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Amycolatopsis alba var. nov DVR D4, a bioactive actinomycete isolated from Indian marine environment

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Abstract

The taxonomic position of a bioactive marine isolate, strain DVR D4 was established using a polyphasic approach. The organism merits species status in the genus *Amycolatopsis* according to the chemical and phenotypic data. Phylogenetic analysis of the strain based on its 16S rDNA sequence shows that there was 100% pair-wise similarity and identity with no nucleotide gaps with the species *Amycolatopsis alba* strain DSM 44262. As the organism was distinguished with substantial differences in some of the phenotypic characteristics and other properties, it was proposed as a strain variety of *Amycolatopsis alba* and designated as *Amycolatopsis alba* var. nov DVR D4 (GenBank accession number of strain D4 is JN872327).

Key words: Amycolatopsis alba, polyphasic taxonomy, phylogenetic analysis, 16S rDNA sequence, bioactive, antagonistic activity.

Introduction

The genus *Amycolatopsis*, assigned to the family *Pseudonocardiaceae* (Warwick et al. 1994; Embley et al. 1988) was proposed by Lechevalier et al. (1986) to accommodate nocardioform actinomycetes having type IV cell composition, type PII phospholipid pattern and lacking mycolic acid. The members of the genus *Amycolatopsis* are Gram-positive, non-acid fast, non-motile actinomycetes that form branched vegetative hyphae that undergo fragmentation into rod-like and squarish elements. The members of this genus have high G+C (>55%) content in the DNA (Cwale et al. 2011).

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Only 10 novel species have been described for this genus until the last decade, But since 2000, many novel species have been described which were isolated from various terrestrial environments (Carlson et al. 2007; Groth et al. 2007; Lee et al. 2006; Tan et al. 2006; Saintpierre-Bonaccio et al. 2005; Kim et al. 2002; Huang et al. 2001; Goodfellow et al. 2001) and clinical material (Huang et al. 2004; Labeda et al. 2003). At the time of writing, the genus contains 48 species (Labeda et al. 2011; Albarracin et al. 2010; Atchareeya et al. 2010; Chen et al. 2010; Duangmal et al. 2010; Tamura et al. 2010; Tang et al. 2010; Liras & Demain 2009; Soon 2006).

Literature suggests that there have been some important novel bioactive compounds isolated from the members of the genus *Amycolatopsis* (Demain & Zhang 2005; Zhang et al. 2005; Trenin et al. 2001; Krishna et al. 1998; Malkova et al. 1991). In an effort to explore the relatively untapped potential members of this genus and investigate the potential application of their secondary metabolites, we attempted to isolate and identify strains having the capability of producing bioactive compounds from the marine sediments of coastal Visakhapatnam, India.

Materials and Methods

Isolation

Sediments from Bay of Bengal (NTPC area, Visakhapatnam, India) were collected by grab sampler. Strain DVR D4 was isolated on a starch casein agar (SCA- soluble starch, 10.0g; vitamin free casein, 0.3g; KNO_3, 2.0g; NaCl, 2.0g; K_2HPO_4, 2.0g; $MgSO_4.7H_2O$, 0.05g; $CaCO_3$, 0.02g; $FeSO_4.7H_2O$, 0.01g; agar, 20.0g; sterilized natural aged sea water, 1L; pH, 7.2; supplemented with rifampicin 2.5µg/ml and cycloheximide 75µg/ml to inhibit bacterial and fungal contamination, respectively) plate, which had been seeded with a sediment sample suspension and incubated at 28^oC for 14 days (Ramesh & Narayanasamy 2009). The isolate was maintained on YEME (Yeast Extract Malt Extract) slants at 4^oC and as a glycerol suspension (20%v/v) at -20^oC (Williams & Cross 1971).

Taxonomy

The cultural and morphology properties were examined by using standard procedures (Holt et al. 1994) and procedures of Williams et

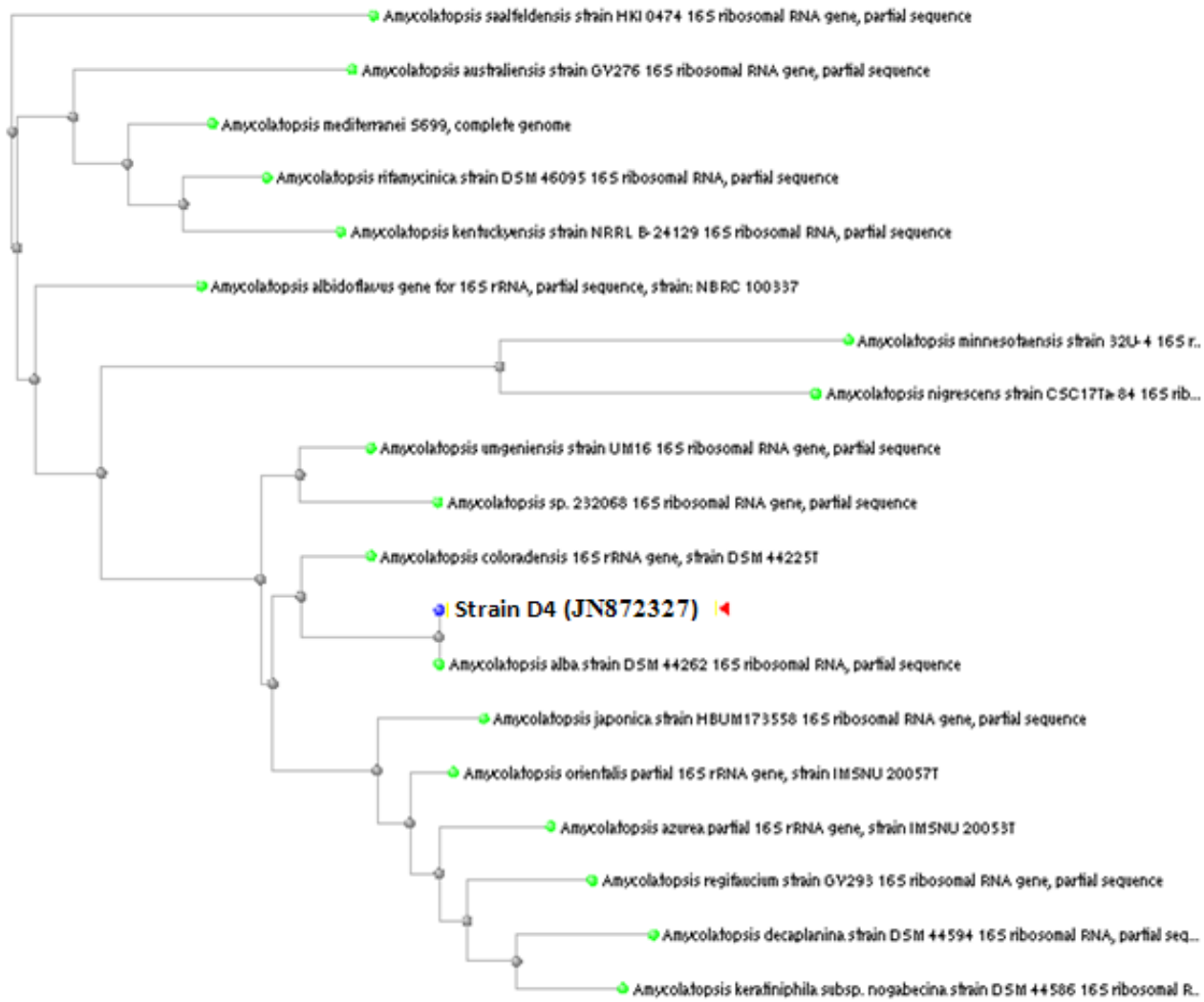

Figure 1: Neighbor-joining tree (Saitou and Nei, 1987) based on almost complete 16S rDNA sequences showing relationships between strain DVR D4 and representatives of the family *Pseudonocardiaceae* and related taxa.

Table 1: 16S rDNA similarity values between strain DVR D4 and the representatives of the genus *Amycolatopsis*

Rank	Name	Strain	Accession	Pair-wise Similarity	Identities	Diff/Total nucleotides
1	*Amycolatopsis alba*	DSM 44262	NR_024888.1	100%	1428/1428	0/1428
2	*Amycolatopsis coloradensis*	DSM 44225T	AJ421142.1	99%	1421/1431	4/1431
3	*Amycolatopsis umgeniensis*	UM16	DQ110876.1	99%	1412/1423	3/1423
4	*Amycolatopsis* sp. 232068	-	GU130127.1	99%	1417/1431	3/1431
5	*Amycolatopsis japonica*	HBUM173558	EU841704.1	99%	1415/1434	8/1434
6	*Amycolatopsis azurea*	IMSNU 20053T	AJ400709.1	99%	1414/1434	9/1434
7	*Amycolatopsis orientalis*	IMSNU 20057T	AJ293755.1	98%	1413/1435	11/1435
8	*Amycolatopsis regifaucium*	GY293	AY129769.1	98%	1409/1435	11/1435
9	*Amycolatopsis decaplanin*	DSM 44594	NR_025562.1	98%	1407/1433	8/1433

al. (1983) after growth on YEME Agar for 7-10 days at 28°C. Phenotypic tests were carried out following the procedures of Goodfellow et al. (1997) and Gordon et al. (1974). Chemotaxonomic characters were determined as described previously (Wu et al. 1989; Collins, 1985; Minnikin et al. 1984; Hasegawa et al. 1983; Lechevalier & Lechevalier 1980; Minnikin et al. 1980).

16S rDNA analysis

Genomic DNA was prepared by using standard methods (Maniatis et al. 1982; Marmur 1961). The G+C 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 (ccgaattcgtcgacaacAGAGTTTGATCATGGCTCAG) and rP1 (cccgggatccaagcttACGGTTACC-TTGTTACGACTT). 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 tree making algorithm used was Neighbor joining (Saitou & Nei, 1987) with Max Seq diff of 0.75. The phylogenetic tree was constructed (newick format) using Tree view software.

Fermentation

A full grown slant culture of the strain DVR D4 on YEME agar slant was transferred to Erlenmeyer flasks containing 50 ml seed medium (composition: soyabean meal, 10.0g; corn steep liquor, 10.0g; glucose, 5.0g; $CaCO_3$, 5.0g; sterilized natural aged sea water, 1.0L; pH 7.0) and incubated for 2 days at 28°C on a orbital shaker (220 rpm). A 5% of this inoculum was transferred into the optimized production medium (composition: D-glucose, 20.0g; malt extract, 40.0g; yeast extract, 4.0g; dipotassium hydrogen phosphate, 5.0g; sodium chloride, 2.5g; zinc sulphate, 0.04g; calcium carbonate, 0.4g; 1.0L sterile distilled water with pH 6.0) (Dasari et al. 2011a,b). The inoculated production flasks were incubated for 96 h at 28°C on a rotary shaker (220 rpm).

Antibacterial activity (cup-plate method)

The culture broth was centrifuged at 4000 rpm for 10 min, at 8°C and clear culture filtrate was separated for their antagonistic activity against the selected Gram-positive and Gram-negative bacteria by cup-plate method. Inoculated with test organisms, cups of 6 mm diameter were prepared in the nutrient agar plates and the cups are filled with the 50 µl of clear culture supernatant; and the diameter of inhibition zones were measured after incubation for 24 h at 37°C.

Table 2: Antibiotic resistance and inhibition similarities of *Amycolatopsis alba* var. nov DVR D4 and *Amycolatopsis alba*

Antibiotic	**Strain D4**	***Amycolatopsis alba* (Frederick and Raymond, 1993)**
Cephalosporin (10 µg/ml)	+	N/A
Tetracycline (30 µg/ml)	+	+
Rifampicin (5 µg/ml)	+	+
Gentamycin (10 µg/ml)	+	-
Lincomycin (2 µg/ml)	+	-
Neomycin (30 µg/ml)	+	+
Penicillin (10 U)	-	-
Vancomycin (30 µg/ml)	-	-
Streptomycin (10 µg/ml)	-	-
Resistant, "+"; Inhibition, "-"		

Results and Discussion

An almost complete 16S rDNA sequence was determined for strain DVR D4 (1428 nucleotides). Comparison of this sequence with those of representation reference strains of the family *Pseudonocardiaceae* shows that the organism belongs to the genus

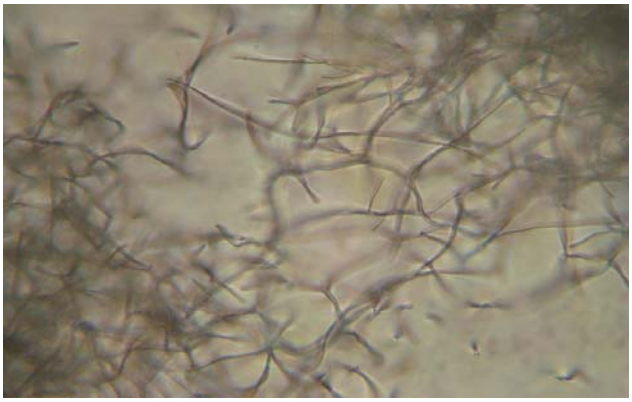

Figure 2: Spore chain structure of *Amycolatopsis alba* var. nov DVR D4; phase contrast microscope X400

Amycolatopsis (Fig. 1). The GenBank accession number for the 16S rDNA sequence of strain D4 is JN872327. The 16S rDNA sequence similarity between the strain DVR D4 and its nearest neighbor, *Amycolatopsis alba*, is 100 %; this value corresponds to 0 differences out of 1428 nucleotide positions compared (Table 1). The chemotaxonomic data of the test strain are consistent with its assignment to the genus *Amycolatopsis* (Lechevalier et al. 1986). Strain DVR D4 contains *meso*-diaminopimelic acid as the wall

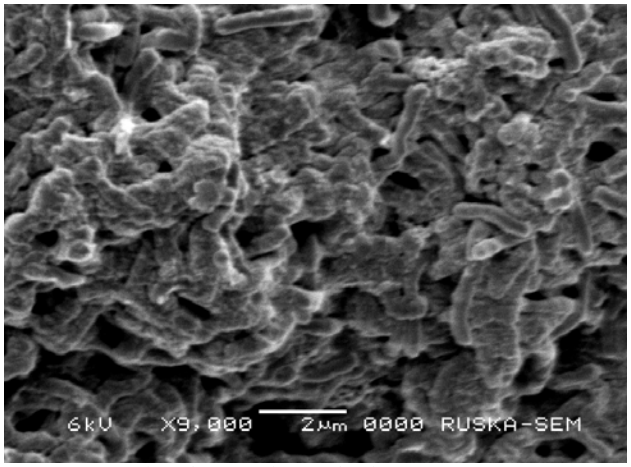

Figure 3: Scanning electron micrograph of *Amycolatopsis alba* var. nov DVR D4 grown on YEME medium for 7 days at 28°C; Magnification, X 9,000; Bar, 2.0µm.

diamino acid, arabinose and galactose as major wall sugars. It also contains phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylinositol diagnostic polar lipids but it lacks mycolic acids; the G+C content of the DNA is 58.9 mol%. This chemical profile serves to distinguish strain DVR D4 from members of all other wall chemotype IV taxa, except those classified in the genus *Amycolatopsis* (Holt et al. 1994; Embley 1992; Goodfellow & Lechevalier 1989; Henssen et al. 1987; Lechevalier et al. 1986). The classification of strain DVR D4 in the genus *Amycolatopsis* is also supported by phenotypic and morphological properties. The organism is aerobic, non-motile, Gram-positive, non-acid alcohol-

fast and produces branching substrate mycelium which fragments into cylindrical elements. Colonies have wrinkled surfaces and are covered with fine white hyphae; the hyphae are segmented into long strands of conidia that are arranged in a typical cobweb morphology characteristic of some nonstreptomycete actinomycetes (Fig. 2). The conidia are cylindrical and have a smooth surface (Fig. 3).

Strain DVR D4 was resistant to the following antibiotics (µg/ml): Cephalosporin (10), Tetracycline (30), Rifampicin (5), Gentamycin (10), Lincomycin (2) and Neomycin (30). It was inhibited by Penicillin (10 U), Vancomycin (30 µg/ml) and Streptomycin (10 µg/ml) (Table 2). The strain DVR D4 can be distinguished from the published *Amycolatopsis* species *Amycolatopsis alba* using a combination of phenotypic properties (Table 3). The chemical, genotypic and phenotypic data show that strain DVR D4 merits species status in the genus *Amycolatopsis.* Even though there was 100% pair-wise similarity and identity with no nucleotide gaps with the species *Amycolatopsis alba* strain DSM 44262, there are substantial differences in some of the phenotypic characteristics (Tables 2 and 3). Therefore, it is proposed that this organism as a strain variety of *Amycolatopsis alba* and designated as *Amycolatopsis alba* var. nov DVR D4.

Table 4 Antibacterial activities of product produced by *Amycolatopsis alba* var. nov DVR D4

Test Organism	Zone of inhibition (mm)
B. subtilis NCIM 2439	18
B. pumilus NCIM 2327	16
B. saccharolyticum NCIM 2238	22
S. aureus NCIM 5021	20
S. griseus NCIM 2622	14
E. coli NCIM 2067	12
A. formicans NCIM 2319	20
P. taetrolens MTCC 1612	20

Table 3 Phenotypic characters of *Amycolatopsis alba* var. nov DVR D4

Character	Result	
	Strain D4	***Amycolatopsis alba* (Frederick and Raymond, 1993)**
Gram staining	+	+
Spore staining	-	-
Motility	-	-
Color of aerial mycelium	White	White
Growth at 15°C	-	ND
Growth at 25°C	-	ND
Growth at 28°C	+	ND
Growth at 30°C	+	+
Growth at 37°C	-	ND
Growth at 42°C	-	ND
Growth at pH 5.0	+	ND
Growth at pH 6.0	+	ND
Growth at pH 6.5	+	ND
Growth at pH 7.0	+	ND
Growth at pH 7.5	+	ND
Growth at pH 8.0	+	ND
Growth at pH 9.0	+	ND
Growth at pH 10.0	+	ND
Growth on NaCl 2%	+	ND
Growth on NaCl 5%	-	+
Growth on NaCl 7%	-	ND
Growth on NaCl 10%	-	ND
Starch hydrolysis	-	ND
Casein hydrolysis	-	ND
Citrate utilization	-	+
Gelatin liquefaction	+	ND
H_2S production	-	ND
MR (Methyl Red)	-	ND
VP (Vogues Proskauer)	-	ND
Nitrate reduction	-	-
Indole	-	ND
Catalase	+	ND
Oxidase	-	ND
Urease	+	+
Acid production from		
Arabinose	-	+
Galactose	+	+
Glucose	+	ND
Mannitol	+	+
Raffinose	-	+
Salicin	-	+
Xylose	+	+
Sucrose	-	-
Rhamnose	-	-
Meso-inositol	-	+
Fructose	+	ND

+, positive or present; *-, negative or absent; ND, not done*

The antagonistic principles secreted by the *Amycolatopsis alba* var. nov DVR D4 exhibited broad antagonistic spectrum against the tested bacterial species studied (Table 4). To the best of my knowledge from literature search, this was the first report of *Amycolatopsis alba* isolation from marine environment and also this was the first bioactive actinomycete strain of *Amycolatopsis alba* isolated in India.

Conclusion

The bioactive actinomycete was aerobic, non-motile, Gram-positive, non-acid filamentous, differentiated into substrate; abundant white aerial mycelium was produced and it also fragments into cylindrical elements. Cylindrical, smooth spores are formed with typical cobweb morphology. It grows on 2 % NaCl at 28^{o}C and in pH range of 5.2 to 10.0. It was positive for urease, catalase and liquefaction of gelatin but negative for indole, oxidase, hydrolysis of starch and casein, utilization of citrate, H_2S production and nitrate reduction. Acid was produced from the following carbohydrates: galactose, glucose, mannitol, xylose and fructose. Acid was not produced from the arabinose, raffinose, salicin, sucrose, rhamnose and *meso*-inositol. The organism contains *meso*-diaminopimelic acid as the wall diamino acid, arabinose and galactose as major wall sugars. It also contains phosphatidylethanolamine, diphosphatidylglycerol and phosphatidylinositol diagnostic polar lipids but it lacks mycolic acids; the G+C content of the DNA is 58.9 mol%.

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