Biotech Health Sci. 5(1):e45306. doi: 10.5812/bhs.45306.

Published online 2017 March 8. **Research Article**

Biodegradation of Endosulfan as an Organochlorine Pesticide with *Pseudomonas plecoglocissida* Transfected by *LinA* Gene

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Received 2017 January 02; **Revised** 2017 January 12; **Accepted** 2017 January 16.

Abstract

Background: Organochlorine pesticides (OC) are applied to soil and agricultural farms and can contaminate sewerage and ditches, which in turn may be transported to rivers, and consequently distributed in the environment and as a pollutant that endangers human health.
Objectives: This study aimed at constructing a genetically engineered *Pseudomonas plecoglossicida* strain enabled to degrade the OC pesticide, endosulfan.
Methods: Transfected *Pseudomonas plecoglossicida* strain was initially made in the following steps, first polymerase chain reaction (PCR) product of gene *LinA* from *Sphingomonas paucimobilis* UT26 was transferred to the pUC57 plasmid, and the plasmid was then transfected to *P. plecoglossicida*.
Results: In endosulfan supplemented carbon-deficient minimal medium, the isolate showed its ability to hydrolyze endosulfan to endosulfan sulphate. The isolate was observed to grow on endosulfan as the carbon source. Thus, the strain was capable of degrading the OC pesticides and utilizing products of their degradation as nutrients.
Conclusions: Ability of transfected *Pseudomonas plecoglossicida* strain in biodegradation of chemicals makes it an excellent candidate for bioremediation of contaminated agricultural and industrial sites.

Keywords: Organochlorine, Biodegradation, *Pseudomonas plecoglossicida*

1. Background

Organochlorine (OC) pesticides are broadly used as pest control in developing countries. Organochlorine degrading gene (linA) encodes hexachlorocyclohexane dihydrochlorinase from the hexachlorocyclohexane-degrading bacteria Sphingomonas paucimobilis UT26, which has the ability to catalyze the conversion of hexachlorocyclohexane (-HCH) to 1,2,4-tri chloro benzene (1,2,4-TCB) via -1,3,4,5,6-penta chlorocyclohexane (-PCCH) (1, 2).

Today, OC pesticides are widely used in developing countries (1) and tremendous development of the chemical industry in the past century has made possible the present quality of life, yet, as a side effect, large amounts of synthetic chemicals have been released to the environment either intentionally (as waste deposits, fertilizers or pesticides) or accidentally. Therefore, today a substantial number of earth-bound and marine habitats are contaminated by various xenobiotic compounds, many of which are harmful to living organisms.

Hexachlorocyclohexane (HCH) among these synthetic chemicals effects the nervous system, liver, and kidneys and accumulates in biological tissues (3). Bacterial enzymatic biodegradation has attracted considerable interest as a strategy to economically and effectively degrade pesticides. Occupational exposure in agricultural industries and self-poisoning with pesticide compounds causes significant health problems (4-7).

Previous studies have shown that *Pseudomonas* species are interesting microorganisms for microbial remediation and degradation (8-10) and within this genus, *Pseudomonas plecoglossicida*, which is a soil habitant has attracted much interest (11).

2. Objectives

This experimental study aimed at constructing a *LinA* transfected *Pseudomonas plecoglossicida* strain capable of degradation of the organochlorine pesticide, endosulfan.

3. Methods

Chemicals, enzymes, and oligonucleotides: all chemical reagents including endosulfan were obtained from

Sigma-Aldrich (Tehran, Iran). Proteinase K was prepared by Roche (Tehran, Iran) and dNTPs by Takapou Zist (Tehran, Iran).

Media for bacterial growth: carbon-deficient minimal medium (CSM) and Luria-Bertani Medium (LB) were prepared as described previously (OD 600 nm) (12). Immediately before inoculation, CSM was supplemented with different concentration (100, 200, 300, and 400 μg/ML) of endosulfan.

Evaluation of degradation effect of transfected *P. plecoglossicida* on endosulfan: To assess the biodegradation effect of transfected *P. plecoglossicida* on endosulfan, the bacterial suspension was spread on CSM agar plates supplemented with increasing concentrations of organochlorine pesticide. Endosulfan concentrations in agar were 100, 200 and 400 μg/ML. The plates were incubated at 37°C for 20 hours.

Degradation of organochlorine pesticide by resting cells: Degradation experiment of organ chlorine pesticide was performed as previously described by Shivaramaiah et al. (13, 14). Decomposition of organochlorine pesticides was monitored by high performance liquid chromatography conditions as described previously (15). Briefly 25 $\times$ 14 mm C18 Hichrom column with 25 $\times$ 14 mm dimension was used; Mobile phase: Pump A: 99% water and 1% phosphoric acid (pH2.0), and Pump B: acetonitril 100%. The gradient was applied with the following condition: 0 to 3 minutes of 60% acetonitrile, 3 to 16 minutes of 60% to 90% acetonitril, 16 to 19 minutes of 90% to 60% acetonitrile, and 19 to 20 minutes of 60% acetonitrile. The flow rate was 1mL/minute and detection was done at absorbance 213 nm. All tests were repeated 3 times.

Cellular DNA was isolated by the Cetyl Trimethylammonium Bromide (CTAB) method as described previously (16) and amplification and insertion of *LinA* gene in *P. plecoglossicida* was assessed by the polymerase chain reaction (PCR) with forward and reverse primers derived from the sequence of the *linA* gene, which was amplified from *Sphingomonas paucimobilis* UT26 with the following PCR primers and condition:

Forward: 5'-GACCATGGTGATGAGTGATCTAG-3' and Reverse 5'-GTAAGCTTTTATGCGCCGGACG-3' (17, 18). The reaction mixture contained 12.5 pmol of each primer, 5 - 10 ng of DNA, 37.5 pmol of $MgCl_2$, 62.5 pmol of each dNTP and 0.625 units of taq polymerase in 10 $\times$ PCR buffer (TAKARA, Japan). The PCR product was analyzed by electrophoresis in 0.8% agarose gel with 1 $\times$ TBE buffer. The obtained DNA fragments were excised from the gel, purified as described, and then 471 bp of *LinA* gene was inserted to the puc57 plasmid by the mentioned protocol in the manual of the Template Generation System II kit (Thermo Scientific, Nedaye Fan, Tehran, Iran), and transfected to *P. plecoglossicida*. Briefly, 4 μL of amplified *LinA* gene DNA, 10 μL of H_2O, 4 μL of 5X reaction buffer, 1μL of MuA Transposase, and 1 μL of Entranceposon (CamR-3) were mixed and incubated for one hour at 30°C and then heat inactivated by incubation at 75°C for 10 minutes. The produced plasmid was then transformed to competent Stellar cells amplified plasmid. Presence of the plasmid in *P. plecoglossicida* cida was confirmed by amplification by the following PCR mixture: 14.4 μL of H_2O, 4 μL of 5X Phire reaction buffer, 0.4 μL of 200 μM each dNTPs (10 mM each), 0.4 μL of pUC forward or pUC reverse primer (25 μM) 0.5 μM, 0.4 μL of Mu end primer (25 μM) 0.5 μM, 0.4 μL of Phire Hot Start II DNA polymerase, and 20 μL of the following primer sequences:

MuEnd primer: 5-GTTTTTCGTGCGCCGCTTCA; pUCRevPrimer:5-TTATGCTTCCGGCTCGTATGTTGTGT-3 or pUCFwd Primer: AGCTGGCGAAAGGGGGATGTG-3 with this cycling system: 98°C for 30 seconds and 30 cycle of 98°C for 5 seconds, and 72°C for 1 minute. A PCR product of 517 bp determined successful insertion.

The SPSS 14 software was used for evaluating statistical tests, such as mean, median, and standard deviation.

4. Results and Discussion

Constructed bacterial strain designated as transfected *P. plecoglossicida* was created by inserting the PCR product of amplification of *LinA* gene of *Sphingomonas paucimobilis* UT26 to pUC57 plasmid and transfection of this plasmid to *P. plecoglossicida*. Pure culturing of transfected strain in endosulfan supplemented CSM medium, caused a color change of the medium to yellow. The isolated bacteria were able to grown on CSM medium agar at 100 to 400 μg/mL. Endosulfan acted as the available carbon source. Degradation of endosulfan during growth of bacterial strains in carbon-deficient minimal medium (CSM) supplemented with 400 mg/l endosulfan is shown in Figure 1. This finding suggests that this strain has the ability of biodegrading the organochlorine pesticides and using the resulting product.

Detection of the gene in the transfected *P. plecoglossicida* was performed according to an established protocol that relies on analytical PCR with *linA* specific primers (23). The PCR on the whole transfected *P. plecoglossicida* genomic DNA yielded a single amplicon of the expected size for each of the primer pairs (Figure 2).

High performance chromatography of endosulfan after 24 hours of inoculation of carbon-deficient minimal medium (CSM) broth containing 400 μg/mL of endosulfan with 0.5 mL of modified *Pseudomonas plecoglossicida* suspension showed that concentrations of endosulfan decreased after incubation with transfected *Pseudomonas plecoglossicida* at the mentioned conditions. These results

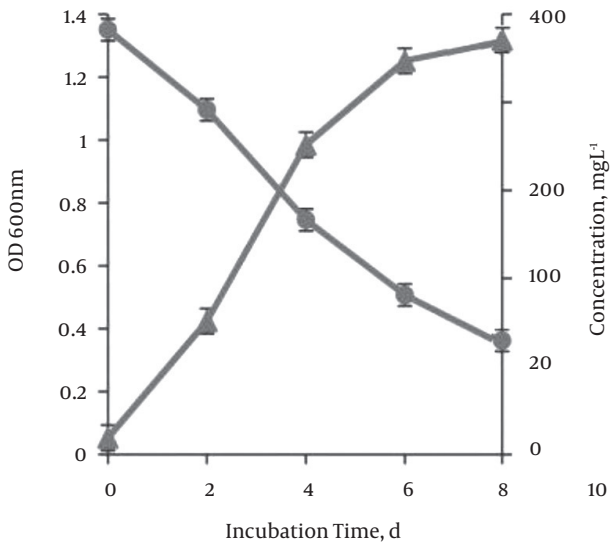

Figure 1. Degradation of Endosulfan During Growth of Bacterial Strains in Carbon-Deficient Minimal Medium (CSM) Supplemented With 400 mg/mL Endosulfan

showed that with increasing incubation time, concentration of the remaining of endosulfan pesticide decreased in culture tubes (Table 1).

Organochlorine pesticides are utilized directly for agricultural farms or soil and can contaminate sewerage and ditches, which in turn may be transported to rivers, and when this substances are distributed in the environment they become a pollutant and require remediation (12-14).

Ability of transfected *P. plecoglossicida* to proliferate in the culture medium containing endosulfan, as an exclusive carbon source, suggests that this strain may have the capability of degradation of endosulfan in the environment, and the discovered biodegradation capabilities of transfected *P. plecoglossicida* may have applications in a variety of tasks. The strain can be used to accelerate internalization of organochlorine pesticides in agricultural systems and sewage water, and to contain leaks of petroleum hydrocarbons and related industrial chemicals.

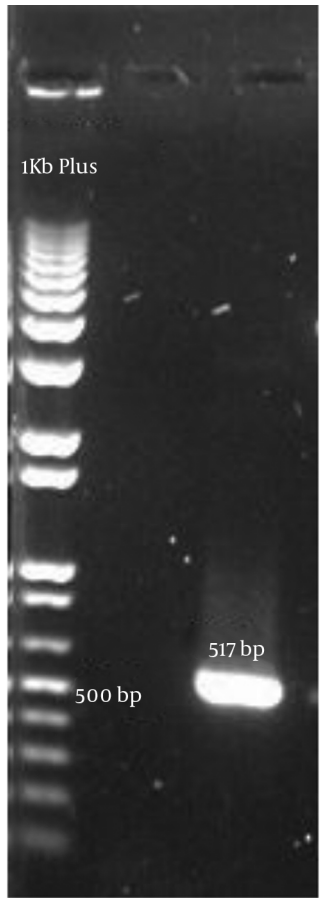

Figure 2. Detection of *LinA* Gene in the Transfected *Pseudomonas plecoglossicida* by Analytical Polymerase Chain Reaction With *LinA* Specific Primers

Acknowledgments

The authors appreciate the Deputy of Research of Qazvin University of Medical Sciences for their financial support.

Footnotes

Authors' Contribution: Taghi Naserpour Farivar designed the research, performed the experiments and analyzed the data. Masoumeh Aslani Mehr and Reza Najafipour analyzed the data. Pouran Johari performed the experiment and analyzed the data. Amir Peymani and Safar Ali Alizadeh designed the research, performed the experiment and analyzed the data.

Financial Disclosure: There was no conflict of interest.

Funding/Support: This study was performed by Qazvin University of Medical Sciences.

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Table 1. Retention Time, Peak Area and Peak Height of Endosulfan After Incubation With *LinA* Transfected *Pseudomonas plecoglossicida* in Deionized Distilled Water[a]

Time of Inoculation, d	0	2	4	8
Retention time	12:20.3 ± 0:1.7	12:51.4 ± 0:6.5	12:20.9 ± 0:2.7	12:27.4 ± 0:3.5
Peak areas	706.9 ± 23.2	552.0 ± 10.4	399.7 ± 6.8	351.9 ± 08.5
Peak height	313.2 ± 6.5	230.4 ± 8.0	214.2 ± 4.1	74.4 ± 8.3

[a] All tests were repeated three times.

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