter rodentium*	ICC168	AB045737	1371/1411 (97%)	40/1411
9	*Citrobacter sedlakii*	YL090822	AF025364	1368/1413 (97%)	45/1413

An almost complete 16S rDNA sequence was determined for strain haritD11 (1409 nucleotides). Comparison of this sequence with those of representation reference strains of the family *Enterobacteriacae* shows that the organism belongs to the genus *Citrobacter* (Figures 4 and 5). However if we consider the GC content, we found that our strain the *Citrobacter freundii* strain haritD11 has a relatively higher percent GC content (55%) as compared to other Citrobacters which generally have an average of 50-52%[2]. The Genbank accession number for the *Citrobacter freundii* haritD11 is KC344791.

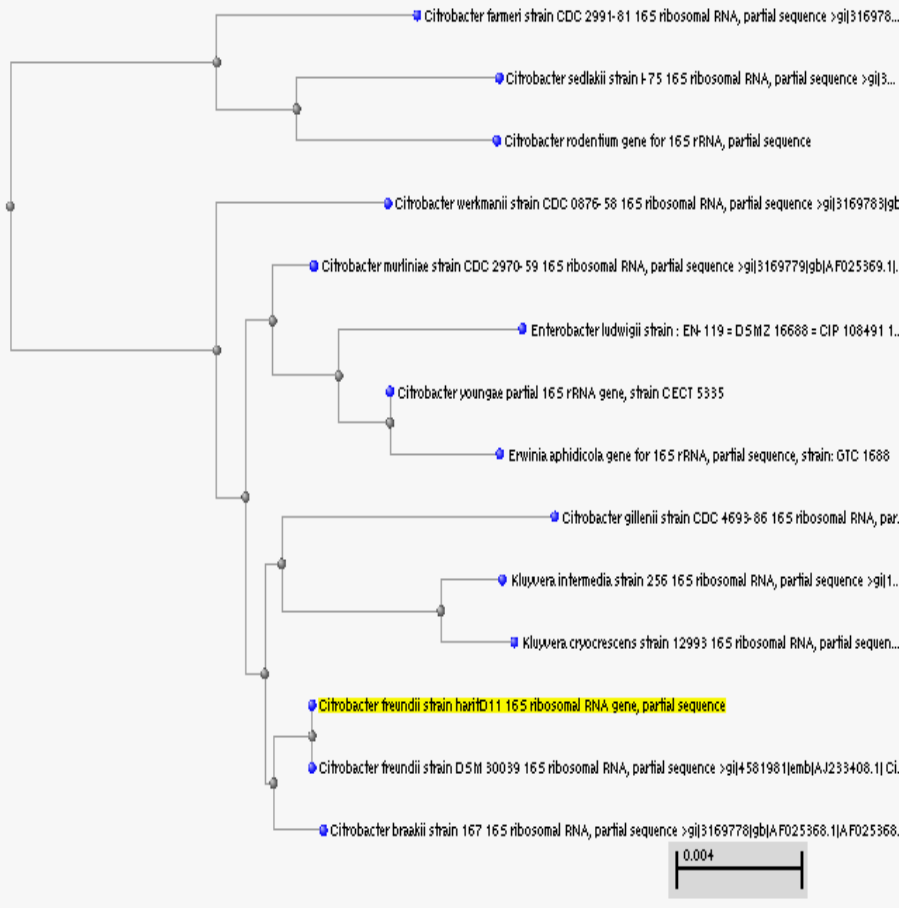

Figure 4
Neighbor-joining tree based on almost complete 16S rDNA sequences showing relationships between strain haritD11 and representatives of the family Enterobacteriacae and related taxa.

Figure 5
The DNA sequence for 16S rRNA gene of Citrobacter freundii strain haritD11

CONCLUSION

The chitinolytic *Citrobacter* was facultatively anaerobic, non-motile, Gram-negative bacterium which grew as smooth, slightly convex, shining white colonies. It grows on 2%-10% NaCl at a temperature of 25˚-37˚C and in the pH range of 8.0-10.0. It was positive for Starch hydrolysis, Oxidase, Catalase and Nitrate reduction tests but negative for Urease, Citrate utilization, MRVP, Indole and Casein hydrolysis. Acid was produced from the following carbohydrates: Fructose, Galactose, Glucose, Mannitol, Sucrose, Rhamnose and Arabinose. Acid was not produced from meso-inositol, Salicin, Xylose and Raffinose. The G+C content of the DNA was 55 mol%. A detailed survey of literature and the genotypic characterization revealed that it was a novel strain of *C. freundii* and was designated as *Citrobacter freundii* str. nov. haritD11. To the best of our knowledge from literature search, this was the first report of chitinolytic *Citrobacter freundii* strain isolated from Indian marine environment.

REFERENCES

1. D Borenshtein and D Schauer. The Genus *Citrobacter*. In: The Prokaryotes: A Handbook on the Biology of Bacteria: Proteobacteria: Gamma Subclass, Dworkin, M., S. Falkow, E. Rosenberg, K.H. Scheleifer and E. Stackebrandt (Eds.). Springer, New York, USA, 2006, pp: 90-98.
2. W Frederiksen, Genus X. Cirobacter Werkman and Gillen. In: Bergey's Manual of Systematic Bacteriolog, Volume 2: The Proteobacteria Part C the α-, β-, δ- and Epsilon proteobacteria, Brenner, D., N. Krieg, J. Staley and G. Garrity (Eds.). Springer, New York, USA., 2006, pp: 651-656.

3. Drelichman V and Band JD, Bacteremias due to *Citrobacter diversus* and *Citrobacter freundii*. Incidence, risk factors, and clinical outcome. Archives of Internal Medicine, 145 (10): 1808–1810, (1985).
4. Badger JD, Stins MF and Kim KS, *Citrobacter freundii* Invades and Replicates in Human Brain Microvascular Endothelial Cells. Infection and Immunity, 67 (8): 4208–15, (1999).
5. Janda JM and Abbott SL, The Enterobacteria. 2nd Edn, ASM Press, New York, USA., ISBN-13: 411,(2006).
6. Kakizaki E, Kozawa S, Tashiro N, Sakai M and Yukawa N, Detection of bacterio plankton in immersed cadavers using selective agar plates. Legal Med., 11: S350-S353 (1999).
7. Whitaker John O, Kathleen Dannelly H, and David A Prentice, Chitinase in Insectivorous Bats. Journal of Mammalogy, 85(1): 15-18, (2004).
8. Irulan A, Nathan PT, Priya Y, Marimuthu G and Elangovan V, Isolation and Characterization of Chitinase Producing Gut Microflora of Insectivorous Bats. Trends in Biosciences, 4(1): (2011).
9. Zarei M, Aminzadeh S, Ghoroghi A, Motalebi AA, Alikhajeh J and Daliri M, Chitinase Isolated from Water and Soil Bacteria in Shrimp farming Ponds. Iranian Journal of Fisheries Sciences 11(4): 911-925 (2012).
10. Maniatis T, Fritsch EF and Sambrook J, Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, (1982).
11. Marmur J, A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3: 208-218, (1961).
12. Saitou N and Nei M, The neighbor joining method: a new method for constructing phylogenetic trees. Mol Biol Evol, 4: 406-425, (1987).
13. Bergey David Hendricks and Holt John G , Manual of Determinative Bacteriology 9th Edn, Lippincot Williams and Wilkins publisher, 190-204, (1994).
14. Altschul SF, Gish W, Miller W, Myers EW, and Lipman DJ, Basic local alignment search tool. Journal of Molecular Biology, 215(3): 403–410, (1990).
15. Priya.CS, Jagannathan N and Kalaichelvan PT, Production of chitinase by *Streptomyces hygroscopicus* VMCH2 by optimisation of cultural conditions. International Journal of Pharma and Bio Sciences, 2(2): 210-219, (2011).