Effect of phenol on ultra structure and plasmid DNA of *Xanthomonas oryzae* pv. *oryzae*

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Most phenolic substances of plant origin are toxic to microorganisms and they confer some degree of protection to plants against phytopathogens. *Xanthomonas oryzae* pv. *oryzae*, bacterial blight pathogen of rice (*Oryza sativa*) was treated with phenol (monohydroxy benzene) and its effects on the morphology and cytological changes of the bacterium were studied. Total lysis of cells occurred with 5 mM conc of phenol while at 2 mM conc, the cell walls became rough and cell contents started shrinking. Plasmids isolated from both treated (2 mM) and control cells did not show any marked difference under electron microscope except that they differed in their quantity and might influence pathogenicity.

Plants synthesize a variety of phenolic substances that include phenols, phenolic acids, flavanoids, aurones, chalcones, tannins, lignins, lignans and anthocyanins. Most phenols are toxic to microorganisms and they confer some degree of protection to plants against phytopathogens1. The mode of action of phenols on microorganisms is reflected by the alteration of various morphological, cytological and physiological functions of microorganisms. Partial detachment of outer membrane resulting in abnormal space between outer membrane and cytoplasmic membrane, and damaged or absence of cytoplasmic membrane has been reported in *Escherichia coli* treated with 8.8 mM p-aminobenzoic acid2. *Lactobacillus plantarum* cells exposed to phenols exhibited an irregular surface covered by projections or granules of different sizes giving them a rough appearance3. *Pseudomonas putida* DOT-T1 cells grown in 10% (v/v) toluene exhibited larger periplasmic space with occasional membrane evagination when compared the control cells4.

Pathogenic bacteria that cause a variety of diseases of plants have not been investigated for their response to phenols, which have been implicated in disease resistance1. *Xanthomonas oryzae* pv. *oryzae* causes bacterial blight of rice (*Oryza sativa*) and is an important disease5. In the resistant rice cultivars, infection by *X. oryzae*, pv. *oryzae* leads to increase in phenolics6. The effect of a simple phenol, monohydroxy benzene on protein and lipid metabolism in *Xanthomonas oryzae* pv. *oryzae* has been reported earlier7,8. This communication deals with the changes in ultra structure of *X. oryzae* pv. *oryzae* in response to treatment with phenol.

*Xanthomonas oryzae*, pv. *oryzae* was obtained from the Culture Collection of Centre for Advanced Studies in Botany, University of Madras, Chennai. Toxicity of phenol on growth of the bacterium was determined by amending the synthetic medium without sucrose with suitable volume of phenol stock solution. Bacterial cells obtained from these cultures were processed for electron microscopic studies after 24 h of treatment.

*Transmission electron microscopic (TEM) observation of X. oryzae*, pv. *oryzae*—The cells were harvested by centrifugation at 10,000 g and washed twice in distilled water. The pellet was suspended in 5 ml of sterile distilled water and 100 µl of this suspension was placed in an embryo cup. To this, 1 ml of 2% phosphotungstic acid solution containing 50 µl of bovine serum albumin (100 µg/ml) was added and stained for 30 min.

A fresh ‘formvar’ coated copper grid was washed in acetone and dried. It was dipped in the bacterial suspension; taken out immediately and the excess water in the grid was drained using a filter paper. The grid was examined in a Philips Transmission Electron Microscope (Model-CM 10) operating at a voltage of 60 kV.

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Isolation of plasmid DNA—Plasmid DNA was isolated from the cells as previously described. Cells, 24 hr old, \(7 \times 10^6\) cells/ml were collected by centrifugation at 10,000 g for 10 min, washed twice in distilled water and once in TE buffer. The pellet was dissolved in 100 \(\mu l\) of lysozyme (10 mg/ml TE), incubated at 30° ± 2°C for 30 min, 200 \(\mu l\) of the mixture of 1% SDS + 0.2 N NaOH (freshly prepared) was added and kept at room temperature till a clear lysate was obtained. An equal volume of TE saturated phenol:chloroform (1:1) was added, mixed gently, centrifuged at 10,000 g for 10 min, the upper clear layer was transferred to a fresh tube and 300 \(\mu l\) of 3 M sodium acetate (pH 5.2) and an equal volume of isopropanol was added. The contents were mixed well; kept at -20°C for 30 min and centrifuged at 10,000 g for 10 min. After discarding the supernatant, the pellet was dissolved in 10 \(\mu l\) of TE. To this, 10 \(\mu l\) of bromophenol blue was added, loaded on to 8% agarose gel and electrophoresed at 50 V for 4 hr, stained in ethidium bromide and examined under UV transilluminator.

TEM examination of DNA/plasmid DNA—DNA isolated from the control and phenol treated cells was processed for EM observation following the droplet diffusion technique. Pictures were taken in a Philips Transmission Electron Microscope operating at a voltage of 60 kV.

Droplet diffusion technique—An aliquat of 0.5 ml of the droplet solution containing 0.9 ml H2O, 0.1 ml NH4Ac buffer and 3 \(\mu l\) 0.1% cytochrome-C was carefully mixed with 1 \(\mu l\) DNA. Using a micropipette, droplets of 30-40 \(\mu l\) were placed on a hydrophobic surface (parafilm), covered with a Petri dish for 15 min, avoiding vibration. The surface film of denatured cytochrome-C with enclosed DNA was transferred onto a carbon coated grid, 400 mesh (Agar Scientific, U.K.) by touching the film side of the grid as parallel as possible to the surface of a droplet either laterally or at its top. The grid was washed for 5 sec on dist water, stained with 5\(\times10^{-5}\) M uranyl acetate (UO2Ac) in 90% ethanol and dehydrated for another 10 sec in 90% ethanol. The grid was dried face down on filter paper and examined in Philips TEM. Several hundred specimens were examined and results representing observations most frequently recorded.

Effect of phenol on cell morphology—Untreated cells were intact with a single polar flagellum with smooth cell wall surface (Fig. 1A). The treated cells had rough surface indicating the damage of cell wall due to phenol treatment at 2 mM (Fig. 1B) and the cells lost their flagella. At high conc (5 mM), disruption of cell wall occurred and the disintegration of cells was complete (Fig. 1C).

Effect on chromosomal and plasmid DNA—The effect of phenol on the yield of plasmid DNA separated on agarose gel electrophoresis revealed that in control cells more plasmid DNA was present than in the treated cells. Agarose gel electrophoresis revealed that the plasmid DNA from phenol treated cells appeared as a faint band whereas the control plasmid appeared as a bright band when viewed under UV light (Fig. 2).

Observation of the plasmid preparation in TEM appeared as circular strand. However, when observed under TEM, no difference was found between plasmids of control and treated cells (Fig. 3A & B).

TEM pictures of chromosomal DNA isolated from control and treated cells showed no differences (Fig. 4A & B).

The site of action of phenol appeared to be the cell wall. The electron microscopic observation of X. oryzae pv. oryzae cells revealed that the untreated cells had a smooth and uniform wall surface whereas the treated cells had rough surface and abnormal space between outer membrane and cytoplasmic membrane due to shrinking of cytoplasmic contents at low conc (2 mM). High conc (5 mM) disrupted the cell wall leading to cell death.

Bacteria respond to treatment with phenols by forming rough surface and holes in the cell wall and also partial detachment of outer membrane resulting in abnormal space between outer membrane and cytoplasmic membrane. P. putida cells exposed to toluene exhibited altered structure, the periplasmic space was usually larger than in the control cells and occasionally membrane evagination was present.

The DNA of X. oryzae pv. oryzae is reported to contain genes required for pathogenicity and plasmids are known to code for a variety of functions including pathogenicity in plant pathogenic bacteria. Our observation of plasmid DNA isolated from both phenol treated and untreated cells showed that the quantity of plasmid decreased in response to exposure to phenol. Whether or not the pathogen-induced altered phenol level in the resistant rice cultivars affects virulence of the pathogen needs further investigation.
Fig. 1-4 — (1) — Electron micrograph of *Xanthomonas oryzae* pv. *oryzae* treated with phenol. (A) Intact cell; (B) Cells treated with 2 mM phenol. CW—Cell wall. (C) Cells treated with 5 mM phenol; (2)—Agarose gel electrophoresis of plasmid preparation from treated and untreated cells. M—Marker λ, *Hind* III. C—Untreated cells, 2 mM—Cells treated with 2 mM phenol; (3)—Electron micrograph of plasmids of *X. oryzae* pv. *oryzae* treated with phenol. (A) Plasmid from untreated cells. (B) Plasmid from treated cells; and (4)—Electron micrograph of chromosomal DNA from *X. oryzae* pv. *oryzae* treated with phenol. (A) DNA from untreated cells. (B) DNA from 2 mM phenol treated cells.
References

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