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PHENOL RESISTANT BACTERIA FROM SOIL: IDENTIFICATION-CHARACTERIZATION AND GENETICAL STUDIES

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Abstract

Phenol and its derivatives are highly toxic and a source of serious environmental concern. The present study was undertaken to isolate and characterize the phenol resistant bacteria from indigenous soil (rhizosphere). Thirty soil bacterial isolates were identified and screened for phenol resistance. Four of these strains (belonging to genera *Staphylococcus, Corynebacterium, Bacillus*, and *Proteus*) were found resistant to 15mM of phenol. Growth kinetic patterns and generation time of these phenol resistant strains were determined. At extremely stressful conditions (glucose starved minimal medium with different molarities of phenol) the generation time was considerably extended. However, *Bacillus subtilis* found an optimum C:N ratio in minimal medium with 0.1mM phenol with a generation time of 174 minutes (several times shorter than the generation time of other sister resistant strains). Location of phenol resistant genes was determined by acridine orange mediated plasmid curing. Interestingly, all the resistant isolates lost the characteristic (to resist phenol) after curing thereby indicating the plasmid genes being responsible for this property. Plasmid DNA isolated from the uncured strains was transferred into the cured competent recipient cells. Stable intrageneric transfer of phenol resistance plasmid gene(s) was observed.

Introduction

Phenols (hydroxy derivatives of benzene) possess antimicrobial, antiseptic, disinfectant or preservative properties depending on the type of compound (Hugo & Bloomfield, 1971). They may be microbistatic or microbicidal (Singleton & Sainsbury, 1988). Phenol or phenolics control microbial activity by affecting bacterial membrane potentials, membrane permeability or by cytoplasmic coagulation and therefore regarded as general protoplasmic poisons (Denyer, 1995). Phenolic wastes (a major class of xenobiotic pollutants from industrial processes such as cooking, industrial resin manufacturing and petroleum-based processing) can be bioremediated by wide spread, naturally occuring microorganisms (Yang *et al*., 1998). Aerobic degradation pathways of various aromatic compounds (benzene, toluene, xylene, naphthalene, biphenyls, polychlorinated biphenyls etc.) have been studied in many bacteria (Kikuchi *et al*., 1994). The ability of soil microorganisms to metabolize biphenyls and polychlorinated biphenyls is well documented (Bedard *et al*., 1987). The genes encoding enzymes that degrade some biphenyls are chromosomal as well as plasmid-borne (Furukawa *et al*., 1989). Ronen & Abeliovich (2000) reported a Gram-negative, aerobic soil bacterium (WH1) which was capable of using non-brominated bisphenol A (BPA) as a sole source of carbon and energy. The initial products of biodegradation of BPA; 4-hydroxybenzoic acid and 4-hydroxyacetophenone were detected in culture medium by gas chromatography-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC). Both these compounds were utilized by WH1 as carbon and

energy source. Li *et al*., (1996) reported an unidentified strain 6; gram variable, flagellated rod with a doubling time of 10 to 11hrs in the presence of phenol. Most likely it is a new species of *Clostridium*. This bacterium has an ability to transform phenol to benzoic acid and 4-hydroxybenzoic acid to phenol and benzoic acid under anaerobic conditions. Different microorganisms (mycobacteria, nonsporulating bacteria, and bacterial spores) vary in their response to antiseptics and disinfectants (Russell, 1996).

Resistance to phenol can be either a natural property of an organism (intrinsic) or acquired by mutation or acquisition of plasmids. Chopra (1987) examined the role of plasmids in encoding resistance to antiseptics and disinfectants. Degradative plasmids enable bacteria to grow on synthetic compounds and may therefore, contribute to the removal of the pollutants. Radnoti *et al*., (1999) suggested that degradation of recalcitrant pollutant in contaminated soils and waters could be facilitated by broadening the degradative capabilities of indigenous microbes by the conjugal transfer of catabolic genes. The use of plasmid curing agents is helpful in the identification of plasmids i.e., in situations in which the phenotypic character is eliminated by the agent at very high frequency. Such studies may also throw some light on biochemical events involved in plasmid maintenance, replication and spread out location of multicopy plasmids (Stewart, 1989).

Keeping in view the ability of naturally occurring microorganisms, the present work was under taken to study the potential of biodegradability/resistance to phenol by bacteria found in the rhizosphere of garden soil. This study includes the determination of phenol resistance profile of the isolates, growth kinetic studies (under normal and phenol stressed conditions) and location of the genetic factors responsible for phenol resistance.

Materials and Methods

Bacterial strains: Thirty strains were isolated from the rhizosphere of garden soil using diluted suspension on nutrient agar (Cappuccino & Sherman, 1999).

Reagents: Nutrient broth (BioM Laboratories, USA) was used for the screening of cultures and for phenol resistance while minimal medium was used for growth kinetic studies. Simmons citrate agar (Difco Laboratories, USA), peptone tryptone broth, Clark's medium, and peptone nitrate broth were used for identification. Brain heart infusion broth (Oxoid, UK) was used for the curing experiments. Acridine orange (Merck, Germany) was used as curing agent. Luria basal agar was used for the bacterial DNA transformation studies. Difco agar (2%) was added in order to solidify the medium. Phenol [Merck (3.3Molar) stock solution)], acridine orange (10mg/ml) and phosphate buffer saline were also used.

Selection of phenol resistant bacteria: Isolated colonies were picked from the nutrient agar plates and screened for their resistance to phenol by replica plate method (Lederberg *et al.,* 1952) on nutrient agar plates containing upto 100 mM phenol.

Studies on growth kinetics of phenol resistant isolates: Overnight culture of phenol resistant bacterial isolates was inoculated in a flask containing fresh 100ml medium to obtain 0.1 O.D. at 460nm. The flasks were incubated in a shakobater (Stewart sc, UK) at 37°C (150 rpm) and O.D.460 was noted periodically until it reached the stationary phase (Madigan *et al.,* 1997).

Plasmid curing studies: Plasmid curing was performed by using acridine orange (Hardy, 1987). Culture was grown in Brain Heart Infusion (BHI) broth for overnight at 37°C. Next day 0.5ml of the culture was inoculated in a 5ml Luria basal broth and incubated at 37°C for 4hrs, in a shaking water bath. Aliquots (2.0ml) of BHI broth containing varying concentrations of acridine orange were inoculated with 30uL of culture grown in LB and incubated in dark for 24 hrs at 37°C. Tubes showing visible growth were selected for serial dilutions. Inoculum from different dilutions was spread on BHI agar plates followed by overnight incubation at 37°C. Isolated colonies were tested for the loss of ability to resist (15mM phenol) present in nutrient broth/agar.

Plasmid DNA isolation: Plasmid DNA was isolated by alkaline lysis method as described by Sambrook *et al.,* (1989).

Transformation of plasmid DNA: Bacterial transformation was carried out according to the $CaCl_2$ protocol (Davis *et al.,* 1986). Cured cultures were used as competent recipient. Transformants were selected on LB agar plates containing phenol (15mM).

Results and Discussion

The garden soil (rhizosphere) was selected for the isolation of phenol resistant bacteria. The rhizosphere is a rich site of phenolic compounds excreted by the plants. Such bacteria may be regarded as phenol resistant. Out of 30 soil isolates, 23 were found to be gram positive while 7 isolates were gram negative. All the isolates were screened for their ability to resist phenol at 10, 15, 20, 50 and 100mM concentrations. Five out of 30 isolates were found to resist 10mM phenol present in nutrient agar except MA-07, which was able to resist 15mM of phenol also (Table 1). The resistant isolates belonging to genera *Staphylococcus*, *Corynebacterium*, *Bacillus* and *Proteus* were identified on the basis of morpho-cultural and biochemical characteristics (Cheesbrough, 1989).

Table 1. Effect of phenol (added in nutrient broth) on the growth of the resistant isolates.

Isolate no.	Growth in Nutrient broth containing phenol (mM)				
	10	**15**	**20**	**50**	**100**
MA-07	+	+	-	-	-
MA-08	+	-	-	-	-
MA-21	+	-	-	-	-
MA-25	+	-	-	-	-
MA-30	+	-	-	-	-

The phenol resistant isolates were subjected to higher molarities of phenol (i.e. isolate no. MA-08, 21, 25 and 30, which were originally resistant upto 10mM, were able to adapt up to 15mM while MA-07 that originally resisted 15mM could not adapt itself beyond this molarity). Growth kinetic studies were carried out by following the method described earlier. All the five phenol resistant isolates were monitored for growth kinetics under various conditions (pertaining to nutrient and minimal medium broth, glucose depletion and phenol replaced conditions). Fig. 1 shows growth kinetics of *Corynebacterium* sp., in nutrient broth, nutrient broth containing 15mM phenol and in minimal medium. Similarly, Fig. 2, 3, 4 and 5 show the kinetics of *S. aureus*, *Proteus* sp.,

Bacillus subtilis and *Staphylococcus* sp., respectively. However, the growth variable conditions in all the microorganisms were kept constant. Growth kinetic studies were also carried out in minimal medium (glucose starved) containing different molar concentrations of phenol as sole source of carbon (data not shown). Generation time of all the five phenol resistant isolates under different conditions was also calculated (Table 2). Generation time of all the phenol resistant isolates (*Corynebacterium* sp., *S. aureus*, *Proteus* sp., *B. subtilis* and *Staphylococcus* sp.) in the nutrient broth ranged between 53 to 72.9 min., with an average of 61.4 min. Nutrient broth is a complete medium containing most of the preformed growth requirements (including organic nutrient sources), which are directly available to the cells (Madigan *et al*., 1997). The generation time of all the 5 phenol resistant isolates in phenol (15mM) containing nutrient broth ranged between 54.6 to 88.1 min., with an average of 77.3 min., which is approximately 1.3 times greater than the generation time in plain nutrient broth. It is evident that the difference in generation time in plain nutrient broth as well as nutrient broth containing phenol is not significant. Probably because phenol may have already bound to the organic matter present in the growth environment. The given results are substantiated with the findings of Watanabe *et al*., (1998). The generation time of the isolates was increased i.e., approximately 7.5 times in plain minimal medium compared to the generation time in plain nutrient broth; a clear indication that the organisms converted mineral salts to the organic compounds before dividing. Furthermore, the maximum amount of growth μ_{max} was lesser in minimal medium than in nutrient broth. This is due to availability of nutrients in limiting concentrations (Tanghe *et al.,* 1999). Interesting results were obtained in the follow up experiments where glucose depleted minimal medium was used. This medium contained phenol in different molar concentrations as sole carbon and energy source. In this situation, the phenol resistant isolates showed extended lag and log phase; hence longer generation time. This situation was reflected in the form of slight increase in the turbidity/$O.D._{460}$ thereby suggesting a possible utilization/biodegradation of phenol by the isolated strains of bacteria. Factor that may be considered for such a pattern of growth kinetics is the C: N ratio. Thus, in general the isolates performed well in minimal medium containing 0.06, 0.08 and 0.14mM phenol (presumably offering optimal C: N ratio). These observations are in agreement with the findings reported earlier (Tanghe *et al.*, 1999). Astonishingly, extended generation time has been witnessed at comparatively higher concentrations of phenol (0.2mM). Probably, this molarity could not offer an optimal C: N ratio. Furthermore, other factors that may contribute towards an extended lag phase and greater generation time include the more time taken by the isolates to open the phenol ring for its subsequent uptake as carbon source. Ronen & Abeliovich (2000) reported growth kinetic pattern with a gram-negative bacterium (strainWH1) isolated from contaminated soil in which phenol derivative BPA (bisphenol A) was used instead of phenol used in our experiment.

Treatment with the fluorescent dyes (e.g., ethidium bromide and acridine orange) and sodium dodecyl sulphate (Tomoeda *et al*., 1968) and physical agents (Novick, 1989) directs the cells to loose their plasmids. This procedure is reliable in order to identify whether the functional gene under investigation was present on plasmid or the chromosome. In the present study, acridine orange was found to be a fluent plasmid-curing agent as it cures one or two resistance determinants effectively (we were concerned with only one marker i.e. phenol resistance). Therefore, we concentrated only for the loss of ability of these cells to resist phenol. After curing all the phenol resistant isolates lost their ability to resist phenol. It indicates that phenol resistance gene are plasmid borne with reference to our isolates.

Table 2. Generation time in minutes of the phenol resistant isolates under different conditions.

Isolates	Nutrient broth	Nutrient broth + phenol	Minimal medium	Minimal medium (glucose minus) + different molarities of phenol (mM)					
				0.06	0.08	0.1	0.14	0.16	0.2
Corynebacterium sp.	59	81.1	353.7	240	360	153.8	540	400.9	642.2
S. aureus	57.14	78.9	453.9	355.5	722	714	371.8	740	876
Proteus sp.	64.5	83.6	410.25	433.7	975.6	1997.8	975.6	750	138.9
B. subtilis	53.6	54.6	481.9	194.2	342.4	174	400	342.4	2006.7
Staphylococcus sp.	72.9	88.1	619.4	497	433.7	744.9	323.9	463.3	873.8

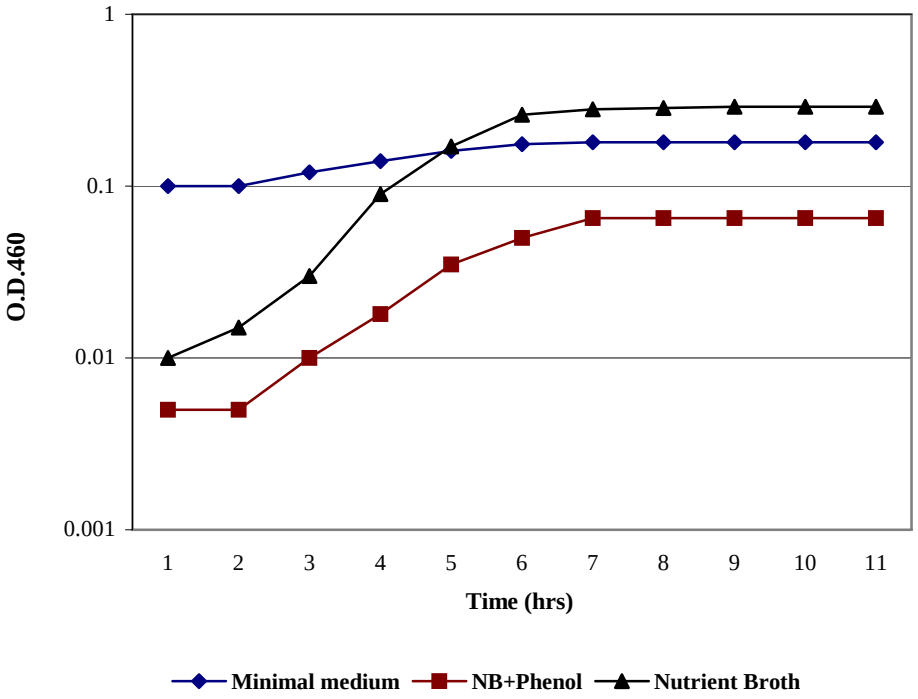

Fig. 1. Growth curve of *Corynebacterium* sp., under different conditions.

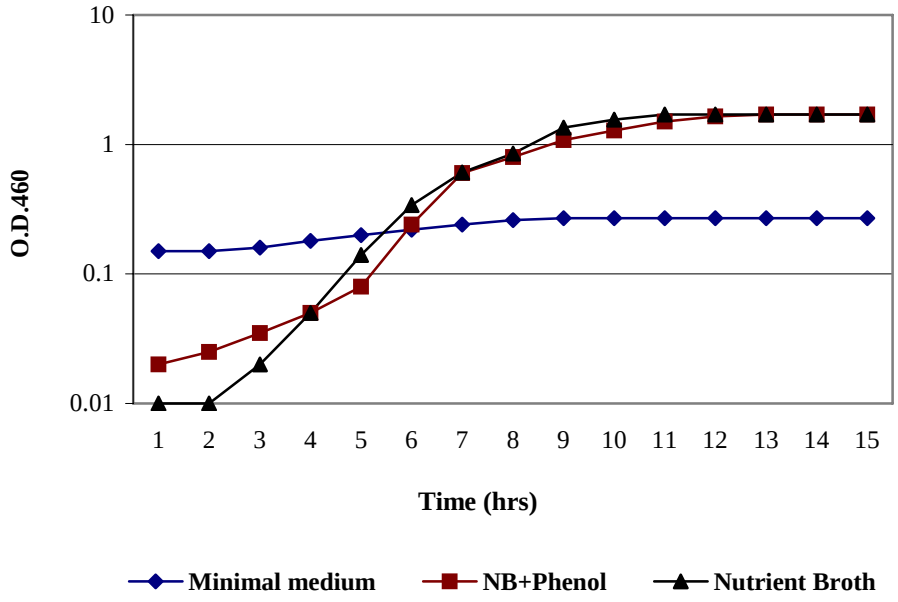

Fig. 2. Growth curve of *Staphylococcus aureus* under different conditions.

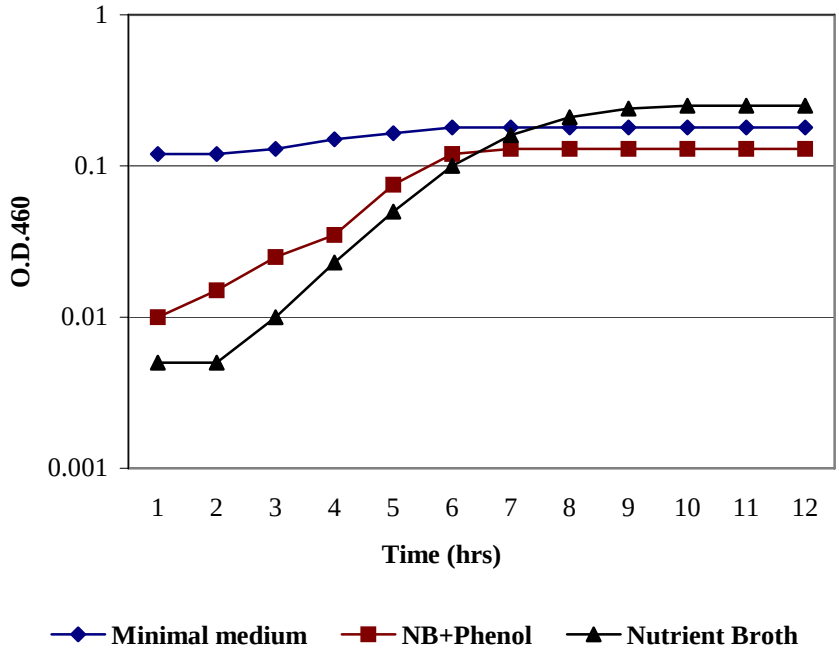

Fig. 3. Growth curve of *Proteus* sp., under different conditions.

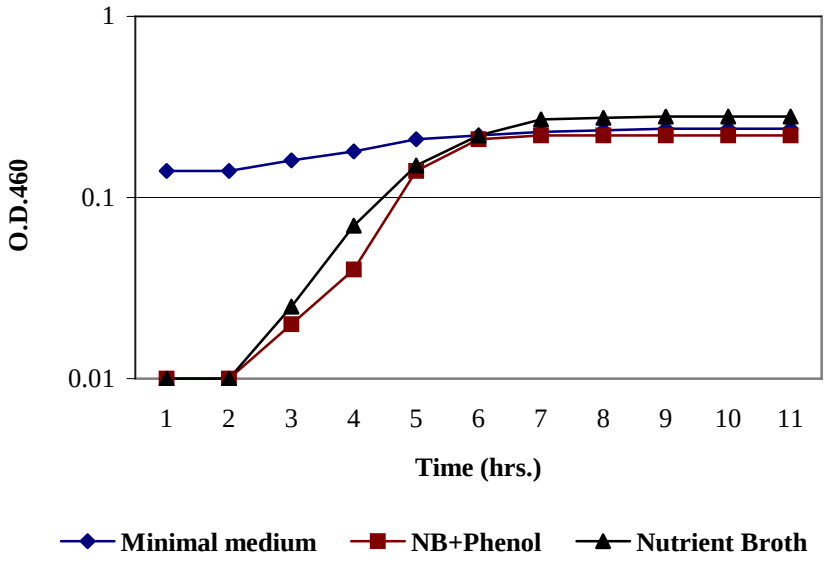

Fig. 4. Growth curve of *Bacillus subtilis* under different conditions

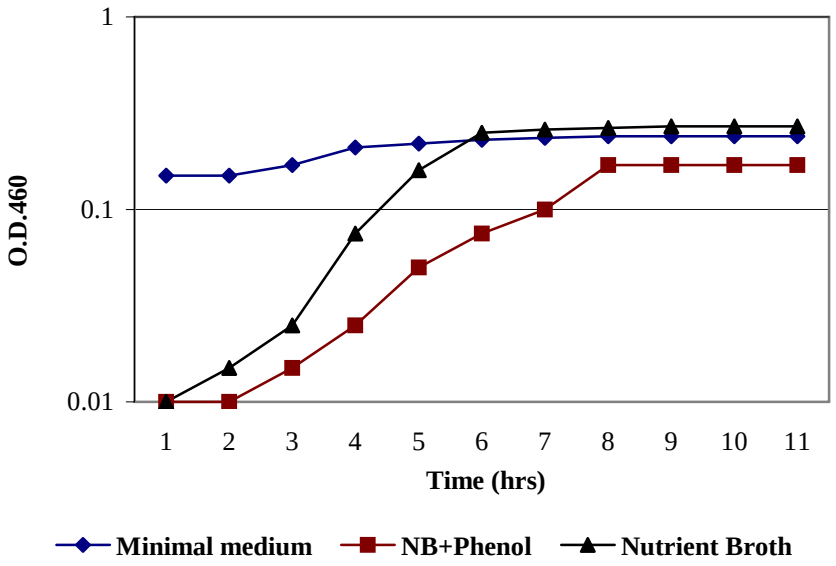

Fig. 5. Growth curve of *Staphylococcus* sp., under different conditions.

Chromosomal or plasmid DNA resistance genes can be transferred from one bacterium to another by *in vivo* gene manipulation including conjugation, transduction, and transformation (Davies, 1994). Plasmid DNA (isolated from uncured phenol resistant strains) was transformed into the same but cured (competent) recipient cells. Accordingly, the plasmid DNA was stably transformed into the cured phenol sensitive recipient turned cells. The transformants (on phenol resistant phenotype) were scored on L.B. agar plates containing 15mM phenol. It is an example of intrageneric transformation of plasmid genes into the cured turned phenol sensitive cells (Table 3). It is interesting that the plasmid borne genetic factors responsible for phenol degradation are transferable to other bacteria. In this way *in vivo* phenol resistance gene transfer could play beneficial dissemination role for phenol degradation in different environments.

The hyper phenol resistant strains that have been reported in this communication may be exploited for the degradation of toxic phenol compounds as contaminants in different environments. We anticipate that the strains can degrade the phenol into environmental friendly compounds that may be utilized by the bacteria as a source of nutrition.

Table 3. Transformation efficiency.

Plasmid (uncured)	Source (Donor)/ recipient (cured)	Transformants/mL (total drug resistant recombinants)	Transformation frequency
pMA-07	*Corynebacterium* sp.	110	110×10^{-7}
pMA-08	*S. aureus*	90	90×10^{-7}
pMA-21	*Proteus* sp.	95	95×10^{-7}
pMA-25	*B. subtilis*	100	100×10^{-7}
pMA-30	*Staphylococcus* sp.	120	120×10^{-7}

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