Serratia ureilytica sp. nov., a novel urea-utilizing species

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A Gram-negative, rod-shaped, urea-dissolving and non-spore-forming bacterium, designated strain NiVa 51^T, was isolated from water of the River Torsa in Hasimara, Jalpaiguri district, West Bengal, India. On the basis of 16S rRNA gene sequence similarity, strain NiVa 51^T was shown to belong to the γ -*Proteobacteria* and to be related to *Serratia marcescens* subsp. *sakuensis* (98.35%) and *S. marcescens* subsp. *marcescens* (98.30%); however, strain NiVa 51^T exhibited only 43.7% similarity to *S. marcescens* by DNA–DNA hybridization. The G+C content of the genomic DNA of the isolate was 60 mol%. Both biochemical characteristics and fatty acid analysis data supported the affiliation of strain NiVa 51^T to the genus *Serratia*. Furthermore, strain NiVa 51^T was found to utilize urea as nitrogen source. The results of DNA–DNA hybridization as well as physiological and biochemical tests allowed genotypic and phenotypic differentiation of strain NiVa 51^T from recognized *Serratia* species. Strain NiVa 51^T therefore represents a novel species, for which the name *Serratia ureilytica* sp. nov. is proposed, with type strain NiVa 51^T (=LMG 22860^T = CCUG 50595^T).

The genus *Serratia* belongs to the family *Enterobacteriaceae* of the γ subclass of the *Proteobacteria*. Some members of this genus produce pigments identified as prodigiosin (Hearn *et al.*, 1970; Gerber, 1975). A spore-forming subspecies of *Serratia marcescens* (*S. marcescens* subsp. *sakuensis*) was recently described by Ajithkumar *et al.* (2003). We have isolated a novel non-pigmented, non-spore-forming strain (isolate NiVa 51^T) from water of the River Torsa, West Bengal, India. The Torsa is an international river traversing three countries, China (Tibet), Bhutan and India, before entering Bangladesh. The strain was isolated in February 2001, when the river water had a high free-ammonia nitrogen content (61.2 p.p.m.) owing to sewage contamination (Bhadra *et al.*, 2003). Strain NiVa 51^T was able to hydrolyse urea to ammonia and to utilize urea as a sole nitrogen source for its growth.

During characterization of nickel-resistant copiotrophic bacteria isolated from water from the River Torsa, strain

NiVa 51^T was recovered on nutrient agar (Himedia) containing 3 mM nickel chloride after incubation at 35 °C for 24 h. Subcultivation was done on nutrient agar at 35 °C for 16–18 h. Strain NiVa 51^T was able to grow on nutrient agar at 10–42 °C, but not at 4 or 45 °C. Growth was also observed at 28 °C on trypticase soy agar (Difco) after incubation for 16–18 h. Basal medium [(g l⁻¹): MgSO₄·7H₂O, 0.5; KH₂PO₄, 0.5; KCl, 0.1; pH 6.5 ± 0.2] containing 50 mM glucose and 0.5 mM ammonium sulfate or urea did not support the growth of NiVa 51^T until supplemented with 0.01% yeast extract. The same basal medium with similar supplementation was used to determine growth curves. In all growth experiments, a 1 ml aliquot of exponentially grown cells in nutrient broth was centrifuged, washed in sterile 0.86 M NaCl, resuspended in 250 µl sterile basal medium and inoculated in 20 ml medium contained in 250 ml Erlenmeyer flasks. Cells inoculated in the same medium, but devoid of urea or ammonium sulfate, served as a negative control. Cultures were grown at 35 °C with shaking. A UV-Vis spectrophotometer (Shimadzu) and a digital pH meter (Systronics) were used to determine OD₅₆₀ and the pH of the culture medium, respectively, at regular intervals. Generation times, calculated from the growth curves (see Supplementary Fig. S1 in IJSEM Online) obtained from the culture media containing urea and ammonium sulfate as a sole nitrogen source, were 120 ± 2 and 90 ± 0.2 min, respectively. For estimation of free-ammonia 'nitrogen', generated in the medium containing urea, a 10 ml aliquot of growing culture (withdrawn at regular

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Figures showing the growth curve of strain NiVa 51^T and phylogenetic trees constructed by using maximum-parsimony, KITSCH and FITCH algorithms, and tables detailing the cellular fatty acid composition of strain NiVa 51^T and related taxa, and similarity coefficients of members of the family *Enterobacteriaceae* are available as supplementary material in IJSEM Online.

intervals) was filtered through a bacterial-filter membrane (diameter 0.45 µm) and then diluted with ammonia-free distilled water. Quantification of free ammonia was done by following standard techniques (APHA, 1985; Manivasakam, 1980). One of the two control sets contained all the chemical ingredients, including urea, without bacterial cells and the other contained heat-killed cells (100 °C for 15 min). In the active culture medium containing 0.5 mM urea as sole nitrogen source and at an initial cell density of 3×10^8 cells ml⁻¹, the rate of free-ammonia 'nitrogen' released into the culture medium in the first 2 h incubation was 0.6 p.p.m. h⁻¹. This reached a maximum rate of 1.4 p.p.m. h⁻¹ after 6–10 h incubation.

Gram staining was performed following the method of Gerhardt *et al.* (1994). Cell morphology was observed under a phase-contrast microscope (Olympus, model M021) at $\times 1000$, with cells grown for 16 h at 35 °C on nutrient agar. The 16S rRNA gene was amplified, cloned and sequenced as described by Carr *et al.* (2003). The sequence obtained was compared with those in GenBank by using the BLAST N program (Altschul *et al.*, 1997). After multiple alignments of the data using the CLUSTAL X program (Thompson *et al.*, 1997), phylogenetic analysis was performed by using the software package PHYLIP 3.6c (Felsenstein, 2002) according to three different methods [neighbour joining (Saitou & Nei, 1987), maximum parsimony (Felsenstein, 1983) and maximum likelihood (Yang, 1997)]. For the purpose of neighbour-joining, KITSCH and FITCH analyses, distances were calculated by using both two-parameter (Kimura, 1980) and one-parameter (Jukes & Cantor, 1969) models in DNADIST. The 16S rRNA gene sequence of *Plesiomonas shigelloides* (the most closely related species in the family Enterobacteriaceae; Brenner, 1992) was used to root all trees.

To determine the confidence values for individual branches, 100 bootstrap replications were done for each generated tree by using SEQBOOT and CONSENSE from the PHYLIP package. Phylogenetic trees obtained by the maximum-likelihood method showed that strain NiVa 51^T branched deeply with the *Serratia* cluster consisting of *S. marcescens*, *Serratia rubidaea* and *Serratia odorifera* (Fig. 1). Similar patterns of branching were noted in trees derived from maximum-parsimony, KITSCH and FITCH analysis (see Supplementary Fig. S2 in IJSEM Online). Sequence similarity, based on pairwise sequence comparisons, was investigated by using complete 16S rRNA gene sequences. The 16S rRNA gene sequence of strain NiVa 51^T showed 96–97% similarity to many species of the genus *Serratia*, and 98.30–98.35% similarity to *S. marcescens* subsp. *sakuensis* and *S. marcescens* subsp. *marcescens*.

Analysis of non-polar and hydroxy fatty acids was performed following techniques described by Pandey *et al.* (2002). The fatty acid profile of strain NiVa 51^T consisted of 12:0 (1.4%), 12:0 2-OH (0.28%), an unknown fatty acid with equivalent length of 13.957 (0.97%), 14:0 (6.28%), an unknown fatty acid with equivalent length of 14.502 (0.85%), 14:0 2-OH (2.21%), 15:0 (0.29%), 16:0 (31.01%), 17:0 cyclo (2.69%), 17:0 (0.35%), 18:1 ω 7c (17.89%), 18:0 (0.46%), 19:0 cyclo ω 8c (0.25%), summed feature 2 (comprising 12:0 alde and 14:0 3-OH/16:1 iso I) (8.01%) and summed feature 3 (comprising 16:1 ω 7c/15 iso 2-OH) (27.07%). The fatty acid profile of strain NiVa 51^T was compared with those of other members of the same *Serratia* cluster (*S. marcescens*, *S. rubidaea* and *S. odorifera*) (see Supplementary Table S1 in IJSEM Online), and was found to have a maximum similarity index of 0.747 with *S. marcescens* GC subgroup B.

Fig. 1. Phylogenetic position of strain NiVa 51^T among related bacteria. The tree was produced by using the maximum-likelihood algorithm and was calculated by using 1430 bases of the 16S rRNA gene sequence. *Plesiomonas shigelloides* was used as the outgroup. Bootstrap values for 100 replications are given at branch nodes (only values of 60 and above are given). Bar, single substitutions per 100 nt.

For determination of the G + C content, DNA was hydrolysed with P1 nuclease, de-phosphorylated with bovine alkaline phosphatase and analysed via HPLC following techniques described by Mesbah *et al.* (1989). The DNA G + C content of strain NiVa51^T was 60 mol%. This value is higher than that reported for *S. marcescens* subsp. *sakuensis* (58 mol%; Ajithkumar *et al.*, 2003), but close to that for *S. marcescens* (57.5–60 %; Grimont & Grimont, 1992).

Results of the physiological characterization are given in the species description below and in Table 1, and were determined with the methods described by Holt *et al.* (1994) and Ajithkumar *et al.* (2003). Strain NiVa 51^T yielded a maximum similarity coefficient (S_{SM} ; 0.87) to *S. marcescens* (see Supplementary Table S2 in IJSEM Online).

In order to confirm the phylogenetic conclusions, levels of DNA–DNA relatedness were studied by using the dot-blot hybridization method with a DIG DNA Labelling and Detection kit (Roche Diagnostics). The percentage hybridization was quantified by using Molecular Analyst software (Bio-Rad). A probe was prepared from genomic DNA of strain NiVa 51^T and approximately 1000-fold of the same was used in hybridizing against frames of three to five target DNAs taken in triplicate quantities on a positively charged nylon membrane. Hybridization was performed at 65 °C and the membrane was washed under high-stringency conditions. NiVa 51^T exhibited a relatively low level of DNA–DNA hybridization with *S. marcescens* subsp. *marcescens* (43.7 %), *S. odorifera* (29.2 %), *S. rubidaea* (27 %), *Pectobacterium carotovorum* subsp. *carotovorum* (21.7 %), *Erwinia amylovora* (20 %), *Citrobacter freundii* (19 %) and *Enterobacter aerogenes* (18.3 %). This indicated strongly that the new isolate represents a novel species of the genus *Serratia*, according to the criteria used for differentiation of bacterial species (Wayne *et al.*, 1987).

From the phenotypic, chemotaxonomic and phylogenetic results presented here, we can conclude that strain NiVa 51^T forms a separate group within the genus *Serratia*. The novel species can be differentiated from *S. marcescens* subsp. *marcescens* based on phenotypic characteristics, such as pigment production, methyl red test, caseinase, urease, arginine dihydrolase, and growth in the presence of adonitol, xylose, oxalate, DL-threonine and L-tryptophan as sole carbon source. Proportions of the fatty acids 17:0 cyclo and 19:0 cyclo ω 8c in NiVa 51^T were approximately four to five times lower than in *S. marcescens*. 16S rRNA gene sequence similarity between *S. marcescens* subsp. *marcescens* and strain NiVa 51^T was 98.3 % and DNA–DNA relatedness was 43.7 %. The unique urea-utilizing characteristic of strain NiVa 51^T therefore supports its classification as a novel species of *Serratia*, for which the name *Serratia ureilytica* sp. nov. is proposed.

Description of *Serratia ureilytica* sp. nov.

Serratia ureilytica [u.re.i.ly'ti.ca. N.L. n. urea -ae urea; N.L. adj. lyticus -a -um (from Gr. adj. lutikos) dissolving; N.L. fem. adj. ureilytica urea-dissolving].

Table 1. Biochemical and physiological characteristics of strain NiVa 51^T and related taxa

Taxa: 1, NiVa 51^T; 2, *S. marcescens* subsp. *marcescens* LMG 2792^T; 3, *S. marcescens* subsp. *sakuensis* JCM 11315^T; 4, *Serratia odorifera* MTCC495; 5, *Serratia rubidaea*. All were oxidase-negative, catalase-positive, rod-shaped and motile and could grow in the presence of 1.4 M NaCl; none was able to produce indole, amylase or H₂S. All strains were positive for the Voges–Proskauer reaction, aesculin hydrolysis, lysine and ornithine decarboxylase, Simmons' citrate and nitrate reduction. All could utilize acetate, succinate, L-arginine and L-histidine as sole carbon source and could produce acids from glucose and sucrose. Characteristics that distinguish strain NiVa 51^T from *S. marcescens* subsp. *marcescens* are indicated by an asterisk (*). +, Positive; –, negative; NA, data not available; ND, not done.

Characteristic	1	2	3†	4	5‡	
Spore	—	—	+	(round)	—	—
Pigment*	—	+	—		—	—
Methyl red*	+	—	—		+	—
Caseinase*	+	—	+		—	—
Urease*	+	—	—		—	—
Lipase (Tween 80)	+	+	+		—	+
Arginine dihydrolase*	+	—	—		—	—
Acid production from:						
Adonitol*	+	—	NA		—	+
Lactose	—	—	—		+	+
Sorbitol	+	+	+		+	—
Arabinose	—	—	—		+	+
Raffinose	—	—	—		ND	+
Rhamnose	—	—	—		+	—
Xylose*	+	—	—		+	+
Melibiose*	+	—	NA		+	+
Utilization of organic acid salts:						
Oxalate*	+	—	+		ND	NA
Lactate	+	+	—		+	NA
Amino acid utilization:						
DL-Serine	+	+	+		ND	NA
L-Ornithine	+	+	—		ND	—
DL-Alanine	+	+	+		+	—
L-Proline	+	+	NA		ND	NA
DL-Threonine*	+	—	NA		—	NA
DL-Phenylalanine	+	+	NA		ND	—
L-Hydroxyproline	+	+	—		+	NA
L-Tryptophan*	—	+	NA		+	NA

†Data taken from Ajithkumar *et al.* (2003).

‡Data taken from Grimont & Grimont (1992).

Cells are facultatively aerobic, Gram-negative, motile, non-fluorescent, non-pigmented, straight rods, 0.7–1.0 µm long and 0.8–1.0 µm wide. Grows between 8 and 43 °C over a pH range of 5–11 in nutrient broth media, and can tolerate up to 1.4 M NaCl. Positive for arginine dihydrolase. DNA G + C content is 60 mol% and major cellular fatty acids are 16:0 (31.01 %), summed feature 3 (comprising 16:1 ω 7c/15

iso 2-OH) (27.07 %) and 18:1 ω 7c (17.89 %). Can use urea as a sole nitrogen source for growth. Other biochemical characteristics are given in Table 1. The isolate has a plasmid of 50 kb of unknown function and can grow in nutrient agar media containing 40 μ g ampicillin ml⁻¹, 15 μ g tetracycline ml⁻¹ and 20 μ g chloramphenicol ml⁻¹.

The type strain, NiVa 51^T (=LMG 22860^T=CCUG 50595^T), was isolated from the River Torsa, northern West Bengal, India.

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