Research Paper 研究报告

微生物学报 *Acta Microbiologica Sinica*
48(1)：45~50; 4 January 2008
ISSN 0001-6209; CN11-1995/Q
http://journals.im.ac.cn

Construction of a genetically engineered and stable strain of degrading γ-hexachlorocyclohexane and carbendazim by transposon mini-Tn5

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Abstract: The complete dehydrochlorinase gene *linA* of a γ-hexachlorocyclohexane (γ-HCH) degrading strain *Sphingomonas* sp. BHC-A, containing promoter and Shine-Dalgarno sequence (SD sequence), was amplified by PCR. The *linA* gene was inserted into *Not*I-cut transposon vector pUT/mini-Tn5 (Km^r) to get a novel transposon vector pUT/mini-Tn5-*linA*. With the helper plasmid RK600, the transposon vector pUT/mini-Tn5-*linA* was introduced into one carbendazim degrading gram-positive strain *Rhodococcus* sp. DJL-6 by triparental conjugation and then the dehydrochlorinase gene *linA* was integrated into the chromosome of *Rhodococcus* sp. DJL-6 by the transposon mini-Tn5. The selected multifunctional genetically engineered strain DJL-6A could degrade γ-HCH and carbendazim simultaneously. The dehydrochlorinase activity of DJL-6A was as strong as that of *Sphingomonas* sp. BHC-A in 0.05 and 5 μg/mL initial γ-HCH concentration. The *linA* of the strain DJL-6A was genetically stabile after successive plating DJL-6A for 30 days on nonselective media.

Keywords: dehydrochlorinase gene *lin*A; transposon vector pUT/mini-Tn5; multifunctional genetically engineered strain

CLC number: Q938 **Document code:** A **Article ID:** 0001-6209(2008)01-0045-06

γ-hexachlorocyclohexane (γ-HCH; γ-BHC; lindane) is a potent organochlorine insecticide employed for agricultural and medical purposes. It has been used on fruit and vegetable crops (including greenhouse vegetables and tobacco), for seed treatment, in forestry (including Christmas tree treatment) and for animal treatment worldwide since the 1940s [1]. Accumulating evidence suggested that it had long persistence in soil and toxicity, which could cause decreased sperm counts, impairment of sperm motility, reduced fertilization ability, producing abnormal sperm in men and wildlife [2]. Because of these, most countries have prohibited the use of γ-HCH. However, there are still many contaminated sites throughout the world [3, 4], where many crops are growing. When using other pesticides to protect the crops, the residues can persist at these sites and the cross contamination will be taken.

Carbendazim (methyl benzimidazol-2-ylcarbamate) is the most widely used benzamidazole fungicide and the major degradation product of other systemic fungicides, as benomyl and thiophanate-methyl *et al*. [5, 6]. It can persist at application sites and easily induce cumulative effects; its residue in fruits, plants and soils could be harmful to human health through food chains. Now, some evidence has shown that γ-HCH residue in some samples, such as tea, coming from carbendazim-contaminated environment could also be detected[7]. The cross pesticides contaminations need to be taken seriously.

Bioremediation is a useful approach to remove these residual pesticides from the contaminated environment. *Sphingomonas* sp. BHC-A is one γ-HCH degrading strain, which can utilize γ-HCH as the sole source of carbon and energy under aerobic conditions[8]. *Rhodococcus* sp. DJL-6 is one carbendazim degrading strain, which can utilize carbendazim as the sole source of carbon and energy [9]. In a previous study, the *linA* gene encoding γ-HCH dehydrochlorinase (LinA) was cloned, which triggered the initial dehydrochlorination of γ-HCH to 1,2,4-trichlorobenzene (1,2,4-TCB) via γ-pentachlorocyclohexene (γ-PCCH) in BHC-A[8]. In this study, the *linA* gene was integrated into the chromosome

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Received: 11 May 2007 / Revised: 5 August 2007

of DJL-6 by the transposon mini-Tn5 to extend the degrading capability of *Rhodococcus* sp. DJL-6. The constructed multifunctional genetic engineered strain DJL-6A could degrade γ-HCH and carbendazim simultaneously.

1 MATERIAL AND METHODS

1.1 Bacterial strains, plasmids, and culture conditions

γ-HCH degrading strain *Sphingomonas* sp. BHC-A (*Amp*^{r}, *Str*^{r}) [8], carbendazim degrading strain *Rhodococcus* sp. DJL-6 (*Ceftazidime*^{r}) [9], *Escherichia coli* HB101 (F$^-$ *hsdS recA ara proA lacy galK rpsL xyl mtl supE*) and *Escherichia coli* SM10 (*thi thr len tonA locy supE recA::RP4-2Tc::Mu λ π R6K*) (*λ pir*) [10] were grown on Luria broth (LB). Cultures were incubated at 30℃ for *Sphingomonas* sp. and *Rhodococcus* sp. and at 37℃ for *E. coli*. The *E. coli* SM10 (*λ pir*) was used as a host strain for transposon vector pUT/mini-Tn5 (derivative of Tn5 *Km*^{r}, *Amp*^{r}, *oriR6K*) [11]. *E. coli* HB101 with plasmid RK600 (*Cm*^{r}) [11] was used as the triparental conjugation helper. Antibiotics were used at final concentrations of 50 μg/ml for ampicillin, streptomycin and kanamycin, 10 μg/ml for chloramphenicol and 40 μg/ml for ceftazidime. The degradation of γ-HCH and carbendazim in microorganism was carried out at 30 °C in mineral salt medium (SM) containing (per liter) 1.0 g of NH_4NO_3, 1.0 g of NaCl, 1.5 g of K_2HPO_4, 0.5 g of KH_2PO_4, 0.1 g of $MgSO_4$.

1.2 Enzymes and reagents

Enzymes and kits necessary for DNA manipulations were purchased from Takara Biotechnology (Dalian) Co., Ltd. and New England Biolabs Co. Beijing, China. IPTG and X-Gal were obtained from Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. γ-HCH and carbendazim were obtained from Sigma- Aldrich USA (Genetimes Technology, Inc. China).

1.3 Preparation of *Sphingomonas* sp. BHC-A genomic DNA

Genomic DNA was isolated from *Sphingomonas* sp. BHC-A by the method of high-salt concentration precipitation [12].

1.4 Construction of suicide vector pUT/mini-Tn5- *linA*

To obtain the complete dehydrochlorinase gene *linA* (GeneBank accession no. AE015451) containing promoter and SD sequence, a specific PCR amplification was performed. The forward primer P_1 was 5′-ATAAGAAT*GCGGCCGC*GATCCTAAACATCAGTTCGC-3′ and the reverse primer P_2 was 5′-ATAAGAAT*GCGGCCGC*TCGCACCTCGATACAAGGAA-3′. The underlined sequence in each primer was a *Not*I site. Amplification was performed in the PCR buffer using about 20 ng of the BHC-A genomic DNA as a template, 25 pmol of each primer and 2 unit of *Taq* DNA polymerase in a total volume of 50 μl by 30 cycles as follows: a denaturing step at 94°C for 30s, primer annealing step at 68°C for 30s, and an extension step at 72°C for 1 min. The 847bp PCR product was digested by *Not*I for 24 hours and then inserted into *Not*I-cut transposon vector pUT/mini-Tn5 (Km^r) to form a novel transposon vector pUT/mini-Tn5-*linA* (Fig. 1). pUT/mini-Tn5-*linA* was transformed into *E. coli* SM10 (*λ pir*) by electroporation.

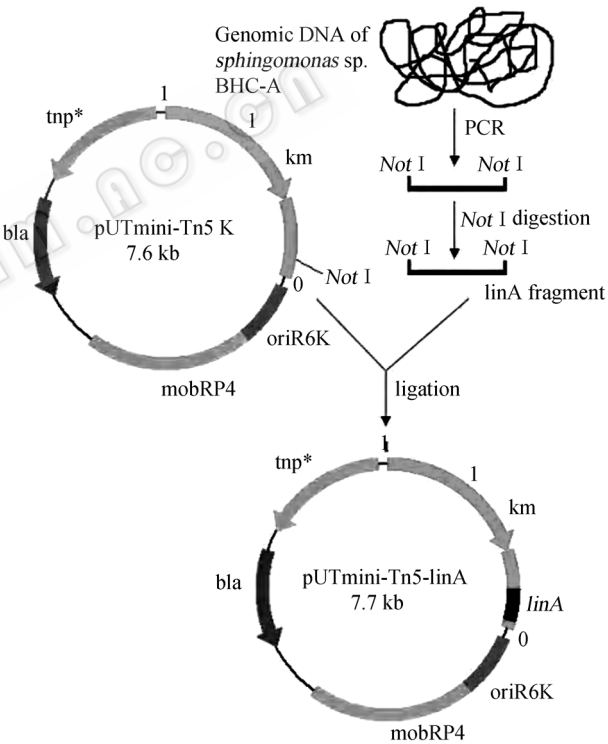

Fig. 1 Overview of construction of suicide vector pUT/ mini-Tn5-*linA*.

1.5 Triparental conjugation

The recipient (*Rhodococcus* sp. DJL-6), donor (*E. coli* SM10-λ*pir* harboring the suicide vector pUT/mini-Tn5-*linA*), and helper (*E. coli* HB101 with RK600) strains were grown individually in LB overnight with the final concentrations of 40 μg/ml for ceftazidime, 50 μg/ml for kanamycin and 10 μg/ml for chloramphenicol respectively. After incubation of the recipient at 42°C for 15 min to temporarily inactivate its restriction systems, 1 ml was mixed with 2 ml of the donor and 2 ml

of the helper. Cells were collected by centrifugation at 6000 rpm in microcentrifuge tubes at room temperature for 5 minutes, washed three times by suspending in 1 ml saline to remove the antibiotics and resuspended in 100 μl of fresh LB, and spotted on an LB plate. After overnight incubation at 30°C, cells were scraped off the plate and resuspended in 1 ml of LB, and serial dilutions were plated on selective LB medium containing 40 μg/ml ceftazidime and 5 μg/ml kanamycin. Ceftazidime^r and kanamycin^r transformants were selected as mini-Tn5-*linA* insertional conjugants. γ-HCH degradation activity was visually assayed by spraying γ-HCH solution (50 mg/ml in ethanol) on the conjugants grown on the LB plates. Genomic DNA of conjugants that could make clear zones on the plates was isolated as template by the method of high-salt concentration precipitation and specific PCR amplifications of the *linA* gene were performed by the primers P_1 and P_2.

1.6 Assay for the dehydrochlorinase activity of conjugants

The degradation of γ-HCH in conjugants, *Sphingomonas* sp. BHC-A and *Rhodococcus* sp. DJL-6 was carried out in mineral salt medium (SM). Precultures of bacteria grown in LB at 1×10^8 CFU/ml were transferred at 2 % (vol/vol) to fresh medium containing 5 μg γ-HCH per milliliter. Each flask containing γ-HCH was incubated at 30°C on a rotary shaker for 2 hours. Aliquots (200 μl) were taken out and extracted with 200 μl of hexane. 1 μl of this extract was analyzed by gas chromatography (GC) with an electron capture detector (ECD).

1.7 Assay for the carbendazim degrading activity of conjugants

The degradation of carbendazim in conjugants, *Sphingomonas* sp. BHC-A and *Rhodococcus* sp. DJL-6 was also carried out in SM. Precultures of bacteria grown in LB at 1×10^8 CFU/ml were transferred at 2 % (vol/vol) to fresh medium containing 5 μg carbendazim per milliliter. Each flask containing carbendazim was incubated at 30°C on a rotary shaker for 24 hours. Aliquots (200 μl) were taken out and extracted with 200 μl of chloroform. The chloroform containing carbendazim was transferred to a fresh tube and dried by blowing nitrogen gas. 200 μl methanol was supplemented and 10 μl of this extract was analyzed by reversed-phase high-performance liquid chromatography (HPLC).

1.8 Selection of the engineered strain

The dehydrochlorinase activity and the carbendazim degrading activity of conjugants were analyzed. Conjugants holding carbendazim degrading activity as DJL-6 and expressing the same dehydrochlorine activity as BHC-A were selected and incubated in mineral salt medium (SM) supplemented with 1% glucose in order to screen and eliminate auxotroph conjugants. As control, DJL-6 was also incubated in same medium. After incubation at 30℃ for 48h, the conjugant holding normal growth as DJL-6 was selected as the engineered strain.

1.9 Testing the dehydrochlorinase activity and the carbendazim degrading activity of the engineered strain in high&low substrates concentration simultaneously

1.9.1 Testing in high substrates concentration: Precultures of the engineered strain grown in LB at 1×10^8 CFU/ml were transferred at 2 % (vol/vol) to fresh SM medium containing 25 μg γ-HCH and 25 μg carbendazim per milliliter. Each flask was incubated at 30°C on a rotary shaker for 24 hours. As control, *Sphingomonas* sp. BHC-A and *Rhodococcus* sp. DJL-6 were tested following the same procedure. For testing the dehydrochlorinase activity, Aliquots (40 μl) were taken out periodically and extracted with 200 μl of hexane. 1 μl of this extract was analyzed by gas chromatography (GC) with an electron capture detector (ECD). For testing the carbendazim degrading activity, aliquots (40 μl) were taken out periodically and extracted with 200 μl of chloroform. The chloroform containing carbendazim was transferred to a fresh tube and dried by blowing nitrogen gas. 200 μl methanol was supplemented and 10 μl of this extract was analyzed by reversed-phase high-performance liquid chromatography (HPLC).

1.9.2 Testing in low substrates concentration: Precultures of the engineered strain grown in LB at 1×10^8 CFU/ml were transferred at 2 % (vol/vol) to fresh SM medium containing 0.05 μg γ-HCH and 0.1 μg carbendazim per milliliter. Each flask was incubated at 30°C on a rotary shaker for 30 min. As control, *Sphingomonas* sp. BHC-A and *Rhodococcus* sp. DJL-6 were tested following the same procedure. For testing the dehydrochlorinase activity, Aliquots (200 μl) were taken out periodically and extracted with 50 μl of hexane. 1 μl of this extract was analyzed by gas chromatography (GC) with an electron capture detector (ECD). For testing the carbendazim degrading activity, aliquots (500 μl) were taken out periodically and extracted with 200 μl of chloroform. The chloroform containing carbendazim was transferred to a fresh tube and dried by blowing nitrogen gas. 50 μl methanol was supplemented and 10 μl of this extract was analyzed by reversed-phase high-performance liquid chromatography (HPLC).

1.10 Testing the genetic stability of the engineered strain

Precultures of the engineered strain grown in LB at 1×10^8 CFU/ml were simultaneously enumerated on both selective (5μg/ml kanamycin) and nonselective (LB) media and cultured at 30°C. The stability of the strain was determined by periodical assay for the dehydrochlorinase activity of the successive plating strains on both selective (5 μg/ml kanamycin) and nonselective media for 30 days. The assay procedure was as 1.6.

1.11 Analytical methods

GC analysis the dehydrochlorinase activity was performed with a Shimadzu GC-14B equipped with an electron capture ^{63}Ni detector and a column containing 3% OV225 (30m×250μm×0.25μm). The column, injector and detector temperatures were maintained at 210℃, 250℃ and 300℃, respectively, with a flow rate of carrier gas (nitrogen) of 40 ml/min.

The carbendazim degrading activity was analyzed by reversed-phase high-performance liquid chromatography (HPLC) using Waters$_{600}$ Instruments C_{18} column (150×2.6 mm). The mobile phase of methanol/water (1:1) was run isocratically at a flow rate of 0.8 ml/min. Column elutions were monitored by measuring A_{280}.

2 RESULTS AND DISCUSSION

2.1 Construction of suicide vector pUT/mini-Tn5-*linA*

`pUT/mini-Tn5 was one of the Tn5-derived mini-transposons that simplified substantially the generation of insertion mutants, in vivo fusions with reporter genes, and the introduction of foreign DNA fragments into the chromosome of a variety of bacteria, including the enteric bacteria and typical soil bacteria like *Pseudomonas* species. pUT/mini-Tn5 (Km^r) consisted of the gene specifying resistance to kanamycin as selection marker and a unique *Not*I cloning site flanked by 19-base-pair terminal repeat sequences of Tn5. This transposon was located on a R6K-base suicide delivery plasmid that provides the IS*50*$_R$ transposase *tnp* gene in *cis* but external to the mobile element and whose conjugal transfer to recipients was mediated by RP4 mobilization functions in the donor [13].

In this study, the complete dehydrochlorinase gene *linA* containing promoter and SD sequence was inserted into the *Not* Ⅰ site of the pUT/mini-Tn5 to form the recombinant transposon pUT/mini-Tn5-*linA* (Fig. 2). pUT/mini-Tn5-*linA* was transformed into *E. coli* SM10 (*λ pir*) by electroporation.

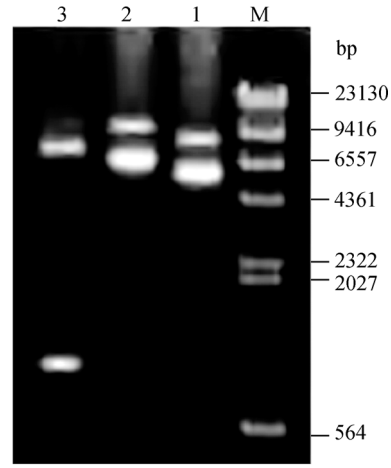

Fig. 2 Construction of pUT/mini-Tn5-*linA*. The *linA* gene was inserted into the *Not* Ⅰ site of the pUT/mini-tn5 (lane 1) to form the new pUT/mini-Tn5-*linA* (lane 2). After the new transposon was digested by restriction with *Not* Ⅰ, the about 874bp fragment was obtained (lane 3). That testified the *linA* gene had inserted into the pUT/mini-Tn5 correctly. M, lambda DNA/*Hind*Ⅲ molecular weight marker.

2.2 Isolation of mini-Tn5-*linA* induced conjugants

Tn5 transpositional recombination by conjugation occurred between gram-negative bacteria was reported widely. In this study, we confirmed that the transpositional recombination by conjugation could also occur in gram-positive bacteria such as *Rhodococcus* sp. Km^r-transconjugants were obtained at a frequency of 2.1×10^{-6} per recipient cell.

Ten conjugants endowed with the dehydrochlorinase activity were picked randomly from 200 transconjugants and were designated DJL-6A, DJL-6B, DJL-6C, DJL-6D, DJL-6E, DJL-6F, DJL-6G, DJL-6H, DGL-6I, and DGL-6J respectively. Specific PCR amplifications were performed in order to check the *linA* gene from these conjugants. The results showed that the *linA* gene could be ampilified from the genome of each conjugant while could not be amplified from the genome of *Rhodococcus* sp. DJL-6 by PCR.

2.3 Selection of the engineered strain from conjugants

More quantitative analysis of the dehydrochlorinase activity of the ten conjugants was performed. As control, *Sphingomonas* sp. BHC-A showed ahout 80% degradation rate of γ-HCH in 2 hours and the concentration of γ-HCH in medium decreased to 1 μg/ml (Fig. 3). While *Rhodococcus* sp. DJL-6 did not show any γ-HCH degrading activity. In these conjugants, one conjugant DJL-6E showed much lower dehydrochlorinase activity than *Sphingomonas* sp. BHC-A, only 30% degradation rate and the concentration of γ-HCH in medium was 3.5

μg/ml after 2 hours. The others showed nearly the same dehydrochlorinase activity as BHC-A, about 80% degradation rate in 2 hours. 1,2,4-TCB as the final dehydrochlorine product was also detected from these conjugants (data not shown).

HPLC analysis of the carbendazim degrading activity of these conjugants was performed simultaneously (Fig. 3). As control, *Rhodococcus* sp. DJL-6 could degrade 5 μg/ml carbendazim completely in 24 hours while *Sphingomonas* sp. BHC-A showed no degradation activity of carbendazim. These conjugants were classified into three groups. The first type, containing DJL-6B, DJL-6C, DJL-6D, DJL-6E, DJL-6G, DJL-6H and DJL-6J, degraded carbendazim much more slowly than *Rhodococcus* sp. DJL-6 and the concentration of carbendazim in medium was more than 1.25 μg/ml after 24 hours. The second type, con taining only one conjugant DJL-6F, showed no degradation of carbendazim. The third type, exemplified by DJL-6A and DJL-6I, could completely degrade 5 μg/ml carbendazim in 24 hours and held high carbendazim degrading activity as *Rhodococcus* sp. DJL-6.

The result of elimination auxotroph showed that the conjugant DJL-6I could not grow in medium while the conjugant DJL-6A could grow as DJL-6. Finally, the engineered strain DJL-6A was selected.

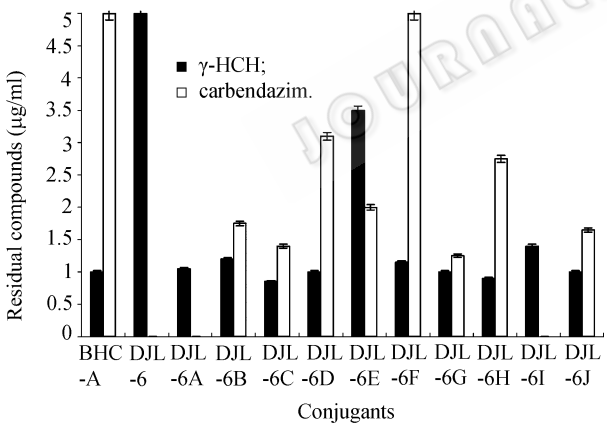

Fig. 3 The degradation of compounds by the conjugants in SM. BHC-A and DJL-6 as control. The error bars indicate standard deviations.

2.4 Testing the dehydrochlorinase activity and the carbendazim degrading activity of the engineered strain in high&low substrates concentration simultaneously

In high substrates concentration testing, DJL-6A showed the same carbendazim degrading activity as DJL-6 (Fig. 4). After 24 hours, only 1.25 μg/ml carbendazim could be detected in medium and the degradation rate was nearly 95%. On the other hand, DJL-6A did not show the strong γ-HCH conversion activity as BHC-A. After 24 hours, BHC-A degrade nearly all γ-HCH while DJL-6A only degrade 60% γ-HCH and 10 μg/ml γ-HCH was detected from medium. It was probably caused by the feedback inhibition of the high concentration of final dehydrochlorine product 1,2,4-TCB.

In low substrates concentration testing, DJL-6A showed strong dehydrochlorinase activity and carbendazim degrading activity (Fig. 5). After 30 min, DJL-6A degraded 0.1 μg/ml carbendazim completely; the degradation rate was the same as that of strain DJL-6. 0.05 μg/ml γ-HCH was also degraded by DJL-6A in 10 min; the conversion rate was equal to that of BHC-A. These results also suggested that γ-HCH did not disturb the degradation of carbendazim in DJL-6A, whenever in high or low substrates concentration testings.]

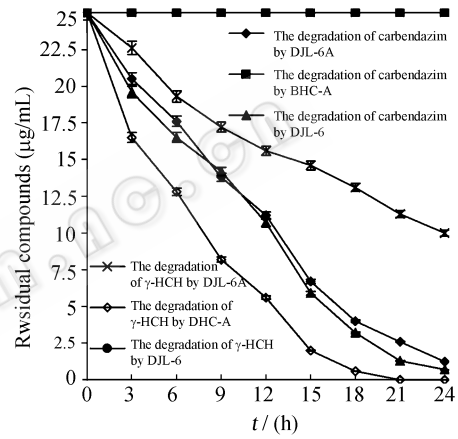

Fig. 4 The degradation of compounds by DJL-6A in high initial substrates concentration. BHC-A and DJL-6 as control. The error bars indicate standard deviations.

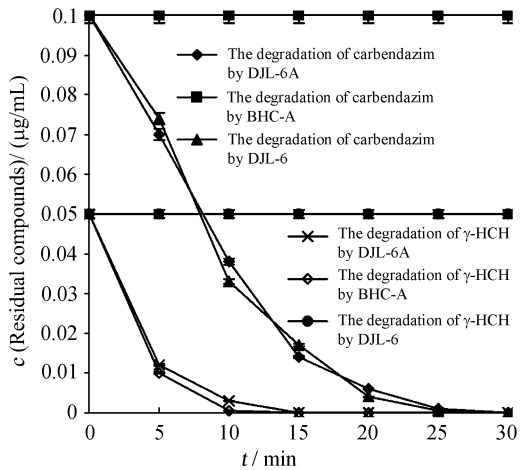

Fig. 5 The degradation of compounds by DJL-6A in low initial substrates concentration. BHC-A and DJL-6 as control. The error bars indicate standard deviations.

2.5 Testing the genetic stability of the engineered strain *Rhodococcus* sp. DJL-6A

The engineering strain was successive cultured for 30 days on both selective (5μg/ml kanamycin) and non-

selective LB plate. GC analyzed the dehydrochlorinase activity. The results showed whether the selective conditions were supplemented into medium or not, the degradation rate of γ-HCH of DJL-6A achieved 83% in 2 hours (data not shown). The dehydrochlorinase activity of DJL-6A was nearly the same level of that of BHC-A. That is to say the dehydrochlorinase activity of DJL-6A did not decrease during the process of culture, which suggested the *linA* gene integrated into the genome of DJL-6A had the high genetic stability.

ACKNOWLEDGEMENTS: This work is supported by the National Natural Science Foundation of China (40471073,30400013), the National Programs for High Technology Research and Development of China [2003AA241150, 2004AA246070, 2004(249) and 2004(514)] and the Hi-tech Research Programs for Science & Technology Department of Jiangsu Province of China (BE2002345,BE2003343,JHZD06-2 and BG2005322)

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遗传稳定型六六六、多菌灵降解基因工程菌构建

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摘要: 通过 PCR 的方法从六六六降解菌 *Sphingomonas* sp. BHC-A 扩增出完整的脱氯化氢酶基因 *linA*。将其克隆到含有 mini-Tn5 的自杀性质粒 pUT4K 上, 构建成质粒 pUT/mini-Tn5-*linA*. 通过三亲杂交, 在辅助质粒 RK600 的帮助下, 将 pUT/mini-Tn5-*linA* 转移到一株高效降解多菌灵菌株 *Rhodococcus* sp. DJL-6 中。利用 mini-Tn5 的转座作用将 *linA* 基因整合到 DJL-6 的染色体 DNA 上, 得到工程菌株 DJL-6A。该工程菌具有同时降解多菌灵和六六六的功能, 且对于初始浓度为 0.05 μg/mL 和 5 μg/mL 的六六六的降解活性与亲本菌株 BHC-A 相当。在不加任何选择压力的条件下工程菌株进行连续传代, 结果证明 *linA* 基因可以持续稳定的存在于宿主的染色体 DNA 上。

关键词: 脱氯化氢酶基因 *linA*; 转座载体 pUT/mini-Tn5; 多功能工程菌

中图分类号： Q938 文献标识码：A 文章编号：0001-6209(2008)-01-0050-06

基金项目: 国家自然科学基金 (40471073 and 30400013); 国家“863 计划”[2003AA241150，2004AA246070，2004(249) and 2004(514)]; 江苏省科技厅高技术研究项目(BE2002345, BE2003343, JHZD06-2 and BG2005322)

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收稿日期: 2007-05-11; 修回日期: 2007-08-05