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Cadmium Resistant Actinomycetes Isolated from Agricultural Fields

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Two actinomycetes strains designated as DAS 131 and DAS 165, were isolated from cadmium contaminated agricultural soil of Gulbarga, Karnataka. The isolates were classified as *Streptomyces* spp. A plate diffusion assays showed to be resistant to cadmium [Cd $(No_3)_2$]. This method allowed a qualitative screen of the effects of metal on growth, on sporulation and in some cases, on secondary metabolic production. The strains have the best growth capacity in presence of 100mM Cdl^{-1} which is representative of their ability to soil colonization.

Keywords: *Streptomyces*; bioremediation; metal resistance.

The use of intensive farming practices including addition of phosphatic fertilizers, sewage sludge input and pesticides treatment are responsible for the pollution of agricultural soils[1]. Although these practices increase significantly the yield by protecting plants and providing them with all the nutrients necessary for a rapid and better growth, they may also introduce large amounts of heavy metals (Cu, Zn, Pb, Cd) and organic pollutants in soil which may then be accumulated by the plants[1]. Hamon *et al.,*[2] shown that the addition of phosphatic fertilizers increased Cd uptake of wheat. A soil cleaning up is difficult using conventional treatments for technical and economical reasons. Phytoremediation could be employed[3], but phytoremediation takes several years, during which no food crop is possible. Therefore an alternative to the cleaning up is the pollutant immobilisation in the soil to avoid its transfer to plants or groundwater. Heavy metals adsorption on mineral or organic amendments has been exploited[4] but cadmium leakage has been observed due to pH change or soil temperature and humidity variations even in the presence of these amendments[5]. Here, the toxicity of the Cd for the microflora is an issue[6-8]. Some species can indeed disappear while resistant strains can proliferate[9]. Microorganism's resistance can be related to the metabolic paths and to the presence of metal-binding proteins or peptides[10-14]. It is also

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dependent on the nature of the medium[15]. Screening of cadmium resistant microorganisms has been realised by some authors[16-18] in order to determine the ability of these strains to biosorb cadmium. Nevertheless the resistance and the accumulation of heavy metals have always been measured on synthetic media adapted to the selected microorganisms[19].

This study deals with cadmium-resistant actinomycetes isolated from agricultural fields exposed to phosphate fertilizers and characterize with respect to their metal resistance. Two strains resistant to cadmium were identified as *Streptomyces* spp.

MATERIAL AND METHODS

Sampling

Soil samples were collected from various locations in agricultural fields in which phosphate fertilizer was frequently used for the isolation of *Streptomyces.* All the samples were kept at 0°C until the use. The soil was chemically characterized according to the methods described by Lister and Jones[20].

Isolation and Characterization

The following screening procedure was adopted for the isolation of *Streptomyces*[21]. The soil was pretreated with $CaCO_3$ (10:1 w/w) and incubated at 37°C for 4 days, and was suspended in sterile Ringer's solution (1/4 strength). The medium used for isolation of actinomycetes was starch-casein agar, composition (per liter): 10g starch, 1g casein dissolved in 0.3M NaOH, 0.5 g K_2HPO_4, 20g agar, pH 7.0-7.5, supplemented with 10µg nalidixic acid ml^{-1}. The medium contained nystatin and cyclohexamide, at 25 µg ml^{-1} and 10 µg ml^{-1} respectively, to minimize the contamination with fungi. Plates were incubated at 28°C and isolated colonies were purified by streaking on ISP Medium 2 (Difco Laboratories, Detroit, MI, USA). Spores stocks were prepared from cultures grown on ISP medium plates, as described by Hopwood *et al.,*[22]. Genus and species confirmation were carried out by cell wall chemotype[23], phenotypic[24-26] and genotypic including, G+C mol % content[27] 16S rRNA gene analysis[28], phylogenetic relationship[29-30] and DNA-DNA hybridization[31].

Isolation of cadmium resistant actinomycetes

ISP medium 2 was used to screen for cadmium resistance. Qualitative assessment of the effect of heavy metals on growth, sporulation and pigment production was determined by using a hot wire, a trough 0.2 by 90mm was cut into agar contained in a square dish which measured by 10 by 10 cm so that it ran parallel to one side approximately 1cm from the wall of the plate into the trough and it was added with 500µl of metal salt solutions of Cd $(No_3)_2$ (100mM). Metal amended plates were then incubated at 28°C for 24h to allow diffusion of metals into the agar by which time a concentration gradient of the metal had been formed. Spore suspension of the test *Streptomyces* species were streaked heavily in a line at right angles to the trough and incubate for 7 days. After incubation, the distance of growth inhibition (in mm) was taken as that from the edge of the trough to the leading edge of the growing mycelium. This was used to determine the metal tolerance of each strain and was expressed as a percentage of the total measured distance on the agar available for growth (90mm). Therefore the greater the distance of the colony from the trough edge, the greater the inhibition excreted by the metal.

Determination of cadmium resistance by an agar diffusion assay

Sensitivity of the strains to cadmium was tested by an agar diffusion assay. Discs (6mm diameter) were saturated with 20µl Cd $(No_3)_2$ solution. Discs were placed on the surface of agar plates inoculated with 10^6 spores of the strains to be tested and zones of inhibition measured after incubation at 28°C for 5 days. Sensitive strain showed zones of inhibition of >10mm, whereas zones of inhibition of resistant strains were <7 mm at 10mM Cd $(No_3)_2$. Growth was compared to the *Streptomyces lividans* TK24-cadmium resistance [2].

Determination of metal toxicity

Spore suspension of the *Streptomyces* strains were inoculated in a liquid defined medium(MM) containing (gl^{-1}) L-asparagine 0.5g; K_2HPO_4 0.5g; $MgSO_4.7H_2O$ 0.2g ; $FeSO_4.7H_2O$ 0.01g; and glucose 10.0g. The MM medium was supplemented with 0.1 to 1.0mM Cd $(No_3)_2$ metal ion solution. Cultures were incubated by shaking

(100rpm) at 28°C for 72h and centrifuge (3000Xg, 10min). After washing the resulting pallet with 25mM Tris EDTA buffer (pH 8.0) the biomass was estimated by drying the pellet to constant weight at 105°C.

Determination of growth

The growth kinetics was followed by estimating the dry weight of the biomass. The medium was filtered through a microporous membrane (Millipore, 0.45µM pore size) and weighed after drying at 105°C for 24h. The cell concentration (dry weight) of bacterial suspensions was determined by measuring the optical density (OD) of the samples at 600nm and following calibration (dry weight (gl^{-1}) = 0.4 ×OD) according to Valentine *et al.,*[32]. Growth rates, µ (h^{-1}) were calculated using the relation = In 2/ρ where ρ (h) is the generation time estimated during the exponential stage of the growth kinetics.

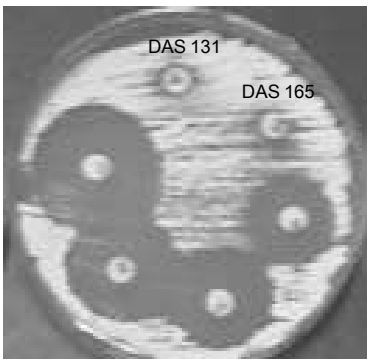
RESULTS AND DISCUSSION

Isolation and screening cadmium resistant actinomyces

Seven isolates resembling actinomycetes were obtained from soil samples collected from agricultural field in which phosphate fertilizer was frequently used for cultivation from Gulbarga region of India, and was chemically analyzed for its components (Table 1). Two strains designated as DAS 131 and DAS 165, were found to be significantly resistant to cadmium nitrate [Cd $(No_3)_2$], using a qualitative trench assay. (Fig.1a&b). Which were confirmed as belonging to the genus *Streptomyces* as it possessed non-fragmented substrate mycelia, aerial hyphae and smooth spores arranged in straight chains, LL-DAP, glycine, no diagnostic sugars were found in the whole cell hydrolysates. DNA G+C content is of 69.8 and 69.7 mol % respectively. More than 1450 bp of the 16S rRNA genes of DAS 131 and DAS 165 were sequenced, 1477bp and 1517bp respectively. Analysis of these 16S rRNA gene sequence revealed that they grouped in the genus *Streptomyces* clade (Fig.2). DNA-DNA hybridization with closest neighbors according to phylogenetic analysis (Fig 2), *Streptomyces venezualae* ISP 5230^T (AY999739) and *Streptomyces tendae* ATCC 19812^T (D63873) discloses that DAS 131 (54%) and DAS 165 (47%) delineation of novel species, for which *Streptomyces gulbargensis* and *Streptomyces tritolerans* sp nov., is proposed[33-34].

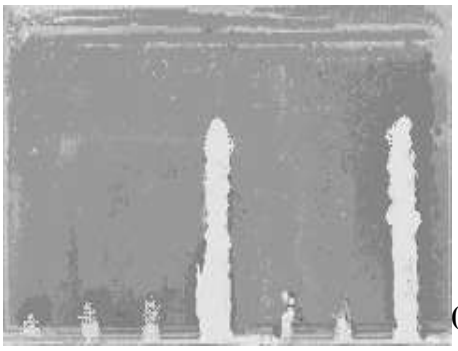
Qualitative analysis of metal tolerance

Seven isolates were selected for qualitative analysis. An inhibition zone of 10mm in diameter was arbitrarily designated as a criterion to determine the metal tolerance of the tested strains. As the result, all the strains tested except DAS 131 and DAS 165 turned out to be sensitive to Cd^{+2} at 10mM or higher concentration.



(A)

Fig. 1(a). Plate assay showing the resistance pattern of isolates DAS 131 and DAS 165 to cadmium metal (10mM).



(B)

Fig. 1(b). Growth of isolates on gradients of Cd $(NO_3)_2$ metals in solid medium.

Note: Growth inhibition [as taken as the distance (mm) from the leading edge of the colony up to the trough and was expressed as a percentage of the total distance on the agar available for growth (90mm)

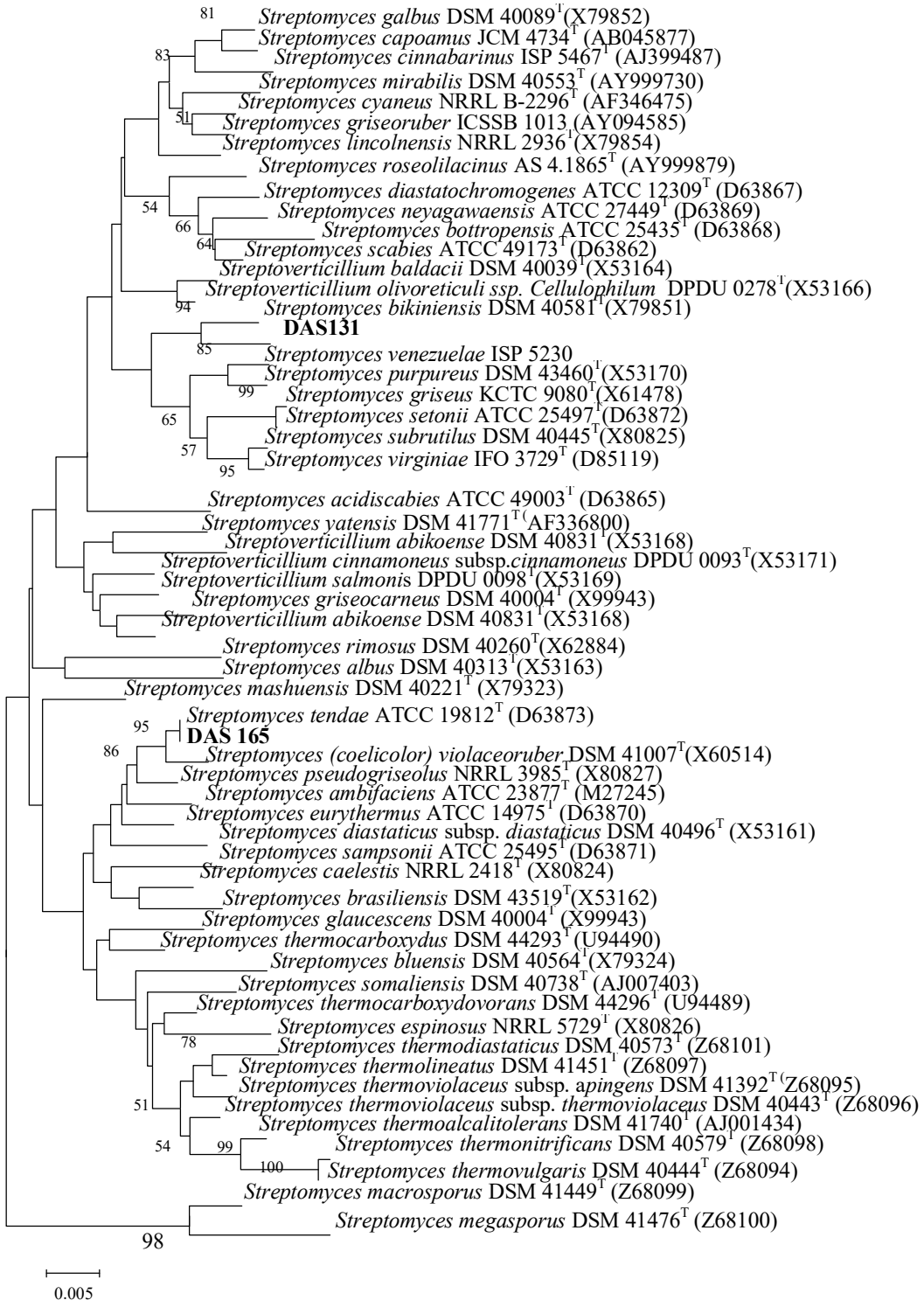


Fig 2. Phylogenetic dendrogram obtained by distance matrix analysis of 16S rRNA gene sequence, showing the position of strain DAS131 and DAS165 among its phylogenetic neighbors. Bar 0.005 substitutions per nucleotide position.

Table 1. Chemical characteristics of the soil

pH	P mg dm^{-3}	OM g kg^{-1}	K^{+}	Ca^{+2}	Mg^{+2}	$H^{+}+Al^{+3}$	CTC	Clay	Silt	Sand
			c mol dm^{-3}						gkg^{-1}	
7.7	2.3	2.6	0.3	2.9	0.4	2.1	5.7	100	30	870

Table 2. Consumption of Cadmium by two novel species of *Streptomyces*

Strain	Specific consumption of different concentration 0.1	0.5	(μ mol mg^{-1}) of metal (mM) 1.0
DAS 131^{T}	0.31	3.41	4.86
DAS 165^{T}	0.50	3.84	6.75
S. lividans TK 24	0.06	0.06	0.06

Specific consumption is defined as metal consumption (μ mol) per biomass (mg)

Streptomyces lividans TK 24, used as the control, was tolerant to 10mM Cd^{+2} but not to higher concentration. The growth inhibition profiles at 100mM concentration revealed two resistant strain DAS 131 and DAS 165 (Fig. 1b) among seven selected wild-type strains. This allowed us to evaluate the range of Cadmium concentration where they were able to multiplicates. We also investigated the cadmium concentration tolerated for the growth of selected strains according to the pH. The maximum biomass production and the growth rates of the two selected strains with respective pH were indicated in table 3.

Determination of sensitivity of the plate diffusion method

The plate diffusion method described in material and methods was to give a rapid but qualitative estimation of the tolerance of the *Streptomyces* isolates. The result described in Fig. 1a & b, yielded qualitative data on metal tolerance with solid medium and as such allowed the screening of large numbers of species to give a rapid indication of those species worthy of further investigation. To validate the sensitivity of this procedure we measured the effects of heavy metals on species.

Table 3. Maximal biomass production (X in gl^{-1}) and growth rates (μ in h^{-1}) of microorganisms in synthetic media

		Cadmium concentration in the medium					
		0 mgl^{-1}		1 mg l^{-1}		10 mg l^{-1}	
Isolates	pH	X	μ	X	μ	X	μ
	5.0	0.276	0.087	0.391	0.032	0.062	0.000
DAS 131	6.0	0.510	0.031	0.061	0.000	0.067	0.000
	7.0	0.591	0.020	0.058	0.000	0.071	0.000
	5.0	0.097	0.039	0.099	0.003	0.094	0.032
DAS 165	6.0	0.128	0.017	0.069	0.003	0.070	0.002
	7.0	0.159	0.038	0.072	0.032	0.081	0.034

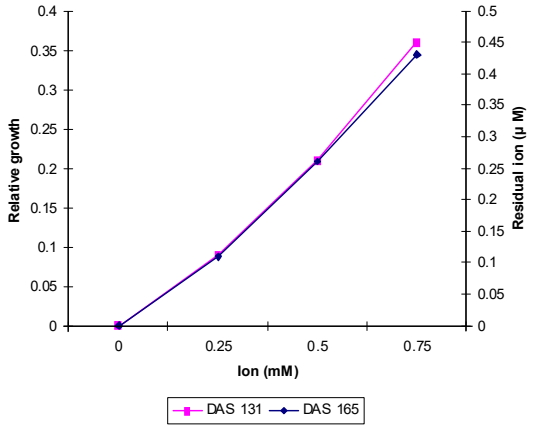


Fig 3. Effect of metal [Cd^{+2}] concentration on the growth of DAS 131 and DAS 165

Growth Kinetics

The uptake analysis of cadmium by the cells showed that, the uptake defined as metal concentration consumed per biomass, increased with initial cadmium fed in the medium, with the exception of *S.lividans* TK 24 (Table 2). However, at the highest Cd^{+2} concentrations (1mM), the range of relative growth was 2-15% of the control growth without metal solution. The toxicities of cadmium were evaluated in MM medium. The relative growth curves showed a hyperbolic response with the increase of Cd^{+2} concentrations in the medium. The growth of the strains in MM medium containing increased cadmium concentration is shown in Fig 3.

The results obtained in this study indicates that metal resistance and metal consuming capability may be widespread among actinomycetes (particularly in *Streptomyces*) growing in contaminated environments. The resistance and sensitivity of selected strains to the heavy metals has been tested. Soil contamination by heavy metals originating from phosphate fertilizers has become a concern in several countries. The heavy metal concentration in phosphate fertilizers is dependent on the type of rock phosphate used as raw material. Soluble phosphorous fertilizers produced from such sources presented Cd concentration ranging from 5.1 to 9.4 $mgkg^{-1}$ [35]. The toxic level of cadmium to *Pseudomonas aeruginosa* and *Aeromonas* sp. in a synthetic medium was reported to be 6.45 and 2.00µM, respectively[36]. Abbas and Edward[37] reported that the growth of *S.coelicolor* was inhibited by 50% after 16h culturing in the presence of 0.14mM Cd^{+2}. Although cadmium has been reported as a very toxic metal for microorganisms. In a selected strain and at all Cd^{+2} concentrations used in the assays, percentages of cadmium remnant in the supernatants were below 0.2% of the initial concentrations (Fig. 3).

The emerging evidence that metal resistance and antibiotic resistance are often found together in many clinical isolates. Since it is thought that in many cases antibiotic resistance genes originate from the producing organisms of which the *Streptomyces* are predominant it should be of interest to monitor their tolerance to heavy metals[38]. By using a simple plate diffusion assay system, we have been able to identify the effects of heavy metals on a *Streptomyces* isolates. It should also be possible to adapt the method to screen the effects of metals on antibiotic production by treating the fully grown metal inhibited mycelium with an agar overlay that contains a susceptible test organism. The method described here should allow the rapid screening of the effects of metals on production of secondary metabolites by commercially important species.

CONCLUSION

Isolation of two novel species of *Streptomyces*, i.e. *Streptomyces gulbargensis,* and *Streptomyces tritolerans,* opens up opportunities to investigate their mechanisms of metal resistance. The metal resistance *Streptomyces* or genes encoding metal resistance isolated from these organisms may be useful in the bioremediation of contaminated environments.

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