Serratia nematodiphila sp. nov., associated symbiotically with the entomopathogenic nematode *Heterorhabditoides chongmingensis* (Rhabditida: Rhabditidae)

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A novel red-pigmented, Gram-negative, motile, fluorescent, rod-shaped strain, DZ0503SBS1^T, with a single lateral flagellum, was isolated from the intestine of the nematode *Heterorhabditoides chongmingensis*. Comparative 16S rRNA gene sequence analysis indicated that the strain is a member of the genus *Serratia*, sharing highest sequence similarities with *Serratia marcescens* subsp. *sakuensis* JCM 11315^T (99.8%), *S. marcescens* subsp. *marcescens* DSM 30121^T (99.5%) and *Serratia ureilytica* LMG 22860^T (98.3%). Similarities between the *rpoB* gene sequence of strain DZ0503SBS1^T and those of *S. marcescens* subsp. *sakuensis* JCM 11315^T, *S. marcescens* subsp. *marcescens* DSM 30121^T and *S. ureilytica* LMG 22860^T were 98.0, 97.4 and 98.3%, respectively. DNA–DNA hybridization values of strain DZ0503SBS1^T with *S. marcescens* subsp. *sakuensis* JCM 11315^T, *S. marcescens* subsp. *marcescens* DSM 30121^T and *S. ureilytica* LMG 22860^T were 68.2, 65.1 and 53.0%, respectively. The major isoprenoid quinone of strain DZ0503SBS1^T was Q-8 and the predominant fatty acids were C_{16:0} (34.76%), cyclo-C_{17:0} (20.03%) and cyclo-C_{19:0}ω8c (17.24%). The cyclo-C_{19:0}ω8c content (17.24%) was significantly different from those found in *S. marcescens* subsp. *sakuensis* JCM 11315^T and *S. marcescens* subsp. *marcescens* DSM 30121^T. Some characteristics of strain DZ0503SBS1^T, i.e. fluorescence and its symbiotic association with nematodes, have not been reported previously in any species of the genus *Serratia*. Phenotypic and biochemical characteristics and molecular data show that strain DZ0503SBS1^T represents a novel species, for which the name *Serratia nematodiphila* sp. nov. is proposed; the type strain is DZ0503SBS1^T (=KCTC 22130^T =CGMCC 1.6853^T).

The genus *Serratia* belongs to the family *Enterobacteriaceae* of the class *Gammaproteobacteria*. Some members of the genus *Serratia* have clinical importance (Grimont &

Grimont, 1992; Brenner, 1984) and other members produce pigments identified as prodigiosin (Hearn *et al.*, 1970; Gerber, 1975). A subspecies of *Serratia marcescens* (*S. marcescens* subsp. *sakuensis*) and a urea-dissolving species (*Serratia ureilytica*) have been described previously by Ajithkumar *et al.* (2003) and Bhadra *et al.* (2005), respectively. In this paper, a red-pigmented, non-spore-forming, fluorescent strain, designated DZ0503SBS1^T, was isolated from the intestine of the nematode *Heterorhabditoides chongmingensis* (Zhang *et al.*, 2008).

The novel strain was associated symbiotically with the entomopathogenic nematode *H. chongmingensis* (Rhabdi-

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The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain DZ0503SBS1^T is EU036987.

A neighbour-joining phylogenetic tree based on partial *rpoB* gene sequences, a fluorescence micrograph showing the location of strain DZ0503SBS1^T in the intestine of the nematode *Heterorhabditoides chongmingensis*, a transmission electron micrograph of strain DZ0503SBS1^T, cellular fatty acid composition data and similarity coefficient values of strain DZ0503SBS1^T and related taxa are available with the online version of this paper.

tida: Rhabditidae) (Zhang *et al.*, 2008) and was obtained from the infective stages of *H. chongmingensis* by two methods. The first method was to crush approximately 100 surface-disinfected infective juveniles, followed by streaking the products on nutrient bromothymol blue agar (NBTA) plates (Akhurst, 1980). The second method was to streak onto NBTA plates a drop of haemolymph harvested from insects parasitized for 24–48 h by *H. chongmingensis* (Bonifassi *et al.*, 1999). To observe the location of the strain in the nematode intestine, 100 adults and 100 infective juveniles of *H. chongmingensis* were selected randomly and collected in a 1.5 ml Eppendorf tube; the nematodes were rinsed three times with Ringer's solution [6 g NaCl, 0.075 g KCl, 0.1 g CaCl₂ and 0.1 g NaHCO₃ (1 H₂O)⁻¹], placed onto slides, covered with a coverslip and then examined live under a fluorescence microscope (Axio Imager A1 microscope; Carl Zeiss).

Genomic DNA used for amplification was extracted from cells grown at 30 °C overnight in TYB agar (0.3 % yeast extract, 0.2 % beef extract, 0.6 % tryptone, 0.3 % NaCl, 0.001 % FeCl₃ and 1.5 % agar) and purified as described previously (Wilson, 2001). The primers used for PCR amplification of the 16S rRNA gene were 5'-AGAGTTTGATCCTGGCTCAG-3' (forward) and 5'-AAGGAGGTGATCCAAGCCGCA-3' (reverse), corresponding to positions 8–27 and 1521–1540 in the 16S rRNA gene sequence of *Escherichia coli* (Brosius *et al.*, 1978). The resultant sequence (1500 bp) of strain DZ0503SBS1^T was compared with those in GenBank by using the program BLASTN (Altschul *et al.*, 1997). A phylogenetic tree of related sequences based on BLAST sequence similarity was inferred by using MEGA 3.1 (Kumar *et al.*, 2001) after multiple alignment of the data by CLUSTAL_X (Thompson *et al.*, 1997). Nucleotide sequence similarities and DNA gaps were calculated and edited by using the program BIOEDIT (Hall, 1999). Distances (using distance options according to the Kimura two-

parameter model; Kimura, 1980, 1983) and clustering were based on the neighbour-joining method (Saitou & Nei, 1987). Bootstrap analysis (1000 resamplings) was used to evaluate the topology of the neighbour-joining tree (Felsenstein, 1985). Sequence similarities, based on pairwise sequence comparisons, were investigated by using complete 16S rRNA gene sequences. Comparisons of 16S rRNA gene sequences showed that strain DZ0503SBS1^T should be positioned within the genus *Serratia*, being related most closely to *S. marcescens* subsp. *sakuensis* JCM 11315^T (99.8 % similarity). The 16S rRNA gene sequence similarities of the novel strain with type strains of other established *Serratia* species were ≤99.5 %: DZ0503SBS1^T shared 99.5 % similarity with *S. marcescens* subsp. *marcescens*, 98.3 % with *S. ureilytica*, 97.9 % with *Serratia odorifera*, 97.9 % with *Serratia entomophila*, 97.8 % with *Serratia ficaria*, 96.9 % with *Serratia plymuthica*, 96.5 % with *Serratia grimesii*, 96.3 % with *Serratia proteamaculans*, 96.1 % with *Serratia fonticola* and 96.1 % with *Serratia rubidaea*. The phylogenetic tree (Fig. 1) showed that strain DZ0503SBS1^T was related most closely to *S. marcescens* subsp. *sakuensis* JCM 11315^T.

The segment of genomic DNA of strain DZ0503SBS1^T encoding the RNA polymerase β -subunit (*rpoB*) gene, corresponding to positions 1468–2114 of the *Escherichia coli* *rpoB* gene, was amplified by a PCR program using primers described by Li *et al.* (2004). A phylogenetic tree based on *rpoB* sequences was constructed by using the same method as that for the 16S rRNA gene. The *rpoB* gene tree showed a similar topology and closer phylogenetic relationship between strain DZ0503SBS1^T and the three most closely related strains than that constructed by using the 16S rRNA gene (see Supplementary Fig. S1 in IJSEM Online); the closest relative of DZ0503SBS1^T was *S. ureilytica* LMG 22860^T and the two strains formed a sister group of the clade comprising *S. marcescens* subsp. *sakuensis* JCM 11315^T and *S. marcescens* subsp. *marcescens*.

Fig. 1. Phylogenetic position of strain DZ0503SBS1^T among related bacteria in a neighbour-joining tree based on analysis of 16S rRNA gene sequence data. 16S rRNA sequences of representative strains of the genus *Serratia* and other members of the family *Enterobacteriaceae* were obtained from GenBank. Bootstrap values (expressed as percentages of 1000 replications) are shown at branch points; only bootstrap values of 50 % and above are shown. Bar, 0.005 substitutions per nucleotide position.

DSM 30121^T. The highest level of *rpoB* sequence similarity was between strain DZ0503SBS1^T and *S. ureilytica* LMG 22860^T (98.3%); strain DZ0503SBS1^T shared 98.0% sequence similarity with *S. marcescens* subsp. *sakuensis* JCM 11315^T and 97.4% similarity with *S. marcescens* subsp. *marcescens* DSM 30121^T. Accordingly, 98–100% intraspecies similarity was observed among enteric strains, whereas 2.0–21.9% interspecies difference was found (Mollet *et al.*, 1997; Li *et al.*, 2004).

Morphological features were examined by light microscopy and transmission electron microscopy (7650; Hitachi) as described by Nedashkovskaya *et al.* (2005). Growth was assessed at 4, 5, 10, 25, 30, 35, 37 and 42 °C on NBTA and TYB agar media. NaCl and pH tolerance were tested by using NBTA and TYB broth containing 0, 1, 2, 3, 5, 6, 7 and 8% NaCl and adjusted to pH 2.0–12.0 (in increments of 1 pH unit) according to Zhou *et al.* (2007). Growth was evaluated on cetrimide agar, Simmons' citrate agar, trypticase soy agar, MacConkey agar and nutrient agar. All media were incubated at 30 °C for 2 days. Gram staining, endospore staining and flagellum staining were performed according to the methods described by Smibert & Krieg (1994). Oxidase activity was tested by determining the oxidation of 1% (w/v) tetramethyl-*p*-phenylenediamine (Merck) and catalase activity was evaluated by determining the production of oxygen bubbles in a 5% (v/v) aqueous hydrogen peroxide solution. Acid production from carbohydrates was determined by using the medium and method described by Yamaguchi & Yokoe (2000). Carbon-source utilization was assessed by using a slightly modified ammonium salt medium [0.2% (NH₄)₂SO₄, 0.05% NaH₂PO₄·H₂O, 0.02% MgSO₄·7H₂O, 0.01% CaCl₂·2H₂O and 0.05% K₂HPO₄] containing 1% carbon source. Hydrolysis of agar, Tween 80, Tween 20 and CM-cellulose was investigated on NBTA and TYB agar after 2 days incubation according to Lányi (1987) and Smibert & Krieg (1994). Urease activity and hydrolysis of casein, gelatin, aesculin, tyrosine and starch were determined on NBTA and TYB agar as described by Cowan & Steel (1965). Nitrate reduction was determined according to the method of Lányi (1987). Antibiotic-sensitivity tests were performed by using the diffusion method, as described by Park *et al.* (2007), on NBTA and TYB agar at 30 °C with filter-paper discs (8 mm diameter; Sanofi Pasteur) containing the following antibiotics (in µg, unless otherwise indicated): streptomycin (10), penicillin G (10 IU), ampicillin (10), chloramphenicol (30), erythromycin (15), tetracycline (30), ofloxacin (5), amoxicillin (10), cefamezin (30), rifampicin (5), gentamicin (10), ceftazidime (30), vancomycin (30), kanamycin (30), carbenicillin (100) and polymyxin B (30). After 2 days incubation, the diameters of the inhibition zones were measured. Phenotypic characteristics of strain DZ0503SBS1^T are given in the species description and in Table 1.

Isoprenoid quinone analysis was performed according to Komagata & Suzuki (1987) using cells harvested from NG

agar (1⁻¹: 8.0 g nutrient broth, 8.0 g glucose, 5.0 g NaCl, 0.5 g yeast extract and 15 g agar) as described by Ajithkumar *et al.* (2003). The major isoprenoid quinone was Q-8, which is consistent with that reported previously for the type strain of *S. marcescens* (Grimont & Grimont, 1992; Collins & Jones, 1981; Hiraishi, 1999). For quantitative analysis of the cellular fatty acid composition, a loopful of cell mass was harvested from an NG agar plate incubated at 30 °C for 2 days and fatty acid methyl esters were prepared and identified by following the instructions of the Microbial Identification system (MIDI), as described by Sasser (1990). The major fatty acids found in strain DZ0503SBS1^T and the type strains of phylogenetically related *Serratia* species are listed in Supplementary Table S1 (available in IJSEM Online). The major cellular fatty acid in strain DZ0503SBS1^T was C_{16:0}, which is in agreement with the profiles of most *Serratia* species.

Genomic DNA was extracted and purified according to Yoon *et al.* (1996) and the DNA G+C content was determined by using the thermal-denaturation method (Mandel & Marmur, 1968). The DNA G+C content of strain DZ0503SBS1^T was 59.52 mol%. This value is higher than that of *S. marcescens* subsp. *sakuensis* JCM 11315^T (58 mol%; Ajithkumar *et al.*, 2003), but similar to that of *S. marcescens* subsp. *marcescens* DSM 30121^T (57.5–60.0 mol%; Grimont & Grimont, 1992; Brenner, 1984).

DNA–DNA hybridizations were determined fluorometrically by the method of Ezaki *et al.* (1989) using photobiotin-labelled DNA probes and microdilution wells. DNA–DNA hybridization of strain DZ0503SBS1^T with *S. marcescens* subsp. *sakuensis* JCM 11315^T, *S. marcescens* subsp. *marcescens* DSM 30121^T and *S. ureilytica* LMG 22860^T revealed relatedness values of 68.2, 65.1 and 53.0%, respectively. This indicated that strain DZ0503SBS1^T represents a novel species of the genus *Serratia*, according to the criteria used for the delineation of bacterial species (Wayne *et al.*, 1987).

Hence, phenotypic and phylogenetic data support the description of strain DZ0503SBS1^T as a novel species within the genus *Serratia*. The novel species can be differentiated from *S. marcescens* subsp. *sakuensis* based on phenotypic characteristics, such as arginine dihydrolase activity, growth in the presence of D-lactose, D-arabinose, raffinose, D-xylose, melibiose, lactate, L-ornithine, L-proline and L-hydroxyproline as sole carbon sources, and the inability to use oxalate as sole carbon source (Table 1). Proportions of the fatty acids cyclo-C_{17:0}, C_{14:0} and cyclo-C_{19:0ω8c} in DZ0503SBS1^T were significantly different from those in *S. marcescens* subsp. *sakuensis* (see Supplementary Table S1 in IJSEM Online). Strain DZ0503SBS1^T yielded a maximum similarity coefficient (*S*_{SM} of 0.82) with *S. marcescens* subsp. *marcescens* DSM 30121^T (see Supplementary Table S2 in IJSEM Online). The fluorescence and symbiotic association with *H. chongmingensis* (Rhabditida: Rhabditidae) (see Supplementary Fig. S2 in IJSEM Online) of strain DZ0503SBS1^T have not been

Table 1. Biochemical and physiological characteristics of strain DZ0503SBS1^T and related taxa

Taxa: 1, DZ0503SBS1^T (=KCTC 22130^T) (this study); 2, *S. marcescens* subsp. *sakuensis* JCM 11315^T (Ajithkumar *et al.*, 2003; this study); 3, *S. marcescens* subsp. *marcescens* LMG 2792^T (Grimont & Grimont, 1992); 4, *S. ureilytica* LMG 22860^T (Bhadra *et al.*, 2005); 5, *S. odorifera* ICPB 3995^T (Grimont *et al.*, 1978); 6, *S. rubidaea* JCM 1240^T (Grimont & Grimont, 1992); 7, *S. entomophila* ATCC 43705^T (Grimont *et al.*, 1988); 8, *S. liquefaciens* ATCC 27592^T (Grimont *et al.*, 1982); 9, *S. grimesii* ATCC 14460^T (Grimont *et al.*, 1982); 10, *S. plymuthica* JCM 1244^T (Spröer *et al.*, 1999); 11, *S. quinivorans* DSM 4597^T (Ashelford *et al.*, 2002); 12, *S. proteamaculans* DSM 4543^T (Grimont *et al.*, 1982); 13, *S. fonticola* DSM 4576^T (Gavini *et al.*, 1979); 14, *S. ficaria* DSM 4569^T (Grimont *et al.*, 1979). All strains were oxidase-negative, catalase-positive, rod-shaped and motile and could grow in the presence of 8% NaCl. None of the strains was able to produce indole or H₂S. All strains were positive for the Voges–Proskauer reaction, aesculin hydrolysis, lysine and ornithine decarboxylase, Simmons' citrate and nitrate reduction. All can utilize succinate, L-arginine and L-histidine as sole carbon source and produce acids from glucose and sucrose. +, Positive; –, negative; NA, data not available.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Fluorescence	+	+	–	–	–	–	NA	NA	–	+	NA	NA	NA	NA
Spore formation	–	+	–	–	–	–	–	–	–	–	–	–	–	–
Pigment production	+	–	+	–	–	+	–	–	–	+	–	–	+	–
Methyl red test	–	–	–	+	+	–	–	+	+	+	–	–	+	+
Caseinase	+	+	–	+	–	–	+	+	+	+	+	+	–	–
Urease	–	–	–	+	–	–	–	–	–	–	–	–	–	–
Lipase (Tween 80)	+	+	+	+	–	+	+	+	+	+	+	+	+	+
Arginine dihydrolase	+	–	–	+	–	–	–	–	+	–	–	–	–	–
Utilization of carbohydrates:														
Adonitol	+	+	–	+	–	+	+	–	–	–	–	–	+	+
D-Lactose	+	–	–	–	+	+	–	–	–	+	–	–	+	+
D-Arabinose	+	–	–	–	+	+	–	–	–	–	–	–	–	–
Raffinose	+	–	–	–	+	+	–	+	–	+	+	+	+	+
D-Xylose	+	–	–	+	+	+	–	+	+	+	+	+	+	+
Melibiose	+	–	–	+	+	+	–	+	+	+	+	+	+	+
Juranose	+	–	+	+	+	+	+	+	+	+	+	+	+	+
Utilization of organic salts:														
Oxalate	–	+	–	+	–	–	NA	–	–	–	–	–	–	–
Lactate	+	–	+	+	+	+	+	+	+	+	+	+	+	+
Amino acid utilization:														
D-Serine	+	+	+	+	–	–	–	–	+	+	–	–	NA	NA
L-Ornithine	+	–	+	+	+	–	–	+	+	+	+	+	+	+
Alanine	+	+	+	+	+	–	+	+	+	+	+	+	+	+
L-Proline	+	–	+	+	+	+	+	+	+	+	+	+	+	+
Threonine	–	–	–	+	–	–	NA	–	–	–	–	–	–	–
Phenylalanine	–	–	+	+	–	–	–	NA	–	–	NA	NA	–	–
L-Hydroxyproline	+	–	+	+	+	–	NA	NA	–	–	+	–	NA	NA
L-Tryptophan	+	+	+	–	+	+	–	+	+	–	+	+	NA	NA
DNA G + C content (mol%)	59.52	58	57.5–60.0	60	NA	NA	58	51.9	NA	NA	53–54	NA	51.5	59.6

reported previously in any species of the genus *Serratia*. The above data and the physiological characteristics (Table 1) provide decisive evidence that strain DZ0503SBS1^T is a member of a different genomic species and, therefore, support its classification as a novel species of the genus *Serratia*, for which the name *Serratia nematodiphila* sp. nov. is proposed.

Description of *Serratia nematodiphila* sp. nov.

Serratia nematodiphila (ne.ma.to.di'phi.la. N.L. n. *nematodum* nematode; Gr. adj. *philos* loving; N.L. fem. adj. *nematodiphila* nematode-loving, referring to its living in the intestine of the nematode *Heterorhabditoides chongmingensis*).

Cells are Gram-negative, short rods, 0.8–1.3 × 0.6–0.7 µm (transmission electron micrographs of cells are given in Supplementary Fig. S3, available in IJSEM Online). Aerobic. Possesses a single flagellum. Motile. Colonies on nutrient agar are red, circular and smooth with entire margins. Grows at 4–42 °C (optimum, 30–37 °C), pH 5–11 (optimum, pH 6.0–10.0) and 2–7% NaCl. Growth occurs on trypticase soy agar, nutrient agar, cetrimide agar, Simmons' citrate agar and MacConkey agar. Catalase-positive and oxidase-negative. β-Galactosidase, ornithine decarboxylase, lysine decarboxylase, arginine dihydrolase and DNase activities are present. Produces amylase. Methyl α-D-glucosidase is present, but phenylalanine decarboxylase, arginine decarboxylase and urease activities are absent. Nitrate is not reduced. Negative for indole and

H₂S production and the methyl red test, but the Voges–Proskauer reaction is positive. Starch, Tween 80, casein, gelatin and Tween 20 are hydrolysed, but cellulose is not hydrolysed. Acid is produced from maltose, D-fructose, D-glucose, sucrose, salicin, L-fucose, D-ribose, xylitol, D-mannose, inositol, D-mannitol, D-galactose and D-sorbitol, but not from D-xylose, D-lactose, starch, di-arabinose, D-rhamnose, L-sorbose, raffinose, L-arabitol, cellobiose, adonite, melezitose, melibiose, amygdalin or D-erythrose. Utilizes adonitol, D-xylose, maltose, D-arabinose, D-fructose, D-glucose, D-lactose, sucrose, D-erythrose, starch, di-arabinose, salicin, L-fucose, raffinose, L-arabitol, cellobiose, D-ribose, D-sorbitol, xylitol, D-mannose, inositol, adonite, melezitose, melibiose, D-mannitol, D-galactose, juranose, amygdalin, erythritol, dextrin, inulin and citrate, but does not utilize acetate, malonate, tartrate or sodium pyruvate. Whole-cell fatty acids are C_{16:0}, cyclo-C_{17:0}, cyclo-C_{19:0}ω8c, C_{14:0}, C_{12:0}, C_{18:1}ω7c, C_{14:0} 2-OH and traces (<1%) of C_{18:0}, C_{10:0} 3-OH, iso-C_{19:0}, C_{15:0}, C_{12:0} 2-OH, C_{12:0} 3-OH, unknown (ECL 14.502), summed feature 2 and summed feature 3. Resistant to ceftazidime and vancomycin, but sensitive to gentamicin, streptomycin, kanamycin, penicillin G, ampicillin, chloramphenicol, erythromycin, tetracycline, ofloxacin, amoxicillin, cefamezin, rifampicin, carbenicillin and polymyxin B. The DNA G + C content of the type strain is 59.52 mol%.

The type strain is DZ0503SBS1^T (=KCTC 22130^T =CGMCC 1.6853^T), isolated from the intestine of *H. chongmingensis* (Rhabditida: Rhabditidae). Experimental support in favour of symbiosis has been demonstrated by Zhang *et al.* (2008).

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