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Enhanced Degradation of Phenol by a New Species of *Rhodococcus*: *R. gordoniae* through *ortho*-pathway

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Abstract: **Degradation of phenol by the new species of *Rhodococcus gordoniae* is studied presently and the bacterium was found to degrade 88% of approximately 25mM phenol (2.5g/L) in minimal medium within 24 hours of incubation. Enzyme assays in the crude cell-free extracts of the bacterium grown on phenol, revealed recruitment of catechol 1,2 dioxygenase (C12O) in this degradation. High specific activity of catechol 1,2 dioxygenase (1.8 U/mg) in the crude cell-free extracts reveal that *ortho*-pathway is the more favoured natural pathway for phenol degradation in this bacterium. Gene encoding C12O in the bacterium was subjected to hybridization between the truncated DNA probe from *phe*B prepared from *Pseudomonas* sp. EST1001 and the HindIII digested total genomic DNA from *R. gordoniae.* The hybridization between the two genes showed non-homology, revealing the fact that the gene sequences of C12O in *R. gordoniae* may be different from the existing *Pseudomonas* gene sequences for the same.**

Keywords: **Catechol 1, 2 dioxygenase, o*rtho*-pathway, *Rhodococcus*, Bioremediation.**

1. INTRODUCTION

The turnover of carbon in nature depends on the catabolic reactions of microorganisms. Their capacity to degrade naturally occurring organic compounds, evolved over millions of years, is now constantly being challenged with synthetic chemicals introduced into the environment deliberately [1] or as the result of accidental spillages, or as bye-products and waste components in the industrial effluents [2]. Phenol and its homologues from the coal carbonization, heavy synthetic chemical, and petrochemical industries are the widespread pollutants frequently found in industrial outfalls, landfill runoff waters [3] at concentrations which may approach 6g/L [4].

As a preparative measure to combat problems due to the release of synthetic compounds, bacteria have largely been employed in the laboratory studies as agents capable of degradation of aromatic compounds with simple and complex structures. Most of these investigations have addressed mainly *Pseudomonas* and *Alcaligenes* species [5]. For phenol degradation, *Pseudomonas* has been exploited in many studies so far [6, 7, 8]. Studies have shown that Gram-positive bacteria, mycobacteria, nocardioform actinomycetes, and streptomycete groups are also known as enhanced degraders of xenobiotic compounds [9]. Thus, these groups of microorganisms may be attractive agents for use in biotechnology. Additionally, they are known for their high biodegradation potential[10] and survival in soil [11]. As a result, they could be good experimental models for the future bioremediation studies as well.

Previous reports indicated degradation of phenol in the range of 2 mM to 20 mM in axenic cultures by different bacteria [12]. We here for the first time report an enhanced degradation (2.5g/L or >25 mM) of phenol up to 88% by *Rhodococcus gordoniae*, identified as a new species [13], in pure cultures within 24h of incubation. Enzyme assays with crude cell-free extract revealed degradation of phenol by the mediation of catechol 1,2-dioxygenase in this bacterium. Plasmid isolation methods and experiments with conjugation indicate absence of catabolic plasmid mediating phenol degradation in the presently studied bacterium or if there is plasmid, it is linear or very large and could not be amenable to extraction using the extant protocols for the same. This supports the view that possibly chromosomal genes mediate phenol degradation in this bacterium.

2. MATERIAL AND METHODS

Rhodococcus gordoniae (previously known as *Nocardia* sp. MTCC 1534), isolated from phenol contaminated soils from near Chandigarh, India, was presently purchased from Microbial Type Culture Collections, Institute of Microbial Technology, Chandigarh, India. Bacterial cells were grown at 28□ C in M_3 medium containing beef extract (1g/L), yeast extract (2g/L), peptone (5g/L) and NaCl (5g/L). Bacterial cells

were also transferred occasionally to solid minimal salts medium containing $MgSO_4$, $7H_2O$ (50mg), Na_2HPO_4 (6.45g), KH_2PO_4 (1.875g) per 100 mL, agar (15g/L) and also trace elements at desired concentrations. The concentration of the aromatic carbon source was always kept at 1-2.5g/L.

Halogenated benzoate, salicylate, hydroxybenzoate, cresol, chlorophenol, phenol, catechol were obtained from Sisco Research Laboratories, BDH India, Bovine Serum Albumin (BSA) was from Sigma, Inc. and Folin's reagent was from Central Drug House, India. Restriction enzymes BamH1 and Hind III were purchased from New England Biolabs. and NADH was from Sigma Chemicals, Inc.

Crude cell extracts were prepared with slight modification of previously described method of Nakazawa and Nakazawa [14] using bacterial cell suspensions containing approximately 50g biomass, which were sonicated at 214 W (6.5 KHz) with a pulse duration of 15 seconds each, keeping the cells on ice. In all, 12 pulses were given at a gap of 1min each, using Sonicator from Heat Systems, Ultrasonics, (Model W-385). The cells were then centrifuged at 10,000g for 30min at 4□ C (Centrifuge, Sorval). The activities of phenol monooxygenase (PMO) and catechol 1,2-dioxygenase (EC 1.13.11.1) were assayed as reported by Beadle and Smith [15] and Nakazawa and Nakazawa [14], respectively in the cell-free enzyme extracts, while the activities of catechol 2,3-dioxygenase was assayed following the method of Nozaki [16]. The cell debris pelleted was processed further for the above enzyme activities, however, no membrane-bound enzyme activities could be detected. The reference cuvette in each reaction contained phosphate buffer, NADH or catechol and crude enzyme extract. The enzymes of the *ortho*-pathway were inactivated by heat treatment before making assay for the *meta*-pathway enzyme to avoid interference in the assay.

The temperature dependence of catechol 1,2 dioxygenase in cell-free crude extract at pH 7.5 was investigated in the range of 5-55□ C.

Several plasmid preparation methods already known [17, 18] were used presently with slight modifications. Total genomic DNA was isolated using Hopwood's protocol [18]. Southern hybridization using digoxigenin (DIG) labelled-UTP-*Phe*B plasmid DNA from *Pseudomonas* sp. strain EST 1001 was used as DNA probe for the identification of gene encoding catechol 1,2 dioxygenase.

Total genomic DNA was digested using Hind III or BamH1. Concentration of phenol in culture medium was determined by high-pressure liquid chromatography (HPLC) on a reverse phase column, RP18 from Jasco, Japan using wavelength 260nm. The mobile phase used contained acetonitrile and water (70:30) and flow rate was kept 0.5-1.0ml/min. Protein concentrations were measured by the method of Lowry et al., [19].

3. RESULTS AND DISCUSSION

R. gordoniae was strictly aerobic, Gram-positive and filamentous with size ranging from more than 10 □ m in length and up to 1 □ m in breadth as observed under preparation SEM (Fig.1). Chlorobenzoates, chlorinated phenol, cresol, hydroxybenzoate and salicylate were not utilized by the bacterium as sole source of carbon and energy. The capability of the strain *R. gordoniae*. to utilize phenol as the sole source of carbon and energy is shown in Fig.2a. The generation time of the bacterium was 4.8 h when it was grown in medium containing phenol. This bacterium degraded phenol (2.5g/L or >25mM) to the extent of 88% within 24h (Fig.2b). The concentrations of phenol higher than 2.5g/L was found to be toxic to the bacterium.

The specific activity of PMO was measured as substrate dependent oxidation of NADH in crude cell-free extract of *R. gordoniae* (Table 1) and that of catechol 1,2-dioxygenase in the crude cell-free extract prepared from phenol grown cells of *R. gordoniae* was determined to be 1.8 units/mg, which is significant as compared to specific activities of other *ortho*-pathway phenol degrading organisms (Table 1). Catechol 1,2-dioxygenase activity of phenol grown cells was present for catechol as the substrate assayed and absent when the cells were grown with chlorocatechols, chlorophenols, cresol and hydroxybenzoate. However, no activity was observed for catechol 2,3-dioxygenase.

On the basis of the found enzyme activities for the oxidation of phenol and catechol, we proposed the *ortho*- pathway for phenol degradation in *Rhodococcus gordoniae* (Fig.2a). The presence of *ortho*-cleaving activities in the cell free extract of phenol grown bacterial cells shows the induction of only one type of catechol dioxygenase.

This catabolic feature of the bacterium resembles that of *Pseudomonas* sp. strain EST 1001, which also degrades phenol through *ortho*-cleavage of catechol [7]. However, the specific activity of catechol 1,2-dioxygenase in the present bacterium is much higher than that of *Pseudomonas* sp. strain EST 1001 [7]. This leads to the view that the present bacterium could possibly be catabolically more potent for phenol degradation.

Genetic studies pertaining to phenol degradation in *R. gordoniae* revealed absence of catabolic plasmid mediating phenol degradation. Degradation of phenol is thus suggested to be mediated by chromosomal genes, which needs to be worked out. Additionally, Southern blot hybridizations using *Phe*B truncated DNA probes from *Pseudomonas* sp. EST 1001 showed no homologies with the genomic DNA of the present bacterium.

Gram-positive bacteria are generally known to degrade aromatic compounds through *ortho*- and *meta*- cleavage of the

aromatic ring, we here did not find any phenol *meta*-cleaving dioxygenase. This corroborates the earlier findings on nocardioform actinomycetes, which have *ortho*-pathway of aromatic degradation a common phenomenon [20, 21] and *meta*- cleaving seems to be a rare event in them, and if it occurs, depends very strongly on the substrates that serve as inducers[21]. Enhanced degradation of phenol via *ortho*-pathway and a very high specific activity of catechol 1,2-dioxygenase in the present study strongly suggests that this bacterium could suitably be applied in bioremediation of sites heavily polluted with phenolic wastes. This is supported by the fact that catechol 1,2-dioxygeanse with significant activities has also been investigated in microorganisms isolated from soils contaminated with polyphenols [22]. Interestingly, the yield coefficient of phenol assimilated via *ortho*-pathway at a given P/Q quotient has been found 9-23% higher than that of *meta*- route [23]. However, the rare availability of natural bacterial strains for phenol degraded via *ortho*-pathway demands that there is a strong need for exploring such flora of microbes and understand their physiology to engineer them to suit to the future biotechnological needs.

4. CONCLUSIONS

An extremely high concentration of phenol (25mM) was found to be degraded through *ortho*-pathway in axenic cultures of *Rhodococcus gordoniae* for the first time. High specific activity of catechol 1,2 dioxygenase is reported in the present bacterium, which could make this organism an attractive agent for biotechnological applications.

5. ACKNOWLEDGEMENTS

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Table 1: Catabolic enzyme activities of microorganisms degrading phenol through *ortho*-pathway

Enzyme and Organism	Compound	Specific activity**	Reference
Phenol mooxygenase/hydroxylase			
Sphingomonas sp.	Phenol	0.0035 units/mg*	Schmidt et al., 1992
A. radioresistens	Phenol	3.8 units/mg	Pessione et al., 1999
Ochromonas danica	Phenol	0.016 units/mg*	Semple and Chain, 1996
R. gordoniae	Phenol	0.62 units/mg	The present paper
Catechol 1,2 dioxygenase			
Pseudomonas Sp. EST 10011	Catechol	0.24 units/mg*	Kivisaar et al., 1989; 1991
R. gordoniae	Catechol	1.8 units/mg	The present paper
Rhodococcus rhodochrous	Catechol	0.3 units/mg	Strachan et al., 1998
Pseudomonas arvilla C-1	Catechol	1.4 units/mg	Nakai et al., 1990

**After conversion as per definition of the specific activity.*
***One unit of enzyme activity is defined as that amount which oxidizes 1□ mole/min NADH at 26ºC (PMO) or catalyzes the formation of 1 □ mole/min cis-cis-muconate at 26ºC (C12O). Specific activity is defined as units per milligram of protein.*

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LEGEND TO THE FIGURES

Figure 1: Scanning Electron Microscopy of *Rhodococcus gordoniae* at X4,500 to observe the bacterial filaments, consisting of coccoid elements. Filaments were measured as >10micrometer long and <1 micrometer broad and represent the aerial mycelium of the bacterium.

Figure 2 (a): Growth of *Rhodococcus gordoniae* in minimal medium containing phenol (2.5g/L) as sole source of carbon and energy. Degradation of phenol was also recorded (88% at 2.5g/L within 24h).

Figure 2 (b): Proposed pathway of phenol degradation in *Rhodococcus gordoniae* based on the assay of catechol dioxygenase (the formation of *cis-cis*-muconate revealed the presence of *ortho*-pathway of phenol degradation).

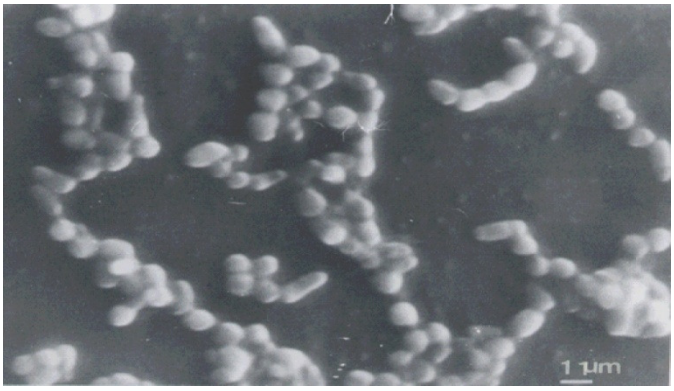

Fig. 1

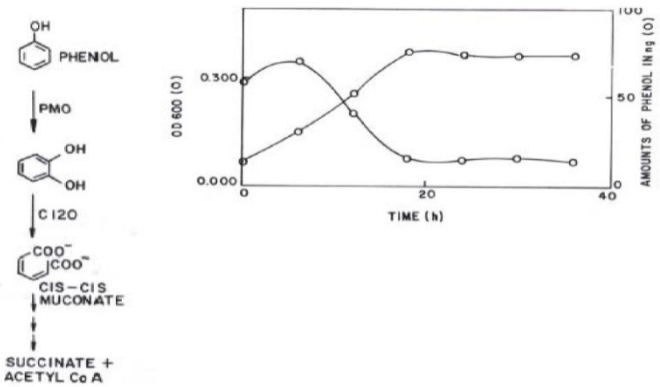

Fig 2.