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Evidence of Indigenous NAH Plasmid of Naphthalene Degrading *Pseudomonas putida* PpG7 Strain Implicated in Limonin Degradation

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A well characterized naphthalene-degrading strain, *Pseudomonas putida* PpG7 was observed to utilize limonin, a highly-oxygenated triterpenoid compound as a sole source of carbon and energy. Limonin concentrations evidenced a 64% reduction over 48 h of growth in batch cultures. Attempts were made to acquire a plasmid-less derivative via various methods (*viz.* Ethidium Bromide, SDS, elevated temperature & mitomycin C), among which the method involving mitomycin C (20 μg/ml) proved successful. Concomitant with the loss of plasmid in *P. putida* PpG7 strain, the cured derivative was identified as a lim$^-$ phenotype. The lim$^+$ phenotype could be conjugally transferred to the cured derivative. Based on the results of curing with mitomycin C, conjugation studies and presence of *ndo* gene encoding naphthalene 1,2 dioxygenase, it was demonstrated that genes for the limonin utilization were encoded on an 83 kb indigenous transmissible Inc. P9 NAH plasmid in *Pseudomonas putida Pp*G7 strain.

Keywords: *Pseudomonas putida* PpG7, plasmid curing, NAH plasmid

The phenomenon of delayed bitterness in certain citrus fruit juices continues to represent an important economic impediment for the citrus industry worldwide, especially with regard to achieving consumer acceptability, of either processed or fresh products. Delayed bitterness has been attributed primarily to the formation of limonin, which is a highly oxygenated triterpene derivative (composed of a furan ring and an epoxide group) and belongs to a class of limonoids. The application of microorganisms as a tool for the conversion of bitter citrus juice to a non-bitter product relies on the existence of enzymes or whole bacterial cells capable of limonin metabolism. The successful use of microorganisms relies on the development of better insight into mechanisms adapted to limonin degradation. A few microorganisms assessed with regard to limonin degradation include *Arthrobacter globiformis*, (Hasegawa *et al.*, 1983), *Pseudomonas* 321-18 (Hasegawa *et al.*, 1974), *Acinetobacter* sp. (Vaks and Lifshitz, 1981), *Corynebacterium facians* (Hasegawa *et al.*, 1985) and *Rhodococcus fascians* (Martinez-Madrid *et al.*, 1989). Nevertheless no genetic characterization of the response of microorganisms to limonin has yet been conducted.

In view of the above we initiated studies of limonin metabolism via the exploration of limonin degradation ability in several strains of *Pseudomonas*, as the metabolic diversity of *Pseudomonas* has been thoroughly documented (Martinez-Madrid *et al.*, 1989). Here, we report for the first time, the ability of a well-characterized strain of *Pseudomonas putida* known as *Pp*G7 to utilize limonin as a sole carbon and energy source. Thus far this species has been shown repeatedly to carry out naphthalene oxidation via plasmid encoded naphthalene degradative pathway (Dunn and Gunsalus, 1973; Yen and Serdar, 1988) and also involved in degradation of other polycyclic aromatic hydrocarbons (PAHs) (Sanseverino, 1993). In this communication we describe our experiment designed to determine whether the indigenous NAH plasmid plays any role in limonin degradation.

Materials and Methods

Microorganism and culture conditions

Freeze dried culture of *Pseudomonas putida* PpG7 (DSM No. 4476) were obtained from Microbial Type Culture Collection and Gene Bank (MTCC1072), Institute of Microbial Technology (IMTECH), Chandigarh, India. The complete medium used for *Pseudomonas*

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putida was Luria-Bertani Agar/Broth and limonin (Sigma, USA) or Naphthalene (Sigma, USA) used as carbon source in the minimal mineral medium 442 (Dunn and Gunsalus, 1973). All incubation was conducted at 30°C.

Growth of Pseudomonas putida PpG7 strain in limonin

Growth curve of *Pseudomonas putida* PpG7 was conducted with limonin (300 parts per million) as sole carbon source over time. *P. putida* was inoculated from log phase pregrown culture to minimal mineral medium containing limonin. Cultures were incubated at 30°C with agitation. Appropriate volumes of the culture were aseptically withdrawn from each set after every 4 h interval. The culture was then centrifuged for 2 min at 10,000 × g. The pellet was then re-suspended in 1 ml of saline and the optical density was recorded at a wavelength of 600 nm.

Residual limonin estimation

Limonin content was spectrophotometrically determined by a modified version of a previously described method (Vaks and Lifshitz, 1981). Aliquots (1 ml) of the culture were collected after regular growth intervals and centrifuged to create a cell-free extract. The 1 ml supernatant taken for estimation was extracted using 1 ml chloroform. The chloroform layer was separated out and kept overnight for drying. 500 µl chloroform was added to the beaker to dissolve limonin and 2.5 ml of Ehrlich reagent was prepared by mixing 0.1 g p-diamino benzaldehyde (Merck, Germany) to 3.0 ml acetic acid in 2.4 ml perchloric acid. The sample was then incubated for 30 min at room temperature and optical density was determined at 503 nm with appropriate controls.

Plasmid DNA extraction and molecular weight determination

The method described by Kado and Liu (1981) was modified for the extraction of indigenous plasmid of *P. putida.* Log-phase bacterial cells were washed in saline (0.85%) and centrifuged for 5 min at 10,000 × g. The cell pellet was resuspended in 100 µl E buffer (50 mM Tris acetate; pH 8.0). 200 µl of lysis buffer (3% SDS, 50 mM Tris; pH 12.0 using 0.1 M NaOH) mixed via gentle inversion. The mixture was incubated at 65°C for 30 min for complete lysis of cells. The crude lysate was then cooled at 4°C. 30 µl of phenol : chloroform : isoamyl alcohol was added to the lysates at a ratio of 24 : 24 : 1 and the tubes were inverted for mixing. Centrifugation was conducted for 25 min at 10,000 × g and 4°C. The supernatant was transferred into another fresh microfuge tube using a truncated tip. Sample (25 µl) was then mixed with 5 µl of loading dye, and subjected to 0.8% agarose gel electrophoresis for 3 h at 50 volts.

Aliquots of plasmid DNA were digested overnight at 37°C with ten units of various restriction enzymes (*EcoR*I, *Sma*I, Promega, USA). The fragments thus generated were then fractionated via electrophoreses on 0.7% agarose gel. The bands were then visualized under UV light, and digitized with the Mol Match program for calculations of molecular mass.

Plasmid curing

Plasmid curing was conducted in accordance with previously described method (Rheinwald *et al.*, 1973). Cells of *P. putida* (10^4 to 10^5 cfu/ml) from an overnight culture were inoculated in several tubes containing 5 ml of Luria broth and mitomycin C (Sigma, USA) in 5 µg increments at concentrations ranging from 5 to 25 µg/ml. Cultures were shaken at 30°C until growth became visible, as evidenced by turbidity. Aliquots from the highest concentration of mitomycin C that still allowed bacterial growth were then serially diluted and spread onto nutrient agar plates. Individual colonies were then tested on solid media for growth via replica plating on minimal media exposed to naphthalene vapors and on minimal media containing limonin (300 ppm). Colonies that proved unable to utilize naphthalene as sole carbon and energy source were tested for the presence of indigenous plasmid and those proved negative were further evaluated with regard to limonin degradation. The stability of the cured strains was monitored via the periodic cycling of cells under nonselective conditions and retesting for the ability to grow on naphthalene.

Calculation of curing frequencies

The frequency with which indigenous NAH7 plasmid of *Pseudomonas* was cured i.e. lost, as a result of mitomycin treatment, was calculated as described previously (Stephens and Dalton, 1987).

Amplification of gene encoding naphthalene 1, 2 dioxygenase located on the NAH7 plasmid

PCR was conducted to determine the presence of a catabolic gene *(ndo),* which is one of the genes responsible for Naphthalene degradation in *P. putida* G7. Primers *ndo* F; 5'-CACTCATGATAGCCTTGATTCCT GCCCCCGGCG-3' and *ndo* R; 5'-CCGTCCCACAA CACACCCATGCCGCTGCCG-3'were used, corresponding to positions 662 to 1663 on *P. putida.* Final concentrations of the reaction mix were 100 mM Tris–HCl (pH 8.0), 800 mM dNTP, 2.5 mM $MgCl_2$, and 2 U Taq polymerase (Amersham), reaction temperatures and times were arranged as follows: 96°C for 2min, 30 cycles at 94°C for 1 min, 55°C for 1 min. 72°C for 1 min and one cycle at 72°C for 7 min.

Generation of spontaneous mutants and conjugation by membrane filter mating

Prior to the conjugal mating between the wild type *P. putida* (donor) and the cured strain of *P. putida* (recipient), spontaneous mutants of wild type and cured *P. putida* were generated for resistance against antibiotic streptomycin (250 µg/ml) and rifampicin (100 µg/ml) respectively in order to generate selectable markers. Freshly prepared donor and recipient cultures were added to 5 ml Luria Bertani (LB) broth in tubes and incubated in a shaker (200 rpm) for 18 h at 30°C. Donor (3 ml) and recipient (2 ml) were mixed on a sterile petri dish and 3 ml were passed through sterile filter assembly with a 0.45 µm pore size membrane filter (Sartorius, Germany). The membrane was then placed onto the surface of Luria agar, and incubated for 24-48 h at 30°C. Mating was disrupted via vigorous shaking in 5 ml of sterile normal saline and serially diluted (10^{-2} to 10^{-8}). Each of the dilutions (0.1 ml) was spread into double antibiotic streptomycin-rifampicin containing Luria agar selective for transconjugants and recipients. The frequency of conjugation was then calculated as number of transconjugants divided by the number of recipient multiplied by the dilution factor. Simultaneously controls for donor and recipient were conducted in order to determine any spontaneous mutants.

Screening of transconjugants

The transconjugants on the Luria agar plates containing the antibiotics streptomycin and rifampicin were then replica plated on minimal media exposed to naphthalene vapors as well as on minimal media containing limonin. The plates were incubated at 30°C. Appropriate controls were utilized. The colonies evidenced growth both on limonin and naphthalene. Some colonies which proved unable to grow on limonin and naphthalene were evaluated for the presence of plasmids.

Results

Limonin utilization pattern by P. putida PpG7

A time course study was conducted to determine the rate of limonin utilization by *P. putida*. Thus, the growth of *P. putida* with limonin as sole carbon source and residual limonin levels in the media were monitored, as is shown in Fig. 1. A significant reduction of limonin content, 64% occurred in 48 h, and the residual limonin was shown to be only 36%. We also noted a proportionate reduction in the limonin content, occurring concomitantly with cell growth.

Interrelationship between optical density and viable cell numbers (biomass) of P. putida PpG7

In order to determine whether the structural gene encoding for enzymes implicated in limonin degradation is encoded for solely by the plasmid, it was essential to cure the wild-type strain of its indigenous NAH plasmid. *P. putida* samples were taken at different stages of growth and after the OD_{600} (LB-grown cultures) was recorded, a serial dilution was prepared and plated out, in duplicate, on LB agar. The number of viable cells was determined to be directly proportional to OD but only up to an OD of 1.0. Cultures with an OD values excess of 1.0 were therefore diluted with the same medium until they evidenced an OD below this threshold. An OD_{600} of 1.0 was determined to be equivalent 5.2×10^8 cells per ml.

Survival curve

An inoculum of *P. putida* (1.2×10^5 cells) was inoculated in LB-containing mitomycin C at different concentrations as well as LB without mitomycin C as a control. For each mitomycin C concentration, two tubes were prepared; one was inoculated with the

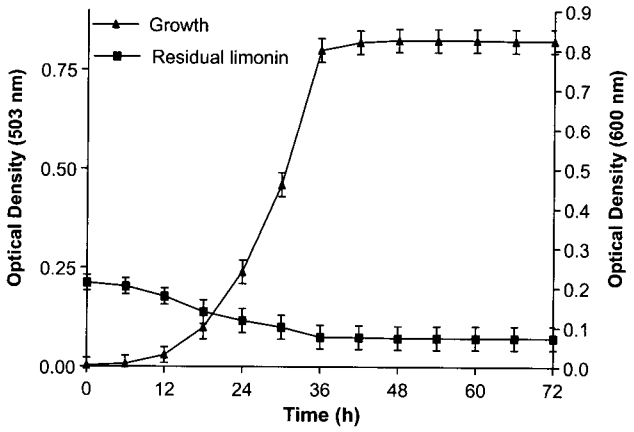

Fig. 1. Growth and limonin degradation profile of *Pseudomonas putida* PpG7.

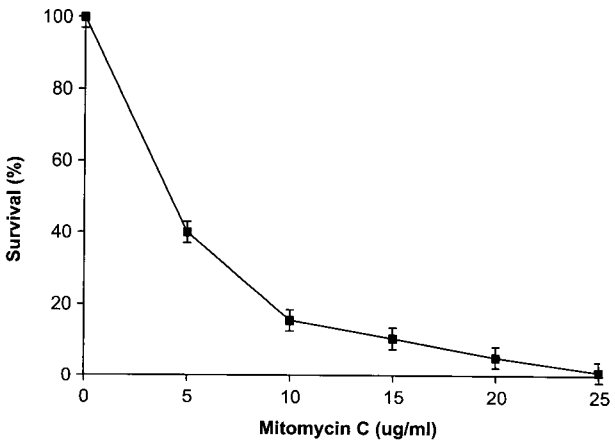

Fig. 2. Survival curve of *Pseudomonas putida* G7 as function of mitomycin C.

Table 1. Profile of four cured mutants

Putative cured derivatives	Growth on naphthalene (After 24 h)	Presence of *ndo* gene	Growth in limonin	Plasmid
PpC1	–	–	–	visible
PpC2	–	+	–	not visible
PpC3	–	–	–	not visible
PpC4	–	+	–	not visible

organism and the other was employed as a blank during the course of the experiment. After inoculation, the cultures were incubated with shaking at 30°C and OD_{600} values were intermittently determined. The number of viable cells was plotted as a function of mitomycin C (Fig. 2). A killing rate of 94.7% (±1.2, n=6) was observed in the presence of 20 ug/ml mitomycin C. Such a rate was considered sufficiently high for screening the remaining viable cells (5.3%) for plasmid loss. One ml of this culture was harvested, washed in 50 mM phosphate buffer pH 7.4, and resuspended in the same buffer to yield an OD_{600} of 1.0. A serial dilution was constructed in the same buffer and plated out on LB-agar plates then incubated overnight at 30°C. Among the resulting colonies, 1600 colonies were screened in terms of their ability to use naphthalene and limonin as sole carbon and energy sources. Among these, four colonies, viz. PpC1–PpC4, were selected. No growth was visible on naphthalene media after 24 h of incubation. However in two (PpC2, PpC4) colonies very faint growth was observed after 48 h. All the four suspected cured derivatives were further analyzed for plasmid loss. In only one of the four strains plasmid was visualized when resolved in agarose gel.

Molecular weight determination of plasmid

Aliquot of wild-type *P. putida* PpG7 indigenous plasmid were digested with two restriction enzymes *EcoR*I and *Sma*I in accordance with the recommended procedures. The resultant fragments were fractionated by agarose gel (0.7%) electrophoresis, as described in Materials and Methods. The fragments representing the genetic fingerprint of the plasmid were then fed into the Mol Match program for the determination of molecular weight, and the results indicated that the indigenous plasmid had a molecular mass in the region of approximately 83 kb (data not shown). This result is fully consistent with the size of NAH plasmid harbored by the previously reported PpG7 strain (Dunn and Gunsalus, 1973; Yen and Serdar, 1988).

Calculation of curing frequency

The data shown in table clearly demonstrate that out of four suspected cured derivatives (PpC1-PpC4); only one was a true cured strain (PpC3) as evidenced by utter loss of both plasmids, as well as its inability to grow on naphthalene as a sole carbon source. The other two cured strains viz. PpC2 and PpC4 appeared to have had the plasmid integrated into the genome, as evidenced by the absence of plasmid, but the very slow rate of growth on naphthalene. PpC1 was essentially a mutant, as the strain harbored the plasmid, but lost the ability to grow on naphthalene for one reason or another. According to these data and figures (Table. 1 and Fig. 2) a curing frequency with a mean of 4×10^{-4} i.e 1 per cell 1.6×10^{2}, (n=6), was calculated. Mitomycin C proved to be a very effective curing agent, as compared to other curing agents viz. EtBr, SDS, elevated temperature (data not shown).

Amplification of plasmid encoded ndo gene in P. putida PpG7

Total genomic DNA of all four suspected cured derivatives, as well as the DNA of the wild-type strain (control), were extracted and employed as templates for detection of of the *ndo* gene via PCR, using specific primers. Fig. 3 shows presence of bands approximately 640 bp in size in wild type and in other two of the four cured strains, viz. PpC2, PpC4. No bands were detected in PpC1 or PpC3. PpC3 was a true cured strain.

Verification of identity of the cured strain and confirmation of plasmid encoded limonin utilization

We then attempted to verify that cured strain was indeed a derivative of *P. putida* PpG7 and to confirm that the degradation of limonin is a plasmid-encoded function. The plasmid was transformed back to the cured strain via conjugation. The transconjugants obtained after screening (those growing on naphthalene) was once again monitored for the physical presence of plasmid as shown in Fig. 4. Also, it was determined to utilize limonin as a sole carbon and energy source.

Discussion

The diversity of complex carbon sources usable by *Pseudomonas* species led us to screen a number of known *Pseudomonas* species with regard to their ability

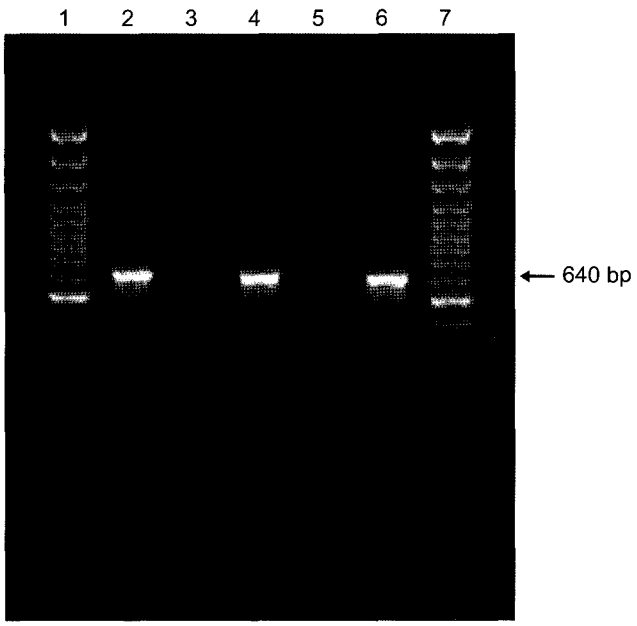

Fig. 3. Agarose (1.5%) gel of PCR products Lane 1, 100 bp ladder. Lane 2, presence of *ndo* gene encoding naphthalene 1, 2 dioxygenase (640 bp fragment) in wild-type PpG7. Lane 3, absence of *ndo* gene in PpC1 derivative. Lane 4, presence of *ndo* gene in PpC2 derivative. Lane 5, absence of *ndo* gene in PpC3 derivative. Lane 6, presence of *ndo* gene in PpC4 derivative. Lane 7, the 100 bp molecular weight marker.

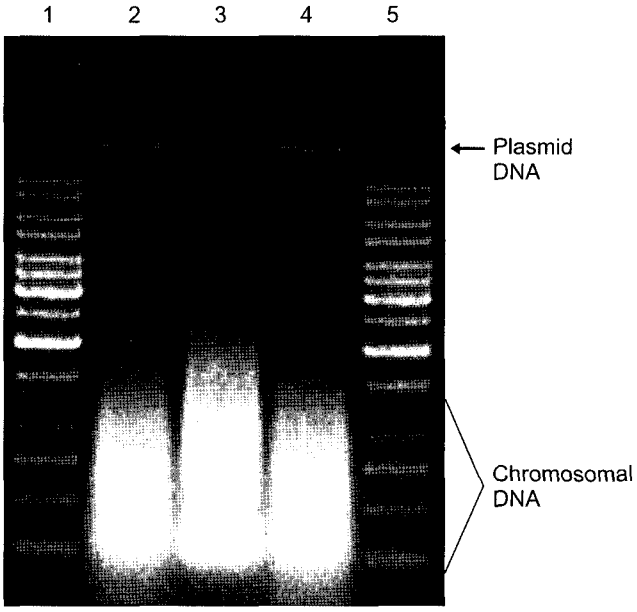

Fig. 4. Visualisation of plasmids in 0.7% agarose gel. Lanel, 1 kb molecular size marker. Lane 2, plasmid DNA of *Pseudomonas putida* G7 (wild-type) visualized by the method of Kado and Liu. Lane 3, DNA of cured strain absence of plasmid band. Lane 4, Plasmid DNA after conjugal transfer into cured derivative. Lane 5, 1 kb molecular size marker.

to utilize limonin as a sole carbon source. Among 14 strains of *Pseudomonas* thus far tested (data not shown) a well characterized strain of *Pseudomonas putida* PpG7 was determined to be the potential strain due to its ability to utilize limonin, a triterpenoid compound as sole source of carbon and energy. A significant reduction (64%) of limonin occurred at 48 h. Maximum reduction appears to occur during the exponential growth phase. However, as growth reaches saturation level and attains stationary phase we assume formation of other metabolites might render the cells unable to further utilize limonin (Furukawa and Chakrabarty, 1982; Chauhan and Jain, 2000; Thakur *et al.*, 2001). It was also previously reported (Williams and Murray, 1974; Chakrabarty, 1976) that plasmids harbored by the members of the genus *Pseudomonas* frequently harbor genetic information for the degradation of variety of organic compounds, thus contributing to the metabolic diversity of their hosts. Many of the exotic *Pseudomonas putida* phenotypes of predicated on plasmid encoded pathways which channelize substrates to metabolites that feed into central metabolic pathways (Timmis, 2001). Thus it became a focus of research to determine whether the indigenous NAH plasmid present in the strain PpG7 might be involved in limonin degradation. Loss of function at a rate higher than the expected rate of mutation and/or the ability to transfer a function to recipient cells are generally accepted properties that provide genetic evidence for the presence of a plasmid involved in conferring a particular function upon a cell. Also, curing of the indigenous plasmid from a bacterial strain is one effective by which the relationship between a genetic trait and the carriage of the specific trait within the plasmid can be substantiated (Trevors, 1986).

In a physical analysis and visualization of plasmid DNA conducted via a previously described method (Sanseverino *et al.*, 1993), a single large plasmid with a size 83 kb was determined to be present in this strain. Besides which, the test organism appeared to harbor no other small plasmid as several attempts with different isolation methods failed to reveal the presence of any other smaller plasmid. The size of the plasmid is also consistent with the earlier reports. It was clear that this plasmid belonged to the class of NAH plasmids and harbored genes that mediate naphthalene degradation pathway (Dunn and Gunsalus, 1973; Yen and Serdar, 1988). Phenotypes that have undergone a plasmid loss in *P. putida* PpG7 strain was easily screened principally on the basis of ability to grow on naphthalene (i.e. Nah^+/Nah^- phenotypes). As mentioned earlier the ability of this strain to utilize naphthalene as sole carbon and energy source is mediated by NAH plasmid. Attempts were made to acquire cured derivatives in order to determine the

role of the plasmid in degradation of limonin. The mutants which failed to grow in naphthalene medium (nah^-) were selected as this inability could be attributed to the loss of nah plasmid. Among variety of methods used for plasmid curing (viz. EtBr, SDS, and elevated temp.) success was only achieved when mitomycin C was used as curing agent (20 μg/ml). Mitomycin C owes its inhibitory activities to metabolic activation via reduction to a corresponding hydroquinone. The hydroquinone undergoes further changes, and generates a reactive intermediate, which can then react with the purine bases due to this type of nucleophilic attack; it inserts alkylating crosslinks between the two strands of double-stranded DNA. Any crosslink is sufficient to block transcription by RNA polymerase.

Concomitant with the loss of plasmid in the test organism *P. putida* PpG7 strain, all the cured derivatives were accessed for growth on limonin, cured derivatives no longer supported growth on limonin i.e. the true cured derivative turned out to be lim- phenotype. However loss of plasmid was encountered in three putative cured derivatives (PpC2, PpC3, PpC4) and nah^- trait was detected in only two of the four putative cured derivatives (PpC1, PpC3) (after 24 h of incubation time). Interestingly, out of these two, PpC1 evidenced the presence of plasmid whereas PpC3 did not. PpC1 appeared to have undergone mutation which may have resulted in the inactivation of the naphthalene catabolic gene. In case of PpC2, PpC4 plasmid remained undetected as they may have integrated into the genome of the test organism; consequently, the low copy number resulted in a reduced naphthalene (nah) catabolic activity. Another clear evidence for the identification of PpC3 as a true cured derivative resulted, when curing was verified in four putative cured derivatives via PCR analysis of total DNA. PCR products (640 bp) were detected in the wild-type as well as in three cured derivatives except for one, PpC3, in which the band corresponding *ndo* gene was absent.

Conjugation studies were conducted in an attempt to obtain Lim^+ phenotype. Transconjugants were obtained at a frequency of approximately 10×10^6 on the selection medium. The plasmid DNA isolated from these transconjugants was found to carry the same plasmid (approximately 83 kb), as was present in the wild type. Limonin degrading-ability was restored after plasmid was conjugally transferred into the cured strain. Therefore, on the basis of mutation, plasmid DNA isolation, conjugation studies and presence of *ndo* gene, it was clearly demonstrated that the genes for the limonin-degrading pathway are encoded on an indigenous NAH plasmid in the *Pseudomonas putida* PpG7 strain.

In conclusion, we report for the first time, the limonin utilization ability by the naphthalene-degrading *Pseudomonas putida* strain PpG7. However, whether direct linkage between plasmid-mediated catabolic genes implicated in naphthalene degradation with that of genes involved in limonin degradation has yet to be established. It appears that the highly oxygenated heterocyclic triterpenoid, limonin, due primarily to its structural resemblance with that of polycyclic aromatic hydrocarbons, may possess such metabolic potential. Moreover this report is of value in further resolving the as-yet-unknown plasmid mediated limonin degradation pathway.

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