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Short Communication

The bacterium associated with the entomopathogenic nematode *Steinernema abbasi* (Nematoda: Steinernematidae) isolated from Taiwan

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ABSTRACT

A symbiotic bacterium of the entomopathogenic nematode, *Steinernema abbasi*, isolated from Taiwan, determined to be a species of *Xenorhabdus* based on its physiological and biochemical characteristics has been determined to be similar to *Xenorhabdus indica* of *S. abbasi* Oman isolate as based on sequence analyses of 16S rDNA.

A bacterium associated with the entomopathogenic nematode, *Steinernema abbasi,* isolated from soil in the Sultanate of Oman, was determined as *Pseudomonas (Flavimonas) oryzihabitans* based on electron-microscopic observations and 16S rRNA analysis (Elawad et al., 1997, 1999; Samaliev et al., 2000). However, the symbiotic bacteria in the taxa *Steinernema* and *Heterorhabditis* are generally included under *Xenorhabdus* and *Photorhabdus* (Bird and Akhurst, 1983). *P. oryzihabitans*, isolated initially from rice paddies and clinical specimens, is a Gram negative and an aerobic bacterium (Kodama et al., 1985). It is a soil-inhabiting and saprophytic bacterium that survives in moist environments and occasionally found in the immunocompromised patients (Decker et al., 1991). *P. oryzihabitans* has a single polar flagellum, and can suppress the activities of the second stage juveniles of *Meloidogyne javanica* (Samaliev et al., 2000), and infect larvae of Lepidoptera and Coleoptera as well (Elawad, 1998). Therefore, *P. oryzihabitans* symbiotic nature of *P. oryzihabitans* with *S. abbasi* remains controversial.

To date, the symbiotic bacteria associated with entomopathogenic nematodes have been identified mainly based on the phenotypic, biological and biochemical characteristics (Akhurst, 1983; Boemare et al., 1993; Akhurst et al., 1996; Suzuki et al., 1996; Givaudan and Lanois, 2000; Walsh and Webster, 2003), and by using genomic DNA, DNA hybridization techniques and/or 16S rDNA analyses (Brunel et al., 1997; Fischer-Le et al., 1998). Although the classification using molecular–biological techniques is being applied widely, examination of phenotypic characteristics, especially those relating to synthesis of lipase, proteinase, crystal proteins, pigments and antibiotics are considered critical in the determination of a bacterium. In addition, the 16S rRNA sequencing is commonly applied to determine the identity of symbiotic bacteria and to work out their phylogenetic relationships (Rainey et al., 1995; Suzuki et al., 1996; Brunel et al., 1997; Liu et al., 1997; Szallas et al., 1997). In this study, therefore, we have adopted biological and biochemical characteristics as well as 16S rDNA analyses to clarify the taxonomic status of the bacterium associated with *S. abbasi*, which was isolated from a sweet-potato field near Hwa-lien County in eastern Taiwan in 1998 by Liao et al. (2001). The nematode has been regularly reproduced in larvae of *Spodoptera litura* as a laboratory culture, since 1998.

Purity of the bacterium isolated from *S. abbasi* was verified by plating on NBTA medium [nutrient agar (NA) supplemented with 0.004% (w/v) triphenyltetrazolium chloride (TTC, Sigma, St. Louis, KS) and 0.0025% (w/v) bromothymol blue (BTB, Sigma. St. Louis, KS)] maintained at 25 °C (Akhurst, 1980). The isolate was examined for key morphological characteristics of *Xenorhabdus* (Fig. 1) following the methods of Boemare and Akhurst (1988). The cell is rod-shaped without endospore and shows primary and secondary forms. The primary form is mobile with peritrichous flagella and is two times larger than the secondary form, while the secondary form is immobile without flagella. This was the same as *X. nematophilus* observed using electron microscopy (Givaudan and Lanois, 2000).

The phenotypic characterization of the symbiotic bacterium was generically referred to that of *Xenorhabdus* by Boemare and

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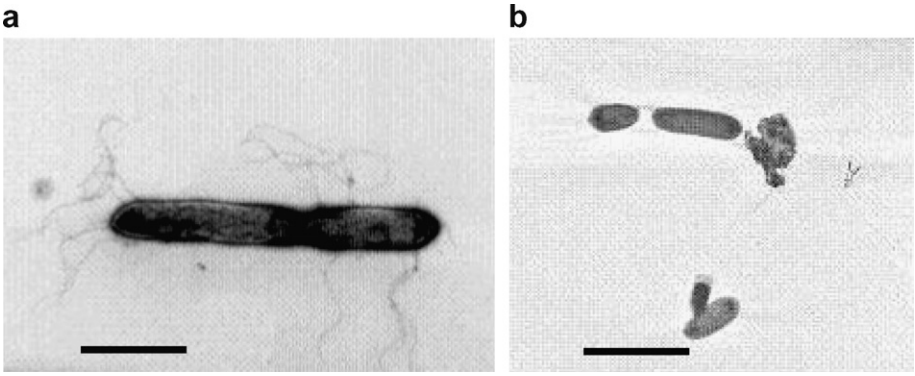

Fig. 1. The transmission electron micrographs of bacterial cells isolated from *Steinernema abbasi*. (a) primary form; (b) secondary form. Bar = 1 μm.

Akhurst (1988). Our bacterial isolate was cultured on NA, NBTA and MacConkey's medium (50 g MacConkey agar powder in l L of distilled water, DIFCO, Sparks, MD) at 25 °C. Their colonies were observed at 72 h after culturing. The primary and secondary forms were observed and photographed in a transmission electron microscope (JEOL 200-CX, Japan) using negative staining. In NA medium, both forms were creamy and beige, revealing that they were unpigmented. Their colonies were flat and glossless. In NBTA medium, colonies of the primary form were flat and cyan with pale blue in the surrounding, indicating that they could absorb and react with bromothymol blue whereas those of the secondary form reacted with triphenyltetrazolium chloride. In MacConkey's medium, the central colony of the primary form was lavender with a creamy halo, whereas colonies of the secondary form were creamy and lavender and flat with a gloss. Both forms did not emit any fluorescence. These results indicated that the colonies of the *S. abbasi* symbiotic bacteria have similar morphology and biological characteristics as those of *Xenorhabdus* (Akhurst and Boemare, 2004).

Physiological and biochemical tests were carried out with the culture of 48 h at 25 °C using API 20E and Biolog GN microplates. Ninety-five carbon sources were examined with Biolog Microstation Reader (Biolog Inc., Hayward, CA), and analyzed using Biolog GN Microplate TM computer software version 4.0. Among them, 13 compounds were utilized by the primary form, including Tween 80, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, α-D-glucose, D-mannose, D-trehalose, methyl pyruvate, D, L-lactic acid, L-asparagine, L-aspartic acid, L-glutamic acid, inosine and glycerol. However, the reactions with Tween 40 and *p*-hydroxy phenylacetic acid appeared insignificant. The similarity between the bacteria of *S. abbasi* and *X. nematophila* is 61% based on the computer analysis (data not shown). In addition, examinations of *S. abbasi* symbiotic bacteria based on Gram negative Enterobacteriaceae using API 20E showed that only the arginine dihydrolase, urea hydrolysis, Voges–Proskauer test, and gelatin hydrolysis tests, were positive (Table 1). Therefore, our results indicated that the symbiotic bacterium of *S. abbasi* isolated from Taiwan is a species of *Xenorhabdus*, in agreement with the Oman isolate reported by Tailliez et al. (2006).

Genomic DNA of the bacterium associated with *S. abbasi* was extracted and purified using QIAmp® DNA Mini Kit (QIA GEN Inc., USA). The 16S rDNA was PCR amplified using the protocol modified from Brunel et al. (1997). A pair of primers, 5′-GGAGAGT TAGATCTTGCCTC-3′ (sense) and 5′-AAGGAGGTGATCCAGCCGCA-3′(anti-sense) was used for characterizing 16S rDNA (Weisburg et al., 1991). The reactions were run on a DNA thermal cycle for a 35-cycle amplification series after initial denaturation of the reaction mixture at 95 °C for 3 min. Each cycle included denaturation at 95 °C for 1 min, re-annealing at 51 °C for 30 s, and extension at 72 °C for 2 min. The final extension was carried out at 72 °C for 5 min. The products were separated in a 1% agarose gel and gel extraction using MinElute TM Gel Extraction Kit (Qiagen Inc., Valencia, CA). After ligaturing, PCR products and TA cloning vector (pOSI-T, GeneMark Technology Co, Ltd., Tainan, Taiwan) were transformed into *Escherichia coli* competent cells (DH5α), which were further cultured in Luria–Bertani (LB) broth (DIFCO, Sparks, MD) for 16 h. The plasmid purification was performed using the protocol included in the High-Speed Plasmid Mini Kit (Geneaid, Taipei, Taiwan). The 16S rDNA sequences were analyzed using the nucleic-acid automatic ordering meter (ABI PRISM 377 DNA sequencer), and finally compared with the database from the sequence gene bank at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST/). We adopted 10 sets of 16S rDNA sequences of *S. abbasi* symbiotic bacterium to align following the PileUP analysis. After alignment, the likelihood and Branch-bound search operational mode in PAUP 4.0 (Swofford, 1998) were operated 500 times for constructing a phylogenetic tree of the similar clusters.

The sequence of 16S rDNA in the bacterium associated with *S. abbasi* was of 1458 bp (not including both sides of primers). A phylogenetic tree was constructed using fragments of 1458 bp comparing them with 16S rDNA obtained from NCBI database (Fig. 2). The symbiotic bacterium of *S. abbasi* Taiwan isolate remained in the same cluster as *X. indica* of the Oman isolate. The similarity between species of *Xenorhabdus* sp. and other related taxa was 93–98%. For example, the sequences producing significant alignments between symbiotic bacterium of *S. abbasi* Taiwan isolate and *X. indica* OM01 (DQ211718) were 98%, while those between symbiotic bacterium of *S. abbasi* Taiwan isolate and *Xenor-*

Table 1
Physiological and biochemical characteristics of *Xenorhabdus* sp. associated with *Steinernema abbasi*

Test item	*Xenorhabdus* sp. primary form	Test item	*Xenorhabdus* sp. primary form
Beta-galactosidase	–	Oxidation	–
Aginine dihydrolase	+	Glucose	–
Lysine dihydrolase	–	Mannitol	–
Ornithine dihydrolase	–	Inositol	–
Citrate utilization	–	Sorbitol	–
H_2S production	–	Rhamnose	–
Urea hydrolysis	+	Sucrose	–
Tryptophan deaminase	–	Melibiose	–
Indole production	–	Amygdalin	–
Voges-proskauer test	+	Arabinose	–
Gelatin hydrolysis	+	Starchc hydrolysis	–

Note: "+" positive reaction; "–" negative reaction.