Identification and Phylogenetic Analysis of New Sulfate-Reducing Bacteria Isolated from Oilfield Samples

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Microbiologically influenced corrosion (MIC) caused by sulfate-reducing bacteria (SRB) has been investigated in an oilfield injection water system. Strain CW-01 was isolated from an oilfield and strain CW-04 was isolated from biofilm dirt of pipeline walls. The strains were facultative anaerobes, non-motile, Gram-positive, pole flagellum, and spore-forming curved rods. The growth was observed over the temperature range 20–70 °C. Strain CW-01 grew optimally at 37 °C. The pH range for growth was 3.0–11, optimal at pH 6.0. Strain CW-04 grew optimally at 48 °C. The pH range for growth was 3.0–10, optimal at pH 7.2. The strains grew at a very broad range of salt concentrations. Optimal growth was observed with 1.5 g/L NaCl for strain CW-01 and 0.7 g/L NaCl for strain CW-04. The strains showed most similarity in physiological characteristics, except for acetone and saccharose. Analysis of the 16S rDNA sequences allowed strains CW-01 and CW-04 to be classified into the genus *Desulfotomaculum*. The corrosion speciality of the strains had been comparatively investigated. Especially SRB's growth curve, bearable oxygen capability, drug fastness and corrosion rate had been analyzed. The results showed that it is difficult to prevent bacterial corrosion caused by these two strains.

Key words: Microbiologically Influenced Corrosion, Sulfate-Reducing Bacteria, 16S rDNA

Introduction

During crude oil exploitation, water is often injected into an underground oil layer to maintain the oilhead pressure (Qiao *et al.*, 2008). Because the injection water contains organic acids such as acetic acid, propionic acid, and butyric acid, and high concentrations of inorganic salts, especially sulfates and carbonates, the biofilm will be formed gradually (Hamilton, 1983; Odom, 1990). A variety of microorganisms are able to grow in biofilms, especially sulfate-reducing bacteria (SRB). Conclusions were drawn considering engenderation (Tamilvanan *et al.*, 2008), configuration (Licina, 1989) and microbe community analyses (Dubiel *et al.*, 2002; Rosnes and Torsvik, 1991): A biofilm: biofilm is not only necessary for microbiologically influenced corrosion (MIC), but also makes corrosion prevention more difficult.

SRB, a group of anaerobic heterotrophs which can be able to reduce sulfate to sulfide, are known to be involved in MIC of metals of pipelines and rigs (Neria-Gonzalez *et al.*, 2006), store tanks space throughout (Starosvetsky *et al.*, 2002), and power generation equipment in the oil and gas industry. SRB can cause corrosion of many kinds of metals including low-grade carbon steels (Castaneda and Benetton, 2008), stainless steels (Antony *et al.*, 2008), and copper alloys (Pak *et al.*, 2003). It has been estimated that MIC causes millions of dollars lost to the production, transport, and oil storage of the US oil industry every year (Eckford and Fedorak, 2002b). In a water supply system, especially in an oilfield injection water system and crude oil transport system, it is very important to prevent of pipelines corrosion effected by SRB. Many bacteria separated from oilfields were reported, including *Desulfotomaculum* (Kleikemper *et al.*, 2002), *Desulfosporosinus* (Watanabe *et al.*, 2002), *Thermodesulfobacterium* (Yumiko and Kazuya, 2003), *Desulfovibrio* (Watanabe *et al.*, 2000) and so on (Bonch-Osmolovskaya *et al.*, 2003; Ma-

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got *et al.*, 2000; Nazina *et al.*, 2000, 2001, 2005). Here, the main goal was to establish efficient prevention countermeasures against SRB corrosion of the injection water system of Zhongyuan Oilfield in China through investigating physiological characteristics and lineages of evolution of strains isolated from biofilms. For this goal, some new analytical methods were set up and optimized. After strains had been enriched, separated and purified, their physiological characteristics, including growth curve, tolerable oxygen capability, drug fastness, and corrosion velocity, were investigated comparatively. At last, phylogenetic analysis of 16S rDNA sequences were done.

Material and Methods

Strain isolation

The samples (injection water and biofilm of pipeline) were collected from Wennan United Management Station in Zhongyuan Oilfield, He'nan Province, P. R. China. For enrichment and pure cultures of SRB, one liter of medium included 1.0 g NH_4Cl, 2.0 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.5 g KH_2PO_4, 1.0 g Na_2SO_4, 1.0 g yeast extract, 3.5 g lactate as well as 1.4 g $(NH_4)_2Fe(SO_4)_2$, 0.1 g sodium thioglycolate, 0.1 g ascorbic acid. Using a sterile hypodermic needle, the bacteria were carefully removed and transferred into sterilized medium. Aliquots of different serial dilutions were inoculated to the isolation medium. The isolated medium contained (per liter of distilled water): 1.0 g glucose, 2.0 g $MgSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2 \cdot 2H_2O$, 0.5 g KH_2PO_4, 1.0 g Na_2SO_4, 1.0 g yeast extract, 3.5 g lactate and 16 g agar added for plates (pH 7.1~7.4). After the medium was sterilized by autoclaving for 20 min at 121 ºC, the plates were prepared under oxygen-free conditions using N_2 mixed with CO_2 [N_2:CO_2 = 4:1 (v/v)] as described by Nazina *et al.* (2005).

Culture conditions and physiological tests

Colonies isolated from the agar plates were inoculated in isolated medium. Proliferation experiments were carried out with cultures undisturbed for some days under dim light and microaerobic conditions at various temperatures with 50 mL tubes containing about 15 mL autoclaved isolated medium. The physiological tests were performed using the methods described in Bergey's Manual of Systematic Bacteriology (Garrity, 2001).

DNA isolation

A 20-mL sample of a fully grown cell culture was pelletted by centrifugation at 10000 × *g*, at 4 ºC for 10 min. The cell pellet was resuspended in 4 mL SE buffer (150 mM NaCl, 100 mM EDTA, pH 8.0) and then centrifuged at 10000 × *g*, at 4 ºC for 10 min. After removing the supernatant, the pellet was resuspended in 2.5 μL SE buffer and 55 μL fresh lysozyme (50 mg/mL) was added at room temperature for 20 min to lyze the cells. The suspension was then mixed with 220 μL sodium dodecyl sulfate (SDS) (25%, w/v) and incubated for 20 min. Total genomic DNA was isolated from lyzed bacterial cells by treatment with proteinase, prior to extraction with phenol/chloroform/isoamyl alcohol and precipitation with ethanol.

Amplification of 16S rRNA genes

The polymerase chain reaction (PCR) was used to amplify 16S rRNA genes from purified genomic DNA. The following degenerated oligonucleotides had been used: forward primer GM3, 5' AGAGTTTGATC(A/C)TGGCTCAG 3', corresponding *Escherichia coli* (8–22); reverse primer U1492r, 5' GGTTACCTTGTTACGACTT 3', corresponding *Escherichia coli* (1492–1511). The PCR medium included 100 μL: 2 μL of genomic DNA (50 ng/μL), 10.4 μL of 10 × PCR reaction buffer, 0.4 μL of *Taq* DNA polymerase, 2 μL of

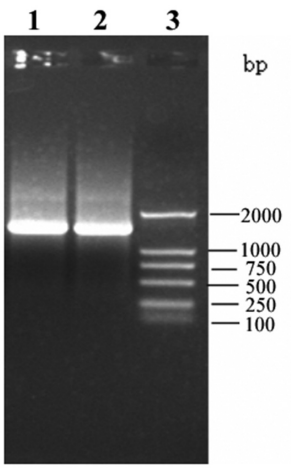

Fig. 1. Analysis of the 16S rDNA genes by gel electrophoresis. Lane 1, PCR product of strain CW-01; lane 2, PCR product of strain CW-04; lane 3, DL2000 DNA marker.

forward primer (25 μM), 2 μL of reverse primer (25 μM), 16.8 μL of dATP (1.25 μM), 16.8 μL of dCTP (1.25 μM), 16.8 μL of dGTP (1.25 μM), 16.8 μL of dTTP (1.25 μM), and 16 μL of pure sterilized water. The 16S rRNA gene was amplified under standard reactions in an automated thermal cycler with the following reaction conditions: 30 cycles of denaturation at 95 ºC for 1 min, primer annealing at 55 ºC for 1 min, and chain extension at 72 ºC for 1 min. This was followed by 7 min at 72 ºC to allow the extension of all molecules to be completed. 16S rDNA PCR products were separated in 1% horizontal agarose electrophoresis gels stained with ethidium bromide (1 μg/mL) in TAE buffer (Tris-acetate/EDTA electropheresis buffer) using the DL2000 DNA marker (Shengshizhongfang Biotech Co., Ltd, Beijing, China) and visualized by UV excitation (see Fig. 1). Amplified 16S rDNA gene products were excised from agarose gel and purified by using the gel extraction kit (Shanghai Shenneng Biotechnology Co., Ltd, Shanghai, China).

16S rDNA gene sequence and phylogenetic analysis

Cleaned products were cloned with a pGEM-T Easy cloning vector kit according to the instructions of the manufacturer (Promega Co., Ltd, Beijing, China), and then shifted into *E. coli* DH5α reception cells. Unique clones were identified and plasmids were purified with a Wizard genomic DNA purification kit (Shanghai Shenyou Bio Co., Ltd, Shanghai, China). Cleaned plasmid preparations were identified by electrophoresis on 0.7% agarose gels, and sequences of plasmids extracted from positive clones were sequenced by Shanghai Sangon Biological Engineering Technology & Service Co., Ltd., China.

An about 1.6 kb 16S rDNA sequence was subjected to comparison analysis in the GenBank database and then, on the basis of primary and secondary structural consideration, aligned to a 16S rRNA database selected from the Ribosomal Database Project (RDP) version 2.2 using

Table I. Physiological characteristics of strains CW-01 and CW-04.

Characteristic	CW-01	CW-04	Characteristic	CW-01	CW-04
Electron donors (with sulfate)					
$H_2 + CO_2$	–	–	Valerate	+	+
H_2 + acetate	–	–	Caproate	+	+
Formate	–	–	Heptanoate	+	+
Acetate	+	+	Octanoate	+	+
Lactate	+	+	Nonadecanoate	+	+
Propionate	–	–	Decanoate	+	+
Butyrate	+	+	Tridecanoate	+	+
Pyruvate	+	+	Pentadecanoate	+	+
Citrate	–	–	Palmitate	+	+
Succinate	+	+	Heptadecanoate	+	+
Fumarate	+	+	Stearate	+	+
Malate	+	+	Benzoate	+	+
Oxalate	–	–	Yeast extract	+	+
Methanol	–	–	Undecanoate	–	–
Ethanol	+	+	Dodecanoate	–	–
Propanol	–	–	Tetradecane	–	–
Butanol	–	–	Crude oil	+	+
Isopropanol	+	+	Fructose	–	–
Diethyl ether	–	–	Saccharose	–	+
Acetone	–	+	Maltose	–	–
Glucose	–	–			
Electron acceptors (with lactate as energy and carbon source)					
Sulfate	+	+	Nitrate	–	–
Sulfite	+	+	Sulfur	–	–
Thiosulfate	+	+			

–, No growth; +, growth.

T-COFFEE (Notredame *et al.*, 2000). Pairwise genetic distances were computed with Mega4 software (Tamura *et al.*, 2007) by using the method of Jukes-Cantor, and unrooted phylogenetic trees were constructed from genetic distances using the neighbour-joining method (Saitou and Nei, 1987) implemented in Mega4. To assign confidence levels to the nodes in the tree, bootstrap analysis with 2,000 replicates was conducted.

Analytical procedures

The isolated colonies were washed and stained on copper grids with 2% (g/g) phosphotungstate solution (pH 7.2). Samples were viewed and photographed using a JEOL model JEM-2000FXII transmission electron microscope (TEM) operating at 120 kV. The electron diffraction measurements on magnetosomes were done with the same microscope operating at 150 kV; the camera constant ($L\lambda$) was 0.00235 nm. The biomass was measured by DCW (dry cell weight). To investigate the optimal temperature, the pH value and ionic concentration of the isolates, the cell population was measured with testing flasks made by Huaxing Chemical Reagent Corporation (Beijing, China).

The optimal pH value, concentration of NaCl, resistibility to 1227 (*i.e.* dodecyl dimethyl benzyl ammonium chloride), degree of enduring oxygen and temperature ranges for the growth of the strains were determined by growing the organisms on sodium lactate and sodium sulfate. The corrosion rate was examined by putting 304 stainless steel slices into cultures of two kinds of SRB cells for 15 d, and then the weight of 304 stainless steel was measured.

Alternative electron donors and acceptors were added to the medium from anoxic sterile stocks to give the concentrations listed in Table I. In all of the growth experiments, the population of cells was measured using testing flasks made by Huaxing Chemical Reagent Corporation.

Results and Discussion

Morphological and physiological characteristics of strains CW-01 and CW-04

The strain CW-01 is curved rod-shaped, 2.3~5 μm in size and Gram-negative, while strain CW-04 is rod-shaped (see Table II). The strains showed almost similarity in their physiological characteristics, except for acetone and saccharose (see Table I). While sulfate and sulfite were used, limited compounds such as acetate, lactate, pyruvate, butyrate, succinate, malate, fumarate, valerate, caproate, heptanoate, octanoate, decanoate, tridecanoate, pentadecanoate, palmitate, heptadecanoate were utilized as electron donors. The following substrates were not utilized: formate, benzoate, undecanoate, dodecanoate, tetradecane, propanol and butanol.

Corrosion rate, capability of enduring oxygen and reagent 1227

1227 is a cationic surfactant belonging to quaternary ammonium bactericides, which was widely used in the prevention of metal corrupted, especially in injection water systems of oilfields (Eckford and Fedorak, 2002a). The tolerance of various SRB strains to 1227 could be measured. After 9.5 mL sterile water and 0.5 mL enrichment strains were added to 20-mL tubes sealed with rubber stoppers, different density gradients of 1227 were added to the tubes at a final content of 40 ppm. The populations of SRB were measured with exhaustible trace dilution methods according to National Standard of China SY/T5329–94. The corrosion rate was examined by hitched slices

Table II. Morphological characteristics of thermophilic *Desulfotomaculum* species.

Characteristic	*Desulfotomaculum* strain CW-01	*Desulfotomaculum* strain CW-04	*Desulfotomaculum nigrificans*[a]
Shape	Curved rods	Rods	Rods to curved rods
Size (width × length in μm)	0.8 × 2.3~5	1.2 × 2.5~4	0.5~1 × 3.5
Motility	+	+	+
Gram stain	+	+	+
Spore	+	+	+
Flagellum	1~2	1~2	No record

[a] Eckford and Fedorak (2002a). + Possessing the character.

Table III. Characteristics of bacterial speciality from Wennan oilfields.

Strain	Enduring oxygen [mg/L]	Population after 1227 treatments	Patch weight [mg]
CW-01	7.0	$6.0 \cdot 10^{-2}$	32.85
CW-04	6.5	$4.0 \cdot 10^{-2}$	22.54

in static state. 304 Stainless steel slices were put into cultures filled with five kinds of SRB cells for 15 days; the mass of 304 stainless steel was measured.

Two kinds of new SRB strains were isolated from Zhongyuan Oilfield: CW-01 cells purified from sewage can endure 7.0 mg/L oxygen; they can survive under 40 ppm of 1227 and consume 32.85 mg stainless steel in 15 days. CW-04 cells can endure 6.5 mg/L oxygen and can consume 22.54 mg stainless steel in 15 days (see Table III). Obviously, the two strains are facultative anaerobes and difficult to control.

Difference in pH value, temperature and ion concentration

As shown in Fig. 2, the growth of the two strains was observed over the temperature range 20 to 70 ºC, with no growth at 75 ºC. For strain CW-01, the optimal growth was at 37 ºC and pH 6.0. Correspondingly, strain CW-04 grew optimally at 48 ºC. The pH range for growth was 3.0–10 with optimal growth at pH 7.2 (see Fig. 2). The strains grew at a very broad range of salt concentrations. Optimal growth was obtained with 1.5 g/L NaCl for strain CW-01 and 0.7 g/L NaCl for strain CW-04, but cells were able to grow without NaCl or with as much as 3.0 g/L NaCl. No growth was observed with 3.5 g/L NaCl. Vitamins were not necessarily required, but they can speed up cell growth.

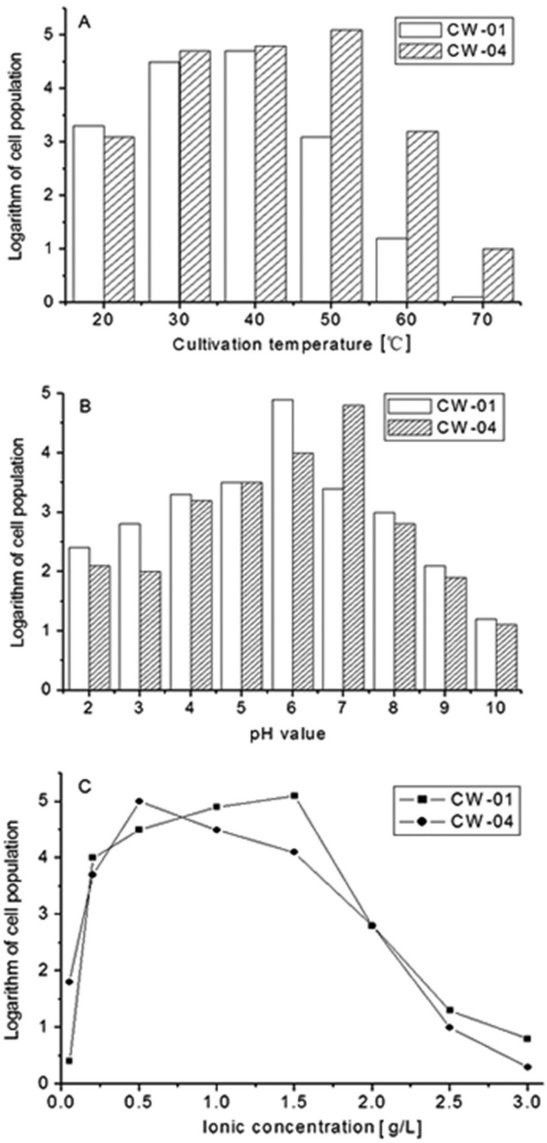

Fig. 2. Effect of (A) different cultivation temperatures, (B) pH values, and (C) ion concentrations on the growth of strains CW-01 and CW-04.

Phylogenetic analysis

For further characterization, homology analysis of the sequences of strains CW-01 and CW-04 had been conducted by the BLAST program in GenBank, and the highest scores were found in genus *Desulfotomaculum*. The sequences with scores of more than 90% were selected to be aligned and to reconstruct the phylogenetic tree. The constructed phylogenetic tree is shown in Fig. 3. Overall sequences similarity values between the two isolated strains and the *Desulfotomaculum* strains already described were 97.0%–98.0%.

In the presented dendrogram, Fig. 3, all *Magnetospirillum* strains form a single line of descent, indicating that all representatives of this genus have a common evolutionary origin. Strain CW-

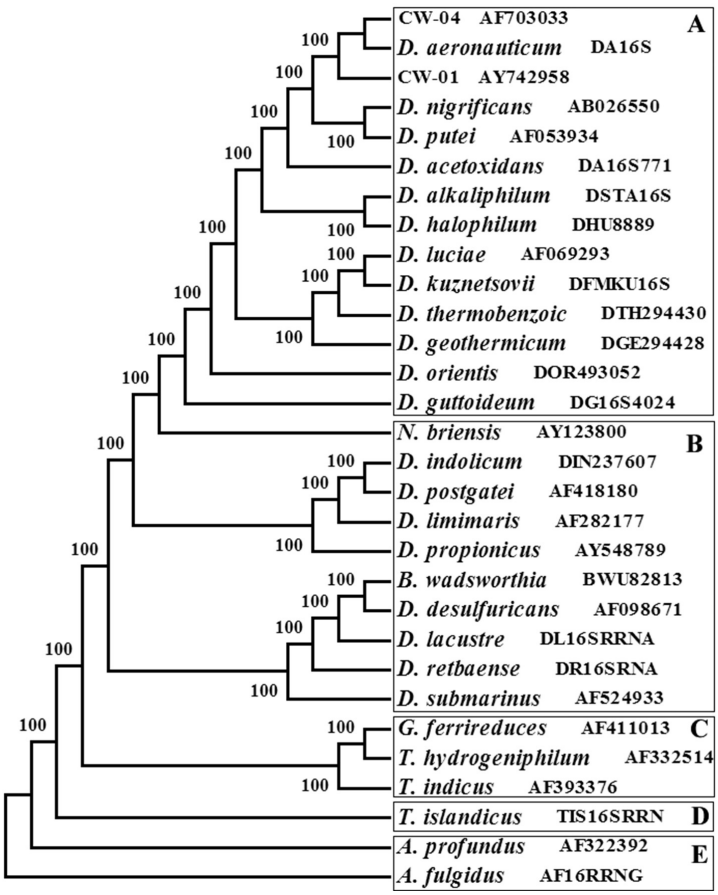

Fig. 3. The phylogenetic tree based on 16S rDNA sequence analysis showing the position of the strains CW-01 and CW-04 among the members of the *Desulfotomaculum* strains. The tree is constructed by a neighbour-joining method using MEGA4 package with 2,000 bootstrap replicates. A, Firmicutes; B, Deltaproteobacteria; C, Thermodesulfobacteria; D, *Nitrospira*; E, Archaea.

01 has only a similarity of 97.3% to *D. aeronauticum* (DA16S). According to the current species concept (Loy *et al.*, 2002), strains having an overall similarity below 97.5% most likely belong to different species, whereas 16S rRNA similarities above this threshold require further investigations to determine the taxonomic status of the strain. The 16S rDNA sequence of strain CW-01 had similarity values <97.5% to the sequences of *D. aeronauticum* (DA16S). Thus, strain CW-01 is likely a new species of the genus *Desulfotomaculum*.

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