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 S12T, 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 S12T showed the highest similarity (98 and 97 %, respectively) to Shewanella baltica and Shewanella putrefaciens. However, the level of gyrB similarity between strain S12T and S. putrefaciens was 87 %. DNA from strain S12T showed 41·8 and 41·9 % DNA relatedness, respectively, to the DNA of S. baltica DSM 9439T and S. putrefaciens DSM 6067T. The DNA G+C content of strain S12T 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 : 1v7c, similar to the profiles of other Shewanella species. On the basis of its physiological and molecular properties, strain S12T appears to represent a novel species of the genus Shewanella, for which the name Shewanella decolorationis sp. nov. is proposed. The type strain is S12T (CCTCC M 203093T = IAM 15094T).

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 (S12T) that was isolated from an activated sludge of a textile-printing waste-water treatment plant in Guangzhou, China.

Strain S12T was isolated from activated-sludge samples collected in 2002 from an aerobic reactor of a printing and dyeing waste-water treatment system in Guangzhou,
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 absorbance maximum (λmax) 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 respective dye was used as reference. Decolorization activity (%) was calculated according to the formula decolorization activity = (A-B)/A × 100, in which A is the initial absorbance and B is the observed absorbance.

Strain S12T 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 S12T 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 S12T and the reference strain DSM 6067T 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 (Ézaki 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 S12T 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 S12T are summarized in the species description below and in Table 1. Strain S12T simultaneously contained both menaquinones and ubiquinones as iso-prenoid quinones. This profile has been also observed in other Shewanella species (Venkateswaran et al., 1999; Bozal et al., 2002). The major menaquinone of strain S12T was MK-7 (98 %), with MK-8 (2 %) as a minor component. The ubiquinones detected in strain S12T 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 16:1o7c. Palmitoleic acid (16:1o7c) was the most abundant monounsaturated fatty acid found in the novel isolate. Strain S12T synthesized large amounts of straight-chain saturated fatty acids followed by terminaly 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 S12T

Table 1. Phenotypic characteristics of various Shewanella species

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<tr>
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<td>Reduction of iron oxide</td>
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</table>
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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> showed 41.8 and 41.9 % DNA relatedness to the DNA of the two phylogenetically closest strains *S. baltica* DSM 9439<sup>T</sup> and *S. putrefaciens* DSM 6067<sup>T</sup>, respectively. These data are in agreement with the conclusion that strain S12<sup>T</sup> 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<sup>T</sup> 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<sup>T</sup> 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 tion.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/DDBJ accession numbers are given in parentheses; the sequences determined in this study are in bold type.](image-url)
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%.

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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References


