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# Plasmid Mediated Degradation of Phenol by Two Bacterial Strains *Pseudomonas* sp. and *Staphylococcus* sp.

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**Abstract:** Two microorganisms, which utilize phenol as a sole source of carbon and energy, were isolated from an enrichment culture. The microorganisms were identified as *Pseudomonas* sp. and *Staphylococcus* sp. The bacterial strains were found to harbor three endogenous plasmids. Two of the plasmids were lost when the microorganisms were treated with ethidium bromide. Loss of the plasmids in the organisms were correlated with the loss of the ability to metabolize phenol. Phenotypic testing of the wild type and cured strains revealed that the gene(s) responsible for phenol degradation may reside upon the plasmids.

**Key words**: Plasmid, Phenol degradation, *Pseudomonas* and *Staphylococcus*

## Introduction

Modern society has introduced a large number of xenobiotics to the environment. Some of these compounds are readily degraded in the environment by microbes where as others are recalcitrant. Soil and water bacteria in general and members of the genus *Pseudomonas*, in particular, able to degrade and use as sources of carbon and energy a wide range of organic compounds, including some that are quite noxious and otherwise biocidal (eg. phenol) and these exotic properties are not found in commensal and parasitic bacteria, such as *Escherichia coli* (Timmis *et al.*,1985).

The degradative plasmids endow on their hosts the ability to utilize rather uncommon organic compounds as their carbon and energy source. Because many such compounds, particularly naturally occurring hydrophobic and synthetic chlorinated hydrocarbons are toxic to the microorganisms, the presence and expression of such plasmids allow the host cells to quickly reduce the toxic concentration of the substrate hydrocarbons (Farrell and Chakrabarty, 1979). Ecologically, plasmid-encoded pathways are advantageous because they provide genetically flexible systems and can be maintained in the population and transferred between bacterial species (Sayler *et al.*, 1990).

Most strains of *Pseudomonas putida* are able to degrade benzoate, p-hydroxy-benzoate and tryptophan via the ortho-cleavage pathway (Palleroni, 1986). Since this capacity is species specific it is most likely chromosomal encoded (Herrmann *et al.*, 1987). Only certain strains of *P. putida* can utilize other aromatic compounds, for example m-toluate, toluene, xylenes, naphthalene or salicylate. Degradation of these substrates occurs via the meta-cleavage pathway (Fig. 1) and the corresponding genes were shown to be located on degradative plasmids (Williams and Murray, 1974; Wong *et al.*, 1978; Dunn and Gunsalus, 1973, Chakrabarty, 1972). Although different strains of P. putida can utilize phenol as the sole carbon and energy source, the genetic basis of this capacity bas been examined so for only in strain PP1-2 (Wong *et al.*, 1978). In this strain phenol is degraded by the products of chromosomal genes via the ortho cleavage pathway.

Herrmann *et al.* (1987) had reported on the isolation of two plasmids with a molecular mass of about 50 kb and 200-220 kb, respectively from P. putida strain H. They presented the evidence that the large one, pPGH I, is involved in phenol degradation via meta cleavage pathway. It has also been reported that the presence of TOL plasmid pWWO facilitates the survival of phenol-utilizing bacteria at higher concentration of phenol (Wong *et al.*, 1978).

In this paper we describe two bacterial strains (*Pseudomanas* sp. and *Staphylococcus* sp.) isolated from an enrichment culture preformed with phenol as the sole carbon and energy source exhibiting phenol degradation. And, we further report that this ability to utilize phenol as sole source of carbon and energy is encoded by the plasmid DNA.

## Materials and Methods

Isolation of phenol degrading microorganisms from enrichment culture. Arable soil (0.5 gm), Gas work soil (0.5 gm), river water (1 ml) and sewage (1 ml) were added to individual 250 ml Erlenmeyer flasks each containing 100 ml of sterile minimal salts (MS) medium supplemented with phenol at a concentration of 0.5 mM. Control flasks without an Inoculum were also prepared to take account of any abiotic disappearance of the phenol. This primary enrichment culture was incubated for several days at 37°C with shaking at 110 rpm on an orbital shaker. Microorganisms capable of degrading phenols were isolated from enrichment cultures (obtained originally from the river water Inoculum) by plating out on minimal salts agar containing phenol (10 mL/100 ml). When the plates were incubated at 37°C, small colonies developed after four to seven days. The isolation of a pure culture of phenol degrading bacteria was achieved by repeated transfer from agar to agar and agar to liquid cultures both containing phenol (10 mL/ml).

**Identification of phenol degrading microorganisms:** For the identification of phenol degrading microorganisms microscopic observation and growth characteristics as well as biochemical tests and fermentative characteristics were studied. The microorganisms were identified according to Bergey's Manual of Systematic Bacteriology.

**Plasmid extraction**: The plasmid DNA of the microorganisms was isolated according to Maniatis *et al.* (1982).

**Plasmid Curing**: Methods were taken from the review on plasmid curing by Trevors (1986). Curing agents used were ethidium bromide, acriflavin and sodium dodecyl sulfate (SDS). In addition, repeated subculture in nutrient broth in the absence of appropriate carbon source was used as a curing strategy.

Table 1a: Microscopic observation and growth characteristics of the isolated

| | Bacterial strain 1 | |
|---|---|---|
| | Tests | Observation |
| Microscopic examination | i. Simple staining | Rod shaped |
| | ii. Gram staining | Gram-negative |
| Growth Characteristics | i. Agar colony | Abundant, moist, creamy |
| | ii. Agar slant | Luxuriant, moist, creamy, speading growth |
| | iii. Nutrient broth | Turbid, viscid-sediment, medium becomes greenish -yellow fluorescent. |

Table 1b: Biochemical and fermentative tests of the isolated bacterial strain 1

| Test | Result |
|---|---|
| Catalase test | + ve |
| Gelatin liquefaction | + ve |
| Test of nitrate | + ve |
| Test of ammonia production | + ve |
| Hydrolysis of starch | Starch hydrolysis weak. |
| Fermentation test. | No acid from glucose, starch, lactose, sucrose, maltose, glycerol or mannitol |

Table 2: Microscopic observation and growth characteristics of the isolated bacterial strain 2:

| | Tests | Observation |
|---|---|---|
| Microscopic | i. Simple staining | Spherical, appeared as pairs and clusters. |
| | ii. Gram staining | Violet color (gram positive) |
| Growth Characteristics | i. Agar colony | Abundant growth, opaque white colonies with rough margin. |
| | ii. Agar slant | Same as agar colony. |
| | iii.Nutrient broth | Turbid, no sediment. |
| | iv. MacConkey agar slant | Growth positive. |
| | v. Blood agar slant | Well growth |

## Results

**Isolation of the phenol degrading bacteria**: By selective enrichment culture for growth on phenol as the only source of carbon and energy, two bacterial strains were isolated.

**Identification of the bacterial strains:** Microscopic observation and growth characteristics al well as biochemical tests and fermentative characteristics of the isolated bacterial strains 1 and 2 were studied and are represented in Table 1a, 1b and 2 respectively.

From the results of the different tests shown in the Table 1a, 1b (according to the Bergey's manual) the isolated bacterial strain 1 seem to be *Pseudomanas* sp. Table 2. From the results of the different tests shown in Table 2 (according to the Bergey's Manual) the isolated bacterial strain 2 seem to be *Staphylococcus* sp.


Fig. 1: Plasmid DNA of wild type Pseudomonas ps. (Lane 1, 2 and 3) and Staphylococcus sp (lane 4 and 5). The DNA was resolved on 1% agarose gel and stained with ethidium bromide 10 µg/ml

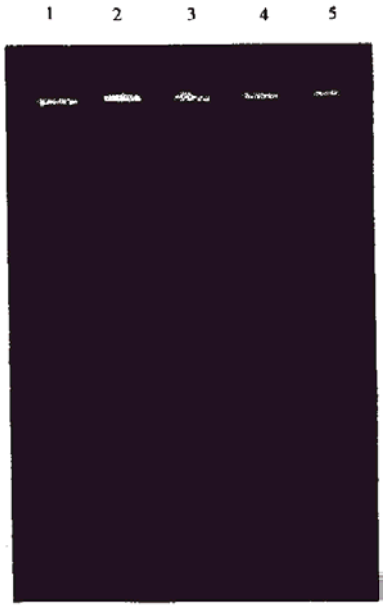


Fig. 2: Cured stains of Pseudomonas sp. (Lane 1, 2 and 3) and cured strains of Staphylococcus sp (lane 4 and 5). In both the organisms (2 bands of) plasmid DNA were lost when treated with Et Br

**Plasmid content of wild type and cured bacterial strains**: To determine the possible role of plasmid DNA in the metabolism of phenol, the plasmid content of wild type and cured strains was examined. The wild type strains of both the bacteria contained three DNA bands of plasmid. The cured strains of both the bacteria were found to have lost two bands of plasmid DNA. The loss of the two bands in the cured strain proved that the bands found on the gel belongs to the DNA carrying the gene(s) responsible for phenol degradation. Thus, the loss of two bands with the treatment of ethidium-bromide (200 mg/ml) suggested that there was strong correlation between the inability to metabolize phenol with the lost DNA bands. This result strongly suggested that the gene(s) responsible for the ability to metabolize phenol might be the plasmid DNA.

## Discussion

In this study, microorganisms capable of using phenol as their sole source of carbon and energy were isolated from the enrichment cultures. Pure culture of phenol degrading bacteria was isolated by plating out on agar solidified MS medium containing phenol. When the physiological and biochemical tests were done, two microorganisms were found; one was gram negative rod shaped *Pseudomanas* sp. and the other was gram positive cocci, identified as *Staphylococcus* sp.

Cook (1987) and Reineke and Knackmuss (1984) used enrichment cultures from sewage or waste water to obtain bacteria capable of degrading S-triazines and chloro-benzene respectively.

In the present study we further tried to find out that whether the ability to degrade phenol by these two bacterial strains was encoded by chromosomal DNA or extra-chromosomal DNA. The two bacterial strains, after the plasmid curing experiment, by using ethidium bromide, lost the ability to grow in phenol suggests the involvement of plasmids.

There is evidence that a number of soil bacteria capable of growth on 2-monocholropropronic acid (2 MCPA) or monochloro acetic acid (MCA), which are metabolized by the enzymes known as dehalogenases (Slater and Bull, 1982) and the dehalogenase capacity could be lost. Six such isolates, from *Pseudomonads* and two *Alcaligenes* sp. have been examined in detail and all found to contain a single plasmid ranging in molecular mass from 109 to 190 MDa (D.J. Hardman, P.G. Gowland and J.H. Slater, unpublished data). Curing the four *Pseudomonads* with ethidium bromide resulted in the concomitant loss of the plasmids and dehalogenase activities, showing that the dehalogenase genes were encoded on plasmids.

The genes of the catabolic pathways of many compounds are coded in plasmids (Chakrabarty, 1972; Farrell and Chakrabarty, 1979) including those for the degradation of Salicylate (Chakrabarty, 1972), Camphore (Rheinwald *et al*., 1973), Octane (Chakrabarty, 1973), Naphthalene (Dunn and Gunsalus, 1973), Toluene and Xylene (Williams and Murray, 1974; Hopper and Kemp, 1980), Carbofuran (Head *et al*., 1989), 2,4-dichlorophenoxyacetate (Chaudhry and Huang, 1988) and Chlorinated aromatic compounds (Ghosal *et al*., 1986).

Agarose gel electrophoresis showed that the wild type organisms carrying three bands of plasmid DNA (Fig. 2) where as the cured ones carrying only one. Since the cured strains were found unable to grow in the medium containing phenol which was the sole source of carbon and energy, the loss of the two bands of plasmid DNA in the cured strains proved that the DNA carrying the gene(s) responsible for phenol degradation.

It is also evident from earlier studies that there are significant number of pathways for the catabolism of aromatic compounds. Timmis *et al*. (1985) analyzed the plasmid encoded pathways for the catabolism of aromatic compounds. Shingler *et al*. (1989) also demonstrated that the growth of *Pseudomonas* strain CF 600 on phenol and 3,4-dimethyl phenol, as sole carbon and energy source, is by virtue of plasmid encoded phenol hydroxylase and involves a meta cleavage pathway.

The role of plasmids in the degradation of phenol has provided a lucrative ground for examining the potential for and mechanisms of bacterial evolution in nature and the practical consequences in terms of pollution control. More research should be performed on the genetic construction of bacteria for expanded catabolic activity, which may involve more than simple combining genes from different catabolic pathways; In addition, gene expression of the enzymes must be properly regulated and unproductive enzyme pathways deactivated in order to form man made bacterial colonies that will clean up a factory's waste before it leaves the plant.

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