Microbial Degradation of Acetamiprid by *Ochrobactrum* sp. D-12 Isolated from Contaminated Soil

Guangli Wang*, Xiao Chen, Wenlong Yue, Hui Zhang, Feng Li, Minghua Xiong

College of Life Sciences, Huaibei Normal University, Huaibei, People’s Republic of China

Abstract

Neonicotinoid insecticides are one of the most important commercial insecticides used worldwide. The potential toxicity of the residues present in environment to humans has received considerable attention. In this study, a novel *Ochrobactrum* sp. strain D-12 capable of using acetamiprid as the sole carbon source as well as energy, nitrogen source for growth was isolated and identified from polluted agricultural soil. Strain D-12 was able to completely degrade acetamiprid with initial concentrations of 0–3000 mg L$^{-1}$ within 48 h. Haldane inhibition model was used to fit the special degradation rate at different initial concentrations, and the parameters $q_{\text{max}}$, $K_1$ and $K_2$ were determined to be 0.6394 (6 h$^{-1}$), 50.96 mg L$^{-1}$ and 1879 mg L$^{-1}$, respectively. The strain was found highly effective in degrading acetamiprid over a wide range of temperatures (25–35°C) and pH (6–8). The effects of co-substrates on the degradation efficiency of acetamiprid were investigated. The results indicated that exogenously supplied glucose and ammonium chloride could slightly enhance the biodegradation efficiency, but even more addition of glucose or ammonium chloride delayed the biodegradation. In addition, one metabolic intermediate identified as N-methyl-(6-chloro-3-pyridyl)methylamine formed during the degradation of acetamiprid mediated by strain D-12 was captured by LC-MS, allowing a degradation pathway for acetamiprid to be proposed. This study suggests the bacterium could be a promising candidate for remediation of environments affected by acetamiprid.

Materials and Methods

Chemicals and Media

Analytical grade acetamiprid (purity, 99%), purchased from Wako Pure Chemical Industries (Osaka, Japan), was used as a standard. Acetamiprid samples (>97% purity) were purchased from Dongfeng Pesticides Factory (Shanghai, China). Chromatographic grade acetonitrile and acetic acid were purchased from Sigma-Aldrich (St. Louis, USA). Molecular biology reagents were purchased from TaKaRa Biotechnology Co., Ltd (Dalian). All other chemicals were of the highest grade that was commercially available.
available. Luria-Bertani (LB) medium (10.0 g NaCl, 10.0 g peptone and 5.0 g yeast extract per litre water, pH 7.0) and mineral salts medium (MSM) (1.5 g K₂HPO₄, 0.5 g KH₂PO₄, 0.2 g MgSO₄.7H₂O, 1.0 g NaCl per litre water, pH 7.0) were utilised in this study. For solid medium, agar powder was added at a concentration of 1.6%. When necessary, acetamiprid was added to the media at an appropriate concentration. All media used in this study were prepared using Milli-Q water (>18.2 MQ) and sterilised by autoclaving at 121°C for 25 min.

Isolation and identification of bacteria

A soil sample was collected from an agricultural field, which had been exposed to acetamiprid for more than 10 years, in the city of Yancheng, China. Enrichment and isolation of degrading bacterial isolates were conducted as described in detail previously [18]. The ability of isolates to degrade acetamiprid was determined by HPLC following the protocol described below. One pure isolate designated D-12 showing the highest degradation activity was selected for further study.

Strain D-12 was characterized based on its morphological, physiological and biochemical properties [20] and genetic analysis based on 16S rRNA gene sequence. The cell morphology was examined by light microscopy (BH-2, Olympus, Japan) and transmission electron microscopy (H-7650, Hitachi High-Tech- nologies Corp., Japan), using cells from an exponentially growing culture. The genomic DNA of D-12 strain was extracted by high- salt precipitation [21]. Pure cultures were phylogenetically characterized using 16S rRNA gene sequencing. Two PCR primers 27F/1492R were designed to amplify the 16S rRNA gene [22]. The 1389 bp 16S rRNA gene sequence was compared to sequences in GenBank using BLAST program. Multiple sequence alignment was carried out using Clustal X 1.8.3 with the default settings. For further phylogenetic analysis, MEGA version 4.0 software [23] was used. Distances were calculated using the Kimura two-parameter distance model. Unrooted trees were built using the Neighbor Joining method. The date set was bootstrapped 1000 times.

Acetamiprid degradation experiments

The isolated strain was grown on LB medium for 16 h at 30°C on a rotary shaker (160 rpm). Then cells were harvested and washed three times with a 0.02 mol·L⁻¹ phosphate buffer (pH 7.0). The washed cells were re-suspended in the same buffer, resulting in a cell suspension with an OD₆₀₀ of 1.0. In addition, a stock solution of acetamiprid (4,000 mg·L⁻¹) was prepared by dissolving the acetamiprid in MSM. After transferring appropriate volume of this stock solution to a 250 mL sterile flask, 100 mL of MSM was added and 1 mL of acetamiprid was added to the media at an appropriate concentration. All media used in this study were prepared using Milli-Q water (>18.2 MQ) and sterilised by autoclaving at 121°C for 25 min.

300 mg·L⁻¹ of ammonium chloride as a nitrogen source was added as sole carbon and nitrogen source set as control. Each treatment was set in triplicate.

Chemical Analysis

Cell growth was monitored by measuring the optical density of culture samples at 600 nm (OD₆₀₀). Non-inoculated medium served as control. For acetamiprid extraction from liquid culture, 5 mL of sample collected from the medium was extracted with 10 mL of dichloromethane. After shaking for 10 min, the dichloromethane phase was dried over anhydrous Na₂SO₄, and the solvent was removed using a stream of nitrogen at room temperature. The residues were dissolved in 200 μL of acetonitrile. Samples in acetonitrile were then filtered through a 0.22 μm Millipore membrane filter. An aliquot of the solution (20 μL) was injected into an HPLC system for detection.

The concentration of acetamiprid was determined by HPLC using a Zorbax C-18 ODS Spherex column (250 mm×4.6 mm). The mobile phase was 65% (volume) water and 35% (volume) acetonitrile as well as 0.01% acetic acid at a flow rate of 1 mL/min. The eluate was monitored by measuring the A242 with a Waters 2487 Dual Wavelength Absorbance Detector, and the injection volume was 20 μL. Recovery efficiency of the stated method was evaluated at the concentrations of 10, 30, 50 and 70 mg·L⁻¹ acetamiprid that appended in MSM.

The metabolites produced during acetamiprid degradation were purified using thin-layer chromatography (TLC) by concentrating the extract on a pre-coated silica-gel TLC plate (silica G, 20×20 cm, 0.25 mm thickness) with chloroform-methanol solution (20:1 by volume). The collected metabolite was dissolved in acetonitrile and centrifuged at 10,000 g to remove the silica. The organic solvents were then removed and the residue was dried under vacuum condition.

The purified metabolites were analysed by standard MS, ionized by electrospray with a positive polarity, and scanned in the normal mass. Characteristic fragment ions were detected with second-order MS.

The MS apparatus was an LC-MSD-Trap-SL system equipped with an electrospray ionization source and was operated in the positive polarity mode. The ES-MS interface was operated using a gas temperature of 35°C and a drying gas flow of 9.0 L min⁻¹. The nebulizer nitrogen gas pressure was 45 psi. Full scan signals were recorded within the m/z range from 30 m/z to 600 m/z. For LC-MS, the spray voltage was 7.0 kV. The sheath and auxiliary gases were nitrogen. The sheath and auxiliary gases were adjusted to 65 and 10 arbitrary units, respectively. Auto Gain Control mode was used to optimize the injection time.

Data Analysis

Results were also assessed by analysis of variance (ANOVA) and statistical analyses were performed on three replicates of data obtained from each treatment. The significance (P<0.05) of differences was treated statistically by single factor analysis of variance using SPSS software packages.

Ethics statement

The location that we collected samples was a very common field, so it did not need specific permission and involve any endangered or protected species. In order to avoid being influenced by plant diseases and insect pests, acetamiprid was used discontinued ten years ago.
Table 1. Recovery efficiency and regression equation determined by different concentrations of AAP for the analytical method evaluation.

| Append concentration (mg·L⁻¹) | Detected concentration | Recovery efficiency (%) | Regression equation |
|-------------------------------|------------------------|-------------------------|---------------------|
| 10                            | 10.44 ± 0.53           | 104                     |                     |
| 30                            | 28.12 ± 1.45           | 94                      | y = 12.224x + 0.0760 |
| 50                            | 43.34 ± 0.32           | 87                      | R² = 0.9982         |
| 70                            | 67.35 ± 2.15           | 91                      |                     |

Regression equation was determined by HPLC using different concentrations of standard AAP; y, AAP concentration; x, the peak area of HPLC; R², correlation coefficient of the regression equation.

doi:10.1371/journal.pone.0082603.t001

Figure 1. Phylogenetic tree constructed by the neighbor-joining method based on 16S rRNA gene sequences of D-12 and type strains of Ochrobactrum species. Bootstrap values, expressed as percentages of 1,000 replications, are given at branching points. Bar, 0.002 nucleotide substitutions per nucleotide position.

doi:10.1371/journal.pone.0082603.g001
**Results and Discussion**

Evaluation of the analytical method for acetamiprid determination

The recovery efficiency of acetamiprid in MSM are arranging from 87% to 104%. The evaluation results displayed that the analytical method applied in this study well satisfied the requirement of the pesticide analysis standard (Table 1; satisfy the pH was at 6.0-9.0, acetamiprid biodegradation was distinctly inhibited, implying that restricted growth of D-12 occasioned at pH 6.0-8.0, more than 95% of 300 mg L⁻¹ acetamiprid could be degraded by D-12 within 16 h. At pH 7.0, acetamiprid was completely degraded in 14 h. However, when the pH was at 4.0, 5.0, 9.0 or 10.0, acetamiprid biodegradation was distinctly inhibited, implying that restricted growth of D-12...
occurred at these pH values. But the temperature has no significant ($P>0.05$). Experiment showed that the optimum temperature for the biodegradation of acetamiprid was 25–35°C. Acetamiprid biodegradation efficiency was the highest at this temperature range; however, acetamiprid biodegradation decreased when the temperature dropped to 20°C or rose to 40°C, indicating that lower and higher temperatures were not beneficial for the biodegradation of acetamiprid by D-12. Therefore, pH 7.0 and 30°C were chosen for all subsequent experiments. At the same time, control experiments were carried out under the same conditions without bacteria. No obvious degradation was detected in the control experiments. The recovery rates of acetamiprid after sample pretreatment were measured between 94% and 102%.

In order to determine the effect of initial acetamiprid concentrations on degrading efficiency, biodegradation of acetamiprid by *Ochrobactrum* sp. D-12 was conducted under acetami-
Acetamiprid concentrations ranging 250, 500, 1000, 1500, 2000, 2500 and 3000 mg·L⁻¹. Strain D-12 grew on acetamiprid up to the concentration, as high as 3000 mg·L⁻¹. As shown in Fig. 4, the lag phase was extended at higher acetamiprid concentration. At concentrations of 250, 500, 1000, 1500 and 2000 mg·L⁻¹, the degradation rates reached 70.80%, 66.80%, 64.00%, 60.27% and 55.60% after 12 hours of incubation, respectively. However, only 50.68% and 39.27% degradation were achieved at higher initial concentrations of 2500 and 3000 mg·L⁻¹, respectively.

The decrease in the specific acetamiprid degradation rate with an increase in the initial acetamiprid concentration suggests that acetamiprid may act as a partial inhibitor to strain D-12. Therefore, the substrate inhibition model Eq. (1) adapted from [35] was used to explain the degradation kinetics of acetamiprid by strain D-12.

\[
q = \frac{q_{max}S}{S + Ks + (S/Ki)}
\]
Effects of different co-substrates on the biodegradation of acetamiprid by strain D-12

Industrial wastes often contain a mixture of recalcitrant compounds as well as easily biodegradable compounds. Investigation of the biodegradation of refractory pollutants in the presence of accessible carbon and nitrogen sources might aid in reducing the toxic and growth-inhibiting effects of xenobiotics on cells, thereby increasing the transformation rates of xenobiotics [36–38]. However, the reverse consequences had been also reported [39–42]. Consequently, effect of the two substrates on degradation of piperazine [43]. Based on these studies, appropriate amounts of ammonium chloride as a second nitrogen source (co-substrate) on the biodegradation of acetamiprid by strain D-12 was investigated.

The acetamiprid degradation efficiency could reach as high as 73% within 12 h when only acetamiprid was added as a sole carbon source (control). Comparatively, after adding 100 mg L\(^{-1}\) glucose as the second carbon source (Fig. 6a), the acetamiprid degradation efficiency was enhanced and 200 mg L\(^{-1}\) acetamiprid was completely degraded within 10 h. However, when more than 300 mg L\(^{-1}\) glucose was added, the acetamiprid degradation efficiency decreased slightly within the same period (Fig. 6a). It has been reported that the addition of glucose promotes the growth of the strain and thus stimulates the degradation of piperazine [43]. The same phenomenon could be observed in the literatures [36–38,44,45].

It was found that the addition of 300 mg L\(^{-1}\) and 600 mg L\(^{-1}\) ammonium chloride could evidently enhance the degradation process and acetamiprid was completely degraded in less 12 h than the control (Fig. 6b), but even more addition (above 900 mg L\(^{-1}\)) of ammonium chloride would delay the biodegradation of acetamiprid by strain D-12. These results are consistent with those of Wang et al. (2012) who found that the addition of 500 mg L\(^{-1}\) ammonium chloride could enhance the biodegradation of 600 mg L\(^{-1}\) nitrobenzene, while the addition of greater amounts of ammonium chloride delayed biodegradation [4]. Luo et al. (2008) found that the degradation of 100 mg L\(^{-1}\) bensulphuron-methyl in the presence of 1000 mg L\(^{-1}\) ammonium chloride was greater than in the sample without it. Moreover [37], Qiao and Wang (2010), demonstrated the opposite effect, who found that 100 mg L\(^{-1}\) ammonium chloride mildly inhibits the biodegradation of 900 mg L\(^{-1}\) pyridine [46]; Cai et al. (2013) illustrated that the addition of ammonium did not have an evident growth-promoting effect while slightly inhibiting the degradation of piperazine [43]. Based on these studies, appropriate amounts of an extra nitrogen source may inhibited the biodegradation of toxic compounds.

Furthermore, when both glucose and ammonium chloride were added as co-substrates, the degradation efficiency of acetamiprid was much higher compared to that with addition of either glucose or ammonium chloride as carbon or nitrogen source. As shown in Fig. 6a and Fig. 6c, when adding both 100 mg L\(^{-1}\) glucose and 300 mg L\(^{-1}\) ammonium chloride, the acetamiprid degradation
efficiency was enhanced by 20% or 50% than that with the addition of same dosage of glucose or ammonium chloride within 10 h, which implied that the performance of biodegradation is likely to be even better in the presence of both carbon and nitrogen sources.

Conclusions

Strain D-12 isolated in the present study appeared to be highly efficient in degrading acetamiprid in different contaminated soils and water resources, thus suggesting the isolate could be a significant potential use for the cleanup of acetamiprid-contaminated soil. Degradation of acetamiprid occurred at 25–35°C and pH 6–8. This is an important feature of a microorganism to be employed for bioremediation of variable environments. Another important feature which is worth mentioning is that the bacterium utilized acetamiprid as the sole carbon source as well as energy, nitrogen source for growth. Moreover, the strain harbors the metabolic pathway for the detoxification of acetamiprid. Finally, this is the first report about biodegradation of acetamiprid by a bacterial strain from the Ochrobactrum genus.

Supporting Information

Figure S1  Transmission electron micrograph of strain D-12. Bar, 1.0 μm.
(TIF)

Figure S2  The degradation products of acetamiprid in the culture extracts were detected by HPLC.
(TIF)

Figure S3  Proposed pathway for acetamiprid degradation by Ochrobactrum sp. strain D-12.
(TIF)

Acknowledgments

The authors would like to thank the academic editor and the two anonymous reviewers for their helpful comments and suggestions. We also gratefully acknowledge B.S. Hao Gao of Huaibei Normal University for excellent assistance in the kinetics analysis.

Author Contributions

Conceived and designed the experiments: GLW, WLY. Performed the experiments: XC, MHX. Analyzed the data: HZ, FL. Contributed reagents/materials/analysis tools: GLW. Wrote the manuscript: GLW.
References

1. Arther RG, Cunningham J, Dorn H, Everett R, Herr LG, et al. (1997) Efficacy of imidacloprid for removal and control of fleas (Ctenocephalides felis) on dogs. Am J Vet Res 58: 649–650.

2. Jacobs DE, Hutchinson MJ, Fox MT, Krueger KJ (1997) Comparison of flea control strategies using imidacloprid or fipronuron on cats in a controlled simulated home environment. Am J Vet Res 58: 1260–1262.

3. Yamamoto I, Casida JE. (1999) Neonicotinoid insecticides and the nicotine acetylcholine receptor. Toxicology. Springer, Tokyo.

4. Wang J, Hirai H, Kawagishi H (2012) Biotransformation of acetamiprid by the white-rot fungus Phanerchnus sudicus YK-624. Appl Microbiol Biotechnol 93: 831–835.

5. U.S. Environmental Protection Agency, http://www.epa.gov/opprd001/factsheets/acetamiprid.pdf. (2002).

6. Singh DK, Kumar S (2008) Nitrate reductase, arginine deaminase, urase and dehydrogenase activities in natural soil (ridge with forest) and in soil after leaching treatments. Chemosphere 71: 412–418.

7. Seccia S, Fidente P, Barbini DA, Morricia P (2005) Multi residue determination of neonicotinoid insecticide residues in drinking water by liquid chromatography with electrospray ionization mass spectrometry. Anal. Chim. Acta 533:21–26.

8. Marino Y, Dai YJ, Chen T, Yuan S, Ni JP, et al. (2008) Persistence of acetamiprid in/on mustard (Brassica juncea L.). Bull Environ Contam Toxicol 76: 356–360.

9. Sanyal D, Chakma D, Alam S (2008) Persistence of a neonicotinoid insecticide, acetamiprid on chilli (Capsicum annuum L.). Bull Environ Contam Toxicol 81: 365–368.

10. Arora PK, Sasikala C, Ramana CV. (2012) Degradation of chlorinated nitroaromatic compounds. Appl Microbiol Biotechnol 93: 2263–2277.

11. Fan X, Liu X, Huang R, Liu Y (2012) Identification and characterization of a novel thermotolerant pyrethroid-hydrolyzing enzyme isolated through metagenomic approach. Microb Cell Fact 11: 33.

12. Zhang HZ, Li J, Hu HY, Xu P (2012) A newly isolated strain of Bacillus licheniformis sp. hydrolyzes acetamiprid, a synthetic insecticide. Process Biochem 47: 1820–1825.

13. Bai Y, Yin Z, Zhang W, Yu C, Ji W, et al. (2010) Biotransformation of thiacloprid by bacterium Stenotrophomonas maltophilia. APSE 101: 3033–43.

14. Dai Y, Zhao Y, Zhang W, Yu C, Ji W, et al. (2010) Biotransformation of thiamethoxam neonicotinoid insecticides: diverse molecular substituents response to metabolism by bacterium Stenotrophomonas maltophilia CGMCC 1.1781. Biorresour Technol 101: 3838–43.

15. Dai YJ, Ji WW, Chen T, Zhang WJ, Liu ZH, et al. (2010) Metabolism of the neonicotinoid insecticides acetamiprid and thiadiazuron by the yeast Rhodosporidium mucilaginosum strain BM-2. J Agric Food Chem 58: 2419–25.

16. Zhu Y, Dai YJ, Chen LL, Sun J, Zhou J, Fan Y, et al. (2009) Hydroxylation of thiadiazuron by bacterium Stenotrophomonas maltophilia (CGMCC 1.1781). Biodegradation 20: 761–768.

17. Chen T, Dai YJ, Ding JF, Yuan S, Ni JP, et al. (2008) N-demethylation of neonicotinoid insecticide acetamiprid by the yeast Rhodosporidium mucilaginosum strain BM-2. J Agric Food Chem 56: 7831–8315.

18. Hu JY, Fan Y, Lin YH, Zhang HB, Ong SL, et al. (2003) Microbial diversity and prevalence of virulent pathogens in biofilms developed in a water reclamation system. Res Microbiol 154: 623–629.

19. Tamura K, Dudley J, Nei M (2007) MEGA 4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596–1599.

20. Fitzgerald J (2004) Laboratory bioassays and field evaluation of insecticides for the control of Anthomus ruhi, Lygus rugulipennis and Chilo suppressalis, and effects on beneficial species in UK strawberry production, Crop Prot 23: 801–809.

21. Yoo KH, Min H, Lu ZH, Yuan HP (2006b) Influence of acetamiprid on soil enzymatic activities and respiration. Eur J Soil Biol 42: 120–126.

22. Danko AS, Liao MZ, Bagwell CE, Brignoni RL, Freedman DL, et al. (2004) Involvement of Linear Plasmids in Aerobic Biodegradation of Vinyl Chloride. Appl Environ Microbiol 70: 6092–6097.

23. Wang JL, Quan XC, Han LP, Qian Y, Werner H, et al. (2001) Biodegradation of nicotine from tobacco waste extract by Ochrobactrum intermedium DNV. J Ind Microbiol Biotechnol 34: 567–570.

24. Qian XL, Bai WQ, Zhong QZ, Li M, He MQ, et al. (2006) Isolation and characterization of a bacterial strain of the genus Ochrobactrum with methyl parathion mineralizing activity. J Appl Microbiol 101: 986–994.

25. Veerranagouda Y, Patal PVE, Gorla P (2006) Complete mineralisation of dimethyldichloroacetamide by Ochrobactrum sp. DGVK1 isolated from the soil samples collected from the coalmine leftovers. Appl Microbiol Biotechnol 71: 369–375.

26. Yuan YJ, Lu ZK, Huang JY, Li Y, Lu FX, et al. (2007) Biodegradation of nicotine from tobacco waste extract by Ochrobactrum intermedium DNV. J Ind Microbiol Biotechnol 34: 567–570.

27. Brunet JL, Badiou A, Belzunces LP (2005) In vivo metabolic fate of [14C]-acetamiprid in six biological compartments of the honeybee, Apis mellifera L. Pest Manag Sci 61: 742–748.

28. Tomizawa M, Lee DL, Casida JE. (2000) Neonicotinoid insecticides: Molecular features conferring selectivity for insect versus mammalian nicotinic receptors. J Agric Food Chem 48 (12): 6016–6024.

29. Ford KA, Casida JE (2008) Comparative metabolism and pharmacokinetics of seven neonicotinoid insecticides in spinach. J Agric Food Chem 56: 10168–10175.

30. Chen J, Dong YH, Chang C, Deng Y, Zhang XF, et al. (2013) Characterization of a novel cyfluthrin-degrading bacterial strain Brevibacterium sp. and its biochemical degradation pathway. Bioresour Technol 132: 16–23.

31. Wang JL, Quan XC, Han LP, Qian Y, Werner H, et al. (2002) Kinetics of co-metabolism of quinoline and glucose by Bacillus pumilus. Process Biochem 37: 83–86.

32. Luo W, Zhao YH, Ding HT, Lin XY, Zheng HB, et al. (2008) Co-metabolic degradation of bensulfouron-methyl in laboratory conditions. J Hazard Mater 150: 206–214.

33. Mohammad ZK, Pijush KM, Suhail S, Vinoth T (2011) Degradation pathway, toxicity and kinetics of 2,4,6-trichlorophenol with different co-substrate by aerobic granules in SBR. Bioresour Technol 102: 7016–7021.

34. Lin Q, Wanga JL (2010) Microbial degradation of pyridine by Paracoccus sp. isolated from contaminated soil. J Hazard Mater 176: 220–225.

35. Xiong RL, Chen LJ, Liu JI (2009) Biodegradation of pyridine with co-substrates by Paracoccus diminutus W12. Journal of Tsinghua University (Science and Technolgy) 49: 826–829.

36. Wang JL, Quan XC, Han LP, Qian Y, Werner H, et al. (2001) Biodegradation kinetics of a mixture containing quinoline and glucose by Bacillus pumilus. Process Biochem 37: 83–86.

37. U.S. Environmental Protection Agency, http://www.epa.gov/opprd001/factsheets/acetamiprid.pdf. (2002).

38. Qiao L, Wang JL (2010) Microbial degradation of pyridine by Paracoccus sp. isolated from contaminated soil. J Hazard Mater 176: 220–225.