A novel native bacillus thuringiensis biology essay

Science, Biology

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5, 6-trichloro-2-pyridyl) phosphorothioate) is one of the most widely used organophosphate insecticides effective against a broad spectrum of insect pests of economically important crops, such as cotton, corn, and fruit trees including oranges and apples (EPA, 2006; Liu, 1993). Although chlorpyrifos was outlawed from residential use in 2001, it is still applied intensively on corn, rice and soya (Pope et al., 2005). The half-life of chlorpyrifos in soil is usually between 60 and 120 days, but can range from 2 weeks to over 1 year, depending on the soil type and other environmental conditions (Howard, 1991). Therefore, wide contamination of water and soils by this insecticide has aroused much public concern (Guinazu et al., 2012; Zhang et al., 2012). Bioremediation, as an ef? cient and cheap biotechnological approach to clean up polluted environments, has received increasing attention (Xu et al., 2008). Many bacteria and fungi capable of degrading chlorpyrifos have been isolated and characterized. Considerable amount of strains, such as Flavobacterium (Mallick et al., 1999), Enterobacter strain B-14 (Singh et al., 2004), Alkaligenes faecalis DSP3 (Yang et al., 2005), Stenotrophomonas sp. YC-1 (Yang et al., 2006), Sphingmonas sp. Dsp-2 (Li et al., 2007), Paracoccus sp. TRP (Xu et al., 2008), Bacillus pumilus C2A1 (Anwar et al., 2009), Synechocystis sp. Strain PUPCCC 64 (Singh et al., 2011), Pseudomonas Iso 1-4, Agrobacterium Iso 5-6, Bacillus Iso 7 (Maya et al., 2011), Aspergillus sp. F1, Penicillium sp. F2, Eurotium sp. F4, Emericella sp. F5 (Maya et al., 2012) and Cupriavidus sp. DT-1 (Lu et al., 2012), have been reported to be capable of degrading chlorpyrifos. In contrast, few studies have been conducted on the degradation of chlorpyrifos by entomopathogen, which were used as bio-pesticide. Despite it has been

reported that many entomopathogen have the potential of degrading chemical wastes (Dave and Dave, 2009; Wang et al., 2008). Thus far, there has been no report of the isolation of chlorpyrifos degrading bacteria shown to be toxic to insects. B. thuringiensis is the most widely used microbial biopesticide. Upon sporulation, it produces insecticidal crystal proteins (ICPs), including Cry and Cyt toxins. These toxins are highly speci? c to their target insect, are innocuous to humans, vertebrates and plants, and are completely biodegradable (Bravo et al., 2007). It is a widespread bacterium in different natural habitats which may indicate a wide diversity and, hence, a large arsenal of hydrolytic enzymes (Raymond et al., 2010). It has reported that B. thuringiensis strains were capable of degrading many kinds of chemical wastes, such as diesel fuel (Kebria et al., 2009), dimethyl phthalate (Brar et al., 2009), pentachlorophenol (Karn et al., 2010), alachlor (Wang et al., 2008), acid red 119 dye (Dave and Dave, 2009), and malathion (Mohamed et al., 2010). However, there has been little information on the combination of its degrading ability and insecticidal activity. The search for native B. thuringiensis strains with both activities against insects and degradation of chlorpyrifos could have a positive impact on the application of B. thuringiensis worldwide. The study here was aimed at isolating chlorpyrifos-degrading B. thuringiensis from a pesticide factory in Fujian province, China. The 16s rRNA, biochemical reactions, antibiotic sensitivity, plasmid profile and crystal protein profile of the selected strains were also studied. 2. Materials and methods2. 1 Chemicals and mediaAnalytical-grade chlorpyrifos (> 99% purity; Sigma-Aldrich, Ltd., China) were used in this study. All other reagents used in this study were of analytical-reagent grade

(Shanghai Chemical Reagent Co. Ltd., China). LuriaeBertani (LB) medium, which contained (g l-1): tryptone 10. 0, yeast extract 5. 0 and NaCl 10. 0, and mineral salts medium (MSM) that contained (g l-1): K2HPO4 1. 5, KH2PO4 0. 5, MgSO4? 7H2O 0. 2, NaCl 0. 5, NH4NO3 1. 5, were used for the isolation of bacterial strains (Li et al., 2008). The initial pH was adjusted to 7. 0. When necessary, chlorpyrifos (200 mg l-1) dissolved in methanol was added to the medium. Peptone medium (PM), which contained (g l-1): tryptone 5. 0, yeast extract 1. 0, glucose 2. 5, KH2PO4 1. 0, ZnSO4? 7H2O 0. 02, MgSO4? 7H2O 0. 3, MnSO4? 7H2O 0. 02, FeSO4? 7H2O 0. 02, pH 7. 0, were used for culture spore and crystal protein (Wang et al., 2012). 2. 2 Sample collection and isolationSamples were collected from an industry that has manufactured organophosphate pesticides for more than 10 years in Sanming, Fujian Province, China (E26��17�� N117��39��). The sampling sites had been used for intensive organophosphate pesticides production for several decades, and thus had been exposed to organophosphate pesticides for long, and were not exposed to any commercial formulation of Bacillus thuringiensis. The procedure used for isolating B. thuringiensis was the same as described previously by Baig and Mehnaz (2010), with some modification. Brie? y, the samples (1 g or 1 ml) were added to 10 ml selecting medium (LB medium with 0. 4 mg ml-1 ampicillin and 0. 15 mol l-1 NaAc pH 6. 8) at 37�� for 4 h in 50 ml ? ask with 150 r min-1. The suspension (1 ml) was then incubated at 80�� for 10 min to eliminate non-sporulated microbes that germinated, and cooled on ice for 5 min immediately. Dilutions of the heated samples were placed onto MSM agar plates containing 100 mg l-1chlorpyrifos as a carbon source and

incubated at 30�� until sporulation. Bacterial colonies with clear haloes, indicating degradation of chlorpyrifos, were selected and puri? ed. Each culture was grown on LB agar plate and examined with a phase contrast microscope for crystal production and morphology. 2. 3 Characterization2. 3. 1 Microscopy and biochemical characterizationIsolated Bacillus thuringiensis strains were identi? ed based on morphological characters with reference to Bergey��s Manual of Determinative Bacteriology (Holt et al., 1994). Biochemical and antibiotic sensitivity tests were analyzed by HX-21A Bacilli analyzer (Heng xing Co., Ltd., China), with corollary reagent plate of Bacillus, according to the instruction manual. 2. 3. 2 Ampli? cation of 16S rRNA genes and sequence analysesTotal DNA extraction was achieved following the method described by Silva-Werneck and Ellar (2008). The 16S rRNA gene was ampli? ed, from total DNA with the universal primers, purified and sequenced as described by Li et al. (2007). The nucleotide sequence coding for 16S rRNA of isolated strain was deposited in the GenBank database. The sequences were compared against the available DNA sequences in GeneBank using the BLASTN tool. Alignment of different 16S rRNA sequences from GenBank was performed using ClustalX software (Larkin et al., 2007). Phylogenesis was analyzed by MEGA, version 4. 1. Distances were calculated using the Kimura two-parameter distance model. An unrooted tree was built by the neighbor joining method. The dataset was bootstrapped 1000 times. 2. 3. 3 Molecular characterization of insecticidal genesThe cry-type insecticidal genes were identified by PCR-RFLP method (Kuo and Chak, 1996; Song et al., 2003). Total DNA from B. thuringiensis was extracted by a simple method, and B. thuringiensis subsp. kurstaki strain HD1 served as control

strains. Pairs of universal oligonucleotide primers designed based on the conserved blocks from known cry-type genes, were used to screen isolated B. thuringiensis strains. The universal primer pairs of K5un2/K3un2 and K5un3/K3un3 (cry1/7/9), S5un2/S3un2 (cry2), S5un3/S3un3 (cry3), S5un4/S3un4 (cry4/10), cry5F/cry5R (cry5), cry6F/cry6R (cry6), cry8F/cry8R (cry8) and cry11F/cry11R (cry11) were designed to detect corresponding genes. PCR ampli? cation was performed as described above. The resulting amplicons were puri? ed with Tiangen Mini Puri? cation Kit (Tiangen, China). The appropriate restriction endonucleases (TaKaRa, China) were selected to digest the PCR fragments and the resulting restriction fragments were separated according to their length by agarose gel electrophoresis (Kuo and Chak, 1996). 2. 3. 4 Insecticidal crystal protein profilesEach strain was grown in 50 ml PM medium at 30�? and shaked at 250 r min-1 for 48 h. Crystals of Cry or Cyt toxins were pelleted at 5, 000 g for 15 min at 4 ??. The pellet was washed three times with 1 M NaCl and then three times with distilled water. The mixture of spores and crystals was then resuspended in lysis buffer (1%Na2CO3, 0. 08% DTT), incubated at 30?? with continuous shaking for 30 min, and centrifuged at 7, 000 g for 15 min at 4??. The soluble proteins in supernatant were further purified by isoelectric precipitation in Na2CO3/Na2Ac buffer with pH 5. 0 (Wang et al., 2012). Protein concentrations were measured by the Bradford Quanti? cation Method (Bradford, 1976). The crystal proteins were analyzed on 10% sodium dodecyl sulfate?Cpolyacrylamide gel electrophoresis (SDS?CPAGE) as described by Laemmli (1970). 2. 3. 5 Plasmid pro? lePlasmid DNA was extracted according to the method described by Ibarra et al. (2003) with

some modi? cations. B. thuringiensis strains were grown in LB to an optical density of 0. 8 at 595 nm. Cells were washed in TE (50 mmol l-1 Tris, 10 mmol l-1 EDTA, pH 8. 0) and incubated for 2 hour at 37�? in TE with 10mg ml-1 lysozyme. After 10 min at 4??, lysis buffer (0. 2 mol l-1 NaOH, 1% sodium dodecyl sulfate) was added and the mixture was incubated for 5 min at 4??. A solution of 3 M sodium acetate, pH 4. 8, was added and stored for 20 min at 4??. Samples were then centrifuged at 13, 000 g, at 4?? for 20 min. Two volumes of ethanol were added, and the mixture was incubated for 20 min at -20?? to precipitate DNA. DNA was centrifuged as above, dissolved in distilled water, and visualized in 0. 6% agarose gels. 2. 4 Chemical analysisThe extraction of pesticides was carried out according to a standard method established by the AQSIQ (2008) with some modi? cations. Remaining chlorpyrifos in the cultures was extracted three times with equal volume of ethyl acetate. The extracts were then dried over anhydrous Na2SO4 and evaporated under reduced pressure using a centrifugal evaporator at 30??. The residual organic material was redissolved in an equal volume of methanol and transferred to vials for GC analysis. Chlorpyrifos was detected with GC-14A (Shimadzu Corporation, Kyoto, Japan) equipped with a HP-5 fused silica capillary column (30 m??0. 53 mm, 1. 5 ??m, Hewlett Packard, Avondale, USA) and ? ame photometric detector (FPD). The injector and detector temperatures were 260??. The temperature program was as follows: 120?? (1 min), 10??/min to 240?? (7 min). Nitrogen carrier gas was used at the ? ow rate of 59. 0 ml/min. Sample solution (2. 0 ??l) was injected in splitless mode, and the quanti? cation of pesticide was performed using an external standard. 2. 5

Degradation studies2. 5. 1 Inoculum preparation for degradation studiesBRC-HZM2 cells were pre-cultured in LB medium, harvested by centrifugation at 6000 g for 5 min and washed three times with sterilized water. For all experiments, cells were used at a concentration of 108 cells per ml and samples were incubated at 30��on a shaker at 150 r min-1 unless otherwise stated. 2. 5. 2 Degradation of chlorpyrifos by BRC-HZM2Shake ? ask studies were carried to work out the chlorpyrifos degrading capacity of the isolated B. thuringiensis strain. LB (100 mL) containing 100 mg l-1 chlorpyrifos were inoculated 2% inoculum and incubated at 30�� and 230 r min-1 for 5 days. At different time points (0 h, 8 h, 12 h, 24 h and 48 h), samples from the culture medium were recovered aseptically and residual pesticide concentration determined as described above in Sections 2. 4. Uninoculated ? asks with LB containing 100 mg l-1 chlorpyrifos served as controls. The experiment was conducted in duplicate. 3. Results and discussion3. 1 IsolationIn this study, colony-forming bacteria were separated by heat-treatment on nutrient medium, among which bacterial isolates forming spore and parasporal crystals were selected for further study. In total, 6 isolates of 220 samples were separated (Table S1). All of these strains were found to be capable of degrading chlorpyrifos in MSM medium. These strains were designated as BRC-HZM1, BRC-HZM2, BRC-HZM3, BRC-HZM4, BRC-HZM6, BRC-HZM7, respectively. Strains BRC-HZM1, BRC-HZM2 and BRC-HZM6 were isolated from water samples, however, strains BRC-HZM3, BRC-HZM4 and BRC-HZM7 were from soil samples (Table S2). The characteristics of spore and parasporal crystals of these isolated strains were identi? ed based on morphological result (Fig S1). The isolation rate of B.

thuringiensis from polluted water and soil of Fujian sannong chemical and pesticide factory is 2. 7%. B. thuringiensis strain BRC-HZM2 was selected for further study. 3. 2 Identi? cation of strain BRC-HZM23. 2. 1 Microscopy and biochemical characterisationB. thuringiensis strain BRC-HZM2 is a gram-positive and rod-shaped bacterium. The morphology of parasporal crystals produced by BRC-HZM2 was diamond. Biochemical tests for strain BRC-HZM2 are described in Table S3. It was showed that both strains HD1 and BRC-HZM2 had similar biochemical reactions. Two strains showed positive reaction in Arginine, Carbamide, Esculin, Glucose, Xylose, Lactose, Sulfureted hydrogen, Ornithine, Rhamnose, Gelatin, but negative in Nitrate, Fructose, Galactose, Maltase, Mannose, Mannitol, MacConkey Agar, Sodium chloride, Amylase, Acetate, Citric acid, SS acid, Sucrose and VP. They only showed difference in Lysine. Meanwhile, 29 antibiotic sensitivity characteristics are shown in Table S4. Both strains were sensitive to Gentamicin, Aikacin, CLDM, Erythrocin, Cprofloxacin, and Ofloxacin. HD1 showed sensitivity to Cefoperazone while strain BRC-HZM2 shows resistance. Strain BRC-HZM2 showed sensitivity to Tobramycin while strain HD1 showed resistance. The two strains also showed difference in reaction to Oxacillin, Cefazolin, Clindamycin, Rifampin and Cefalotin. 3. 2. 2 Ampli? cation of 16S rRNA genes and sequence analysesThe 16S rRNA gene sequence of BRC-HZM2 has been uploaded in GenBank of NCBI database under the accession numbers GQ140344. A BLAST search revealed that BRC-HZM2 is closely related to Bacillus thuringensis. The phylogenetic tree illustrating the results of the 16S rRNA analysis is presented in figure 1. The 16S rRNA sequence of BRC-HZM2 showed a highest similarity to Bacillus thuringiensis strains,

indicating that strain BRC-HZM2 belongs to the Bacillus thuringiensis, just the same as microscopy. To our knowledge, this is the ? rst report of a strain of Bacillus thuringiensis which was capable of degrading chlorpyrifos. 3. 2. 3 Identi? cation of cry-type genes by PCR–CRFLPThe PCR-RFLP identi? cation system was used to identify cry genes within this strain. Ten pairs of universal oligonucleotide primers were used to detect cry genes, of which the primer pairs of K5un2/K3un2 and K5un3/K3un3, S5un2/S3un2, S5un3/S3un3, S5un4/S3un4, cry5F/cry5R, cry6F/cry6R, cry8F/cry8R and cry11F/cry11R produced PCR products. The PCR amplicons were digested with pairs of restriction endonucleases to further characterize the type of cry gene present (Fig. 2). The results indicated that B. thuringiensis BRC-HZM2 harbor cry1Aa, cry1Ab, cry1Ac, cry2Ab and cry1Ea genes. Although the result of 16S rRNA identification of B. thuringiensis BRC-HZM2 was serotyped as ssp. kurstaki, it encoded different toxin gene-types to that of the reference strain (HD1). This unusual multiple gene type might result from gene flow of plasmids, which contained insecticidal gene between strains in the natural environment. For other virulence factors, the presence of chi, inhA, aiiA and vip3A genes was con? rmed in BRC-HZM2 as deduced by PCR ampli? cation products (Fig. 3). However, vip1 and vip2 genes were not found in BRC-HZM2. 3. 2. 4 Insecticidal crystal protein profilesBRC-HZM2 displayed almost the same profile with HD1, with major proteins of 130 and 60 kDa. As shown in Fig. 4, HD1 produces toxins, which include Cry1Aa, Cry1Ab, Cry1Ac and Cry2Ab, which was consistent with the report. BRC-HZM2 displayed almost the same profile as HD1, with major proteins of 130 and 60 kDa. 3. 2. 5 Plasmid pro? leSamples were analyzed by agarose gel

electrophoresis and a complex plasmid array was found. As Figure 5 shows, many plasmid patterns were detected in BRC-HZM2. Six plasmids were detected in HD1 and were used as a standard marker for BRC-HZM2. Seven plasmids were detected in BRC-HZM2 and shown high resemblance with pattern of plasmid from HD1. 3. 3 Degradation of chlorpyrifos by B. thuringiensis BRC-HZM2The residue of chlorpyrifos in LB medium was measured and the residual rate of chlorpyrifos with the external standard method. The result is shown in Fig. 6. Each time interval, chlorpyrifos in control samples basically have no degradation. In samples with BRC-HZM2 high degradation activity to chlorpyrifos was observed. The degradation at 8 h, 12 h, 24 h, 48 h rate were 39. 91%, 50. 00%, 83. 94%, 88. 99%, respectively. The degradation activities mainly appear during first 24 h. This may have some correlation with the degradation mechanism. 4. DiscussionIn this study, the modified sodium acetate and antibiotics heat treatment method was used to isolate B. thuringiensis from the samples which were polluted by organophosphorus pesticide. Sodium acetate can restrain the germination of B. thuringiensis spore (Travers et al., 1987), but has no effect on other Bacillus. Meanwhile, the ampicillin and heat treatment would kill other bacteria which existing as vegetative cells. However, B. thuringiensis spores, which were resistant to such condition, will survive after such treatment. Finally, the strains, which were capable of degrading chlorpyrifos, will form colony on the MSM plate that contain chlorpyrifos. To our knowledge, this is the ? rst report for biodegradation of chlorpyrifos by B. thuringiensis, an entomopathogen. However, the separation rate of the samples collected in this study is relative lower than that reported before

(Zhang et al., 2007). The inhibition of the growth of bacteria by chemical pollutant may contribute to such result. The results of phylogenetic tree, based on the 16S rRNA gene sequences, shows that BRC-HZM2 belonging to the Bacillus cereus group, which include Bacillus cereus, Bacillus thuringiensis and Bacillus anthracis. The bacteria in Bacillus cereus group are genetically very close, as shown by gene sequence analysis and multilocus enzyme electrophoresis (Helgason et al., 2000; Vilas-Boas et al., 2002). B. cereus and B. anthracis are human pathogenic bacteria, and B. thuringiensis is an entomopathogen and has been extensively used as a biopesticide for over 50 years (Bravo et al., 2007). However, B. thuringiensis strains can be distinguished from B. cereus and B. anthracis strains by light microscopic detection of parasporal crystals (Vilas-Boas et al., 2002). Thus, the results of phylogenetic tree and microscopy indicate that BRC-HZM2 belongs to B. thuringiensis group. There are, however, some differences in the result of biochemical and antibiotic sensitivity tests of these strains indicating that these strains may belong to different subspecies. The classic differentiation into subspecies is based on the flagellar serotype (H), and we will identify the Serotyping of these strains in further study. However, serotyping only describes one phenotypic trait of an isolate, and serotyping cannot be used as a way of predicting other phenotypic abilities (Lecadet et al., 1999; Soufiane and Cote, 2009). So, studies on protein and plasmid profiles, PCR analyses and bioassay of these specific isolate were also necessary. All data can be a theory basis for further application of this strain. There are many reports about bioremediation of B. thuringiensis to pollutant. However, this study first found that B. thuringiensis can degrade organophosphorus

pesticide. It is possible that B. thuringiensis can be used for bioremediation of pesticide pollution. Major degradation products of chlorpyrifos include TCP and DEP (Lu et al., 2012). High levels of TCP will produce an inhibitory effect on the microorganisms (Li et al., 2007). The test did not provide enough evidence to show that B. thuringiensis can degrade the TCP. It needs to verify it by using HPLC-MS to analyze metabolism products in the further study. The test results show that the strain degrade organophosphorus pesticide on a broad spectrum. Therefore, it is likely to carry a gene or enzyme which can degrade high concentration organophosphorus pesticide. This is what we want to study in the next step. The results of microscopic examination show that treatment group was not into the sporangium period while the spores of the control group strains have released. It may be that chlorpyrifos or its degradation products affected the expression of the genes related to spore forming. But mechanism and the extent of the interaction is not clear. It needs to be confirmed by using the scanning electron microscope and atomic force microscope in further research. These results also indicate the potential of the complementary relationship between B. thuringiensis and chlorpyrifos. On one hand, B. thuringiensis is widely distributed, harmless to mammals, and was able to colonize plants, that will provide a long term affect to protect them. However, it has low activity to non-target insect, such as Nephotettix cincticeps, Nilaparvata lugens, and Empoasca vitis Gothe. On the other hand, chlorpyrifos is a broad-spectrum organophosphorus insecticide, which was quite toxic to many insect species, but is harmful to the environment and human health. If we can put these two things together in order to complement each other's advantages, it is

reasonable for us to believe that B. thuringiensis can be developed to become pesticide fertilizer which will not only kill target pests, but also degrade organophosphorus pesticide. It can make B. thuringiensis become a multipurpose agricultural microorganisms and expand the application field of B. thuringiensis, and the application cost of crop protection will reduce in the near future. Compared with other pesticide degrading bacterium, B. thuringiensis has the following advantages: firstly, there are safety problems about the bacterium with degradation ability. For example, some are human pathogens and are not suitable for releasing to the environment. And B. thuringiensis is the most widely used microbial pesticides and harmless to animals and people. The biological security problems are limited. Secondly, it may reduce the cost of bioremediation. Many bacteria with degradation ability already discovered are anaerobic microbes, which were hard to produce in mass quantities. In contrary, the production process of B. thuringiensis is quite mature. If it can be successfully used in the bioremediation of environment pollutants, the cost of control of environmental pollution would be reduced. Last but not least, many reported bacteria with degradation ability have good performance in the laboratory, but the effect is not ideal once applied in the field. B. thuringiensis is expected to reach a good application effect because it is long-term used in the field. It can adapt to the climate, the pH of ecological soil and temperature. 5 ConclusionsIn summary, we have successfully isolated six B. thuringiensis strains capable of degrading chlorpyrifos from industry manufacturing organophosphate pesticides in Sanming, Fujian Province, China. This is the ? rst report of B. thuringiensis with chlorpyrifos degrading

ability combined with its insecticidal activity and proposed its application in field. The bacteria may be useful for the bioremediation of field which are contaminated with chlorpyrifos, and possibly provide new insights into microbial degradation of organophosphorus pesticide. Further studies to elucidate the enzymes and the genes involved in the degradation are now being undertaken.