

# Spore-forming *Serratia marcescens* subsp. *sakuensis* subsp. nov., isolated from a domestic wastewater treatment tank

Bindu Ajithkumar,<sup>1</sup> Vasudevan P. Ajithkumar,<sup>1</sup> Ryozo Iriye,<sup>1</sup>  
Yukio Doi<sup>1†</sup> and Tadashi Sakai<sup>2</sup>

## Correspondence

Ryozo Iriye

kn53278@gipmc.shinshu-u.ac.jp

<sup>1</sup>Ecological and Toxicological Laboratory, Department of Bioscience and Biotechnology, Faculty of Agriculture, Shinshu University (United Graduate School of Agriculture, Gifu University), 8304 Minamiminowa, Kamiina, Nagano 399-4598, Japan

<sup>2</sup>UltraStructure Research Laboratories Ltd, Tochiku, Yokohama 224-0007, Japan

A strain (KRED<sup>T</sup>) that formed endospores and produced the pigment prodigiosin was isolated from activated sludge. The presence of spores in cells of strain KRED<sup>T</sup> was evident upon electron microscopy examination, heat treatment and the detection of dipicolinic acid in the cells. Biochemical characteristics, and 16S rDNA sequence and DNA–DNA homology data identified strain KRED<sup>T</sup> as *Serratia marcescens*. The major respiratory quinone of strain KRED<sup>T</sup> was found to be ubiquinone Q-8. The formation of endospores by Gram-negative bacteria has not been observed previously, and has never been reported in any species of *Serratia*. Here, it is shown that strain KRED<sup>T</sup> (JCM 11315<sup>T</sup> = CIP 107489<sup>T</sup>) represents a novel subspecies of *S. marcescens*, for which the name *Serratia marcescens* subsp. *sakuensis* is proposed.

## INTRODUCTION

When the bacterial flora of activated sludge in a domestic wastewater treatment tank in Saku, Japan, was analysed, co-workers Doi and Iriye isolated a spore-forming bacterium (strain no. 9; KRED<sup>T</sup>) that produced a red-coloured pigment and endospores, and hydrolysed cooked meat. Due to the presence of endospores, the spore-forming bacterium was tentatively identified and reported to be a *Bacillus* sp. (Doi *et al.*, 1998). However, the DNA G + C content, production of red pigment and biochemical characteristics of the bacterium were not in agreement with those of the genus *Bacillus*, but instead resembled the characteristics of the genus *Serratia*. The genus *Serratia* belongs to the *Enterobacteriaceae*, and some members of this genus produce pigments identified as prodigiosin (Hearn *et al.*, 1970; Gerber, 1975). Some members of the genus *Serratia* also have clinical importance (Grimont & Grimont, 1992; Brenner, 1984). Production of spores among *Serratia* spp. has never been observed, but the strain (KRED<sup>T</sup>) described in this study produced spores. Therefore, the spore-forming

strain isolated from the wastewater treatment tank posed a difficult question regarding its taxonomic position. Polyphasic identification of the novel bacterium, including 16S rDNA sequence phylogeny and genetic relatedness to confirm the identity of the isolate, and evidence for spore formation by the bacterium are presented in this work.

## METHODS

**Isolation and culture conditions.** The domestic wastewater was favourably treated at Komaba sewerage facility, Saku, Nagano, Japan (Doi *et al.*, 1998) (actual residence time 24 h; typical quality of treated water: biological oxygen demand, 4.6 mg l<sup>-1</sup>; suspended solids, 4 mg l<sup>-1</sup>; total nitrogen, 7.5 mg l<sup>-1</sup>; total phosphate, 2.5 mg l<sup>-1</sup>). The gross yield coefficient of the sludge was below 8%, and the concentration of mixed-liquor suspended solids (MLSS) in the treatment tank was high (5500 mg l<sup>-1</sup>). To elucidate the bacterial flora of the activated sludge, bacteria in the suspended liquor in the first aerobic tank were analysed by the dilution heterotrophic plate-count method. The suspended liquor was diluted with 0.5% NaCl (1 × 10<sup>4</sup> dilution) and plated onto culture plates (NG agar, described below); the cultures were incubated for 24 h at 32 °C and then at room temperature for 6 days.

Culture medium (NG broth) was composed of 8.0 g nutrient broth (CM-1; Oxoid), 8.0 g glucose, 5.0 g NaCl and 0.5 g yeast extract (Difco) in 1 l distilled water. Agar (15 g) was added to NG broth for plate culture (NG agar).

**Cell morphology and characterization.** Tests for the utilization of various carbon compounds by the novel bacterium and other biochemical tests were carried out following standard methods (Smibert

Published online ahead of print on 18 October 2002 as DOI 10.1099/ijs.0.02158-0.

<sup>†</sup>Present address: Fujiclean Engineering Company Ltd, Chigusa, Nagoya 464, Japan.

**Abbreviations:** DPA, dipicolinic acid (pyridine-2,6-dicarboxylic acid); *t<sub>R</sub>*, retention time.

The DDBJ accession number for the 16S rRNA gene sequence of *Serratia marcescens* subsp. *sakuensis* KRED<sup>T</sup> is AB061685.

& Krieg, 1994). Cooked-meat hydrolysis was tested by the decrease in the amount of cooked meat (200 mg; Oxoid) present after incubation in 0.5% NaCl (6 ml) at 32 °C for 10 days.

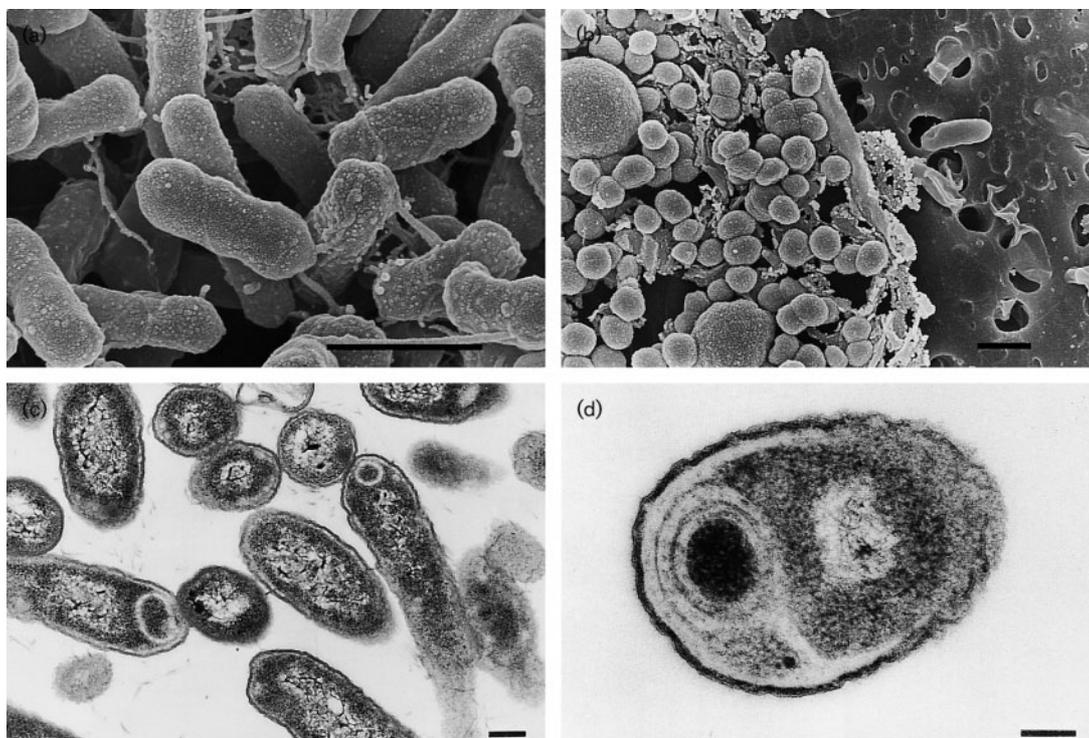
**Heat-resistance test.** Seven-day- and 3-month-old cultures of strain KRED<sup>T</sup> on NG agar were subjected to a heat-resistance test. Each culture was suspended in 0.5% NaCl (1 ml) or NG broth (5 ml) (OD<sub>660</sub> 0.05) and heated at 60, 62, 65, 70 and 75 °C for up to 20 min. Each culture heated in 0.5% NaCl was shaken at 32 °C in NG broth (5 ml) or incubated on an NG agar slant at 32 °C. Each culture heated in NG broth was incubated at 32 °C with shaking or incubated on an NG agar slant at 32 °C. *Serratia marcescens* JCM 1239<sup>T</sup> (=ATCC 13880<sup>T</sup>) and *Bacillus subtilis* IFO 13719<sup>T</sup> (=ATCC 6051<sup>T</sup>) were similarly treated and used as controls.

**Analysis of dipicolinic acid (DPA).** DPA was extracted from strain KRED<sup>T</sup> according to published methods (Janssen *et al.*, 1958; Powell, 1953). Cells of strain KRED<sup>T</sup> (0.2 g wet weight) that had been cultivated for 1 week on NG agar plates and washed with water were heated in 2 ml water at 100 °C for 20 min. The suspension was cooled and centrifuged; the resulting supernatant was condensed to dryness. The residue was dissolved in 0.1 ml water and then filtered through a 0.45 µm filter disc. The filtrate (0.05 ml) was analysed using an LC-MS spectrometer [column AQ-312 (ODS 6 × 150 mm), YMC, Kyoto, Japan; solvent 10 mM CH<sub>3</sub>COONH<sub>4</sub>; flow rate 1 ml min<sup>-1</sup>]. Authentic DPA could be detected at retention time (*t<sub>R</sub>*) 3.45 min (*m/z* values of 168, 124 and 80) [LC-MS spectra were measured with a Hitachi M-1200AP LC-MS spectrometer; flow rate 1 ml min<sup>-1</sup>; atmospheric pressure chemical ionization (APCI) positive mode].

**Scanning electron microscopy.** Electron micrographs of strain KRED<sup>T</sup> incubated on NG agar for 7 days at 20 °C (cells) and on NG agar for 18 days at 20 °C (spores) were taken at UltraStructure Research Laboratories; the cells were pre-fixed in 2% glutaraldehyde and fixed with 1% osmic acid. A Hitachi S-4500 scanning electron microscope was used.

**Transmission electron microscopy.** A study of the ultrastructure of the endospores of strain KRED<sup>T</sup> was carried out at UltraStructure Research Laboratories. Strain KRED<sup>T</sup> was cultivated on NG agar at 32 °C for 20 h and successively for 40 h at 20 °C (liberation of spores was observed with a phase-contrast microscope). Cells of strain KRED<sup>T</sup> were pre-fixed with 2% glutaraldehyde in phosphate buffer and fixed with 1% osmic acid (Fig. 1c) and successively stained with 4% uranyl acetate (Fig. 1d). Epoxy resin was used for embedding the cells. Sections (800 Å) of the cells were prepared with an LKB U5 ultramicrotome (Amersham Pharmacia) and examined with a JEOL JEM 800a electron microscope.

**Sequencing of 16S rDNA.** Extraction of DNA from strain KRED<sup>T</sup> and analysis of the sequence of its 16S rDNA was carried out at NCIMB, Japan, using the PrepMan method (DNA extraction; Applied Biosystems), and a Microseq full gene 16S rDNA bacterial sequencing kit (Microseq system; Applied Biosystems) and an ABI model 310 automated DNA sequencer (Applied Biosystems). The 16S rDNA sequence of strain KRED<sup>T</sup> was compared to available sequences using the BLAST program from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) and the Ribosomal Database Project (Maidak *et al.*, 1996). An evolutionary



**Fig. 1.** Scanning and transmission electron micrographs of strain KRED<sup>T</sup>. (a) Strain KRED<sup>T</sup> after incubation for 7 days on NG agar. Bar, 1 µm. (b) Spores of strain KRED<sup>T</sup> after incubation for 18 days on NG agar. Bar, 1 µm. (c) Endospores of strain KRED<sup>T</sup>, after incubation for 16 h at 32 °C and 40 h at 20 °C on NG agar, and pre-fixed with 2% glutaraldehyde and fixed with 1% osmic acid (bar, 0.2 µm), and (d) successively stained with 4% uranyl acetate (bar, 0.1 µm).

distance tree and sequence similarities were calculated using the programs TREEVIEW and CLUSTAL W, respectively (Saitou & Nei, 1987; Thompson *et al.*, 1994).

**DNA–DNA homology.** DNA was extracted from bacteria by the method of Marmur (1961). DNA–DNA homology was determined by fluorometric hybridization in microdilution wells [Black Cliniplate; Labsystems, catalogue no. 95029120 (enhanced binding type)] according to the method of Ezaki *et al.* (1989), using biotinylated DNA. DNA–DNA hybridization was performed at 50 °C for 2 h in 2 × SSC containing 45 % formamide. Fluorescence intensity was measured with a FP 3000 Fluorolite microplate reader (Shinseirika, Tokyo) at 360 nm for excitation and 450 nm for emission.

**Assay for antibacterial sensitivity.** Antibacterial sensitivity profile of strain KRED<sup>T</sup> was determined on NG agar plates using antibacterial discs [Showa Disc (Showa Yakuhin Kako) supplied by Nissuiseiyaku, Tokyo].

**Cellular fatty acid analysis.** Cells of strain KRED<sup>T</sup> that had been cultured on NG agar (4 days) were washed with distilled water. Wet cells (200 mg) were refluxed in 10 % KOH/65 % ethanol (5 ml) for 1 h under an N<sub>2</sub> atmosphere. The reaction mixture was acidified with 1 M H<sub>2</sub>SO<sub>4</sub> and fatty acids were extracted with ethyl acetate. The organic layer was washed with aqueous NaCl and then dried over MgSO<sub>4</sub>. After evaporation of solvents, the resulting fatty acid mixture in ether was methylated with diazomethane and analysed using a GC-MS spectrometer by EI positive mode. The *t<sub>R</sub>* values of the fatty acid methyl esters (FAMES) were compared with those of reference bacterial FAMES purchased from Supelco. The molecular formula of each peak was determined using high-resolution GC-MS analysis (Pizzimenti *et al.*, 1999). Low- and high-resolution GC-MS spectra were measured by using a JEOL JMS-700 spectrometer with the JEOL data processing system and a Hewlett Packard 5890 gas chromatograph [column DB-1 (HP, non-polar); temperature 140–220 °C (6 °C min<sup>-1</sup>) and kept at 220 °C for 20 min; injection temperature 240 °C; flow rate 7.2 ml min<sup>-1</sup> (He gas)].

**Isoprenoid quinone analysis.** Cells of strain KRED<sup>T</sup> that had been cultured on NG agar (4 days) were washed with distilled water. Isoprenoid quinones were extracted from 500 mg wet cells with 150 ml acetone (three times) by stirring for 2 h each at room temperature. After centrifugation, the supernatant was condensed to dryness. The extracts dissolved in acetone were applied to preparative silica-gel TLC, and then developed with benzene. The isoprenoid quinones were detected under an UV lamp. The bands due to menaquinones and ubiquinones were collected and extracted with acetone. Each fraction was analysed using an LC-MS spectrometer [Column Zorbax-ODS (4.5 × 250 mm), solvent methanol/diisopropyl ether (4:1, v/v), flow rate 1 ml min<sup>-1</sup> (Collins & Jones, 1981; Hiraishi, 1999)]. LC-MS spectra were measured using a Hitachi M-1200AP LC-MS spectrometer (flow rate 1 ml min<sup>-1</sup>; APCI positive mode).

**Pigment analysis.** Cells of strain KRED<sup>T</sup> that had been cultured on NG agar (2 days) were washed with distilled water. Pigments (66 mg) were extracted from 8 g wet cells with 150 ml ethyl acetate in a separating funnel. The organic layer was washed twice with saturated NaCl solution and then dried over MgSO<sub>4</sub>. After evaporation of the solvents, the extracts (66 mg) were separated by silica-gel TLC (benzene/acetone, 5:1). The red-coloured band (*R<sub>f</sub>* 0.35–0.6) was collected and extracted with ethyl acetate/ethanol (5:1). After evaporation of the solvents, the pigment (4.3 mg) was dissolved in 5 ml ethyl acetate containing a few drops of 2 M HCl and shaken. The organic layer was washed with saturated NaCl solution and then dried over MgSO<sub>4</sub>. After evaporation of the solvent, the pigment was analysed by using a Bruker DRX 500 spectrometer with tetramethylsilane as an internal standard and Bruker's Pulse programme

(<sup>1</sup>H at 500.13 MHz and <sup>13</sup>C at 125.77 MHz). The red pigment was extracted from *S. marcescens* JCM 1239<sup>T</sup> using the same method as described above and analysed using an NMR spectrometer.

## RESULTS

### Cell morphology and biochemical characteristics

Cells of strain KRED<sup>T</sup> (Fig. 1a) were rods (0.5–0.6 × 1.3–2 μm) that formed granules (generally 0.4–0.6 μm, but some measured 1.5–1.8 μm; Fig. 1b) identified as endospores (Fig. 1c, d). Colonies of strain KRED<sup>T</sup> were smooth and round, and on nutrient agar the strain produced a bright-red pigment. Because of endospore formation, some cells of strain KRED<sup>T</sup> were found to be swollen when first isolated, though such swollen cells could not be observed after successive transfers and maintenance. The biochemical characteristics of strain KRED<sup>T</sup> are given in Table 1, and they were found to be very similar to those of *S. marcescens*. Strain KRED<sup>T</sup> differed from the type strain of *S. marcescens* in terms of its methyl red test [negative on sucrose (5 days), D-sorbitol (7 days) and L-arabitol, whereas *S. marcescens* was positive on sucrose, D-sorbitol and L-arabitol (7 days)]. Strain KRED<sup>T</sup> did not produce acid from L-arabitol, whereas *S. marcescens* did.

### Elucidation of the ultrastructure of strain KRED<sup>T</sup>

Endospores of strain KRED<sup>T</sup> were observed by transmission electron microscopy analysis (Fig. 1c, d). Cells of strain KRED<sup>T</sup> had an outer membrane, a peptidoglycan layer and a cell membrane (not visible), all characteristics of Gram-negative bacteria (Fig. 1c). Inside the cells, granules with cortex layers were observed (Fig. 1c, a white ring), indicating that endospores were present. Although a spore coat and spore membrane could not be detected in the cells under the conditions tested, spore coats could be clearly observed from some endospores in which a cortex layer was covered with the peptidoglycan layer of the cell, by staining with uranyl acetate (Fig. 1d). The spore membrane of strain KRED<sup>T</sup> endospores could not be observed, and was presumed to be very thin. The structure of the endospores of strain KRED<sup>T</sup> was similar to that of an endospore of *Bacillus megaterium* (Ralph *et al.*, 1992).

### Heat resistance

Cultures of strain KRED<sup>T</sup> that had been incubated for 7 days and 3 months, respectively, survived heat treatment at 62 °C for 15 min in 0.5 % NaCl and 75 °C for 20 min in NG broth. *B. subtilis* IFO 13719<sup>T</sup> incubated for 10 days survived similar heat treatment, but *S. marcescens* JCM 1239<sup>T</sup> did not survive heat treatment at 60 °C for 15 min. Growth from cultures of strain KRED<sup>T</sup> that had been subjected to heat treatment could be observed after 24 h, whereas growth of the controls (not subjected to heat treatment) was observed after 12 h. The heat resistance of strain KRED<sup>T</sup> could be attributed to it forming spores.

**Table 1.** Biochemical characteristics of *Serratia marcescens* subsp. *sakuensis* and differentiation of this novel subspecies from *S. marcescens* subsp. *marcescens*

Strains: 1, *S. marcescens* subsp. *sakuensis*; 2, *S. marcescens* subsp. *marcescens*. Both subspecies of *S. marcescens* were rod-shaped organisms that were motile by means of peritrichous flagella. Both subspecies were able to grow in the presence of 7% NaCl, were Voges–Praskauer-positive and had a fermentative type of metabolism (O/F test).

Characteristic	1	2
Size (µm)	0.5 × 1.3	0.5–0.8 × 0.9–2.0*
Spore	+ (Round)	–
Motility	+	+*
Anaerobic growth	+	+*
Casein hydrolysis	+	+*
Tween 80 hydrolysis	+	+*
Catalase	+	+*
NO <sub>3</sub> reduction to NO <sub>2</sub>	+	+*
NH <sub>3</sub> utilization	+	+*
Indole production	–	–*
Starch hydrolysis	–	–*
Growth on and acid production from:		
Sucrose	+†	+
D-Glucose	+†	+†
D-Sorbitol	+†	+
L-Arabitol	+‡	+
Hydrolysis of cooked meat (%)§	80	73
G + C content (mol%)	58 (HPLC)	57.5–60 (T <sub>m</sub> , buoyant density)*

\*Data are from Grimont & Grimont (1984).

†Methyl red test negative within 7 days.

‡Does not produce acid.

§Decrease in the amount of cooked meat present after 10 days incubation.

## DPA

DPA could be detected [10 µg (g bacteria)<sup>-1</sup>] by LC-MS analysis [*t*<sub>R</sub> 3.5 min; *m/z* 168 (*M*<sup>+</sup>H)<sup>+</sup>, 124 (*M*<sup>+</sup>H–CO<sub>2</sub>)<sup>+</sup> and 80 (*M*<sup>+</sup>H–2CO<sub>2</sub>)<sup>+</sup>].

## DNA G + C content

DNA G + C content (as determined by HPLC) of strain KRED<sup>T</sup> was 58 mol%, and agreed with the published values for *S. marcescens* (Grimont & Grimont, 1992; Brenner, 1984).

## Phylogenetic analysis and DNA–DNA homology

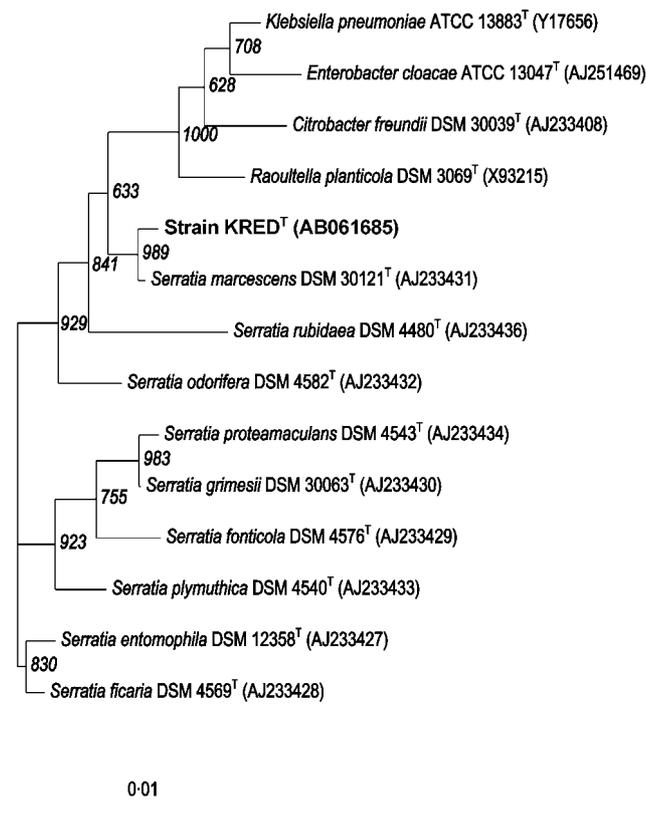
An almost complete sequence (1532 bp) of the 16S rDNA of strain KRED<sup>T</sup> was determined (DDJB accession no. AB061685). A phylogenetic tree of related sequences based on BLAST sequence homology (Altschul *et al.*, 1990) was constructed using the DDBJ data analysis server (CLUSTAL

w) and TREECON software (Van de Peer & De Wachter, 1997); the tree is shown in Fig. 2. The 16S rDNA sequence of strain KRED<sup>T</sup> showed 99.6% similarity with *S. marcescens*, 98.2% with *Serratia entomophila*, 98.1% with *Serratia ficaria*, 98.0% with *Serratia odorifera*, 97.8% with *Serratia rubidaea*, 97.4% with *Enterobacter cloacae*, 97.4% with *Klebsiella pneumoniae* and 97.4% with *Citrobacter freundii*.

The level of DNA–DNA hybridization between strain KRED<sup>T</sup> and *S. marcescens* JCM 1239<sup>T</sup> was found to be 97%.

## Fatty acids

Whole-cell fatty acids of strain KRED<sup>T</sup> were detected as saturated fatty acids C<sub>12:0</sub> (1.25%), C<sub>15:0</sub> (0.58%), C<sub>16:0</sub> (33.2%), C<sub>17:0</sub> (0.66%) and C<sub>18:0</sub> (1.93%), mono-hydroxylated fatty acids C<sub>14:0</sub> (2.83%) and C<sub>16:0</sub> (0.14%), and unsaturated fatty acids C<sub>12:2</sub> (2.94%), C<sub>14:1</sub> (9.81%), C<sub>17:1</sub> (28.31%), C<sub>18:2</sub> (0.77%) and C<sub>19:1</sub> (19.94%).



**Fig. 2.** Phylogenetic placement of strain KRED<sup>T</sup> within the genus *Serratia*, based on 16S rRNA gene sequence analysis. The significance of each branch is indicated by bootstrap values at the nodes. The GenBank accession number for the 16S rDNA sequence of each reference species is shown in parentheses. Bar, estimated substitutions per nucleotide position.

The profile of strain KRED<sup>T</sup> was similar to that of *S. marcescens* (Grimont & Grimont, 1992; Pizzimenti *et al.*, 1999).

### Isoprenoid quinones

Ubiquinone (Q-8;  $t_R$  10.8 min) was detected as the major respiratory quinone of strain KRED<sup>T</sup>, along with very small amounts of menaquinones [MK-8, MK-8(H<sub>2</sub>), MK-8(2H<sub>2</sub>);  $t_R$  16.1–16.3 min]. The presence of Q-8 as the major quinone in the type strain of *S. marcescens* has been reported previously (Grimont & Grimont, 1992; Collins & Jones, 1981; Hiraishi, 1999).

### Antibacterial sensitivity

Strain KRED<sup>T</sup> was resistant to tetracycline, erythromycin, polymyxin, lyncomycin and benzylpenicillin, but sensitive to ampicillin (MIC 50.0 µg ml<sup>-1</sup>), chloramphenicol (MIC 6.25 µg ml<sup>-1</sup>), gentamicin (MIC 6.25 µg ml<sup>-1</sup>), kanamycin (MIC 12.5 µg ml<sup>-1</sup>), streptomycin (MIC 50.0 µg ml<sup>-1</sup>) and carbenicillin (MIC 12.5 µg ml<sup>-1</sup>).

### Pigment analysis

The pigment produced by strain KRED<sup>T</sup> was identified as prodigiosin by high-resolution MS (calculated for C<sub>20</sub>H<sub>25</sub>ON<sub>3</sub>, found 323.1998, measured 323.1990) and on the NMR spectra with the authentic sample extracted from the type strain of *S. marcescens*.

## DISCUSSION

Strain KRED<sup>T</sup> was isolated from the bacterial flora of activated sludge in a domestic wastewater treatment tank in Saku, Japan. It was present in the sludge at  $2.25 \times 10^4$  c.f.u. ml<sup>-1</sup>, whereas *Bacillus* spp. and total bacteria were present at  $2.3 \times 10^6$  c.f.u. ml<sup>-1</sup> and  $5 \times 10^6$  c.f.u. ml<sup>-1</sup>, respectively (Doi *et al.*, 1998). From transmission electron microscopy analysis of strain KRED<sup>T</sup>, cells of the novel strain were found to possess an outer membrane, a peptidoglycan layer and a cell membrane (not visible), all characteristics of Gram-negative bacteria. The presence of endospores in cells of the novel strain could be confirmed due to the presence of a granule with a cortex layer and a spore coat formed inside cells of strain KRED<sup>T</sup>. The structure of the spores of strain KRED<sup>T</sup> was similar to that of spores of *B. megaterium*, while some endospores of strain KRED<sup>T</sup> were wrapped in the peptidoglycan layer of the cell. The spore membrane of strain KRED<sup>T</sup> was presumed to be very thin and it could not be observed under the conditions described above. The formation of endospores by strain KRED<sup>T</sup> was also confirmed by the results of the heat-resistance test and by the presence of DPA in the cells, although the temperature of heat resistance was lower than that of *Bacillus* spp. On the basis of the above results, cells of strain KRED<sup>T</sup> were shown to form endospores which possessed similar characteristics to spores of *Bacillus* spp. Strain KRED<sup>T</sup> was identified as *S. marcescens*

on the basis of FAME analysis, quinone profiling, pigment analysis and DNA analysis. The 16S rDNA sequence of strain KRED<sup>T</sup> showed 99.6% similarity with the 16S rDNA sequence of *S. marcescens*. The close relationship of the novel strain with *S. marcescens* was further confirmed by DNA–DNA homology analysis. Although DNA–DNA homology and 16S rDNA sequence analyses showed strain KRED<sup>T</sup> to be very similar to *S. marcescens* JCM 1239<sup>T</sup>, the unique spore-forming characteristic of strain KRED<sup>T</sup> supported its classification as a novel subspecies of *S. marcescens*, for which we propose the name *Serratia marcescens* subsp. *sakuensis*.

Endospore formation is known to be a characteristic of some Gram-positive bacteria, including those grouped in genera such as *Acetonebma*, *Bacillus*, *Clostridium*, *Desulfotomaculum*, *Sporomusa*, *Sporosarcina* and *Thermoactinomyces* (Edward, 1997; Sneath, 1989). The work presented here is the first report of the isolation of a spore-forming bacteria belonging to the *Enterobacteriaceae*, and is a deviation from the general concept regarding endospore-forming bacteria. We propose that either (i) *S. marcescens* strains that possess the gene related to endospore formation may be present in nature but have been unreported until now or (ii) endospores were present in strain KRED<sup>T</sup> due to gene transfer. The latter explanation is more acceptable at this moment in time, since endospore-forming *S. marcescens* have not been found in nature and the gene related to spore formation has not been reported for *S. marcescens*. The similarity of the biochemical characteristics of strain KRED<sup>T</sup> to those of *S. marcescens* also support the hypothesis for gene transfer. The activated sludge from the wastewater treatment facility at Komaba, Japan, had abundant *Bacillus* spp. and high concentrations of magnesium and silicate present (22.5 mg Mg<sup>2+</sup> l<sup>-1</sup> and 31.9 mg silicate l<sup>-1</sup> in pipe water, and 26.1 mg Mg<sup>2+</sup> l<sup>-1</sup> and 32.7 mg silicate l<sup>-1</sup> in the supernatant of the first aerobic treatment tank). The possibility of gene transfer between *Bacillus* and *Serratia* spp. should be investigated in activated sludge containing high concentrations of Mg<sup>2+</sup> and silicate ions.

### Description of *Serratia marcescens* subsp. *sakuensis* subsp. nov.

*Serratia marcescens* subsp. *sakuensis* (sa.ku.en'sis. N.L. adj. *saku* referring to Saku, Nagano, Japan, where the strain was isolated).

Cells are Gram-negative, short rods (0.5 × 1.3 µm). Facultatively anaerobic. Motile. Forms round endospores. Colonies on nutrient agar produce the pigment prodigiosin. Catalase-positive and oxidase-negative. Hydrolyses casein, cooked meat and soybean oil, but not starch. Reduces nitrate to nitrite. Indole is not produced. Can grow in 7% NaCl. Utilizes glucose, sucrose, D-sorbitol, L-arabitol, 4-hydroxybenzoic acid, gentisic acid and *m*-erythritol as sole carbon sources, but not D-tartrate, L-rhamnose, L-arabinose, D-xylose, lactose, benzoic acid, 3-hydroxybenzoic acid, trigonelline or raffinose. Produces acids from sucrose,

D-glucose and D-sorbitol, but not from L-arabitol. Fermentative on glucose. Methyl red test is negative on sucrose (5 days), glucose (7 days), D-sorbitol (7 days) and L-arabitol. DNA G+C content is 58 mol%. Other characteristics of the species can be found in Table 1. The type strain of *Serratia marcescens* subsp. *sakuensis* is KRED<sup>T</sup> (JCM 11315<sup>T</sup> = CIP 107489<sup>T</sup>). Isolated from the suspended water of a domestic wastewater treatment tank in Komaba, Saku, Nagano, Japan.

## ACKNOWLEDGEMENTS

We thank the Mitsui Engineering and Ship Building Co. Ltd, and The Society of New Activated Sludge Technology/Research (Professor T. Kitao, Toyohashi University of Technology, Japan) for financial support, and Professor Hans Trüper (University of Bonn, Germany) for his advice regarding the Latin epithet. The assistance of Associate Professor Shinichiro Tabata (Shinshu University, Japan) with scanning electron microscopy is gratefully acknowledged.

## REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Brenner, D. J. (1984). Family I. *Enterobacteriaceae* Rahn 1937, Nom. Fam. Cons. Opin. 15, Jud. Comm. 1958, 73; Ewing, Farmer and Brenner 1980, 674; Judicial Commission 1981, 104. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 408–420. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Collins, M. D. & Jones, D. (1981). Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implication. *Microbiol Rev* **45**, 316–354.
- Doi, Y., Lee, B.-S., Iriye, R., Tabata, S. & Tateishi, K. (1998). Dominantly growing bacteria in malodorless domestic sewage treatment tanks and their biochemical characteristics. *J Antibact Antifungal Agents (Japan)* **26**, 53–63.
- Edward, R. L. (1997). Prokaryotic diversity: form, ecophysiology, and habitat. In *Manual of Environmental Microbiology*, pp. 14–24. Edited by J. H. Christon, R. K. Guy, J. M. Michael, D. S. Linda & V. W. Michael. Washington, DC: American Society for Microbiology.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid-deoxyribonucleic acid hybridization in microdilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Gerber, N. N. (1975). Prodigiosin-like pigments. *C R C Crit Rev Microbiol* **3**, 469–485.
- Grimont, F. & Grimont, P. A. D. (1992). The genus *Serratia*. In *The Prokaryotes*, vol. 3, 2nd edn, pp. 2822–2848. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.
- Grimont, P. A. D. & Grimont, F. (1984). Genus VIII. *Serratia* Bizio 1823, 288<sup>AL</sup>. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 477–484. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Hearn, W. R., Elson, M. K., Williams, R. H. & Medina-Castro, J. (1970). Prodigiosin [5-(2-pyrryl)-2,2-dipyrrylmethene] and some substituted prodigiosenes. *J Org Chem* **35**, 142–145.
- Hiraishi, A. (1999). Isoprenoid quinones as biomarkers of microbial populations in the environment. *J Biosci Bioeng* **88**, 449–460.
- Janssen, F. W., Lund, A. J. & Anderson, L. F. (1958). Colorimetric assay for dipicolinic acid in bacterial spores. *Science* **127**, 26–27.
- Maidak, B. L., Olsen, G. J., Larsen, N., Overbeek, R., McCaughey, M. J. & Woese, C. R. (1996). The Ribosomal Database Project (RDP). *Nucleic Acids Res* **24**, 82–85.
- Marmur, J. (1961). A procedure for the isolation of deoxyribonucleic acid from microorganisms. *J Mol Biol* **3**, 208–218.
- Pizzimenti, F. C., Nostro, A., Marino, A., Villari, A., Verzera, A., Trozzi, A. & Alonzo, V. (1999). Fatty acid profiles in pigmented and non-pigmented strains of *S. marcescens*. *New Microbiol* **22**, 91–98.
- Powell, J. F. (1953). Isolation of dipicolinic acid (pyridine-2, 6-dicarboxylic acid) from spores of *Bacillus megaterium*. *Biochem J* **54**, 205.
- Ralph, A., Slepecky, H. & Hemphill, E. (1992). The genus *Bacillus* – nonmedical. In *The Prokaryotes*, vol. 2, 2nd edn, p. 1673. Edited by A. Balows, H. G. Trüper, M. Dworkin, W. Harder & K.-H. Schleifer. New York: Springer-Verlag.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Smibert, R. M. & Krieg, N. R. (1994). Phenotypic characterization. In *Methods for General and Molecular Bacteriology*, pp. 607–651. Edited by P. Gerhardt, R. G. E. Murray, W. A. Wood & N. R. Krieg. Washington, DC: American Society for Microbiology.
- Sneath, P. H. A. (1989). Endospore-forming Gram-positive rods and cocci. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1104–1105. Edited by P. H. A. Sneath, N. S. Mair, M. E. Sharpe & J. G. Holt. Baltimore: Williams & Wilkins.
- Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**, 4673–4680.
- Van de Peer, Y. & De Wachter, R. (1997). Construction of evolutionary distance trees with TREECON for Windows: accounting for variation in nucleotide substitution rate among sites. *Comput Appl Biosci* **13**, 227–230.