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Description of *Xenorhabdus khoisanae* sp. nov., the symbiont of the entomopathogenic nematode *Steinernema khoisanae*

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Bacterial strain SF87^T, and additional strains SF80, SF362 and 106-C, isolated from the nematode *Steinernema khoisanae*, are non-bioluminescent Gram-reaction-negative bacteria that share many of the carbohydrate fermentation reactions recorded for the type strains of recognized *Xenorhabdus* species. Based on 16S rRNA gene sequence data, strain SF87^T is shown to be closely related (98 % similarity) to *Xenorhabdus hominickii* DSM 17903^T. Nucleotide sequences of strain SF87 obtained from the *recA*, *dnaN*, *gltX*, *gyrB* and *infB* genes showed 96–97 % similarity with *Xenorhabdus miraniensis* DSM 17902^T. However, strain SF87 shares only 52.7 % DNA–DNA relatedness with the type strain of *X. miraniensis*, confirming that it belongs to a different species. Strains SF87^T, SF80, SF362 and 106-C are phenotypically similar to *X. miraniensis* and *X. beddingii*, except that they do not produce acid from aesculin. These strains are thus considered to represent a novel species of the genus *Xenorhabdus*, for which the name *Xenorhabdus khoisanae* sp. nov. is proposed. The type strain is SF87^T (=DSM 25463^T=ATCC BAA-2406^T).

Xenorhabdus species are bound in obligate alliance of mutual benefit with specific species of entomopathogenic nematodes of the family Steinernematidae (Thomas & Poinar, 1979). Thus far, 65 *Steinernema* species have been described worldwide, of which *Steinernema khoisanae* (Nguyen *et al.*, 2006) was isolated in the Western Cape province of South Africa. Thomas & Poinar (1979) were the first to describe symbiotic bacteria in *Steinernema* as members of the genus *Xenorhabdus*. Since then, 23 species in the genus *Xenorhabdus* have been described as listed in Fig. 1 and Table 1. In this paper, we describe a novel symbiont, *Xenorhabdus khoisanae* sp. nov., associated with the entomopathogenic nematode *S. khoisanae* collected in South Africa.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *dnaN*, *gltX*, *gyrB*, *recA* and *infB* gene sequences of *Xenorhabdus khoisanae* sp. nov. SF87^T are HQ142625, AB685733–AB685736 and JX623984, respectively. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, *recA*, *dnaN*, *gltX*, *gyrB* and *infB* gene sequences of *X. khoisanae* sp. nov. SF 80, 106-C and SF362 are JX623966–JX623983, respectively.

Seven supplementary figures are available with the online version of this paper.

Bacterial strains SF87^T, SF80, SF362 and 106-C were isolated from different *S. khoisanae* nematode populations, as previously described (Malan *et al.*, 2006, 2011). Cultures were obtained indirectly from the nematodes by sampling the haemocoel of wax moth larvae (*Galleria mellonella* L.), which was plated onto nutrient agar (Biolab Diagnostics), supplemented with 0.004 % (w/v) triphenyltetrazolium chloride and 0.025 % (w/v) bromothymol blue (NBTA) according to the procedure described by Akhurst (1980). Plates were incubated at 26 °C for 72 h and blue–green colonies were randomly selected for identification and further characterization. Isolates of *Xenorhabdus* species were also routinely cultured in trypticase soy broth (TSB; Beckton Dickinson) and Luria broth (LB; Biolab), and stored in 40 % (v/v) glycerol at −80 °C. *Xenorhabdus miraniensis* DSM 17902^T, *Xenorhabdus hominickii* DSM 17903^T and *Bacillus subtilis* subsp. *subtilis* DSM 10^T were obtained from the DSMZ. *Escherichia coli* transformants were cultured in LB containing 100 μg ampicillin ml^{-1}. *Bacillus subtilis* subsp. *subtilis* DSM 10^T was grown in nutrient broth (NB; Biolab).

Cell size measurements were taken by means of a Leica DM2000 research microscope (Leica Microsystems) equipped

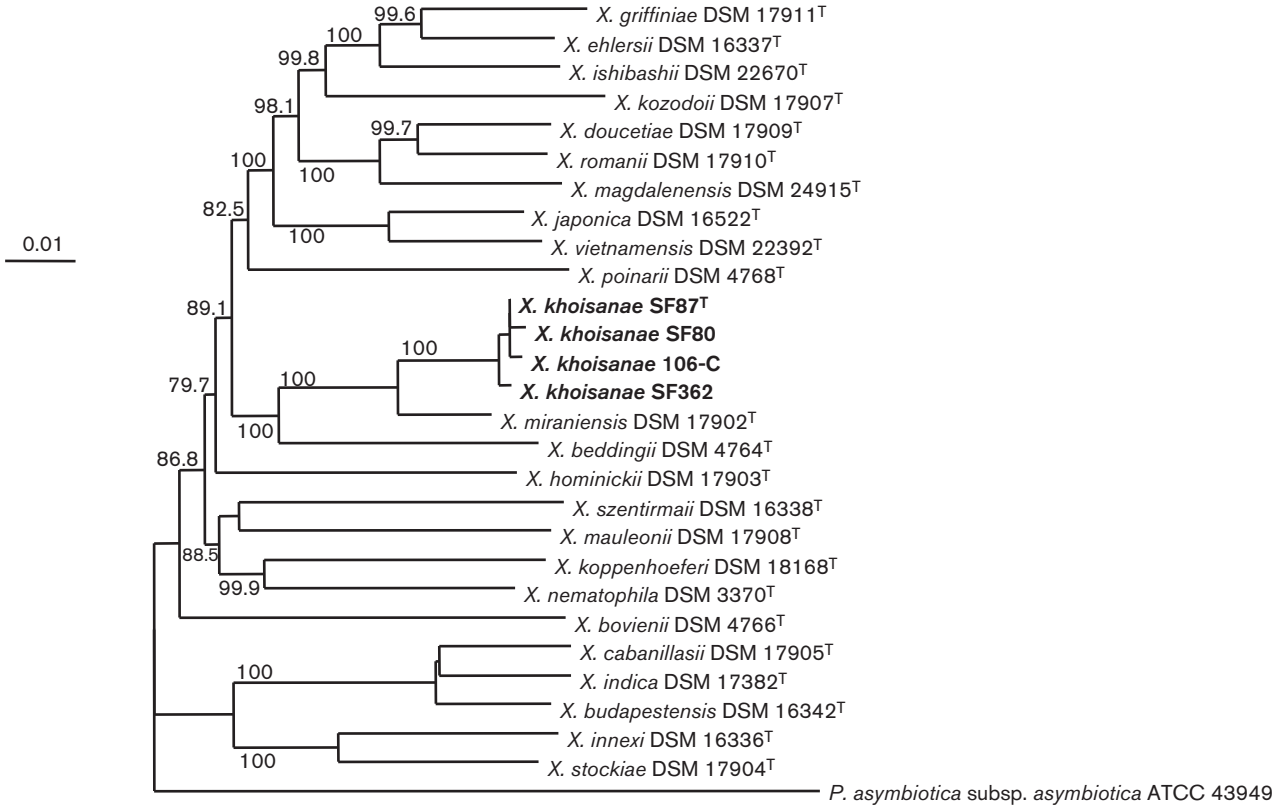

Fig. 1. Phylogenetic relationship of *X. khoisanae* strains SF87^T, SF80, 106-C and SF362 to known *Xenorhabdus* species based on concatenated (16S rRNA, *recA*, *dnaN*, *gltX*, *gyrB* and *infB*) gene sequences. The tree was constructed by the neighbour-joining method. *Photorhabdus asymbiotica* subsp. *asymbiotica* ATCC 43949 was used as an outgroup species. Bootstrap values above 70 % are given at branch points. Bar, 10 % sequence divergence.

with a camera, computer and digital image software [Leica Application Suite (LAS), version 3.5.0]. Several phenotypic characteristics were determined, including Gram reactions. For colony pigmentation, isolates were streaked onto NBTA and MacConkey Agar (Biolab), and incubated for 48 h at 30 °C. To determine optimum temperature requirements for growth, isolates were grown overnight in LB at 26–42 °C (1 °C increases). Optimum growth temperature for strain SF87^T was also determined in NB and TSB. Growth was measured spectrophotometrically at 600 nm. Sensitivity to ampicillin in LB was determined as described by Somogyi *et al.* (2002). Antimicrobial activity was tested by overlaying 48 h cultures of strains SF87^T, SF80, SF362, 106-C, *X. hominickii* DSM 17902^T and *X. miraniensis* DSM 17903^T with an active growing culture of *B. subtilis* subsp. *subtilis* DSM 10^T. Lipase, DNase and lecithinase activities were determined as described by Ferreira *et al.* (2013). Haemolysis was observed by streaking the strains on agar plates containing either 10 % (v/v) sheep blood or 5 % (v/v) horse blood (National Health Laboratory Services). Plates were incubated at 30 °C for 48 h. All tests were conducted in duplicate. Biochemical properties were recorded using Biolog GN microplates, and API 20 NE and API 50 CH test strips (bioMérieux). Test strips were incubated at 30 °C for 10 days as indicated by Boemare & Akhurst (1988). *X. miraniensis* DSM 17902^T and *X. hominickii* DSM 17903^T were included as reference strains in carbohydrate (API) reactions and agar plate phenotypic tests, except for ampicillin sensitivity. The possible presence of bioluminescence was determined by scanning the colonies with the Xenogen *in vivo* imaging system (IVIS; Caliper Life Sciences).

Total genomic DNA of overnight cultures of strains SF87^T, SF80, SF362 and 106-C was extracted using a ZR fungal/bacterial DNA kit (Zymo Research). Isolates were initially identified by amplifying the 16S rRNA gene using primers as described by Brunel *et al.* (1997). Based on previous phylogenetic studies, the recombinase A (*recA*), DNA polymerase III beta chain (*dnaN*), glutamyl-tRNA synthetase catalytic subunit (*gltX*), DNA gyrase subunit B (*gyrB*) and initiation factor B (*infB*) genes were amplified with primers recA1(F) and recA2(R), dnaN1(F) and dnaN2(R), gltX1(F) and gltX2(R), 8SF_gyrB(F) and 9Rev_gyrB(R), and infB1(F) and infB2(R), respectively (Tailliez *et al.*, 2010, 2012). An initial denaturation step of 94 °C for 4 min was used, followed by 35 cycles of 94 °C for 1 min, the appropriate temperature for each primer pair for 30 s and 72 °C for 1 min. Final extension was at 72 °C for

Table 1. Comparison of acid production and assimilation by the four novel strains and the type strains of recognized *Xenorhabdus* species after 10 days incubation at 30 °C

Strains: 1, SF 87^T; 2, SF 80; 3, SF 362; 4, 106-C; 5, *X. beddingii* DSM 4764^T; 6, *X. bovienii* DSM 4766^T; 7, *X. budapestensis* DSM 16342^T; 8, *X. cabanillasii* DSM 17905^T; 9, *X. doucetiae* DSM 17909^T; 10, *X. ehlersii* DSM 16337^T; 11, *X. griffiniae* DSM 17911^T; 12, *X. hominickii* DSM 17903^T; 13, *X. indica*, DSM 17382^T; 14, *X. innexi* DSM 16336^T; 15, *X. ishibashii* DSM 22670^T; 16, *X. japonica* DSM 16522^T; 17, *X. koppenhoeferi* DSM 18168^T; 18, *X. kozodoii* DSM 17907^T; 19, *X. magdalenensis* DSM 24915^T; 20, *X. mauleonii* DSM 17908^T; 21, *X. miraniensis* DSM 17902^T; 22, *X. nematophila* DSM 17382^T; 23, *X. poinarii* DSM 4768^T; 24, *X. romanii* DSM 17910^T; 25, *X. stockiae* DSM 17904^T; 26, *X. szentirmaii* DSM 16338^T; 27, *X. vietnamensis* DSM 22392^T. Data for the reference type strains are from Akhurst & Boemare (1988), Kuwata *et al.* (2012), Lengyel *et al.* (2005), Nishimura *et al.* (1994), Somvanshi *et al.* (2006), Tailliez *et al.* (2006, 2010, 2012) and Thomas & Poinar (1979). +, Carbohydrate utilized; −, not utilized; w, weak reaction; v, variable reaction.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
Acid production from (API 50CH E):																											
D-Ribose	+	+	+	+	+	v	−	−	v	v	+	v	−	+	v	+	−	+	−	+	+	v	−	−	+	v	−
Inositol	w	w	w	w	−	v	+	v	v	−	−	v	+	v	−	−	−	−	−	+	−	v	−	w	+	v	−
D-Sorbitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	v	−
N-Acetylglucosamine	+	+	+	+	+	+	v	+	v	v	−	+	v	+	+	+	−	−	−	+	+	+	+	+	+	v	+
Aesculin	−	−	−	−	+	−	−	−	v	v	+	+	v	−	+	−	−	+	+	+	+	−	v	−	+	+	+
Maltose	+	+	+	+	+	v	−	v	v	v	−	+	−	−	−	+	+	+	+	+	+	v	+	+	+	+	+
Trehalose	+	w	w	+	+	v	−	−	+	−	−	−	−	v	+	−	−	+	+	+	+	v	v	+	−	−	+
Gluconate	+	+	+	+	+	v	−	−	−	−	−	−	−	+	−	−	−	−	−	+	w	−	−	w	+	−	−
5-Keto gluconate	w	w	w	w	+	v	+	v	−	v	w	+	+	+	w	+	+	−	−	w	w	v	v	w	w	−	+
Assimilation of: (API 20 NE)																											
Glucose	+	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
Mannose	+	−	+	+	+	+	+	+	+	v	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
N-Acetylglucosamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	v	+	+	+	+	+	+	+	−	+	−	+	+	+	+	+	+	+	+	+	+
Gluconate	+	+	+	+	+	+	v	+	+	v	+	+	+	+	+	+	−	v	−	+	+	v	+	+	+	+	+

7 min. TaKaRa Ex Taq, 10× Ex Taq buffer and dNTP mixture (Takara Bio) were used at concentrations recommended by the manufacturer. Amplified products were purified using a QIAquick PCR Purification kit (Qiagen), ligated into pGEM T-Easy vector (Promega), transformed into *Escherichia coli*, and plasmid preparations sequenced (DNA Sequencing unit, Central Analytical Facility, University of Stellenbosch) using the BigDye Teminator V3.1 sequencing kit (Applied Biosystems). Sequences were analysed using the BLAST program (NCBI).

The 16S rRNA gene sequences of strains SF87^T, SF80, SF362 and 106-C and related type strains were aligned using the program CLUSTAL X ver. 1.18 (Thompson *et al.*, 1997). Sequences of the closest relatives were retrieved from DDBJ. The Kimura two-parameter model was used to calculate distance matrices for the aligned sequences (Kimura, 1980) and bootstrapping with 1000 replicates was done to estimate the robustness of the individual branches (Felsenstein, 1985). For the 16S rRNA gene sequences, phylogenetic trees were reconstructed using the neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood methods in PHYLIP ver. 3.65 software (Felsenstein, 2005). Partial *recA*, *dnaN*, *gltX*, *gyrB* and *infB* gene sequences were analysed using the neighbour-joining method. DNA–DNA hybridization between strain SF87^T and *X. miraniensis* DSM 17902^T was performed according to Huss *et al.* (1983).

Phenotypic characteristics of isolates SF87^T, SF80, SF362 and 106-C

All strains tested were Gram-reaction-negative and absorbed dye. Colonies were dark blue–green on NBTA and light reddish brown on MacConkey agar. None of the strains displayed bioluminescence. All the isolates were catalase- and oxidase-negative, and did not reduce nitrate. Optimum growth temperature in LB was 31–32 °C, while a maximum growth temperature of 42 °C was observed for strain SF87^T in NB. Strains SF87^T, SF80, SF362 and 106-C were inhibited by ampicillin in solid medium at concentrations higher than 50 μg ml^{-1}, while slight variations in liquid medium were observed (SF87^T, no growth above 50 μg ml^{-1}; SF80 and 106-C, no growth at 25 μg ml^{-1}; SF362, no growth at 12.5 μg ml^{-1}). No lecithinase activity was observed for strains SF87^T, SF80, SF362 and 106-C, but positive reactions were recorded for the reference strains. All four novel strains tested were negative for DNase activity. Lipase activity was not detected on plates containing Tween 20 or Tween 40, and variable results were recorded for plates containing Tween 60 and Tween 80. Total haemolysis was observed for all strains on sheep and horse blood. All strains showed strong antimicrobial activity against *B. subtilis* subsp. *subtilis* DSM 10^T.

Biolog GN microplate reactions indicated that strain SF87^T utilized *N*-acetylglucosamine, DL-lactic acid, bromosuccinic

acid, L-alanine, L-alanine glycine, glycyl L-glutamic acid, L-histidine and L-serine. This matches Biolog GN results for most of the strains studied by Somvanshi *et al.* (2006). Strain SF87^T had a weak affinity for D-mannose, uridine, *p*-hydroxyphenylacetic acid and trehalose. Reactions recorded by Somvanshi *et al.* (2006) indicated that all strains tested utilized D-mannose, most strains utilized uridine and trehalose, while few utilized *p*-hydroxyphenylacetic acid.

According to the API 50 CH system, strains SF87^T, SF80, SF362, 106-C, *X. miraniensis* DSM 17902^T and *X. hominickii* DSM 17903^T produced acid from *N*-acetylglucosamine, D-fructose, D-glucose, glycerol, maltose, D-mannose and ribose. Acid production from inositol and 5-ketogluconate was weak. Acid production from trehalose was positive for strains SF87^T, 106-C and the control strains, but only weakly positive for strains SF80 and SF362. Weak positive reactions on starch were recorded for strains SF87^T and 106-C, while strains SF80 and SF362 were negative. A comparison of API results of strains SF87^T, SF80, SF362 and 106-C with previously published data is presented in Table 1. Results from API 20 NE showed that all four novel strains and the two reference strains assimilated glucose, mannose, *N*-acetylglucosamine, maltose and gluconate.

Phylogenetic position of strains SF87^T, SF80, SF362 and 106-C

According to the Kimura two-parameter model, strains SF87^T, SF80, SF362 and 106-C aligned with 97 % similarity to the 16S rRNA gene sequences of several *Xenorhabdus* type strains, clearly indicating that they belong to the same genus (Fig. S1, available in IJSEM Online). A similar tree topology was obtained by using maximum-likelihood analysis, with highest similarity (98.1 %) to *X. hominickii* DSM 17903^T (Fig. S2).

In addition to 16S rRNA gene sequence analysis, several recent studies have adopted a multi-gene approach to distinguish between *Xenorhabdus* species (Kuwata *et al.*, 2013; Lee & Stock, 2010; Tailliez *et al.*, 2010, 2012). Lee & Stock (2010) analysed the 16S rRNA gene and two housekeeping genes, phosphoserine aminotransferase and *recA*, while Tailliez *et al.* (2010, 2012) and Kuwata *et al.* (2013) used the genes *recA*, *dnaN*, *gltX*, *gyrB* and *infB*. In this study, partial *recA*, *dnaN*, *gltX*, *gyrB* and *infB* gene sequences of strains SF87^T, SF80, SF362 and 106-C were analysed using the neighbour-joining method. The results show that these strains are phylogenetically closely related to *X. miraniensis* DSM 17902^T (96–97 % similarity; Figs S3–S7). This was confirmed by groupings obtained from concatenated sequences (5500 bp) of the six genes (96–97 % similarity; Fig. 1).

Strain SF87 shared only 52.7 % DNA homology with *X. miraniensis* DSM 17902^T by DNA–DNA hybridization analysis. This is below the 70 % DNA–DNA relatedness threshold proposed by Wayne *et al.* (1987). Strains SF87^T, SF80, SF362 and 106-C are thus regarded as isolates of a novel species of the genus *Xenorhabdus*, for which the name *Xenorhabdus khoisanae* sp. nov. is proposed.

Description of *Xenorhabdus khoisanae* sp. nov.

Xenorhabdus khoisanae (khoi.sa′na.e. N.L. gen. n. *khoisanae* of the Khoisan people, i.e. isolated from the lands these people occupy).

Cells are Gram-reaction-negative, catalase- and oxidase-negative, and rod-shaped (2–3 × 0.2–0.7 μm). Growth is aerobic. Maximum growth temperature recorded is 42 °C in NB and 37 °C in TSB. Colonies on MacConkey agar are light reddish–brown. Acid is produced from *N*-acetylglucosamine, D-fructose, D-glucose, glycerol, maltose, D-mannose and ribose. Acid production from trehalose and starch is variable and negative from aesculin.

The type strain, SF87^T (=DSM 25463^T=ATCC BAA-2406^T), was isolated from the nematode *Steinernema khoisanae*. SF 80, 106-C and SF362, isolated from the same source, are additional strains of the species.

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