

Plasmid Encoded Toluene and Xylene Degradation by Phyllosphere Bacteria

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Abstract

Air pollution by monoaromatic hydrocarbons (MAH) is a highly concerned great threat in modern world due to the high carcinogenicity and genotoxicity to all living beings. Most of these MAH releasing processes are oil refining processes and vehicular emission. These air trapped pollutants deposit on ground level and phyllosphere takes special place as a ground level exposure surface for these pollutants. Continuous deposition lead to make a MAH degrading microbial consortium in the phyllosphere and these microorganisms can be used as an efficient bioremediators in remediating MAH contaminants which is an environmental friendly solution compared to chemical remediations. The phyllosphere of plant species *Ixora chinensis*, *Ervatamia divaricata*, *Hibiscus rosa-sinensis* and *Amaranthus cruentus* which are highly abundant along the roadsides of polluted areas in Sri Lanka are rich with several species of bacteria belong to many genera and they were able to degrade toluene and xylene efficiently. The species of *Alcaligenes faecalis*, *Alcaligenes* DN25 *Bacillus cereus* and *Bacillus methylotrophicus* were able to degrade toluene and xylene efficiently. All these strains harbor plasmids conferring them resistance to ampicillin. Curing of the plasmids of *A. faecalis* and *Alcaligenes* sp. DN25 drastically reduced the ability in degrading these toluene and xylene. Upon transformation of plasmids of these two *Alcaligenes* sp. into *E. coli* JM109 enabled it to degrade the two hydrocarbons efficiently. But transformation and curing process of two *Bacillus* sp. into the *E. coli* JM109 was unsuccessful. Plasmid encoded toluene and xylene degradation of two *Alcaligenes* sp. suggested the presence of required catabolic genes in these plasmids. PCR amplification with degenerate primers and comparison of their nucleotide sequences with Genbank sequences indicated that plasmids of *A. faecalis*, *Alcaligenes* sp. DN25 and *B. cereus* harbor the genes *xylQ* involved in toluene and xylene degradation. RFLP and nucleotide sequence comparisons of *xylQ* amplicons revealed that both of these genes in two bacterial strains (*A. faecalis* and *Alcaligenes* sp. DN25) are homologous. But that was heterologous to the *xylQ* gene of *B. cereus*. Hence the results clearly showed the potential of *A. faecalis*, *Alcaligenes* sp. DN25 and *B. cereus* in degrading toluene and xylene and also the potential of using them in remediating aromatic hydrocarbonic contaminants.

Keywords: Bioremediation; Phyllosphere; *Bacillus* sp.; Catabolic plasmid; RFLP pattern

Introduction

Toluene and xylene are major air pollutants which give great risk to all living beings due to their high carcinogenicity and genotoxicity [1]. Plant leaf surfaces are the major exposing surfaces to these aromatic hydrocarbons when they deposit on ground level by wet and dry deposition. These plant surfaces are called phyllosphere and it is a great niche to microorganisms belongs to several genera of bacteria, fungi, algae, protozoans and nematodes. Out of them bacteria and fungi are the predominant microorganisms and those who abundant in the phyllosphere of polluted sites had special ability in aromatic hydrocarbon degradation. The results of Undugoda et al. [2], revealed phyllosphere fungi and bacteria isolated from polluted sites of Sri Lanka had naphthalene and phenanthrene like polyaromatic hydrocarbon degradation ability. Aromatic hydrocarbon degradation ability of most of bacteria is a plasmid encoded character. According to the results of Assinder and Williams, 1990 TOL plasmid pWW0 of *Pseudomonas putida* harbored genes which can encode enzymes to degrade toluene and xylene. *Xyl* genes harboured in this TOL plasmid pWW0 are organized into two operons as the upper and meta operons which are positively regulated by *xylR* and *xylS* genes [3]. Two plasmid-encoded operons account for the entire metabolic process that leads to biodegradation of such aromatic substrates [4]. The upper operon encode the enzymes to convert m-xylene or toluene into 3-methylbenzoate or benzoate, respectively, and the lower operon enzymes determine the conversion of 3 MBz into 3-methylcatechol or benzoate into catechol and its ensuing meta ring cleavage, which ultimately leads to intermediates of the TCA cycle [5,6].

This research was attempted to identify the plasmid encoded toluene and xylene degradation ability of the isolated phyllosphere bacterial species which are highly abundant in the phyllosphere of polluted sites in Sri Lanka. And also availability of toluene and xylene degrading some selected genes in these plasmids of these bacteria was determined and further characterization was done following sequencing and RFLP results.

Materials and Methods

Aromatic hydrocarbon degrading *Alcaligenes faecalis* (KT356811), *Alcaligenes* sp. DN25 (KT356809), *Bacillus methylotrophicus* (KT356809) and *Bacillus cereus* (KT356812) were isolated from the leaf samples collected from the four plant species, *Ixora chinensis*, *Ervatamia dervaticata*, *Amaranthus cruentus* and *Hibiscus rosasinensis* inhabit the five polluted sites, Colombo fort, Maradana, Panchikawattha, Sapugaskanda and Orugodawattha in Sri Lanka [2].

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Plasmid isolation

1.5 ml of overnight bacterial culture was spun for 1 minute in a micro-centrifuge to make the pellet. The bacterial pellet was resuspended in 200 µl of ice-cold solution containing 0.04 M Trisacetate, pH 8.0 (adjust pH with glacial acetic acid) and vortexed vigorously. 400 µl of lysis buffer (0.05 M Tris, 3% SDS, pH 12.50, adjusted with 2 N NaOH) was added into each bacterial suspension and mixed well. Then it was incubated at 60-68°C for 30-45 min. 1000 µl of phenol/chloroform (1:1) was added into the hot sample and mix gently to complete emulsification. Then the emulsion was centrifuged at 14000 rpm for 2 minutes at 4°C in a microfuge. The aqueous upper layer was transferred to a fresh tube. Plasmid DNA was precipitated from the supernatant by adding 600 µl of 95% ethanol at room temperature. The plasmid DNA was dissolved in 100 µl of TE (pH 8.0) containing 20 µg/ml DNase-free RNase A (pancreatic RNase). The solution was vortexed gently for a few seconds. The plasmid DNA was stored at -20°C.

Determination of plasmid based toluene and xylene degradation of selected two bacteria

Isolated plasmids were transformed to a *E. coli* JM109 following the method of Sambrook et al. [7] and toluene and xylene degradation ability of transformants were checked using HPLC method described in Undugoda et al. [2]. Then again plasmids of transformants were cured following Undugoda et al. [2], and again their toluene and xylene degradation ability was checked.

PCR amplification of *xylQ* and *xylM* regions in catabolic plasmid

The gene sequences of *xylQ* gene of *P. putida* pWWO and *Pseudomonas migulae* (GenBank accession nos NC003350, and NC021250, respectively) have been used to design the degenerate primers to amplify *xylQ* gene. Then *xylM* gene sequence of *P. putida* pWWO53 (GenBank accession no NC008275) has been used to design the degenerate primers to amplify *xylM* gene. Sequence of these primers are, *xylQ* F 5' GGCCATATGAGCAAGAACTC 3', *xylQ* R 5' CGATGTCGGCGAATTCGGGC 3', *xylM* F 5' GAT GCC TTC GCT CTT TGT GT 3', *xylM* 5' GAT GCC TTC GCT CTT TGT GT 3'. Plasmid DNA templates of *Alcaligenes faecalis*, *Alcaligenes* sp. DN25, *Bacillus methylotrophicus* and *Bacillus cereus* were amplified using above primers. Then PCR products were visualized and their size estimation was done by gel electrophoresis.

Sequencing of PCR products

Automated sequencing was carried out using applied Biosystems automated sequencer (ABI3730XL) at Macrogen, Seoul, Korea. Purified PCR products of *xylM* and *xylQ* gene segments were sequenced directly using appropriate primers.

Restriction digestion of PCR products

PCR amplicons of *xylM* and *xylQ* genes were digested using *HindIII* restriction enzyme to determine their RFLP patterns.

Results

According to the Undugoda et al. [2], *A. faecalis*, *Alcaligenes* sp. DN25, *B. methylotrophicus* and *B. cereus* were the best toluene and xylene degraders isolated from the selected four plant species inhabit the five polluted sites of Sri Lanka. Plasmid isolation and ampicillin resistancy test of all four bacterial strains revealed they harboured plasmids. To check whether this toluene and xylene degrading ability is

a plasmid encoded or not plasmid transformation and plasmid curing process was done. Eventhough according to the gel electrophoresis results (Figure 1) transformation of plasmids of *Alcaligenes faecalis* (plasmid 1) and *Alcaligenes* sp. DN25 (plasmid 2) into *E. coli* JM109 was successful, it was unable to transform plasmids of *B. cereus* (plasmid 3) and *B. methylotrophicus* (plasmid 4) in to *E. coli* JM109 successfully.

According to the HPLC analysis results *E. coli* JM109 was unable to degrade toluene and xylene efficiently. But after getting plasmid 1 and plasmid 2 into *E. coli* JM109, it was able to degrade toluene and xylene almost close to the plasmid harboured original bacterial strains, *Alcaligenes faecalis* and *Alcaligenes* sp. DN25 (Figure 2). In fact, *Alcaligenes faecalis* was able to degrade 63.64% of xylene in the medium and *E. coli* JM109 transformants with plasmid of *Alcaligenes faecalis* was able to degrade 50.95% of xylene in the medium. These results convinced toluene and xylene degradation ability of *A. faecalis* and *Alcaligenes* sp. DN25 is a plasmid encoded character. It was again confirmed by curing the plasmids of transformant using SDS treatment.

After curing the plasmids of transformants, toluene and xylene degrading ability of them were lost. HPLC results revealed after the curing process, xylene degrading ability of plasmid 1 harboured *E. coli* JM109 was declined into 2.01% (Figure 3). As well as their toluene degradation ability was declined into 1.54% (Figure 4). These results convince toluene and xylene degradation ability is encoded by the plasmids located in these efficient phyllosphere degraders, *A. faecalis* and *Alcaligenes* sp. DN25.

Transformation and plasmid curing results confirmed xylene and

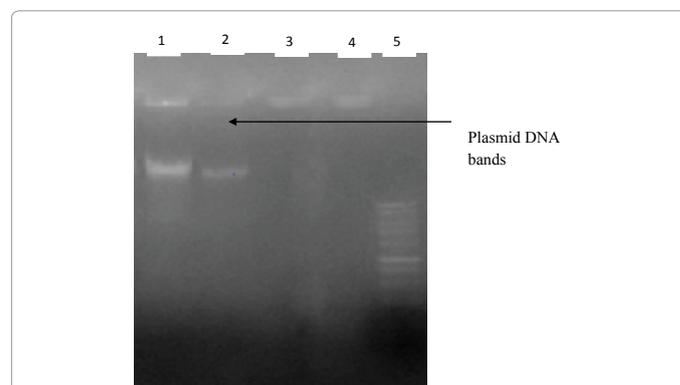


Figure 1: Gel electrophoresis of plasmid DNA from *E. coli*, JM109, transformants. Lane 1. Plasmid DNA of *A. faecalis*, transformed into *E. coli* JM109 Lane 2. Plasmid DNA of *Alcaligenes* sp. DN25 transformed into *E. coli* JM109 Lane 3. Plasmid DNA of *B. cereus* transformed into *E. coli* JM109, Lane 4. Plasmid DNA of *B. methylotrophicus* transformed into *E. coli* JM109 Lane 6. Lamda *Hind III* ladder.

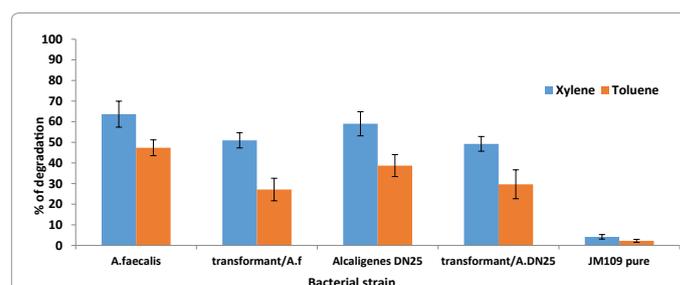


Figure 2: AH degradation (%) by two *Alcaligenes* sp. and two *E. coli* strains transformed with plasmids from two *Alcaligenes* spp. as assayed by HPLC method. N=6.

toluene degradation ability of these phyllosphere bacteria, *Alcaligenes faecalis* and *Alcaligenes* sp. DN25 is a plasmid encoded character. Though transformation and plasmid curing of *B. cereus* and *B. methylotrophicus* were unsuccessful, they showed higher toluene and xylene degradation ability as those two *Alcaligenes* sp. Therefore, PCR amplification was done using *xylQ* and *xylM* specific primers to check, whether these xylene and toluene degrading genes are located on the plasmids isolated from AH degrading four phyllosphere bacterial species. 654 bp size PCR amplicons were observed with plasmid DNA templates of *Alcaligenes* sp. DN25, *Alcaligenes faecalis* and *Bacillus cereus* (Figure 5). This gel electrophoresis results revealed except *B. methylotrophicus*, other three bacterial strains harboured *xylQ* gene in their plasmids.

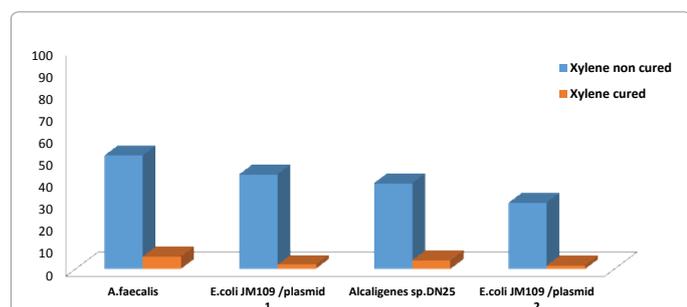


Figure 3: Percentage of xylene degradation by each bacterial strain, before and after curing of plasmid as assayed by colorimetric method. N=6.

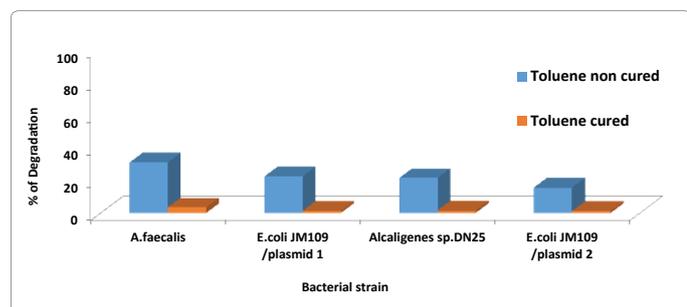


Figure 4: Percentage of toluene degradation by each bacterial strain, before and after curing of plasmid as assayed by colorimetric method. N=6.

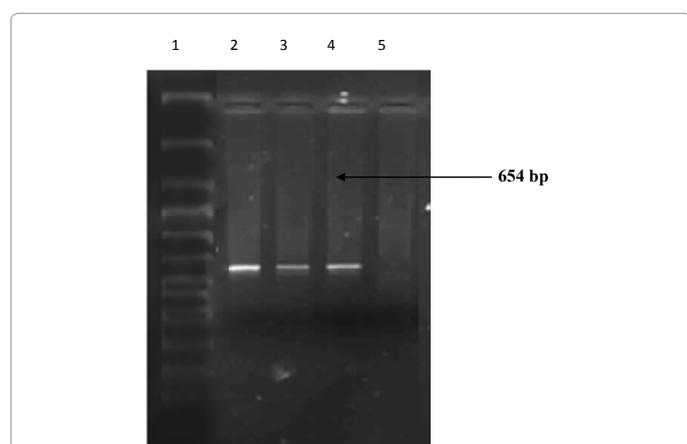


Figure 5: Gel electrophoresis of PCR products obtained by amplification of plasmid DNA templates with primers *xylQ* for gene *xylQ*. Lane 1- Lambda *HindIII* ladder, Lane 2- *Alcaligenes faecalis*, Lane 3- *Alcaligenes* sp. DN25, Lane 4 -*Bacillus cereus*, Lane 5- *B. methylotrophicus*.

Partial nucleotide sequence of *xylQ* gene of *Alcaligenes faecalis* showed 83% similarity to the *xylQ* gene located in the pDK1 plasmid of *P. putida*. Nucleotide sequence of *xylQ* gene of *Alcaligenes* sp. DN25 showed 85% similarity to the *xylQ* gene located in the pWW53 plasmid of *P. putida* MT53. Nucleotide sequence of *xylQ* gene of *Bacillus cereus* showed 98% similarity to the *xylQ* gene located in the pNL1A plasmid of *Sphingomonas aromaticiviran*. Both *Alcaligenes* sp. showed higher similarity with same gene located in the plasmids of *Pseudomonas* sp.

The same gene in different organisms may exist as different alleles with slight variations in their nucleotide sequence. Specially, the catabolic genes are highly variable due to the harsh conditions in the environment. Therefore, the same catabolic gene has so many different alleles. These genetic variations can be used to identify genetic relationships among phyllosphere microorganisms. Therefore, RFLP patterns and nucleotide sequences of these catabolic genes were used to determine phylogenetic relationships existing among AH degrading phyllosphere bacterial strains. The *xylQ* gene amplicon obtained from PCR was digested with *HindIII* enzyme and RFLP pattern of *xylQ* gene was visualized using gel electrophoresis (Figure 6).

HindIII digestion of *xylQ* gene gave two distinct RFLP patterns under *Bacillus* sp. and *Alcaligenes* sp. However, RFLP pattern of *Alcaligenes faecalis* and *Alcaligenes* sp. DN25 was similar to each other (Figure 5). But RFLP patterns of *Alcaligenes* sp. and *Bacillus* sp. are significantly different to each other. In fact, *xylQ* gene of *Alcaligenes* sp. was digested into two DNA fragments by *HindIII*, but *xylQ* gene of *Bacillus* sp. was digested into three DNA fragments. Therefore, same gene has different number of restriction sites. It reveals that the same gene has different alleles when they exist in different genera of phyllosphere bacteria. But the particular gene is homologous within similar species. Therefore, the plasmid harbored xylene degrading phyllosphere bacterial strains have two different allele types of *xylQ* gene.

Discussion

The TOL plasmid pWWO of *Pseudomonas putida* mt-2 is a 117-kilobase (kb) catabolic plasmid which has ability to encode all enzymes necessary for bacterial utilization of toluene (Tol), m- and p-xylene (Xyl), 3-ethyltoluene, and 1,2,4-trimethylbenzene and carboxylic acid derivatives, via a meta cleavage pathway [8]. Most

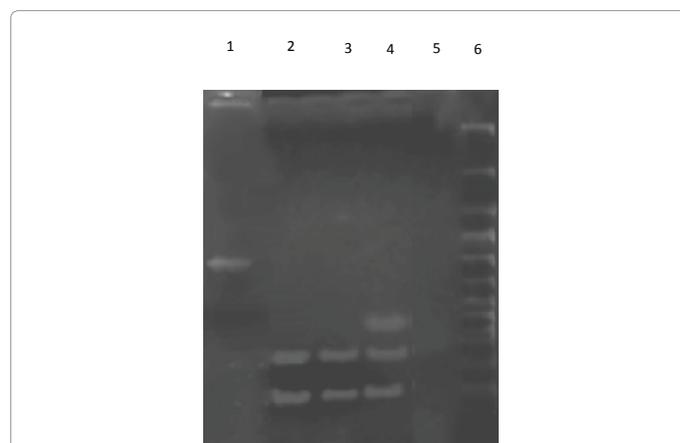


Figure 6: Gel electrophoresis of digested *xylQ* gene fragment containing PCR product, Lanes 1- Non digested 654 bp *xylQ* PCR product from *A. faecalis*, Lane 2-digested PCR product from *Alcaligenes* sp. DN25, Lane 3 - digested PCR product from *Alcaligenes faecalis*, Lane 4- Digested PCR product from *Bacillus cereus*, Lane 6 - molecular weight marker (100-bp ladder).

often *Pseudomonas* sp. was able to degrade toluene and xylene and degradation ability of them were plasmid encoded character. But in this study the best toluene and xylene degraders were two *Alcaligenes* spp. and their degradation is a plasmid encoded character. Even though two *Bacillus* sp. were harbored plasmids and degrade toluene and xylene, it was unable to determine it as a plasmid encoded character due to the unsuccessful transformation and curing process. But PCR amplification results of *B. cereus* revealed it has *xylQ* gene located on its plasmid.

Many genes responsible for the AH degradation are located on the plasmids. Nevertheless, some genes are located on the chromosome of the bacteria. Occasionally, such genes are genomic based and their regulatory genes are plasmid based. Then, plasmid or chromosomal genes cannot express AH degrading character autonomously [1]. The catabolic plasmids which encode the enzymes to initiate pathways for the catabolism of toluene, m-xylene, and p-xylene via aromatic ring meta cleavage is referred to as TOL plasmids [5]. TOL plasmids have mostly been found in representatives of the genus *Pseudomonas*. As an example, pWWO plasmid of *P. putida* harbored *xyl* genes which encoded the xylene and toluene degrading enzymes [9]. Besides toluene degradation, this plasmid has been shown to mediate the degradation of m- and p-toluene, m- and p-xylene and related compounds [4] to acetaldehyde and pyruvate via a bifurcating pathway. Furthermore, the plasmids pWW53, pDK1, and pWW15 also located in *Pseudomonas* sp. which carried many *xyl* genes [10]. But phyllosphere samples collected from five polluted sites were rich in *Alcaligenes* sp. and their plasmid-encoded toluene and xylene degradation ability was high. Nucleotide sequence of *xylQ* gene of both of these *Alcaligenes* sp. had more than 80% sequence similarity to the *xylQ* gene harboring plasmids of *Pseudomonas* sp. Therefore, there should be close phylogenetic relationship among the *xylQ* gene of *Alcaligenes* sp. and *xylQ* gene of *Pseudomonas* sp.

Bacillus cereus isolated in this work harbored *xylQ* gene on its catabolic plasmid and it was able to degrade these AHs efficiently. Although, most of the *xyl* genes carrying catabolic plasmids are found in Gram negative bacterial strains, *Bacillus cereus*, a Gram positive strain also has *xyl* gene on its plasmid. The catabolic plasmids of *Alcaligenes faecalis*, *Alcaligenes* sp. DN25 and *Bacillus cereus* harbored *xylQ* genes and they lack *xylM* genes. RFLP patterns of PCR amplified *xylQ* gene segment revealed *Alcaligenes faecalis* and *Alcaligenes* sp. DN25 had same RFLP pattern of *xylQ* gene and this pattern is different from RFLP pattern of *xylQ* gene of *Bacillus cereus* (Figure 5). When same

gene locates in same genus, they are similar to each other controversy when it locates in the plasmids of different genus, they are different to each other. Therefore same *xylQ* gene has two different alleles.

Conclusion

Toluene and xylene degradation ability of *A. faecalis* and *Alcaligenes* sp. DN25 is a plasmid encoded character. *XylQ* genes of them were located in their catabolic plasmids. Out two AH degrading *Bacillus* sp. (*Bacillus methylotrophicus* and *B. cereus*) *B. cereus* harbors *xylQ* gene on its catabolic plasmid. *XylQ* gene of two *Alcaligenes* spp was different from that of *Bacillus cereus*. That mean *xylQ* gene has two allele types when they exist in the environment.

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