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 feacalis, 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. feacalis 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. feacalis, 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. feacalis 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. feacalis, 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 pWWO 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 dervatica, Amaranthus cruentus and Hibiscus rosasinensis inhabit the five polluted sites, Colombo fort, Maradana, Panchikawattha, Sapugaskanda and Orugadawattha in Sri Lanka [2].

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Received March 06, 2018; Accepted March 17, 2018; Published March 22, 2018

Citation: Undugoda LJS, Kandisa RV, Kannangara S, Sirisena DM (2018) Plasmid Encoded Toluene and Xylene Degradation by Phyllosphere Bacteria. J Environ Anal Toxicol 8: 559. doi: 10.4172/2161-0525.1000559

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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 an 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 pWWO33 (GenBank accession no NC008275) has been used to design the degenerate primers to amplify xylM gene. Sequence of these primers are, xylQ F 5’ GGGCATATGAGCAAGAAACTC 3’, xylQ R 5’ CTATGCATTCTGGCAGCAGGCAAGAAGAACCTC 3’, xylM F 5’ GATGCC TTCGCT TTG GT3’, xylM R 5’ GATGCC TTCGCT TTG GT 3’. Plasmid DNA templates of Alcaligenes faecalis, Alcaligenes sp. DN25, Bacillus methilotrophicus 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. methilotrophicus 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 amplicillin resistance 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. Even though 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. methilotrophicus (plasmid 4) into 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...
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 haboured _xylQ_ gene in their plasmids.

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 aromatica vir_._  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 between _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 encodes 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
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, pWW53 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-toluate, 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 methylotrphicus* 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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