

# Isolation, characterization and phylogenetic analysis of a bacterial strain capable of degrading acetamiprid

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**Abstract:** An aerobic bacterium, capable of degrading the new chloronicotine pesticide acetamiprid, was isolated from the sludge of pesticide factory after successive enrichment cultures and named strain FH2 which is a Gram-negative, rod-shaped, obligate aerobic organism with  $((0.5-0.7) \times (1.5-3.0))\mu\text{m}$  of cell size and with monotrichous flagellum. It was identified as a member of *Pseudomonas* sp. based on morphology, physio-biochemical properties, Biolog GN2, 16S rDNA sequence and phylogenetic characteristic analysis. The isolate could grow optimally at pH 7.0 and 30°C in acetamiprid-mineral medium with 800 mg/L concentration. About 53.3% acetamiprid was degraded by strain FH2 after incubation for 14 d in acetamiprid-mineral medium and nearly 96.7% degraded when incubated in acetamiprid-yeast mineral medium at 30°C for 14 d. This paper describes phylogenetic and degradation characterization of a pure bacterium being able to mineralize acetamiprid for the first time.

**Keywords:** acetamiprid; biodegradation; characterization; isolation; *Pseudomonas* sp.; 16S rDNA

## Introduction

Pesticides have been found as important chemical pollutants in soils and groundwater (Miles, 1992) and have caused considerable environmental and public health consequences all over the world (Richardson, 1998). There have been great concerns on how to degrade the pesticides using microorganism. Moreover, some processes have been made in research works on biodegradation of the rudimental worldwide-used pesticides, such as organochlorine and organophosphorous pesticides. However, new pesticides are being produced and used day by day now. Therefore, there is a serious need to develop remediation processes to eliminate or minimize some new pesticide contamination in soil and groundwater.

Acetamiprid, [(E)-N<sup>1</sup>-(6-chloro-3-pyridyl) methyl-N<sup>2</sup>-cyano-N<sup>1</sup>-methylacetamidine], a kind of new insecticide, was developed by Soda Co. Ltd, Japan, for control of Hemiptera, mainly aphids, thysanoptera and lepidoptera on various crops, especially vegetables, fruits and tea (Roberts and Hutson, 1999). Acetamiprid is being more competitive than conventional insecticide due to its special acting characteristics (Yamamoto *et al.*, 1998) and has been considered as an important substitute to the organophosphate insecticides which have caused severe environmental pollution and pesticide-resistance of insects, banned in many countries by now. Although chemical and physical processes may be involved in the removal of this compound, biodegradation is reported to be the most significant mechanism for its dissipation from

soil (<http://www.epa.gov/opprd001/factsheets/>). To our knowledge, there are few reports concerning the microbial degradation of acetamiprid up to now. Therefore, the goal of this paper was to isolate and identify an acetamiprid-degrading bacterium from acetamiprid-producing factory sludge, and characterize its degradation.

## 1 Materials and methods

### 1.1 Sludge samples

The sludge used for isolation was collected from Dongfeng Pesticide Factory, Shanghai, China.

### 1.2 Chemicals

The standard acetamiprid (99.99% purity) and the technical-grade acetamiprid (97% purity) were purchased from Dongfeng Pesticide Factory (Shanghai, China). All other chemicals used in the work were analytical grade from China Chemical Medicine Co. Ltd.

### 1.3 Media

The liquid mineral medium contained (in 1 L distilled water): 1 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 0.5 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g NaH<sub>2</sub>PO<sub>4</sub>, 0.1 g MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.05 g CaCl<sub>2</sub>·2H<sub>2</sub>O, 0.02 g FeCl<sub>3</sub>, adjusted pH to 7.0. Agar 2% was added for solid mineral medium. Media were autoclaved at 121°C for 20 min. Then some volume of filter-sterilized (0.2- $\mu\text{m}$  pore-size cellulose nitrate membrane) acetamiprid solution was supplemented to mineral medium after being cooled, according to experimental requirement. In order to accelerate degradation and growth, 0.25% yeast extract was

added to acetamiprid-mineral medium.

#### 1.4 Enrichment and isolation

Acetamiprid-degrading bacteria were enriched from sludge continuously for more than 2 months with mineral media containing 200 mg of acetamiprid per liter. Amount of acetamiprid in enriched media was fortified every week by addition of 100 mg of acetamiprid per liter. Sample from the enrichment culture was streaked on the solid acetamiprid-mineral medium plate and incubated at 30°C. Colonies developed after incubation for 4 d were picked up and purified repeatedly. The isolate capable of growing on medium containing acetamiprid as sole carbon source, named strain FH2, was obtained.

#### 1.5 Optimal growth conditions

Cells cultured in an acetamiprid-mineral medium collected by centrifugation, and washed three times using 15 mmol/L phosphate buffer with pH 8.0. The washed cells were inoculated into the acetamiprid mineral medium contained 0, 200, 400, 600, 800, 1000, and 1200 mg/L acetamiprid, respectively. The same amount cells were inoculated into the acetamiprid(800 mg/L) mineral medium, pH of which was adjusted to 5.0, 5.5, 6.0, 6.5, 7.0 and 7.5, respectively. Cultivation was incubated at 30°C in a rotary shaker (150 r/min) for 5 d. The OD<sub>600</sub> was measured and optimal pH was determined. The same amount cells were inoculated into the acetamiprid-mineral medium (pH 7.0) and incubated at 4, 20, 25, 30, 35 and 41°C, respectively, at 150 r/min for 5 d. The OD<sub>600</sub> was measured and optimal growth temperature was determined.

#### 1.6 Degradation of acetamiprid by strain FH2

The washed cells were inoculated into the acetamiprid (800 mg/L) mineral medium with or without yeast extract and incubated at 30°C and 150 r/min for 14 d. The cell biomass and acetamiprid concentration was determined at 2 d intervals. The non-inoculated medium was served as the negative control. All treatments were triplicated.

#### 1.7 Analytical methods

The acetamiprid concentration in the liquid cultures was analyzed by HPLC. The culture broth was extracted with the twice volume of dichloromethane and dried with flushing nitrogen gas, then re-dissolved in methanol. Amount of acetamiprid in the extract was determined by high-pressure liquid chromatography(Agilent 1100 Series, USA) equipped with ODS C18 capillary column (Agilent Inc., USA, 4.6 mm i.d. × 150 mm). A mixture of methanol and ddH<sub>2</sub>O (60%/40%, v/v) at a flow rate of 0.8 ml/min was used as the mobile phase and wavelength for the

detection was 254 nm(Tokieda *et al.*, 1998; Bi, 2002; Obana *et al.*, 2002). The cell biomass of the cultures was estimated by measuring optical density (OD) at 600 nm using a 752 spectrophotometer.

#### 1.8 Identification and characterization

To identify the isolate, biochemical detection experiments were performed as references(Holt *et al.*, 1994; Li *et al.*, 1996; Dong and Cai, 2001). In addition, the biolog microstation (GN2) (Biolog Hayward, CA, USA) was used to identify carbon source utilization patterns of the isolate. The cell of strain FH2 was negatively stained with 1% phosphotungstate and its morphological characteristic was observed using transmission electron microscope (JEM-1200EX, Japan).

The cells of strain FH2 were harvested from cultures grown in Luria-Bertani medium (LB) at 30°C for 24 h and its genomic DNA was extracted by the method of Yoon *et al.*(1996). The G+C mol% content in DNA of the isolate was measured by thermal denaturation method (Marmur and Doty, 1962). DNA from *Escherichia coli* strain K-12 was used as a reference. The 16S rRNA gene was amplified by PCR using the universal primers 5'-AGAGTTTGATCC-TGGCTCAG-3'(16S rRNA gene position 8—27 of *E. coli*) and 5'-AAGGAGGTGATCCAGCCGCA-3' (16S rRNA gene position 1522—1541 of *E. coli*) (Devereux and Willis, 1995). QIAGEN DNA purification Kit was used to purify PCR products. Sequencing of PCR products was accomplished by Shanghai BioAsia Bio-Technology Co. Ltd.

#### 1.9 Sequence alignment and phylogenetic tree construction

The 16S rDNA sequence of the isolate was deposited in GenBank (No. AJ868441). The 16S rDNA sequences of other 16 *Pseudomonas* sp. were obtained from the GenBank database (National Central for Biotechnology Information, NCBI) (Wheeler *et al.*, 2001) using the BLAST search program (Altschul *et al.*, 1997). The 16S rDNA sequence of the strain FH2 was aligned with reference sequences obtained from GenBank database using CLUSTAL W program(Thompson *et al.*, 1997), and a phylogenetic tree was generated using the DNAMAN software 5.22(Lynnon Biosoft, Quebec, Canada).

## 2 Results and discussion

### 2.1 Morphological and biochemical properties of strain FH2

A bacterial strain, named FH2, capable of growing on acetamiprid mineral agar plate, was isolated and purified. Colonies of strain FH2 grown on

LB plate were circular, 2–3 mm in diameter, rumple surfaces, convex with undulate margins, opaque, and buff, and also circular, but only 1–2 mm in diameter and white at first, then became to yellow about 2 weeks later on acetamiprid-mineral agar plate. Strain FH2 was a Gram-negative, rod-shaped, obligate aerobic organism with  $(0.5\text{--}0.7 \times 1.5\text{--}3.0) \mu\text{m}$  of cell size and with monotrichous flagellum(Fig.1).

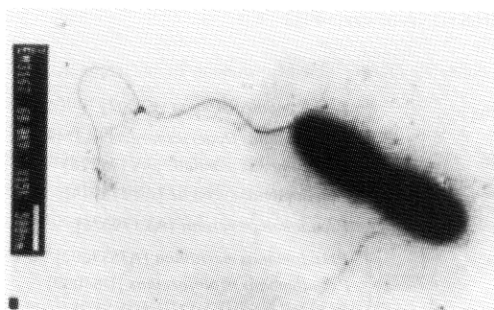


Fig.1 Electron micrograph of strain FH2 cells ( $\times 15000$ )

The isolate could grow on mineral medium only containing simple carbon source without any growth factor. The conventional biochemical test results are summarized in Table 1.

Table 1 Results of conventional biochemical tests of strain FH2

Biochemical reactions	Result	Biochemical reactions	Result
Oxidase	+	V. P reaction	-
Catalase	+	M. R reaction	-
Lysine decarboxylase	-	Indole test	-
Arginine dihydrogenase	+	Growth at 41 °C	+
Ornithine decarboxylase	-	Growth at 4 °C	-
Phenylalanine deaminase	-	Gelatin hydrolysis	+
Nitrate reaction	+	Starch hydrolysis	-
Tween 80	+	Tryptophan deaminase	-
Fermentation/oxidation of dxylose	+*	Fermentation/oxidation of glucose	+*
L-rhamnose	+*	D-mannose	+*
Fructose	+*	Glycerol	+*
D-mannitol	+**		

Notes: \* With production of acidic matter; \*\* without production of acidic matter

An appropriate Biolog GN2 MicroPlate was applied to identify strain FH2 which could utilize very strongly or strongly 53 of 95 substrates and utilize weakly the other 14 substrates in nutrition pools of Biolog system after incubation for 16 h. The Biolog GN data was in an excellent match to the Biolog database descriptions of genus *Pseudomonas*, but the oxidase of which is positive.

## 2.2 G+C mol% in DNA

The G+C content in DNA of strain FH2 is 67.1 mol%, which lies just within the scope of 58–70 mol% of G+C content in *Pseudomonas* (Dong and Cai, 2001).

## 2.3 16S rDNA sequence alignment and phylogenetic tree construction

The 16S rDNA of strain FH2 was amplified(Fig. 2) and partial nucleotide acids sequence of that was sequenced successfully(Fig.3), and the accession number of the sequence deposited in GenBank is AJ868441.

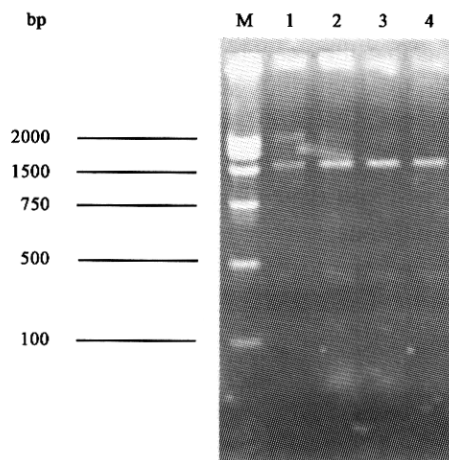


Fig.2 Agarose electrophoresis of 16S rDNA of PCR product

Based on the comparison of a partial 16S rDNA gene sequences from the GenBank Database, the 16S rDNA sequence of strain FH2 exhibited relatively high similarity, more than 97%, with that of many species in genus *Pseudomonas*, and even up to 99% of similarity with that of a strain of *Pseudomonas citronellolis*.

A phylogenetic tree(Fig.4) was constructed based on 16S rDNA sequences of strain FH2 and 16 other members of genus *Pseudomonas*.

Strain FH2 was lied within *Pseudomonas* sp., according to above results demonstrated by phenotypic characteristics, Biolog GN2 and alignment of the 16S rDNA sequencer using Blast program. Fig.2 shows that strain FH2 is closely related with *Pseudomonas citronellolis* strain ATCC 13674<sup>T</sup> and the sequence similarity reaches highly up 99.5%.

Bacteria in *Pseudomonas* are commonly found in water, soil and other environments where have been polluted by different toxic xenobiotic compounds, and many strains which can degrade insecticide have been isolated(Martens, 1976; Larkin and Day, 1986; Dumas *et al.*, 1989; Chapalmadugu and Chandhry, 1993; Chaudhry *et al.*, 1998). Therefore, it is not surprising acetamiprid-degrader, FH2, was a member of the

GCAAGTCGAGCGGATGAAGGGAGCTTGCTCCCGGATTCAGCGGCGGACGGGTGAGTAATGC  
CTAGGAATCTGCCTGGTAGTGGGGGACAAACGTTCCGAAAGGAGCGCTAATACCCGCATACGT  
CCTACGGGAGAAAAGTGGGGGATCTTCGGACCTACGCTATCAGATGACTAGGTCCGGA T T  
AGCTAGTAGGTGGGGTAATGGCTACCTAGGCGACGATCCGTAACCTGGTCTGAGAGGAT GA  
TCAGTCACACTGGAAGTGAACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT AT  
TGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGTCTTCGGATT  
GTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACC  
AACAGAATAAGCACC GGCTAACTTCGTGCCAGCAACCGCGGTAATACGAAGGGTGCAAGCG  
TTAATCGGAATTACTGGGCGTAAAGCGCGGTAGGTGGTTTTGGTAAGATGGATGTGAAATCC  
CCGGGCTCAACCTGGGAAGTGCATCCATAACTGCCTGACTAGAGTACGGTAGAA

Fig.3 The partial nucleotide acids sequence of 16S rDNA of strain FH2

genus *Pseudomonas*. However, the identification of species in this genus is difficult as the differentiation among some species in genus *Pseudomonas* is not so significant that they can be easily distinguished. The techniques used here only allow identification at genus level, thus it is not sure which species the strain FH2 is.

#### 2.4 Growth and degradation under various nutritional conditions

The growth of strain FH2 under different culture conditions is shown in Fig.5.

Strain FH2 could grow well if the acetamiprid concentration in medium was from 200 to 800 mg/L, and would be restrained otherwise. And it grew rarely at pH 5.0 and very well at pH 7.0. When pH was 7.5, however, the growth dropped significantly. That the gradual decomposition of acetamiprid at alkaline situation (<http://www.epa.gov/oppr001/factsheets/acetamiprid.pdf>) may cause the growth decline of strain FH2 remarkably. Strain FH2 could not grow at 4°C and 41°C in acetamiprid mineral medium but well at 30°C with growth range of 20°C to 35°C.

Strain FH2 could grow on mineral medium

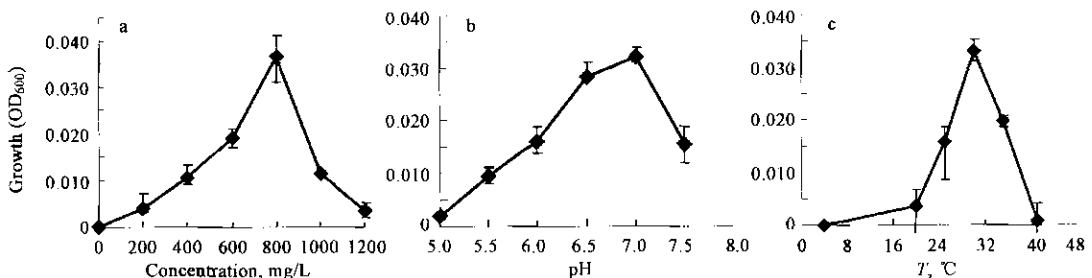


Fig.5 The growth of strain FH2 under different culture conditions  
a. acetamiprid concentration; b. initial pH; c. culture temperature

containing acetamiprid as sole carbon source and degrade acetamiprid, but both of the rates of degradation and growth were relatively slow (Fig.6). Acetamiprid was slowly degraded from 800 mg/L down to 426.27 mg/L, only decrease of about 53.3%, within 18 d. The cell biomass of strain FH2 in acetamiprid mineral medium was a little and the OD<sub>600</sub>

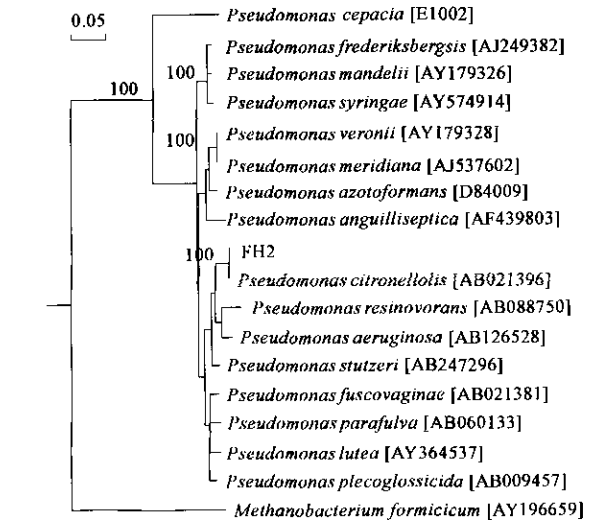


Fig.4 Phylogenetic tree based on the 16S rDNA sequences of strain FH2 and other *Pseudomonas* sp. The GenBank accession number for each microorganism used in the analysis is shown in parentheses after species name. The tree was generated using the Neighbor-joining method in DNAMAN. Horizontal distances are proportional to sequence distances (see scale bar); vertical distances are arbitrary. The numbers at each branch indicate the percentage of 1000 bootstrap, which supports the grouping at that node

was rarely more than 0.12 after incubation for 18 d. This result was well consistent with the results obtained by Berger *et al.* (1998) and Braschi *et al.* (2000) that different bacteria or fungal strains would have the low degrading ability when related compounds were supplemented as sole carbon source. Some organic matter might be added to medium to

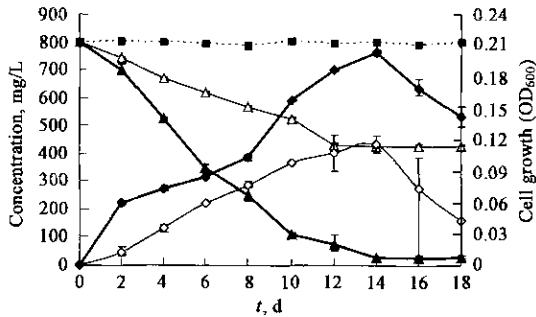


Fig.6 The degradation of acetamiprid and growth of strain FH2 in acetamiprid-mineral medium with or without yeast extract, respectively ■. acetamiprid concentration in non-inoculated cultures; ▲. acetamiprid concentration in the medium with yeast extract; ◆. OD<sub>600</sub> of culture in medium with yeast extract; △. acetamiprid concentration in the medium without yeast extract; ◇. OD<sub>600</sub> of culture in medium without yeast extract

increase the degrading rate of acetamiprid.

Yeast extract could stimulate effectively the growth and degrading rate of acetamiprid (Fig.5a). The result indicated that almost all of acetamiprid could be utilized by FH2 in yeast-acetamiprid mineral medium within 14 d. Growth reached the first stationary phase at the day 6 and began the second growth after the day 6. Acetamiprid was rapidly degraded (decrease rate of about 68.5%) within the initial 8 d with concomitant increase of biomass. The residual acetamiprid (about 27.8%) was degraded by strain FH2 following further cultivation for 6 d. This might be explained by the fact that the nutrients in the media supported good growth of the cells, as was clearly confirmed by cell density at OD<sub>600</sub>. And indeed, the more abundant cellular biomass permitted the attack of molecule in a non-specific way by constitutive enzymes (Daffonchio *et al.*, 1999). It was obviously that organic matter might accelerate degradation of acetamiprid by strain FH2 under the described experimental conditions.

### 3 Conclusions

Acetamiprid-degrading bacteria were isolated from sludge of pesticide factory. The isolate was identified as *Pseudomonas* sp. based on morphology, physio-biochemical properties and sequencing of partial 16S rDNA. The optimal initial growth pH of medium and temperature was 7.0 and 30°C, and the optimal acetamiprid concentration in medium was 800 mg/L. Strain FH2 could degrade about 53.3% acetamiprid in mineral medium containing 800 mg/L acetamiprid, while could degrade nearly 96.7% acetamiprid in acetamiprid mineral medium added 0.25% yeast extract, indicating yeast extract may stimulate degradation of acetamiprid and growth of

strain FH2.

Degradation pathway and secondary products of acetamiprid were not detected in the work. Shelton *et al.* (1996) reported that some toxic products or refractory compounds could be produced during pesticide biotransformation by microorganisms. It is very important that the remained pesticide residues are completely degraded or mineralized for bioremediation of contaminated environments. Further research works are necessary, interesting and challenging on identification of these metabolites, detection of the possible degradation pathway, correlative catabolic enzymes and genes.

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