Shewanella decolorationis sp. nov., a dye-decolorizing bacterium isolated from activated sludge of a waste-water treatment plant

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A highly efficient dye-decolorizing bacterium, strain S12^T, was isolated from activated sludge of a textile-printing waste-water treatment plant in Guangzhou, China. The cells were Gram-negative and motile by means of a single polar flagellum. The strain was capable of anaerobic growth either by fermentation of glucose or by anaerobic respiration and utilized a variety of electron acceptors, including nitrate, iron oxide and thiosulfate. The physiological properties, tested by using the Biolog GN2 system, were similar to those of the genus of *Shewanella*. Analysis of the nearly complete 16S rRNA gene sequence of strain S12^T showed the highest similarity (98 and 97 %, respectively) to *Shewanella baltica* and *Shewanella putrefaciens*. However, the level of *gyrB* similarity between strain S12^T and *S. putrefaciens* was 87 %. DNA from strain S12^T showed 41.8 and 41.9 % DNA relatedness, respectively, to the DNA of *S. baltica* DSM 9439^T and *S. putrefaciens* DSM 6067^T. The DNA G + C content of strain S12^T was 49.3 mol%. The predominant menaquinone was MK-7 and the predominant ubiquinones were Q-7 and Q-8. The dominant fatty acids were 15 : 0, 16 : 0, iso-15 : 0 and 16 : 1 ω 7c, similar to the profiles of other *Shewanella* species. On the basis of its physiological and molecular properties, strain S12^T appears to represent a novel species of the genus *Shewanella*, for which the name *Shewanella decolorationis* sp. nov. is proposed. The type strain is S12^T (= CCTCC M 203093^T = IAM 15094^T).

Synthetic dyes are manufactured and used in large quantities. These xenobiotic compounds are generally not degraded in conventional waste-water treatment systems when they are used in the textile-dyeing and -printing industry (Conneely *et al.*, 1999; Jian *et al.*, 2000). Beside the unpleasant appearance of dye-polluted waste water, most dyes and their potential breakdown products are toxic (Conneely *et al.*, 1999). As a result, considerable work has been done to assess the use of micro-organisms to deal with such industrial pollutants (Yatome *et al.*, 1991; Nigam

et al., 1996; Zissi *et al.*, 1997; Jian *et al.*, 2000). Members of the genus *Shewanella* are widely distributed in nature, especially in aquatic environments, such as freshwater and the ocean (Bowman *et al.*, 1997; Ivanova *et al.*, 2001; Bozal *et al.*, 2002). A notable feature of members of this genus is their ability to use a variety of different electron acceptors such as manganese and iron oxides, uranium, thiosulfate and elemental sulfur (Venkateswaran *et al.*, 1999). Most recognized species of the genus have the potential to mediate the co-metabolic bioremediation of halogenated organic pollutants and to reduce heavy metals (Venkateswaran *et al.*, 1998a; Myers & Nealson, 1988; Lovely & Phillips, 1988; Perry *et al.*, 1993; Moser & Nealson, 1996). In this study, we describe a dye-decolorizing bacterial strain (S12^T) that was isolated from an activated sludge of a textile-printing waste-water treatment plant in Guangzhou, China.

Strain S12^T was isolated from activated-sludge samples collected in 2002 from an aerobic reactor of a printing and dyeing waste-water treatment system in Guangzhou,

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *gyrB* gene sequences of strain S12^T are AJ609571 and AJ609572, respectively.

Detailed fatty acid composition data of various *Shewanella* species and an electron micrograph of strain S12^T are available as supplementary material in IJSEM Online.

China. The activated-sludge samples were inoculated into medium NM9, which is modified M9 medium (Sambrook *et al.*, 1989), containing 10 % nutrient broth (Sambrook *et al.*, 1989) and incubated on a rotary shaker at 150 r.p.m. overnight at 30 °C. The broths were subcultured to the dye medium which was prepared by the addition of appropriate concentrations of the dyes to medium NM9 (medium DNM9). By regular subculturing (5-day intervals) 10 % (v/v) to a fresh DNM9 medium for a period of 3 months, the enriched populations were serially diluted and plated onto DNM9 agar plates for single-colony isolation. The morphologically distinct colonies showing clear zones around them were purified further and stored on DNM9 agar slants. The morphologically distinct bacterial isolates capable of decolorizing the dye on DNM9 agar plates were then cultivated under aerobic conditions at 30 °C overnight in nutrient broth. Cells were harvested by centrifugation. The pellets were washed twice in 10 ml PBS (pH 8.0) and resuspended in 10 ml PBS. Cell suspensions were transferred to fresh DNM9 in replicate tubes to reach an initial cell mass of 0.5–0.6 g (litre dry cell weight)⁻¹ and incubated statically at 30 °C for dye-decolorization tests. Every hour, 5 ml samples were taken out aseptically and centrifuged at 8000 g for 10 min. The supernatants were used to determine the percentage decolorization of the respective dyes. The decolorizing activities were expressed in terms of percentage decolorization, which was determined by monitoring the decrease in absorbance at absorption maximum (λ_{max}) of the respective dyes (i.e. 503 nm for Fast Acid Red GR, C.I. 27290; 603 nm for Reactive Brilliant Blue, C.I. 612051). The uninoculated DNM9 supplemented with the respective dye was used as reference. Decolorization activity (%) was calculated according to the formula decolorization activity = $(A - B)/A \times 100$, in which *A* is the initial absorbance and *B* is the observed absorbance.

Strain S12^T elicited more than 90 % decolorization activity of Fast Acid Red GR and Reactive Brilliant Blue within 12 h and was selected and maintained on nutrient broth slopes at 4 °C or stored at -20 °C in nutrient broth supplemented with 20 % (v/v) glycerol for further studies.

Strain S12^T was tested for a number of key characteristics by using standard procedures (Gerhardt *et al.*, 1994), such as the Gram reaction, cell size and morphology (electron microscopy after negative staining with 4 % phosphotungstic acid, pH 7.0) and tests for cytochrome oxidase and catalase. Acid production from carbohydrates was tested on oxidation-fermentation medium (Atlas, 1993). Furthermore, hydrolysis of starch, gelatin and nitrate reduction were tested following Cowan & Steel (1993). The temperature range for growth was determined on nutrient agar incubated for 7 days at 4, 10, 15, 20, 25, 30, 37, 40 and 42 °C. The pH range for the growth of the strain was determined in nutrient broth with pH values of separate batches of medium adjusted to 5, 6, 7, 8, 9, 10 and 11 with 1 M HCl or 1 M NaOH. The pH was not controlled during growth. At the end of exponential phase of growth, the pH was

analysed. Salt-tolerance tests were performed in nutrient broth with the NaCl concentration ranging from 0 to 10 % (w/v) on a rotary shaker at 150 r.p.m. for 24 h at 30 °C.

To test anaerobic respiration, the strain was inoculated into nutrient broth containing an electron acceptor (KNO₃, NaNO₂, Na₂S₂O₃ or FeCl₃) at a final concentration of 10 mmol l⁻¹. In addition, H₂S production was determined with triple-sugar iron agar. Denitrification was tested in nutrient broth by adding nitrate at 20 mmol l⁻¹ (Brettar *et al.*, 2002). Additional phenotypic characteristics were determined by using the Biolog microbial identification system (Venkateswaran *et al.*, 1999). Dissimilatory iron reduction was tested on a defined medium supplemented with 30 mM lactate as carbon and energy source and 50 mM Fe(III) citrate as terminal electron acceptor (Lovely & Phillips, 1988).

Fatty acid analysis was carried out according to Song *et al.* (2000). Cells were cultivated overnight in liquid Luria-Bertani (LB) broth medium (Sambrook *et al.*, 1989) at 30 °C with vigorous shaking. Cellular fatty acid methyl esters were extracted with solution I (45 g NaOH in 150 ml methanol and 150 ml H₂O) and boiling for 30 min, solution II (190 ml HCl in 275 ml methanol and 135 ml H₂O) at 80 ± 1 °C for 10 min, solution III (200 ml hexane and 200 ml ether) with vigorous shaking for 10 min and then cooled on ice. Extracts were analysed by using a Hewlett Packard model HP6890 gas chromatograph equipped with a flame-ionization detector HP CHEMSTATION version A 5.01 and an Ultra-2 column (0.2 mm i.d.; 25 m long). Hydrogen was used as the carrier gas. Fatty acid peaks were identified and quantified by comparing the results with the patterns from other micro-organisms, using MIDI System software (version 3.2).

For quinone analysis, strain S12^T and the reference strain DSM 6067^T were grown in 500 ml fresh LB medium and incubated with shaking at 150 r.p.m. for 24 h at 30 °C. Cells were twice extracted with chloroform/methanol (2:1, v/v) by lyophilization by the method of Minnikin *et al.* (1984). Quinones were separated by HPLC and individually identified by MS (Nishijima *et al.*, 1997).

Chromosomal DNA was isolated and purified according to the method of Marmur (1961). The G+C content was determined by thermal denaturation (Marmur & Doty, 1962). Levels of genetic relatedness were determined by the fluorometric microdilution plate method (Ezaki *et al.*, 1988; Sawabe *et al.*, 1998). 16S rRNA and *gyrB* gene sequences were determined as reported previously (Yamamoto & Harayama, 1995). The sequences amplified from strain S12^T were compared with those in the GenBank nucleotide database by using online BLAST searches. Phylogenetic trees were constructed according to three different methods (BIONJ, maximum likelihood and maximum parsimony). For the neighbour-joining analysis, a distance matrix was calculated according to Kimura's two-parameter correction model. Bootstrap resampling was done using 1000

replications, BIONJ and Kimura's two-parameter correction model. BIONJ was used according to Gascuel (1997) and the maximum-likelihood and maximum-parsimony programs were from the PHYLIP package, version 3.573c (Felsenstein, 1995). The phylogenetic trees were drawn using NJPLOT (Perrière & Gouy, 1996) and ClarisDraw software for Apple Macintosh. The domains used to construct phylogenetic trees were regions of the small-subunit rRNA gene sequences available for all sequences and excluding positions likely to show homoplasy.

Morphological, physiological and biochemical characteristics of strain S12^T are summarized in the species description below and in Table 1. Strain S12^T simultaneously contained both menaquinones and ubiquinones as isoprenoid quinones. This profile has been also observed in other *Shewanella* species (Venkateswaran *et al.*, 1999; Bozal *et al.*, 2002). The major menaquinone of strain S12^T was

MK-7 (98 %), with MK-8 (2 %) as a minor component. The ubiquinones detected in strain S12^T were Q-6 (4 %), Q-7 (30 %) and Q-8 (66 %). The fatty acid composition of the novel isolate is available as supplementary material in IJSEM Online. The fatty acid profile observed for the novel strain is in accordance with the profiles of other *Shewanella* strains (Ziemke *et al.*, 1998; Venkateswaran *et al.*, 1999; Ivanova *et al.*, 2001; Brettar *et al.*, 2002). The major fatty acids of the novel isolate were 15:0, 16:0, iso-15:0 and 16:1 ω 7c. Palmitoleic acid (16:1 ω 7c) was the most abundant monounsaturated fatty acid found in the novel isolate. Strain S12^T synthesized large amounts of straight-chain saturated fatty acids followed by terminally branched saturated and monounsaturated fatty acids, as found in *Shewanella woodyi* (Venkateswaran *et al.*, 1999; Makemson *et al.*, 1997).

A 1448 bp fragment of the 16S rRNA gene of strain S12^T

Table 1. Phenotypic characteristics of various *Shewanella* species

Species: 1, *Shewanella decolorationis* sp. nov. strain S12^T; 2, *S. baltica* NCTC 10735^T (Ziemke *et al.*, 1998); 3, *S. denitrificans* OS217^T (Brettar *et al.*, 2002); 4, *S. frigidimarina* ACAM 591^T (Venkateswaran *et al.*, 1999; Bowman *et al.*, 1997); 5, *S. japonica* KMM 3299^T (Ivanova *et al.*, 2001); 6, *S. livingstonensis* LMG 19866^T (Bozal *et al.*, 2002); 7, *S. olleyana* ACEM 9^T (Skerratt *et al.*, 2002); 8, *S. oneidensis* ATCC 700550^T (Venkateswaran *et al.*, 1999); 9, *S. putrefaciens* ATCC 8071^T (Nozue *et al.*, 1992; Venkateswaran *et al.*, 1999; Satomi *et al.*, 2003). ND, No data.

Characteristic	1	2	3	4	5	6	7	8	9
Cell shape	Straight rod	Straight rod	Straight or curved rod	Straight or curved rod	Rod	Rod	Rod	Straight rod	Straight rod
DNA G+C content (mol%)	49.3	45.7–46.8	46.8–48.1	40–43	43–44	41	44	45	43–47
Optimal growth temp. (°C)	20–30	ND	20–25	20–22	20–25	ND	20–22	25–35	25–35
Optimal pH for growth	7.0–10	ND	ND	ND	6.0–9.0	ND	ND	7–8	7–8
Growth at:									
4 °C	+	+	+	+	–	+	+	ND	+
35 °C	+	ND	–	–	+	–	–	+	+
40 °C	+	–	–	–	ND	–	–	+ / –	–
Pigment	Slightly pinkish	–	–		Slightly pinkish	Red–brown	Tan	Pinkish	–
Production of:									
Amylase	–	ND	+	–	+	ND	+	–	–
Gelatinase	+	ND	+	ND	+	+	+	+	–
Fermentation of D-glucose	+	–	–	+	+	+	–	–	–
Utilization of:									
D-Galactose	–	ND	ND	–	+	+	+	+	+
D-Fructose	+ / –	ND	ND	–	+	ND	+	–	–
D-Glucose	+	ND	+	+	+	+	–	ND	–
Sucrose	+	+	ND	+	–	+	+	–	–
Citrate	–	+	ND	ND	–	ND	–	–	–
D-Mannitol	–	ND	ND	+	–	+	–	–	–
N-Acetylglucosamine	+	+	+	–	+	+	–	ND	+
Propionate	+	ND	ND	+	ND	ND	ND	ND	–
L-Leucine	+	ND	ND	+	ND	ND	ND	ND	–
Succinate	+	ND	ND	+	+	+	+	+	+ / –
DL-Lactate	+	ND	ND	+	ND	+	ND	+	+ / –
Reduction of iron oxide	+	+	ND	+	–	+	+	+	+

was amplified and sequenced. This sequence was compared with those of the type strains of 20 *Shewanella* species. The phylogenetic analysis showed that strain S12^T is most closely related to *Shewanella baltica* and *Shewanella putrefaciens*, with 98 and 97 % sequence similarity, respectively. Moreover, some previous workers have reported that the identity of 16S rRNA gene sequences is not a sufficient criterion to guarantee species identity for some bacteria such as those belonging to the genera *Bacillus*, *Vibrio*, *Pseudomonas*, *Aeromonas* and *Shewanella* (Fox *et al.*, 1992; Yamamoto & Harayama, 1995; Venkateswaran *et al.*, 1998b, 1999; Yáñez *et al.*, 2003). On the other hand, the *gyrB* gene has been employed as a high-resolution molecular identification marker for distinguishing phylogenetic relationships at the species level (Venkateswaran *et al.*, 1999; Satomi *et al.*, 2003; Yáñez *et al.*, 2003). Therefore, the *gyrB* sequence data were employed to analyse the phylogenetic position of the novel isolate. BLAST searches showed that the novel isolate was positioned in the *S. putrefaciens*–*Shewanella oneidensis* cluster. Analysis of *gyrB* sequences indicated that 87 % similarity was seen between strain S12^T and *S. putrefaciens*. Venkateswaran *et al.* (1999) considered a species cut-off value for *gyrB* sequences is 90 %. Phylogenetic trees constructed by using the neighbour-joining method and 16S rRNA and *gyrB* gene sequences are shown in Fig. 1.

The DNA G+C content of strain S12^T was 49.3 mol% (Table 1), which is within the range described for the genus *Shewanella* (39–55 mol%; Venkateswaran *et al.*,

1999). DNA from strain S12^T showed 41.8 and 41.9 % DNA relatedness to the DNA of the two phylogenetically closest strains *S. baltica* DSM 9439^T and *S. putrefaciens* DSM 6067^T, respectively. These data are in agreement with the conclusion that strain S12^T belongs to a novel species of the genus *Shewanella*, on the basis that strains with DNA reassociation values of less than 70 % belong to separate species (Wayne *et al.*, 1987).

Strain S12^T can be distinguished easily from *S. baltica* (Ziemke *et al.*, 1998) and *S. putrefaciens* (Venkateswaran *et al.*, 1999) by its growth at 40 °C and its ability to ferment glucose. The strain can also be differentiated from *S. baltica* by its inability to utilize citrate and D-mannitol and from *S. putrefaciens* by its ability to produce gelatinase and utilize D-glucose, sucrose, propionate and L-leucine and its inability to utilize D-galactose (Table 1). Thus, phylogenetic analyses of 16S rRNA and *gyrB* gene sequences are consistent with the results of DNA–DNA hybridization experiments and phenotypic characteristics in suggesting that strain S12^T is a representative of a novel species of the genus *Shewanella*, for which the name *Shewanella decolorationis* sp. nov. is proposed.

Description of *Shewanella decolorationis* sp. nov.

Shewanella decolorationis (de.co.lo.ra.ti.on'is. L. gen. n. *decolorationis* of discoloration).

Fig. 1. Phylogenetic trees of the genus *Shewanella* based on 16S rRNA and *gyrB* gene sequences. The trees were constructed by using the neighbour-joining method. GenBank/EMBL/DBJ accession numbers are given in parentheses; the sequences determined in this study are in bold type.

Cells are Gram-negative, polarly flagellated with a single flagellum, straight rods that are 0.6–1.0 µm in width and 1.0–4.0 µm in length. Cells occur singly or in pairs and filaments 7 µm long are observed after 24 h incubation in liquid medium. Neither endospores nor capsules are formed. Oxidase- and catalase-positive. Young colonies are circular, transparent and non-pigmented to slightly pinkish on nutrient broth agar. The pH range for growth is 7.0–10.0, with an optimum at pH 8.0. The growth temperature range is 4–40 °C, with an optimum around 20–30 °C. NaCl supports growth but is not required. Growth is observed at salinities from 0 to 5%. Positive for hydrolysis of gelatin, Tween 80 and Tween 40. H₂S is produced from thiosulfate. Cells are able to reduce nitrate, nitrite, ferric compounds and thiosulfate with lactate or acetate as electron donor. Fermentation of D-glucose is observed. Growth occurs on D-glucose, sucrose, cellobiose, N-acetylglucosamine, propionate, L-leucine, methyl pyruvate, succinate, mono-methyl succinate, formic acid, DL-lactate, bromosuccinic acid, glycyl-L-aspartate and glycyl-L-glutamate. Both menaquinones and ubiquinones are present. Predominant menaquinone is MK-7 and predominant ubiquinones are Q-7 and Q-8. The main fatty acids are 15:0, 16:0, iso-15:0 and 16:1 ω7c. Straight-chain saturated fatty acids are the major fatty acids, followed by terminally branched saturated and monounsaturated fatty acids.

The type strain is S12^T (= CCTCC M 203093^T = IAM 15094^T). The G + C content of its DNA is 49.3%. Isolated from activated sludge of a textile-printing waste-water treatment plant in Guangzhou, China.

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