World Journal of Microbiology & Biotechnology 10, 207–210

Xenorhabdus japonicus sp. nov. associated with the nematode *Steinernema kushidai*

Y. Nishimura,* A. Hagiwara, T. Suzuki and S. Yamanaka

A new species, *Xenorhabdus japonicus*, is proposed as the bacterial symbiont of *Steinernema kushidai* isolated from field soil in Shizuoka Prefecture, Japan. *Xenorhabdus japonicus* could be distinguished phenotypically and genetically from other *Xenorhabdus* spp. The type strain of the species, SK-1, a Gram-negative, facultative anaerobe and peritrichously flagellated rod, has colonies with primary and secondary forms. The strain can be differentiated from the type strain of *Xenorhabdus nematophilus* by several characters, including the formation of arginine dehydrolase, phenylalanine deaminase and lysine decarboxylase, the assimilation of inosine and L-proline and acid production from inositol. The major cellular fatty acids are 16:0, cyclo 17:0 and 18:1. The ubiquinone system is Q-8. The G + C content of DNA is 45.9 mol%. The DNA of strain SK-1 has 20 to 58% homology with that of the type strains of other *Xenorhabdus* spp.

Key words: Field soil, *Steinernema kushidai*, symbiont, *Xenorhabdus*, *Xenorhabdus japonicus*.

Xenorhabdus spp. are bacteria symbiotically associated with the entomopathogenic nematodes *Steinernema* and *Heterorhabditis*. Thomas & Poinar (1979) proposed the new genus, *Xenorhabdus*, based on the type species *Xenorhabdus nematophilus* (basionym *Achromobacter nematophilus* Poinar & Thomas 1965) associated with the nematode *Neoaplectana* (= *Steinernema*) *carpocapsae* (Wouts *et al.* 1982). Since then, *X. luminescens* (Thomas & Poinar 1979), *X. nematophilus* subsp. *bovienii*, *X. nematophilus* subsp. *poinarii* (Akhurst 1983) and *X. nematophilus* subsp. *beddingii* (Akhurst 1986) have been described, after isolation from *Heterorhabditis bacteriophora*, *Steinernema feltiae*, *S. glaseri* and *Steinernema* sp., respectively. Akhurst & Boemare (1988) proposed that the three subspecies be elevated to species level (*X. bovienii*, *X. poinarii* and *X. beddingii*) as the result of numerical analysis of their conventional taxonomic features.

Xenorhabdus spp. inhabit the intestine of juvenile nematodes. Infected nematodes can enter their host insects through natural body openings and then penetrate into the haemocoele where they release the bacterial symbionts. The bacteria then proliferate, killing the insect, providing a food source for the reproducing nematodes and suppressing growth of competing microorganisms. There is considerable interest in the use of bacteria-carrying nematodes for the biological control of insect pests.

A symbiotic bacterium, *Xenorhabdus* sp. SK-1, isolated from a new species of nematode, *Steinernema kushidai* (Kushida *et al.* 1987; Mamiya 1988), can be distinguished phenotypically from other *Xenorhabdus* spp. Intrahaemocoelic injection of strain SK-1 cells or of supernatant solutions from liquid cultures of the strain into sixth instar larvae of *Spodoptera litura* were not pathogenic (Yamanaka *et al.* 1992).

In the present study, the physiological and chemotaxonomic characteristics of strain SK-1 and the homology between the DNA of strain SK-1 and that of other *Xenorhabdus* spp. strains was further investigated. On the basis of the phenotypes and DNA homology, we propose a new species, *Xenorhabdus japonicus*, to accommodate strain SK-1.

Materials and Methods

Xenorhabdus *Strains*

Strain SK-1 and the type strains *X. nematophilus* ATCC 19061, *X. poinarii* DSM 4768, *X. beddingii* DSM 4764, *X. bovienii* ATCC 35271 and *X. luminescens* ATCC 29999 were used. Strain SK-1 was isolated directly from infected juveniles of *Steinernema kushidai*. The nematodes were first surface-sterilized in 0.1% (w/v) merthiolate for 3 h, then washed three times with sterilized water

Y. Nishimura, A. Hagiwara and T. Suzuki are with the Department of Applied Biological Science, Science University of Tokyo, Noda 278, Japan, and SDS Biotech K.K., Tsukuba Technology Centre, Tsukuba 300-26, Japan; fax: 471 23 9767. S. Yamanaka is with SDS. *Corresponding author (at the Science University of Tokyo).

and ruptured by sonication for 2 min. The sonicate was then plated out on a nutrient agar plate.

Media and Cultivation

Stock cultures of the strains were maintained on nutrient agar slants at 10°C and sub-cultured monthly. The cells used for chemical analysis were obtained by shaking cultures with nutrient broth at 28°C for about 24 h.

Taxonomic Analysis

The physiological and biochemical characteristics were tested according to the methods of Akhurst (1983) and cellular fatty acid compositions and ubiquinones were examined by the methods used in previous studies (Nishimura *et al.* 1979, 1983).

The G + C contents (mol%) were analysed by HPLC using a 5 cm column of Shim-Pack WAX-1 (Shimadzu, Kyoto, Japan) after hydrolysis of the purified DNA by nuclease P1 (Yamasa Shoyu, Choshi, Japan) (Nishimura *et al.* 1986). Phosphate buffer (50 mM, pH 2.83) was used as eluent. DNA–DNA hybridization tests were as described previously (Nishimura *et al.* 1987). Purified DNA was labelled *in vitro* with ^{3}H-deoxycytidine 5'-triphosphate (ammonium salt) by nick translation (Rigby *et al.* 1977), using a commercial kit (Nippon Gene Co., Toyama, Japan). The reassociation of hot and cold DNA was carried out at 60°C for 48 h and radioactivity measured in a liquid scintillation counting system (LSC-700; Aloka Co., Tokyo, Japan).

Results

Strain SK-1 was Gram-negative, rod-shaped, a facultative anaerobe and motile (peritrichously flagellated). Cell size was variable, (between 2 and 10 μm in length) and filaments longer than 10 μm were often observed. The strain showed two forms of colony, primary and secondary, on nutrient agar. As in the case of other species of *Xenorhabdus*, the primary-form cells adsorb bromothymolblue (BTB) on NBTA medium (Akhurst 1980), forming blue colonies. The secondary-form cells, however, do not adsorb BTB. Other characteristics of both forms are shown in Table 1. Starch hydrolysis, lecithinase, DNase, proteolysis of skim milk and gelatin, lipolysis of tributyrin and production of antibiotic were positive in the primary form. Phenylalanine deaminase and arginine dihydrolase were positive in the secondary form. Strain SK-1 gave negative catalase, cytochrome oxidase, urease and nitrate reduction reactions and did not produce indole or hydrogen sulphide. The primary-form cells were generally more active in the biochemical reactions than secondary-form cells. On the basis of these characteristics, strain SK-1 was recognized as a member of the genus *Xenorhabdus*. Other physiological and biochemical characteristics are listed in a description of the new species, in which a positive result applies to either or both forms of the species.

The distinguishing characteristics of strain SK-1 and the type strains of other *Xenorhabdus* species are shown in Table 2. Strain SK-1 could be distinguished from the type strain of *Xenorhabdus nematophilus* in the formation of arginine dihydrolase (positive), phenylalanine deaminase (positive) and lysine decarboxylase (negative), the utilization of inosine (negative) and L-proline (negative), and acid production from inositol (negative). The major cellular fatty acids were 16:0 (37%), cyclo 17:0 (11%) and 18:1 (10%). The major ubiquinone was Q-8 (94%).

Table 1. Characteristics of two colony forms of *Xenorhabdus japonicus* strain SK-1.

Characteristic	Primary form	Secondary form
Catalase	–	–
Urease	–	–
Phenylalanine deaminase	–	w
Tryptophane deaminase	w	w
Lysine decarboxylase	–	–
Arginine dihydrolase	–	+
Phosphatase	–	–
Escline hydrolysis	–	–
Soluble starch hydrolysis	w	–
DNase	+	–
Proteolysis:		
Skim milk agar	+	1
Gelatin	+	–
Egg yolk agar:		
Halo precipitation (lecithinase)	+	–
Pearly layer (lipase)	–	–
Lipolysis:		
Tween 20	+	+
Tween 40	+	w
Tween 60	+	w
Tween 80	–	–
Tributyrin	+	–
MR test	w	w
VP test	–	–
OF test	F	F
Nitrate reduction	–	–
Production of antibiotic*	+	–

* Inhibited growth of *Micrococcus luteus*
+—Positive, w—weak positive, – —negative.

The G + C content of DNA in strain SK-1 was 45.9 mol%. The results of DNA–DNA hybridization are shown in Table 3. For the ^{3}H-labelled DNA, that from strain SK-1 showed 20 to 50% homology with that of the other reference strains at a reassociation temperature of 60°C. The labelled DNA of the *X. nematophilus* type strain showed 29% DNA homology with that of the unlabelled DNA of strain SK-1. The DNA of strain SK-1 was more homologous with that of *X. nematophilus*, *X. poinarii*, *X. beddingii* and *X. bovienii* than with that of *X. luminescens*.

Discussion

Steinernematid nematodes (*S. kushidai*) collected from the field soil of Hamakita, Shizuoka Prefecture, Japan, killed

Table 2. Differential characteristics of strain SK-1 and type strains of *Xenorhabdus* spp.

Characteristic	SK-1	*X. nematophilus* ATCC 19061	*X. bovienni* ATCC 35271	*X. poinarii* DSM 4768	*X. beddingii* DSM 4764	*X. luminescens* ATCC 29999
Pigmentation on nutrient agar	Yellowish-brown	Buff	Yellow	Brown	Light brown	Brown
Growth at 37°C	–	–	–	+	+	+
Esclin hydrolysis	–	–	–	–	+	–
Nitrate reduction	–	–	+	–	w	–
Arginine dihydrolase	+	–	–	–	–	–
Starch hydrolysis	w	+	+		–	+
Phenylalanine deaminase	w	–	–	–	w	w
Lysine decarboxylase	–	+	w	–	–	–
Acid production on:						
Ribose	–	–	+	–	–	+
Inositol	–	+	w	–	–	+
Sorbitol	–	–	w	–	–	–
Glycerol	–	–	+	+	–	–
Assimilation of:						
Inosine	–	+	+	+	+	+
L-Proline	–	+	+	+	+	+
Ribose	–	–	+	+	+	+
Associated nematode	*S. kushidai*	*S. carpocapsae*	*S. feltiae*	*S. glaseri*	*S.* sp.	*H. bacteriophora*

+—Positive, w—weak positive, –—negative.

Table 3. DNA–DNA hybridization among *Xenorhabdus* species.

Source of unlabelled DNA	G + C content (mol %)	Reassociation (%) at 60°C with labelled DNA from:	
		X. nematophilus ATCC 19061	Strain SK-1
Strain SK-1	45.9	29	100
X. nematophilus ATCC 19061*	45.6	100	45
X. bovienii ATCC 35271*	44.3	24	51
X. poinarii DSM 4768*	42.6	29	29
X. beddingii DSM 4764*	45.5	33	58
X. luminescens ATCC 29999*	44.3	9	20

* Type strain.

white grubs in laboratory tests (Kushida *et al.* 1987). Koizumi *et al.* (1988) considered that this species of nematode was a promising biological control agent for white grubs and we thought it was time to study the entomopathogenic bacterium of this nematode. *In vitro*, the primary-form cells of the bacterium were more active in biochemical reactions than the secondary-form cells, as also observed for other *Xenorhabdus* strains. The *in vivo* form of strain SK-1 is probably the primary one, because only the primary form was recognized when the strain was isolated from the nematode. The relatively high biochemical activity of the *in vivo* form helps to provide the nematodes with nutrients and to repress the growth of other contaminant bacteria.

Strain SK-1 isolated from *S. kushidai* has the characteristics of a member of the genus *Xenorhabdus* and can be differentiated from known *Xenorhabdus* species (Table 2). Strain SK-1 is comparatively inactive in terms of acid production from several carbohydrates and the utilization of carbon sources. Moreover, the level of DNA homology between strain SK-1 and the type strains of other *Xenorhabdus* species is low (Table 3) and bacterial symbionts of nematodes tend to be host-specific. We therefore propose that SK-1 is of a new species in the genus *Xenorhabdus*.

Description of Xenorhabdus japonicus *sp. nov.*

Xenorhabdus japonicus *(ja.po'ni.cus. M.L. adj. japonicus, of Japan)*

Cells are rods 0.5 to 1.0 μm by 2.0 to 10 μm. Non-sporeforming, non-capsulated, motile, non-acid fast, Gram-negative, facultative anaerobe. The colonies occur in two forms. Those of the primary form on nutrient agar are

smooth, entire, convex, glistening, opaque, and yellowish brown. The primary form cells adsorb BTB, form blue colonies on NBTA medium and form lecithinase, DNase and protease. The colonies of the secondary form are slightly wider and flatter than those of the primary form. The secondary form cells do not adsorb BTB and do not form lecithinase, DNase or protease. Catalase and cytochrome oxidase are not produced. Nitrate is not reduced. Indole and hydrogen sulphide are not produced. Tween 20, 40, and 60 are hydrolysed. Arginine dihydrolase and gelatinase are produced. Starch hydrolysis is weakly positive. Lysine decarboxylase, urease and phosphatase are not produced. Escline is not hydrolysed. Good growth occurs at 22 to 27°C. There is no growth at 37°C. Acids are produced from fructose, glucose, mannose, *N*-acetylglucosamine, rhamnose, and trehalose. D-Glucosamine, glycerol, glucose, maltose, mannose, sucrose, trehalose, citrate, ethanol, glycine and asparagine are utilized. Arabinose, galactose, lactose, raffinose, ribose, xylose, formate, inosine and L-proline are not utilized.

The major cellular fatty acids are 16:0, cyclo 17:0 and 18:1. The ubiquinone system is Q-8. The G + C content of DNA is 45.9 mol%.

The type strain is strain SK-1, which was isolated from the nematode *Steinernema kushidai*.

References

Akhurst, R.J. 1980 Morphological and functional dimorphism in *Xenorhabdus* spp., bacteria symbiotically associated with the insect pathogenic nematodes *Neoaplectana* and *Heterorhabditis*. *Journal of General Microbiology* **121**, 303–309.

Akhurst, R.J. 1983 Taxonomic study of *Xenorhabdus*, a genus of bacteria symbiotically associated with insect pathogenic nematodes. *International Journal of Systematic Bacteriology* **33**, 38–45.

Akhurst, R.J. 1986 *Xenorhabdus nematophilus* subsp. *beddingii (Enterobacteriaceae)*: a new subspecies of bacteria mutualistically associated with entomopathogenic nematodes. *International Journal of Systematic Bacteriology* **36**, 454–457.

Akhurst, R.J. & Boemare, N.E. 1988 A numerical taxonomic study of the genus *Xenorhabdus (Enterobacteriaceae)* and proposed elevation of the subspecies of *X. nematophilus* to species. *Journal of General Microbiology* **134**, 1835–1845.

Koizumi, C., Kushida, T. & Mitsuhashi, J. 1988 Preliminary field tests on white-grub control by an entomogenous Nematode, *Steinernema* sp. *Journal of Japan Forest Society* **70**, 417–419.

Kushida, T., Mamiya, Y. & Mitsuhashi, J. 1987 Pathogenicity of newly detected *Steinernema* sp. (Nematode) to scarabacid larvae injurious to tree seedlings. *Journal of Applied Entomology and Zoology* **31**, 144–149.

Mamiya, Y. 1988 *Steinernema kushidai* n. sp. (Nematoda: Steinernematidae) associated with scarabaeid beetle larvae from Shizuoka. *Journal of Applied Entomology and Zoology* **32**, 313–320.

Nishimura, Y., Kanbe, K. & Iizuka, H. 1986 Taxonomic studies of aerobic coccobacilli from seawater. *Journal of General and Applied Microbiology* **32**, 1–11.

Nishimura, Y., Kano, M., Ino, T., Iizuka, H., Kosako, Y. & Kaneko, T. 1987 Deoxyribonucleic acid relationship among the radiation-resistant *Acinetobacter* and other *Acinetobacter*. *Journal of General and Applied Microbiology* **33**, 371–376.

Nishimura, Y., Yamanaka, H. & Iizuka, H. 1979 Taxonomical studies of *Acinetobacter* species—cellular fatty acid composition. *Zeitschrift für Allgemeine Mikrobiologie* **19**, 307–308.

Nishimura, Y., Yamanaka, S. & Iizuka, H. 1983 Ubiquinone, fatty acid, polar lipid and DNA base composition in *Pseudomonas radiora*. *Journal of General and Applied Microbiology* **29**, 421–427.

Poinar, G.O., Jr. & Thomas, G.M. 1965 A new bacterium *Achromobacter nematophilus* sp. nov. (*Achromobacteriaceae: Eubacteriales*) associated with a nematode. *International Bulletin of Bacteriological Nomenclature and Taxonomy* **15**, 248–252.

Rigby, P.W., Dieckmann, M., Rhodes, C. & Berg, P. 1977 Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase. *Journal of Molecular Biology* **113**, 237–251.

Thomas, G.M. & Poinar, G.O. 1979 *Xenorhabdus* gen. nov., a genus of entomopathogenic bacteria of the family *Enterobacteriacae*. *International Journal of Systematic Bacteriology* **9**, 352–360.

Wouts, W.M., Mráček, Z., Gerdin, S. & Bedding, R.A. 1982 Neoaplectana Steiner, 1929 A junior synonym of *Steinernema* Travassos, 1927 (Nematoda-Rhabditida). *Systematic Parasitology* **4**, 147–154.

Yamanaka, S., Hagiwara, A., Nishimura, Y., Tanabe, H. & Ishibashi, N. 1992 Biochemical and physiological characteristics of *Xenorhabdus* species, symbiotically associated with entomopathogenic nematodes including *Steinernema kushidai* and their pathogenicity against *Spodoptera litura* (Lepidoptera: Noctuidae). *Archives of Microbiology* **158**, 387–393.

(Received in revised form 29 August 1993; accepted 2 September 1993)