Research Papers

Indian Journal of Agricultural Sciences **82** (10): 831–4, October 2012

β-Cyfluthrin biodegradation gene(s) associated in plasmid of *Pseudomonas stutzeri* (strain S1)

JITENDRA SINGH[1], DILEEP K SINGH[2], KIRAN VEER SANDHU[3], SUNIL KUMAR JHA[4] and MADHUBAN GOPAL[5]

Indian Agricultural Research Institute, New Delhi 110 012

Received: 4 February 2011; Revised accepted: 8 June 2012

ABSTRACT

Pseudomonas stutzeri strain S1 was found capable for biodegradation of β-cyfluthrin. Results indicated that the β-cyfluthrin degrading gene(s) of *P. stutzeri* strain S1 were located on a plasmid. This plasmid already had the gene(s) for nalidixic acid resistance. Another gene, Tn5 was inserted into the plasmid from *Escherichia coli* S17. The mutated *P. stutzeri* acquired resistance for nalidixic acid and kanamycin antibiotics and became incapable to degrade β-cyfluthrin. Probably insertion of Tn5 distressed the sequence of β-cyfluthrin degrading gene. This mutant was assayed to locate the β-cyfluthrin biodegrading gene. The mutant was able to transfer the Tn5 inserted plasmid in another strain of *E. coli* DH5α, which was confirmed by acquiring kanamycin resistance in *E. coli* (DH5α) and PCR analysis. Plasmid of the *P. stutzeri* S1 was eliminated using ethidium bromide along with heat shock treatment, and then this strain lost biodegradation activity of β-cyfluthrin. Thus, it has been validated that the biodegradation gene(s) were present on the plasmid. The plasmid was identified as a DNA circular molecule of 18.0 kb size.

Key words: β-cyfluthrin, Biodegradation, Plasmid DNA, *Pseudomonas stutzeri*

β-Cyfluthrin [α-cyano-4-fluoro-3-phenoxybenzyl-3-(2,2-dichlorovinyl)-2,2-dimethy-l-cyclopropane carboxylate] is a synthetic pyrethroid. The unique feature in this molecule over earlier compound cypermethrin is the presence of a carbon fluorine bond that helps in insect resistance management (Saikia *et al.* 2005). Application of synthetic pyrethroids has increased at the cost of older organochlorine pesticides used during last decade and at present β-cyfluthrin is one of the popular synthetic pyrethroids used in India. The environmental hazards due to this pyrethroid have also been identified in USA (Raloff 2006). It is evident, from the fact that the use of synthetic pyrethroids in the country has increased by 42 per cent during the last five years.

The presence of the toxic residues over maximum residue limit (MRL 0.1-0.5 mg/kg) of this insecticide and other pyrethroids in fruits, vegetables and green leaves has been reported (Codex Alimentarius Commission 2004, Frederick 2011). Therefore, it may have hazardous impact on environment, human and animal health. There is a need to evolve methods for improving the level of degradation and detoxification of the pesticides to render it harmless so that those consuming contaminated food commodities are not harmed and also to render it less deleterious to the environment.

Several reports of biodegradation of pyrethroids by microorganisms are available (Grant and Betts 2004, Saikia and Gopal 2004). The involvement of *P. stutzeri* in biodegradation of β-cyfluthrin has been reported (Saikia *et al*. 2005). Therefore, there is a need to explore various approaches including the genetics and molecular biology of *P. stutzeri* to study the biodegradation pathways involved in the breakdown of β-cyfluthrin. There is also need to identify gene(s) and confirm the products formed as metabolites and study their regulation in associated pathways prior to exploitation of microbial biodegradation for detoxification of food commodities from the residues of environmentally deleterious health pollutants.

[1] Senior Scientist (e mail: drjsento@gmail.com), Central Institute of Post Harvest Engineering and Technology, Abohar 152 116, Punjab;

[2] Associate Professor (e mail: dileepksingh@gmail.com), Department of Zoology, University of Delhi, Delhi 110 007;

[3] Ph D Student (e mail: kv.sandhu7@gmail.com), Otto von Guericke University of Magdeburg, Germany;

[4] Assistant Professor (e mail: sunil_env@yahoo.co.in), Department of Biotechnology, Ranchi University, Ranchi 834 002, Jharkhand;

[5] National Fellow and Principal Scientist (e mail: madhubangopal@gmail.com), Division of Agricultural Chemicals

MATERIALS AND METHODS

Present study was carried out at Division of Agricultural Chemicals, IARI, New Delhi from 2005 to 2008. *P. stutzeri* was isolated from a β-cyfluthrin contaminated soil and purified under aseptic conditions. Biparental cross between *P. stutzeri* S1 and *E. coli* S17 strains was carried out to transfer Tn5 from *E. coli* S17 (donor) to *P. stutzeri* S1 (recipient). Ten µl broth cultures of donor and recipient were mixed on TY media plates and incubated overnight at 30°C. The growth was scrapped and suspended in isotonic buffer (pH 7). Then 0.1 ml mixture was spread evenly on nutrient agar media plates, supplemented with nalidixic acid (25 µg/ml) and kanamycin (50 µg/ml) antibiotics, as *E. coli* S17 and *P. stutzeri* S1 strains were sensitive to nalidixic acid and kanamycin, respectively. Similarly, *E. coli* S17 and *P. stutzeri* S1 strains are resistant with kanamycin and nalidixic acid, respectively. Therefore, only exconjugants (mutants) resistant to both kanamycin and nalidixic acid would grow and survive. Each purified exconjugants was grown on similar medium and then replica plated to minimal salt medium supplemented with β-cyfluthrin as the sole carbon source. The exconjugants showing negligible growth were supposed as mutants deficient for β-cyfluthrin utilization, which were further assayed.

P. stutzeri strain S1 was found resistant to 50 µg/ml chloramphenicol, 25 µg/ml nalidixic acid and 50 µg/ml ampicillin. Similarly, *E. coli* strain S17 is kanamycin resistant and harbours the plasmid pSUP5011 and *E. coli* DH5α is nalidixic resistant. *E. coli* DH5α was developed for rifampicin resistance by selection of spontaneously mutated cells, from its fully grown broth culture. For screening spontaneously mutated cells, TY-medium supplemented with 50 µg/ml rifampicin was used and about 6-8 colonies resistant to rifampicin were identified. More than one thousand mutants, derivative of *P. stutzeri* S1 were tested for the degradation of β-cyfluthrin. Only four mutants out of them, numbering 22, 303, 554 and 638 were found lacking in the ability for biodegradation of β-cyfluthrin. These were also resistant to nalidixic acid and kanamycin due to insertion of Tn5.

Table 1 Bacterial strains and plasmids used in the experiment

Bacteria	Strains	Genotype	Reference
P. stutzeri	S1	Chl^R, Nal^R, Amp^R	Laboratory isolate
E. coli.	S 17	Km^R, Str^R, has a suicidal plasmid	Simon (1984)
	DH5α	Nal^R, Tet^R,	Laboratory collection
	DH5α-1	Nal^R, Rif^R, Tet^R	Spontaneously mutated isolation in the laboratory
P. stutzeri S1 mutants derivatives	22, 303, 554 and 638	Chl^R, Nal^R, Amp^R, Km^R	Laboratory isolates
Plasmid			
pSUP5011		Km^R, Tet^R	Simon (1984)
pJDG-1		Nal^R, Km^R	Identified in laboratory

Culture media were prepared separately in double distilled water as per standardized procedure. Media were sterilized by autoclaving at 1.05 kg/cm^2 pressure and 121°C temperature for 20 min and cooled down to about 48°C. Approximate 25 ml culture medium was poured in pre-sterilized glass Petri-plates and allowed to solidify. Luria agar and luria broth medium were used for *E. coli,* and similarly nutrient agar and nutrient broth medium were used for *P. stutzeri* multiplication. Nutrient agar medium supplemented with kanamycin (50 µg/ml) and nalidixic acid (25 µg/ml) antibiotics was used for the screening of Tn5 exconjugants and preparation of minimal salt medium and standard procedure was followed (Saikia *et al.* 2005) to provide β-cyfluthrin (50 µg/ml) as sole carbon source to the mutants in minimal medium.

The elimination of the plasmid from *P. stutzeri* S1 (wild) was achieved in nutrient broth medium by combined treatment of ethidium bromide (EtBr, 500 µg/ml) and heat shock (42°C) for 4 hr at log phase of culture. Then contents were incubated for 24 hr at 30°C and similar procedure was followed without application of EtBr and heat shock in another culture for comparative studies of plasmid elimination efficiency. The combined treated culture was washed, serially diluted (10^6) and cultured on nutrient agar media plates for 24 hr at 30°C. The colonies were first replica plated on the same medium supplemented with chloramphenicol, nalidixic acid and ampicillin to isolate plasmid eliminated colonies, and second replica plated on minimal medium supplemented with β-cyfluthrin only. Some colonies missed to grow in presence of chloramphenicol, nalidixic acid and ampicillin and also on minimal media. Therefore, plasmid eliminated colonies were identified and taken from the mother plate and assayed to observe the presence of plasmid, using plasmid DNA isolation protocol. No DNA was found after complete electrophoresis. Thus above antibiotics resistant genes were located in plasmid(s). Similarly without any antibiotic treatment, growth of plasmid cured and normal cultures were compared in minimal medium (broth) supplemented with β-cyfluthrin as the sole carbon source. Then growth was found only in normal culture.

Similar procedure was followed for the mutants 22, 303, 554 and 638. The heat-shocked broth cultures were spread on complete agar medium supplemented with chloramphenicol, kanamycin, nalidixic acid and ampicillin. Resistance and sensitivity to these antibiotics were recorded. Plasmid cured and normal colonies were grown separately in nutrient broth medium and plasmid DNA was isolated from these by the alkali lysis method. The physical loss of plasmid in the cured derivatives was confirmed by agarose gel electrophoresis of the plasmid DNA after preparation of respective

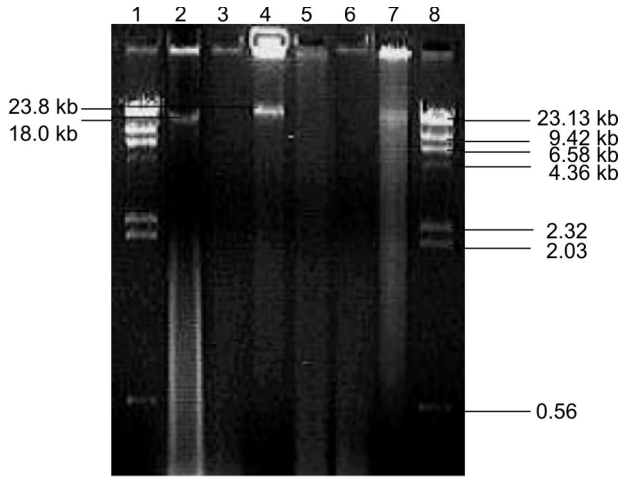

Fig 1 Gel showing the plasmid DNA with the β-cyfluthrin degrading gene(s); lane 1-8; 1 and 8-Hind III λ-DNA molecular ladder, 2 – plasmid of *P. stutzeri* S1 and 3- plasmid cured *P. stutzeri*, 4-plasmid of mutant 303, 5-plasmid cured mutant, 6-*E. coli* DH5α Rif^R, 7-*E. coli* DH5A Rif^R Km^R exconjugant with plasmid of mutant 303

cultures (Fig 1). λ Hind III molecular marker ladder was used for determination of the molecular weight of plasmid.

DH5α rifampicin resistant strain of *E. coli*, previously developed was mated with the mutant 303, on a nutrient agar medium. A visible patch was formed after 24 hr of incubation. The growth was scraped and serial dilutions were made and these were spread on nutrient agar medium supplemented with rifampicin and kanamycin for selection of the exconjugants. The recipient count was determined on nutrient agar medium supplemented with 50 µg/ml rifampicin. The percentage of the recipient acquiring the kanamycin resistance was determined to represent the plasmid transfer frequency.

Minimal medium with β-cyfluthrin as the sole carbon source was inoculated with wild (normal) and mutant strains separately and incubated at 30°C with aeration. Optical densities of the each sample were recorded at different time intervals of growth (8, 16, 24, 32, 40 and 48 hr) at 540 nm. The values of OD were plotted against the period of incubation and the growth curve of parental and mutant strains was obtained (Fig 2).

PCR amplification of inserted Tn5 was carried out using an iCycler (Bio-Rad) PCR instrument. The reaction mixtures containing, 5 µl template plasmid DNA, 0.2 µM each deoxynucleotide triphosphate, 0.5 units of *Taq* DNA polymerase (Fermentas) 1 µl of 10 pM of both forward (5'TAGACTGGGCGGTTTTATGGACAG 3') and reverse (5'AACTCCGCGAGGTCGTCCAGCCTC3') primers, 2.5 µl of 25 mM $MgCl_2$ and 2.5 µl of 10x *Taq* buffer were added and final volume was adjusted to 25 µl. They were processed through a 30-cycle programme consisting of a 4 min at 94°C and 1 min denaturation period at 94°C, 1 min annealing period at 64°C, 1.20 min elongation period at 72°C and a final elongation for 10 min. PCR products were electrophoresed on 1% agrose gels and stained with EtBr.

RESULTS AND DISCUSSION

Growth studies of the strains

The growth of mutant 303 and the parental strain were compared on minimal medium with β-cyfluthrin as the sole carbon source. The parental strain entered the log phase after 8 hr of incubation and reached the stationary phase in 40 hr. The mutant, however, registered almost nil growth (Fig 2). This is obvious and well known phenomenon when the transposons are inserted in a gene sequence, disruption in gene sequence and dysfunction or inactiveness may occurr. Therefore, such a gene(s) is involved in the biodegradation of β-cyfluthrin, will lack the ability to degrade β-cyfluthrin molecule and could not use as carbon source.

Plasmid elimination studies

Ethidium bromide was found more efficient in the elimination of the plasmid since 75 per cent of the cells lost the plasmid when put in its contact along with heat shock, whereas the loss was only 4 percent in the only heat shocked bacterial cells. The elimination of the plasmid led to loss of its ability for degrading β-cyfluthrin and also loss of nalidixic acid resistance in the S1 strain. The physical loss of plasmid in the cured derivatives was confirmed by agarose gel electrophoresis of the plasmid DNA after preparation of respective cultures (Fig 1). The colonies, which were lacking the ability to degrade β-cyfluthrin, were also nalidixic acid sensitive and *vice versa*. The genes for nalidixic acid resistance and biodegradation of β-cyfluthrin were thus present on a plasmid, hereafter referred to pJDG-1.

Plasmid elimination studies of mutant 303 lead to similar observation since the kanamycin sensitive colonies produced by plasmid elimination were also simultaneously defective

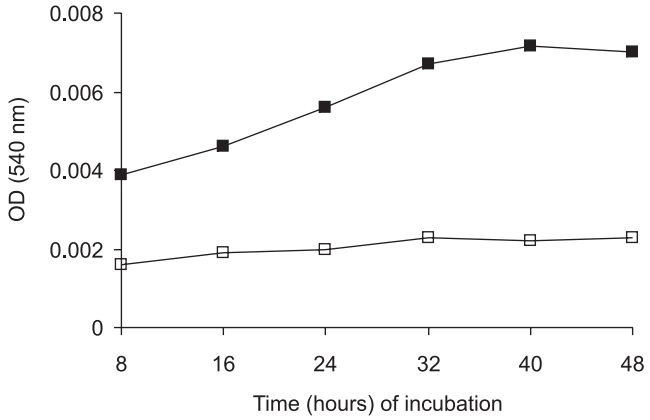

Fig 2 Growth of *P. stutzeri* strain S1 and its mutant 303 derivative on medium with β-cyfluthrin as the sole carbon source (–■– *P. stutzeri*, –□– mutant)

in β-cyfluthrin biodegradation, and nalidixic acid sensitive. The observations were consistent with others. Shahid and Malik (2004) reported the loss of antibiotic resistance in *Pseudomonas aeruginosa* after plasmid curing. Plasmids having several of genes involved in mobilization and determination of fertility controlling cell structure and genes determining metal tolerance supported these observations. Our observations are thus consistent with earlier reports (Williams 2004, Jorge *et al.* 2006).

Plasmid transfer studies

Bacterial biparental cross studies indicated the transfer of only kanamycin resistance from the mutant 303 to *E. coli* DH5α Rif^R strain. The transfer frequency of the plasmid to the recipient was 38 per cent. The observations, along with plasmid elimination and the demonstration of the presence of Tn5 on the plasmid using suitable primers in PCR amplification studies, have indicated the presence 1.2 kb of neomycin/kanamycin resistance gene on the plasmid of mutated *P. stutzeri* S1, *E. coli* S17 and *E. coli* DH5α Km^R (Fig 3). Since the kanamycin resistance is due to Tn5 insertion, which is responsible for the mutant phenotype (Simon 1984, Das *et al*. 2006) it can thus be concluded that the gene(s) for β-cyfluthrin degradation are present on the plasmid pJDG-1. It is a well known fact that *Pseudomonas* spp. carries large number of plasmids with genes possessing variety of functions to degrade complex molecules such as naphthalene (Jorge *et al.* 2006). Plasmids have also been reported to carry the genes for dimethoate degradation by *P. aeroginosa* and earlier reports also supported this fact. Although not much information on the number and nature of plasmid of *P. stutzeri* is currently available. However, we report here of a mobilizable plasmid pJDG-1 of 18.0 kb size of *P. stutzeri* and its mutant carrying Tn5 insertion with total 23.8 kb size of plasmid (Fig 1). Varieties of genes have been reported to be carried by plasmids and due to their wide mobility across microbes are responsible for the magnification of diversity of the microbes over a short time period.

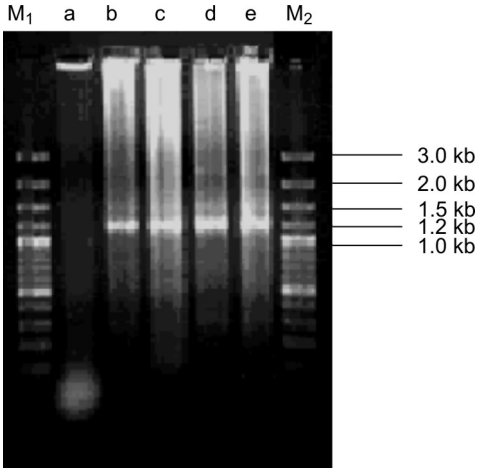

Fig 3 Agarose gel showing the amplification of neomycin resistant insert with the Tn5 in plasmid (pJDG-1) of bacterial strains *Pseudomonas stutzeri* S1, wild type a, mutants; b 22, c 303, *Escherichia coli* S17 and DH5α (Km^r) are in d and e respectively.

ACKNOWLEDGEMENTS

This work was financially supported by Council of Scientific and Industrial Research, New Delhi, India, under a pool scheme for Scientists, Grant No. 13(7984-A)/2005 pool, which is heartily acknowledged. The assistance provided by Mr. Aneet Pal Singh and Mr. Vipul Puri, undergraduate students of Delhi University for conducting the experiments during early period are also acknowledged.

REFERENCES

Codex Alimentarius Commission. 2004. Joint FAO/WHO food standards programme draft and proposed draft maximum residue limits in foods and feeds CX/PR04/5.157 Part A; Thirty-sixth Session, N. Delhi, pp 19–24.

Das S K, Gautam U S, Chakrabartty P K and Singh A. 2006. Characterization of a symbiotically defective serine auxotroph of *Mesorhizobium ciceri*. *FEMS Microbiology Letters* **263**(2): 244–51.

Frederick A A. 2011. Assessment of pesticide residues in vegetables at the farm gate: Cabbage (*Brassica oleracea*) cultivation in Cape Coast, Ghana. *Research Journal of Environmental Toxicology* **5**: 180–202.

Grant R J and Betts W B. 2004. Mineral and carbon usage of two synthetic pyrethroid degrading bacterial isolates. *Journal of Applied Microbiology* **97**: 656–62.

Jorge L, Antoni B, Rafael B, Elena G V, and Norberto J P. 2006. Biology of *Pseudomonas stutzeri* 68, 400–405. *Microbiology and Molecular Biology Reviews.* **70**(2): 510–47.

Klemba M, Jakobs B, Wittich R M and Pieper D. 2000. Chromosomal integration of tcb chlorocatechol degradation pathway genes as a means of expanding the growth substrate range of bacteria to include haloaromatics. *Applied Environmental Microbiology*. **66**: 3 255–61.

Raloff J. 2006. A little less green studies challenge the benign image of pyrethroid insecticides. *Science News,* Feb 4.

Saikia N and Gopal M. 2004. Biodegradation of β-cyfluthrin by fungi. *Journal of Agriculture and Food Chemistry* **52**: 1 220–3.

Saikia N, Das S K, Patel B K, Niwas R, Singh A and Gopal M. 2005. Biodegradation of beta-cyfluthrin by *Pseudomonas stutzeri* strain S1. *Biodegradation* **16**: 581–9.

Shahid M and Malik A. 2004. Plasmid mediated amikacin resistance in clinical isolates of *Pseudomonas aeruginosa. Indian Journal of Medical Microbiology* **22**: 182–4.

Simon R. 1984. High frequency mobilization of gram-negative bacterial replicons by the in vitro constructed Tn5-Mob transposon. *Molecular General Genetics* **196**: 413–20.

Sinha S and Gopal M. 2002. Evaluating the safety of beta-cyfluthrin insecticide for usage in eggplant (*Solanum melongena* L.) crop. *Bulletin of Environnemental Contamination and Toxicology* **68**: 400–5.

Williams P A, Jones R M and Zylstra G J. 2004. Genomics of catabolic plasmids, (*In*) *Pseudomonas*, vol. 1. J L Ramos (ed.), Kluwer Academic/Plenum Publishers, New York, NY, pp 165–96.