Appl Microbiol Biotechnol (2010) 85:1175–1188
DOI 10.1007/s00253-009-2289-9

ENVIRONMENTAL BIOTECHNOLOGY

Characterization of corrosive bacterial consortia isolated from petroleum-product-transporting pipelines

Aruliah Rajasekar · Balakrishnan Anandkumar · Sundaram Maruthamuthu · Yen-Peng Ting · Pattanathu K. S. M. Rahman

Received: 7 July 2009 / Revised: 1 September 2009 / Accepted: 4 October 2009 / Published online: 21 October 2009

Abstract Microbiologically influenced corrosion is a problem commonly encountered in facilities in the oil and gas industries. The present study describes bacterial enumeration and identification in diesel and naphtha pipelines located in the northwest and southwest region in India, using traditional cultivation technique and 16S rDNA gene sequencing. Phylogenetic analysis of 16S rRNA sequences of the isolates was carried out, and the samples obtained from the diesel and naphtha-transporting pipelines showed the occurrence of 11 bacterial species namely *Serratia marcescens* ACE2, *Bacillus subtilis* AR12, *Bacillus cereus* ACE4, *Pseudomonas aeruginosa* AI1, *Klebsiella oxytoca* ACP, *Pseudomonas stutzeri* AP2, *Bacillus litoralis* AN1, *Bacillus* sp., *Bacillus pumilus* AR2, *Bacillus carboniphilus* AR3, and *Bacillus megaterium* AR4. Sulfate-reducing bacteria were not detected in samples from both pipelines. The dominant bacterial species identified in the petroleum pipeline samples were *B. cereus* and *S. marcescens* in the diesel and naphtha pipelines, respectively. Therefore, several types of bacteria may be involved in biocorrosion arising from natural biofilms that develop in industrial facilities. In addition, localized (pitting) corrosion of the pipeline steel in the presence of the consortia was observed by scanning electron microscopy analysis. The potential role of each species in biofilm formation and steel corrosion is discussed.

Keywords Carbon steel API 5 L-X60 · Petroleum product pipeline · Bacterial community · 16S rDNA analysis · Microbiologically influenced corrosion

A. Rajasekar · Y.-P. Ting (✉)
Department of Chemical and Biomolecular Engineering,
National University of Singapore,
4 Engineering Drive 4,
Singapore 117576, Singapore
e-mail: chetyp@nus.edu.sg

B. Anandkumar
Department of Biotechnology, Sourashtra College,
Madurai 625004, India

S. Maruthamuthu
Biocorrosion Group, Corrosion Protection Division,
Central Electrochemical Research Institute,
Karaikudi 630 006, India

P. K. S. M. Rahman
Chemical and Bioprocess Engineering Group,
School of Science and Technology, University of Teesside,
Middlesbrough TS13BA Tees Valley, UK

Introduction

Microbiologically influenced corrosion (MIC) is an electrochemical process where microorganisms initiate, facilitate, or accelerate a corrosion reaction on a metal surface. When present, microorganisms may produce diverse effects due to their interactions with the environment surrounding the metal surface. MIC occurs in virtually all industries, including paper and pulp, sugar, dentistry, shipping, and gas and petroleum industries (Beech and Sunner 2004; Jan-Roblero et al. 2004; Critchley et al. 2004; Bermont-Bouis et al. 2007; Lopes et al. 2006; Neria-Gonzalez et al. 2006). MIC is a well-documented phenomenon which causes deterioration of petroleum product pipelines and storage tanks. Corrosion affects the operation and maintenance costs of the pipelines, and many oil pipelines face severe corrosion and microfouling problems (Benka-Coker et al. 1995). It has been estimated that 40% of all internal pipeline corrosion in the petroleum industry can be attributed to MIC (Graves and Sullivan 1996). Carbon steel is a commonly used engineering

material of construction, and leakage due to internal corrosion of steel tanks has been documented in USA, France, Sweden, Switzerland, and India (Stapleton 1987; Pim 1988; Muthukumar et al. 2003a). In oil pipelines, hydrocarbon and water stratify at the bottom of the line when the velocity is less than that required to drain the liquids through the pipeline, and hydrocarbon degradation by microbes occurs easily at the liquid interface (Muthukumar et al. 2003b). Jana et al. (1999) analyzed crude oil pipelines at Mumbai offshore, India, and concluded that the combined effect of CO_2, sulfate-reducing bacteria (SRB), and chloride in the low-velocity area caused severe corrosion and failure of the pipelines.

Generally, the major bacteria involved in the corrosion of petroleum production systems are the anaerobic SRB (Von Wolzogen Kuhr and Vander Klugt Walker 1934; Benka-Coker et al. 1995). However, aerobic bacteria and fungi may also participate in the corrosion process (Zhu et al. 2003; Jan-Roblero et al. 2004). These microorganisms influence corrosion by altering the chemistry at the interface between the metal and the bulk fluid (Bond et al. 2002; Little and Ray 2002). Although most research on biocorrosion has been focused on SRB, recent studies suggest that other types of bacteria such as iron-oxidizing bacteria, manganese-oxidizing bacteria, acid-producing bacteria, and methanogens may also be involved (Zhu et al. 2003; Muthukumar et al. 2003a; Maruthamuthu et al. 2005; Jan-Roblero et al. 2008). The present work describes 16S rDNA gene sequencing and the identification of bacterial communities present in petroleum-transporting pipelines. As the identification of bacteria in such pipelines in tropical countries appears lacking, this work would be useful for the selection of suitable biocides/inhibitors in the control of corrosive bacteria in petroleum product pipelines. Research on the understanding of the diversity of bacterial species involved in corrosion would be useful for the development of a new approach for the detection, monitoring, and control of MIC.

Materials and methods

Sample collections (diesel and naphtha pipeline)

India transports petroleum products such as kerosene, petrol, and diesel via a cross-country pipeline with a length of 1,400 km and with intermittent product delivery and pressure-boosting stations at several locations. Samples (diesel and corrosion products) were collected from the cross-country pipeline in North India. The corrosion products were pushed out of the pipeline by pigs (a term used for cylindrical devices that move with the flow of oil and clean the inner portion of the pipelines), which were introduced into the pipeline in a preceding station and received at a succeeding station. The water contamination level in the transporting pipeline was observed at about 2–11% *v*/*v*. Carboxylic acid and ester-based inhibitor were added in the petroleum product pipeline to control corrosion and bacterial proliferation. Despite the presence of the inhibitor, about 200–400 kg of corrosion products was accumulated within 2 months over 200 km of the pipeline during pigging, with about 70% of the products being iron oxides (Maruthamuthu et al. 2005). Another cross-country pipeline in Southwest India transports white petrol (naphtha) for electric power generation. Corrosion products of about 10 kg were collected from the 5.5-km pipeline for every 2 months. Large quantity of sludge was found in the naphtha storage tank.

Bacteriological analysis of diesel and naphtha pipeline

Isolation of bacteria

Samples of corrosion products were collected using sterilized conical flasks at various sites at several stations during pigging of the diesel and naphtha-transporting pipeline and were transported in iceboxes to the transit Central Electrochemical Research Institute microbiology laboratory at pipeline stations. The samples were serially diluted (tenfold) with sterile distilled water, and the samples were plated using pour plate technique for the isolation of aerobic bacteria.

The nutrient agar medium consists of (grams per liter): peptic digest of animal tissue 5 g, NaCl 5 g, beef extract 1.5 g, yeast extract 1.5 g, and agar 15 g. Iron-oxidizing medium consists of (grams per liter): part A $(NH_4)_2SO_4$ 3 g, KCl 0.1 g, K_2HPO_4 0.5 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, calcium nitrate 0.01 g, and part B $FeSO_4$ 44.220 g and agar 15 g. Acid producer medium (*Thiobacillus* broth) consists of (grams per liter): $(NH_4)_2SO_4$ 0.40 g, $KHPO_4$ 4 g, $CaCl_2$ 0.25 g, $FeSO_4$ 0.5 g, $MgSO_4$ 0.5 g, $NaSO_4$ 5 g, and agar 15 g. Mn agar base consists of (grams per liter): beef extract 1 g, yeast extract 0.075 g, $MnCO_3$ 2 g, $Fe(NH_4)_2(SO_4)_2$ 0.15 g, sodium citrate 0.15 g, and agar 15 g. The media were used to enumerate the heterotrophic bacteria, iron bacteria, acid producers, and manganese-oxidizing bacteria, respectively.

To obtain the anaerobic bacterial enrichment cultures, all the pipeline samples were immediately added to a flask containing 20 ml of API broth (cat. no. M310, Himedia Laboratories) recommended in American Petroleum Institute (1959), which consists of (grams per liter): yeast extract 1.0 g, ascorbic acid 0.1 g, $MgSO_4{\cdot}7H_2O$ 0.2 g, K_2HPO_4 0.01 g, Fe $(NH_4)_2(SO_4)_2{\cdot}6H_2O$ 0.1 g, and NaCl 10.0 g. The 4.0 ml of sodium lactate was added as a carbon source to 1 l of API broth. SRB was enumerated by the most probable number technique (Oblinger and Koburger 1975; Postgate 1984). To ensure complete anaerobiosis,

strict precautions were taken in the preparation and handling of the tubes. Incubation was carried out in an anaerobic jar (Anaerocult Merk) at 30±1.0°C for 28 days. The afore-mentioned media were procured from Himedia Laboratories, Mumbai, India.

Partial biochemical characterization of the isolates

The dissimilar aerobic bacteria isolated from all types of medium were identified according to *Bergey's Manual of Determinative Bacteriology* (Holt et al. 1994). The isolated bacterial cultures were identified up to genus level by their morphological and partial biochemical characterization using the following: (1) Gram staining, (2) motility test, (3) indole production, (4) methyl red test, (5) Voges-Proskauer test, (6) citrate utilization test, (7) H_2S production test, (8) carbohydrate fermentation test, (9) catalase test, (10) oxidase test, (11) starch, (12) gelatin, and (13) lipid hydrolysis (Holt et al. 1994). In addition, citrate agar was used to detect the iron-reducing activity of the isolates.

The hydrocarbon utilization ability of the isolates was studied using Bushnell-Hass broth which consists of (grams per liter) $MgSO_4 \cdot 7H_2O$ 0.20 g, $CaCl_2 \cdot 2H_2O$ 0.02 g, KH_2PO_4 l.0 g, K_2HPO_4 l.0 g NH_4NO_3 l.0 g, and $FeCl_3$ 0.005 g, with diesel/naphtha 1 g, as carbon source. Manganese oxidation was confirmed by employing Leukoberbelin blue (LBB) spot test (Nealson 1992). The pure culture of all manganese oxidizers of individual isolate was streaked repeatedly with an inoculation loop on pre-prepared sterile Mn agar medium plates separately. Plates were wrapped in plastic bags to prevent desiccation after a few days and were incubated at 37°C for 10 days. Brown colonies observed in the petri plates were counted as "manganese oxidizer." The colonies were picked up and smeared on a filter paper (Whatman no. 1) using a sterile inoculation loop. Then, 0.04% of LBB was added on the filter paper, followed by subsequent spreading of the smear with the inoculation loop. A blue coloration on the bacterial colonies was formed after a few minutes, which indicated the accumulation and conversion of manganese to manganese dioxide by manganese oxidizers.

Molecular identification of bacteria

Genomic DNA of the bacterial isolates was extracted according to Ausubel et al. (1988). Amplification of gene encoding small subunit ribosomal RNA was carried out using eubacterial 16S rDNA primers (forward primer 5′-AGAGTTTGATCCTGGCTCAG-3′ (*E. coli* positions 8 to 27) and reverse primer 5′-ACGGCTACCTTGTTACG ACTT-3′ (*E. coli* positions 1494 to 1513); Weisburg et al. 1991). Polymerase chain reaction (PCR) was performed with a 50-µl reaction mixture containing 2 µl (10 ng) of DNA as the template, each primer at a concentration of 0.5 µM and 1.5 mM $MgCl_2$ and each dNTPs at a concentration of 50 µM as well as 1 µl of *Taq* DNA polymerase and buffer as recommended by the manufacturer (MBI Fermentas). PCR was carried out with a Mastercycler Personal (Eppendorf) with the following program: initial denaturation at 95°C for 1 min; 40 cycles of denaturation (3 min at 95°C), annealing (1 min at 55°C), and extension (2 min at 72°C); followed by a final extension (at 72°C for 5 min). The amplified product was purified using GFX™ PCR DNA and Gel Band Purification kit (Amersham Biosciences) and cloned in pTZ57R/T vector according to the manufacturer's instruction (InsT/Aclone™ PCR Product Cloning Kit, MBI Fermentas), and transformants were selected on LB medium containing ampicillin (100 µg/ml) and X-gal (80 µg/ml). DNA sequencing was carried out using ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems). For sequencing reaction, Big Dye Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Perkin-Elmer) was used.

Phylogenetic analysis of the isolates

The sequences obtained were analyzed with BLAST search version 2.2.20 (Altschul et al. 1990) and tools of Ribosomal Database Project II Release 10 (http://rdp.cme.msu.edu) for taxonomic hierarchy of the sequences. Multiple sequence alignments were performed using CLUSTAL *X*2 (Larkin et al. 2007) with a collection of taxonomically related sequences obtained from the National Center for Biotechnology Information (NCBI) Taxonomy Homepage (http://www.ncbi.nlm.nih.gov/Taxonomy/taxonomyhome.html/) and Ribosomal Database Project-II Release 10 (http://rdp.cme.msu.edu). Phylogenetic and similitude analyses were done with the common 16S rRNA gene regions, and all alignment gaps were treated as missing data. The paired similitude and pairwise distance calculations using the transversion/transition weighting (R=s/v) and the Kimura two-parameter model (Kimura 1980) were performed with the MEGA version 4.1 program (Tamura et al. 2007). The phylogenetic trees were constructed (neighbor-joining method), and 1,000 bootstrap replications were carried out to validate internal branches (Hillis and Bull 1993). MatGAT v. 2.01 software (Campanella et al. 2003) was used to calculate the similitude percentages among sequences.

Chemical analysis of corrosion products collected from diesel and naphtha pipelines

Five-gram sample of corrosion products was mixed with 100 ml of triple-distilled water and agitated for 2 h using a shaker. After shaking, the samples were filtered, and the filtrates were analyzed for chloride and sulfate using

Mohr's method and a gravimetric method, respectively. X-ray diffraction (XRD) of the corrosion product was analyzed using an X-ray diffractometer (Model JEOL JDX-8030) and scanned with CuKα radiation (Ni filter) in the range 10–85° at the rating of 40 kV, 20 mA. Corrosion products collected from the pipelines were dried and crushed to a fine powder and analyzed using XRD to determine the oxides present in the corrosion products

Biocorrosion studies and surface analysis

API 5 L-X60 carbon steel (C 0.29 max., S 0.05 max., P 0.04 max., Mn 1.25 max.) was used to evaluate the MIC behavior of the bacterial consortia. Coupons with size of 2.5×2.5 cm were mechanically polished to mirror finish and then degreased using trichloroethylene. The polished coupons were introduced into a 1,000-ml Erlenmeyer flask consisting of 500-ml diesel with 2% water containing 120 ppm chloride as the control system, while a 500-ml diesel with 2% water containing 120 ppm chloride inoculated with 2 ml of mixed consortia (about 10^8CFU/ml) was used as the experimental system. After 7 days, the coupons were removed and pickled in Clark solution, washed in deionized water, and dried with an air drier. Duplicate experiments were made for each system. Final weights of the six coupons in each system were taken, and the average corrosion rates were calculated. The surface morphological characteristics of the control and experimental coupons were observed under a scanning electron microscope (SEM; Hitachi model S-3000 H) at magnification ranging from ×50 to ×200 and operated at an accelerating voltage of 25 kV.

Nucleotide sequence accession numbers

The 16S rRNA sequence data reported in this paper have been deposited in the GenBank database, under the accession numbers: EF535590 to EF535597, DQ207558 to DQ207562, DQ092416, and AY912105.

Results

Bacteriological analysis of diesel and naphtha pipeline

SRB was not detected in the corrosion products collected from both pipelines. Preliminary identification of the bacteria by biochemical test indicated that the isolates belonged to the genera *Bacillus*, *Pseudomonas*, *Klebsiella*, and *Serratia.* The phenotypic profiles of the diesel and naphtha strains are shown in Tables 1 and 2. The formation of blue coloration by diesel isolates (ACE2 and ACE4) and naphtha isolates (AR3 and AR4) confirmed the presence of manganese oxide deposition. The redox stain LBB indicated the accumulation and conversion of manganese by bacteria. The diesel bacterial isolates AI1, ARI2, and ARI4 and naphtha isolates AR1, AR2, AR3, and AR4 deposited the ferric hydroxide precipitate around the colony and formed as rust-red color.

Molecular identification and phylogenetic analysis of the isolates

The sequences obtained were submitted to a BLAST search to retrieve the corresponding phylogenetic relatives. The phylogenetic affiliations (Firmicutes and Gamma proteobacteria) were confirmed by analyses of all related species recognized by the taxonomic and classification hierarchy done with the NCBI Taxonomy Homepage and Ribosomal Database Project-II Release 10. Three neighbor-joining phylogenetic trees were constructed for the *Bacillus* genera (Fig. 1), Class Enterobacteriales (Fig. 2), and Pseudomonadales (Fig. 3) to analyze the relationships among the sequences of the ribosomal library and related organisms from the GenBank database. The similarity and species identified with the phylogenetic analysis have been given in Table 3. In the *Bacillus* genera tree, most of the isolates belonging to *Bacillus* genus exhibited a high nucleotide sequence similarity with *Bacillus cereus* species (99.5%). Similarly, in the tree corresponding to the *Bacillus* genus, most isolates were related with *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus carboniphilus*, *Bacillus litoralis*, and *Bacillus megaterium* with high similarity values (98%). In the Classes Enterobacteriales, two isolates of the genus *Serratia* exhibited high sequence similarity with *Serratia marcescens* (99%) and one isolate of *Klebsiella* genus (*Klebsiella oxytoca*). Two isolates of genera *Pseudomonas* had high similarities with *Pseudomonas aeruginosa* and *Pseudomonas stutzeri* (98%) in the Pseudomonadales tree.

Chemical analysis of corrosion products collected from diesel and naphtha pipelines

Table 4 shows the estimated chloride and sulfate concentration in the corrosion product samples collected from diesel and naphtha pipeline. The chloride content ranged between 85 and 172 mg/l, while sulfate was approximately 0.23–0.29 mg/l in all the corrosion product samples collected from diesel pipeline. In naphtha pipeline, the chloride concentration in water ranged from 7 to 175 mg/l while the sulfate concentration ranged from 155 to 198 mg/l. Figure 4a, b shows the XRD data corresponding to the phases present in the corrosion product samples collected at the diesel and naphtha pipeline. Ferric, manganese complex, and Fe_2O_3 were observed in the corrosion product in diesel pipeline. Iron and manganese oxides

Table 1 Partial biochemical characterization of isolates from diesel pipeline

Characteristics	ACE2	ACE4	AR12	AR4	AI1	ACP	AP2	AN1	AN4	AN5
Cell morphology										
Gram stain	Negative	Positive	Positive	Positive	Positive	Negative	Positive	Positive	Positive	Positive
Shape	Rod	Rod	Rod	Rod	Rod	Small rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+	+	+	+	+	+
Sporulation	−	+	+	+	+	−	+	+	+	+
Growth at										
20°C	+	+	+	+	+	+	+	+	+	+
30°C	+	+	+	+	+	+	+	+	+	+
40°C	+	+	+	+	+	+	+	+	+	+
Hydrocarbon utilization										
Diesel	+	+	+	+	+	+	+	+	+	+
Biochemical reaction										
Indole production test	−	+	−	−	−	−	+	+	−	+
Methyl red test	−	−	+	+	+	−	−	−	+	+
Voges Proskauer test	+	−	−	−	−	+	−	−	−	−
Citrate utilization test	+	−	+	+	+	+	−	+	+	−
Oxidase test	+	+	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+	+	+
Production of acid from										
Glucose	+	+	+	+	+	+	+	+	+	+
Galactose	−	−	+	+	−	+	−	−	−	−
Fructose	+	+	+	+	+	+	+	+	+	+
Sucrose	−	+	−	−	−	−	+	−	−	−
Mannitol	−	−	−	−	−	+	−	−	+	−
Lactose	−	−	+	+	+	−	−	+	+	+
Cellobiose	−	−	+	+	+	−	−	+	+	+
Adonitol	−	−	−	−	−	+	−	−	−	−
Arabinose	−	+	−	−	+	−	+	−	−	+
Raffinose	−	−	−	−	−	+	−	−	+	−
Inositol	−	−	−	−	−	+	−	+	−	−
Hydrolysis of										
Starch	−	+	+	+	+	+	+	+	+	+
Cellulose	−	−	−	−	−	+	+	−	+	−
Casein	−	+	−	−	−	+	+	+	−	+
Gelatin	−	+	+	+	+	−	−	+	+	+
Urea	−	+	−	−	−	−	+	−	−	−
Tween 80	+	+	−	−	−	+	+	+	+	+

ACE2 S. marcescens, *ACE4 B. cereus*, *AR12 B. subtilis*, *AR14 B. cereus*, *AP2 P. stutzeri*, *ACP K. oxytoca*, *AI1 P. aeruginosa*, *AN1 B. litoralis*, *AN4 B. cereus*, *AN5 Bacillus* sp.

and silicon dioxide (i.e., Fe_2O_3, Fe_3O_4, Mn_3O_4, SiO_2) were noted in the corrosion product samples collected from naphtha pipeline.

Steel surface analysis by scanning electron microscopy

The surface morphological characteristics of the control and experimental steel API 5 L-X60 was observed under SEM. Figure 5 shows the SEM micrograph of the coupons after 7 days of exposure in the bacterial consortia and uninoculated system after removing the corrosion products and biofilm. Unlike the control system where uniform corrosion was noted (Fig. 5a), severe pitting attacks were observed over the surface of steel (Fig. 5b) in the experimental system. Figure 5c (at a higher magnification of ×8,000) shows a pit size of more than 2 μm in diameter.

Table 2 Partial biochemical characterization of isolates from naphtha pipeline

Characteristics	AR1	AR2	AR3	AR4	AR5
Cell morphology					
Gram stain	Negative	Positive	Positive	Positive	Positive
Shape	Rod	Rod	Rod	Rod	Rod
Motility	+	+	+	+	+
Sporulation	−	+	+	+	+
Growth at					
20°C	+	+	+	+	+
30°C	+	+	+	+	+
40°C	+	+	+	+	+
Hydrocarbon utilization					
Naphtha	+	+	+	+	+
Biochemical reaction					
Indole production test	−	+	−	−	−
Methyl red test	−	−	+	−	+
Voges Proskauer test	+	−	−	+	−
Citrate utilization test	+	−	+	−	−
Oxidase test	+	+	+	+	+
Catalase test	+	+	+	+	+
Production of acid from					
Glucose	+	+	+	−	+
Fructose	+	+	+	+	+
Sucrose	+	+	−	+	−
Mannitol	−	−	−	−	−
Lactose	−	−	+	+	−
Cellobiose	−	−	+	+	−
Adonitol	+	+	+	+	+
Arabinose	+	+	+	+	+
Raffinose	+	+	+	+	+
Inositol	−	−	−	−	−
Hydrolysis of					
Starch	−	+	+	+	−
Cellulose	−	−	−	+	+
Casein	−	+	−	+	+
Gelatin	−	+	+	+	+
Urea	−	+	−	+	+
Tween 80	+	+	−	+	+

AR1 S. marcescens, *AR2 B. pumilus*, *AR3 B. carboniphilus*, *AR4 B. megaterium*, *AR5 B. cereus*

Discussion

Microbial contamination of fuels has been a cause of intermittent operational problems throughout the world, and over the years the frequency and severity appear to have been increasing dramatically (Hamilton 1985; Muthukumar et al. 2003b). Microbial activity can result in inhibitor/fuel degradation that leads to problems including unacceptable level of turbidity, filter plugging, corrosion of storage tanks and pipelines, and souring of stored products. The degradation of diesel and crude oil has been studied in oil-spilled soil by Delille (2000). Lloyd-Jones and Trudgill (1989) isolated polycyclic-hydrocarbon-utilizing consortia *Rhodococcus* sp., *Flavobacterium* sp., and *Pseudomonas* sp. from oil refinery soil. Samant and Anto (1992) and Jana et al. (1999) reported the presence of heterotrophic SRB and studied the impact of microbes on the Indian pipelines. Obuekwe and Westlake (1987) also reported that intermediate hydrocarbon degradation products served as an energy source for the physiological activities of the SRB (*Desulfovibrio* sp.). This supply of utilizable hydrocarbon degradation products may well explain why corrosion is intense in the Pembiana field (Canada) crude oil pipeline. Samant and Anto (1992) reported the presence of SRB in an oil pipeline and the interaction between chloride ion and the SRB on corrosion. Several investigators have also isolated SRB

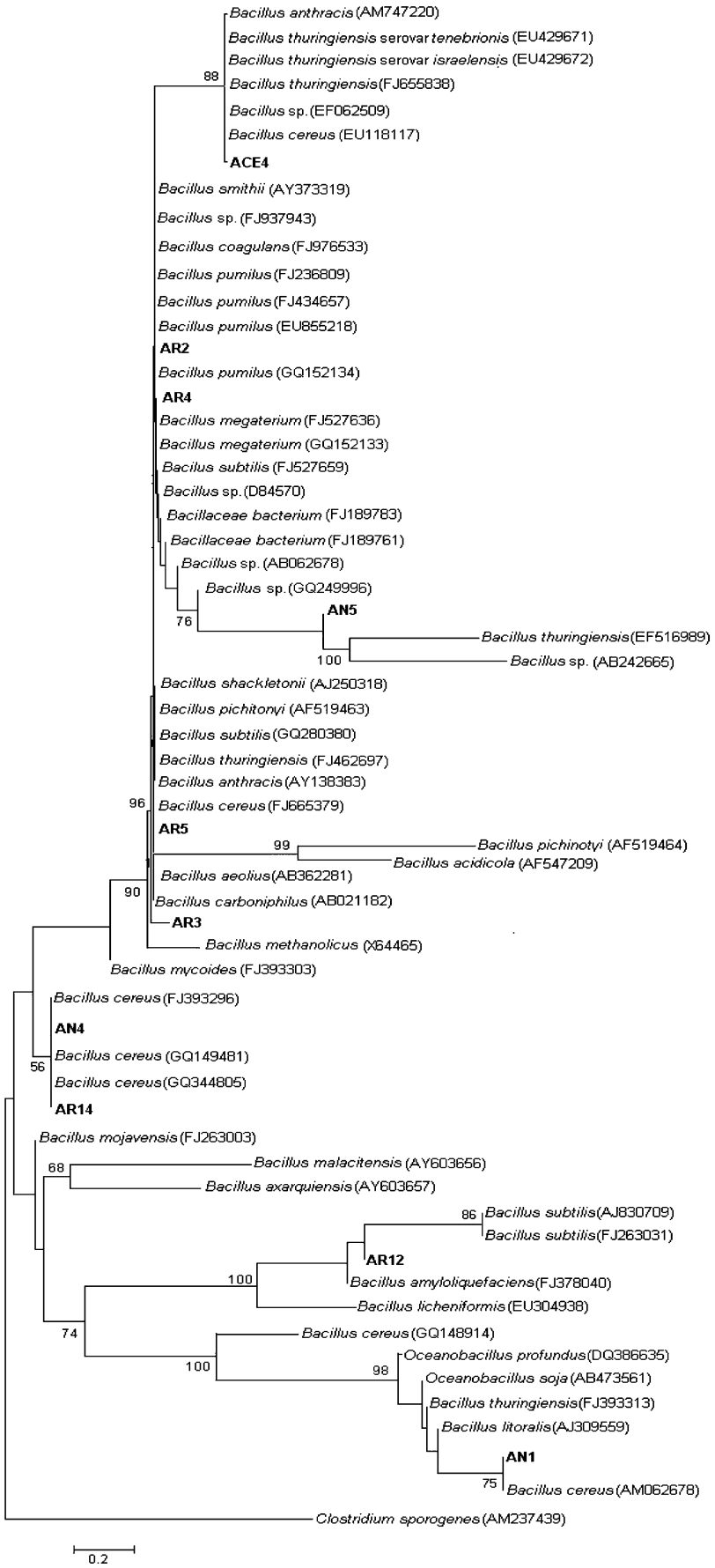

Fig. 1 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the bacterial phylum Firmicutes (*Bacillus*-related species). *Clostridium sporogenes* was used as a bacterial out-group. Numbers at nodes indicate bootstrap values >50% from 1,000 replicates. GenBank accession numbers are given in *parentheses*. The *scale bar* indicates sequence divergence. *ACE4*, *B. cereus*; *AR2*, *B. pumilus*; *AR4*, *B. megaterium*; *AN5*, *Bacillus* sp.; *AR5*, *B. cereus*; *AR3*, *B. carboniphilus*; *AN4*, *B. megaterium*; *AR14*, *B. cereus*; *AR12*, *B. subtilis*; *AN1*, *B. litoralis*

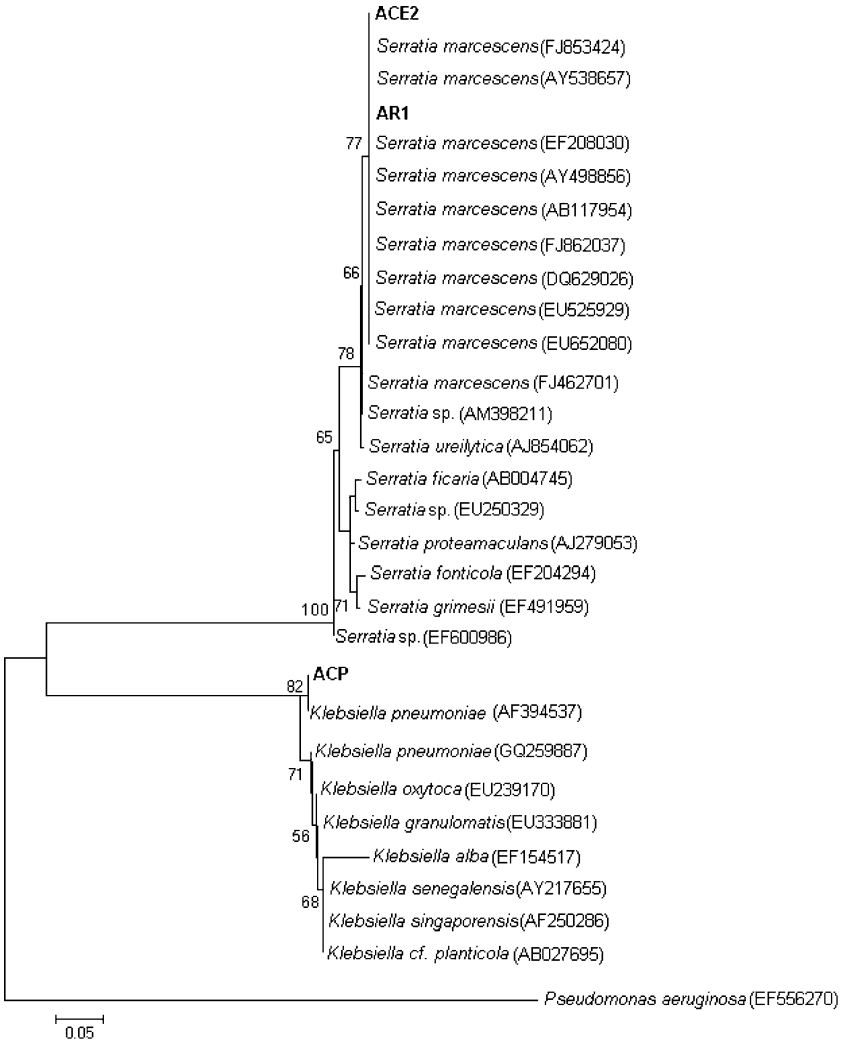

Fig. 2 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the Order Enterobacteriales (phylum Proteobacteria). *P. aeruginosa* was used as the out-group sequence. *Numbers* at nodes indicate bootstrap values >50% from 1,000 replicates. GenBank accession numbers are given in *parentheses*. The *scale bar* indicates sequence divergence. *AR1*, *S. marcescens*; *ACE2*, *S. marcescens*; *ACP*, *K. oxytoca*

(*Desulfovibrio* sp.) from the microbial communities involved in MIC in gas- and oil-transporting pipelines (Miranda-Tello et al. 2003, 2006; Jan-Roblero et al. 2004; Mora-Mentdoze et al. 2003).

Among the heterotrophic bacteria isolated, Gram-positive bacteria were more dominant than Gram-negative bacteria by 30%. Generic distribution was found to be *B. cereus* ACE4 (30%), *S. marcescens* ACE2 (10%), and 10% of each species of *B. subtilis* AR12, *P. aeruginosa* AI1, *K. oxytoca* ACP, *P. stutzeri* AP2, *B. litoralis* AN1, and *Bacillus* sp. AN5. Gram-positive bacteria are more actively involved in the corrosion of API 5 LX steel. The presence of chloride in diesel pipeline indicates (Table 4) the likelihood of water being present in the corrosion product sample. It is known that the contamination of water level vary from about 2% to as high as 11% *v/v* in the pipeline (Muthukumar et al. 2003a; Maruthamuthu et al. 2005). Although sulfate was present in the pipeline, SRB was not detected in all the corrosion product samples. These results corroborate the observations by Zhu et al. (2003) and Jan-Roblero et al. (2004; 2008). In the present study, the presence of acid-forming bacteria *K. oxytoca* ACP, *B. cereus* AN4, *P. stutzeri* AP2, and *S. marcescens* ACE2 in the samples suggests that acid-producing bacteria may play a key role in corrosion. *K. oxytoca* ACP, which was detected only in the corrosion product sample collected from the diesel pipeline, is often present in soil and water and fixes nitrogen under anaerobic or microaerobic conditions (Holt et al. 1994). It produces nitrates and/or nitric acid that may contribute to metal corrosion (Zhu et al. 2003). These results lend further evidence to the hypothesis that acid-forming bacteria play a key role in MIC. The involvement of *S. marcescens* ACE2 is unexpected as this species has not previously been reported to be associated with MIC in petroleum-transporting pipelines. *P. stutzeri* AP2 and *K. oxytoca* ACP have minimal nutritional requirements and are often present in aquatic environments that are rich in organic pollutants such as gasoline and solvents (Zhu et al. 2003). In addition, *P. stutzeri* AP2 contribute to biofilm formation by producing exopolysaccharides and facilitating the attachment

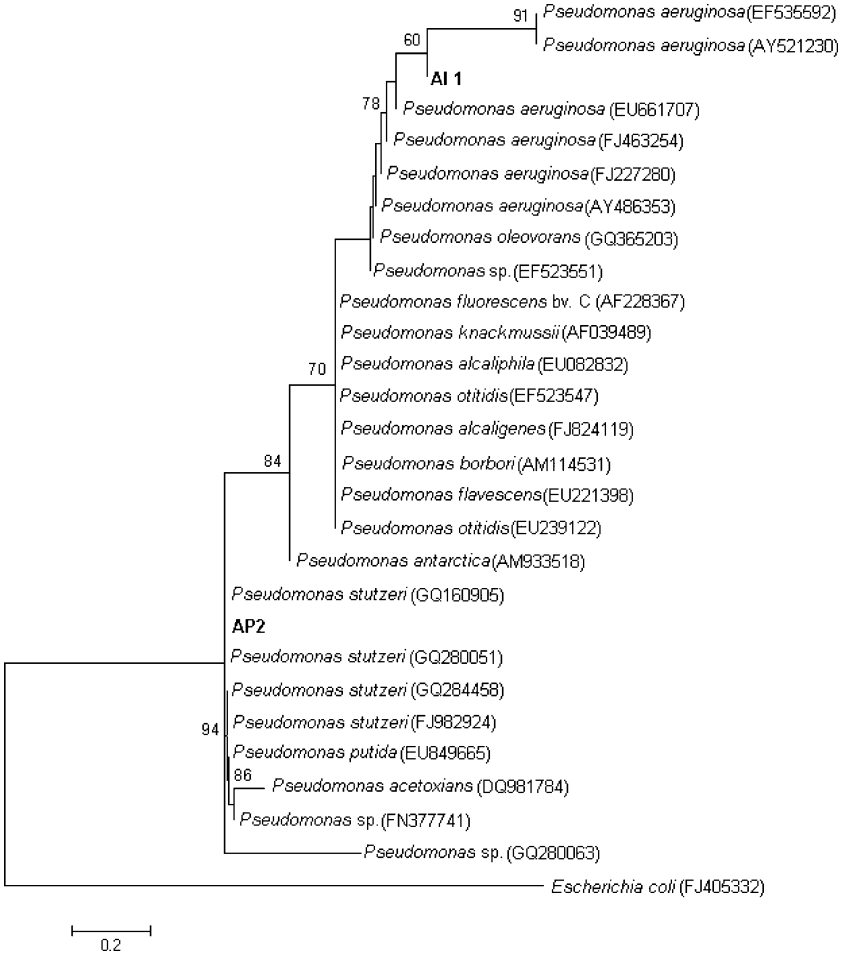

Fig. 3 Neighbor-joining tree based on 16S rRNA gene sequences, showing phylogenetic relationships between sequences of the Order Pseudomonadales (phylum Proteobacteria). *E. coli* was used as the out-group sequence. *Numbers* at nodes indicate bootstrap values >50% from 1,000 replicates. GenBank accession numbers are given in *parentheses*. The *scale bar* indicates sequence divergence. *AI 1*, *P. aeruginosa*; *AP2 P. stutzeri*

Table 3 16S rRNA sequence analysis of the bacterial isolates

Name of the isolates	Taxonomic phylum	Closest relationship in GenBank	Similarity (%)	Microbial group identified
ACE4	Firmicutes	*B. cereus* (EU118117)	99.5	*B. cereus*
AN4		*B. cereus* (FJ393296)	99.5	*B. cereus*
AR14		*B. cereus* (GQ344805)	99.4	*B. cereus*
AR5		*B. cereus* (FJ665379)	99.5	*B. cereus*
AR12		*B. subtilis* (FJ263031)	98.5	*B. subtilis*
AN1		*B. litoralis* (AJ309559)	99	*B. litoralis*
AN5		*Bacillus* sp. (AB242665)	97.3	*Bacillus* sp.
AR2		*B. pumilus* (EU855218)	98	*B. pumilus*
AR3		*B. carboniphilus* (AB021182)	98.4	*B. carboniphilus*
AR4		*B. megaterium* (FJ527636)	98	*B. megaterium*
ACE2	Proteobacteria	*S. marcescens* (FJ853424)	99	*S. marcescens*
AR1		*S. marcescens* (EF208030)	99	*S. marcescens*
AI1		*P. aeruginosa* (EU661707)	98.4	*P. aeruginosa*
AP2		*P. stutzeri* (GQ160905)	98	*P. stutzeri*
ACP		*K. oxytoca* (EU239170)	97.8	*K. oxytoca*

Table 4 Chloride and sulfate concentrations in water/sludge/corrosion product samples collected at diesel and naphtha pipelines and naphtha storage tank

Location and sample	Chloride (mg/L)	Sulfate (mg/L)
Diesel pipeline		
Station 1	111	0.29
Station 2	85	0.23
Station 3	172	0.24
Naphtha pipeline		
Corrosion products from filter 1	7	26
Corrosion products from filter 2	7	23
Naphtha storage tank		
Water from storage tank 1	7	155
Water from storage tank 2	175	198
Sludge collected from storage tank 2	12	60

of other microorganisms (LeChevallier et al. 1988) and hence accelerates the corrosion process (Batista et al. 2000). Pseudomonads are also capable of both complete and incomplete denitrification (Cuypers and Zumft 1993; Drysdale et al. 1999; Bothe et al. 2000). The survival and increase in acid bacteria, iron bacteria, and manganese-oxidizing bacteria are due to the existence of favorable condition and the ability of these organisms to utilize hydrocarbon as nutrients. Strains such as *Bacillus* sp. AN5 and *P. aeruginosa* AI1 use ferrous ions as electron donors and gain energy from the oxidation of Fe^{2+} to Fe^{3+} (Westlake et al. 1986). The free energy change in Fe^{2+} oxidation at low pH is large enough to be coupled to ATP synthesis; it is rather low at neutral pH, and iron bacteria cannot grow at pH above 4. The reaction is given as

$$Fe^{2+} + {}^{1}/_{4}\,O_2 + H^{+} \rightarrow Fe^{3+} + {}^{1}/_{2}\,H_2O$$

In the present study, the oxidation of ferrous to ferric ion by *P. aeruginosa* AI1, *B. subtilis* AR12, and *B. cereus* AR14 indicates that the bacteria promotes ferric formation at low pH. XRD data (Fig. 4a) confirm that the ferric oxides are more dominant components present in the corrosion product. Since carboxylic acid and ester-based corrosion inhibitor were added in the pipeline at one point in the pipeline, the formation of microacidic environment created by carboxylic acid opens up avenues for the progress of the above-mentioned reaction (Maruthamuthu et al. 2005). In the laboratory, inhibition at 100 and 200 ppm in water resulted in pH of about 5.5 and 4.2, respectively. Water stratifies in a pipeline when the velocity is slow (typically 3–4 ft/s) or if the pipeline is operated in a "stop–start" mode. Hence, the presence of the inhibitor may have reduced the pH at various sections in the pipeline, thus leading to a low pH and proliferation of iron oxidizers, manganese oxidizers, and acid producers and the absence of SRB. The acid-producing bacteria *P. stutzeri* AP2 and *K. oxytoca* ACP create a microacidic environment which is favorable for *Bacillus* sp. Moreover, *Gallionella* sp., being chemolithotrophic, microaerophilic, and acidophilic, produces hydrogen peroxide (H_2O_2) as an outcome of its metabolism and needs a scavenging mechanism to overcome this toxic product. Here, the role played by the manganese-oxidizing bacteria

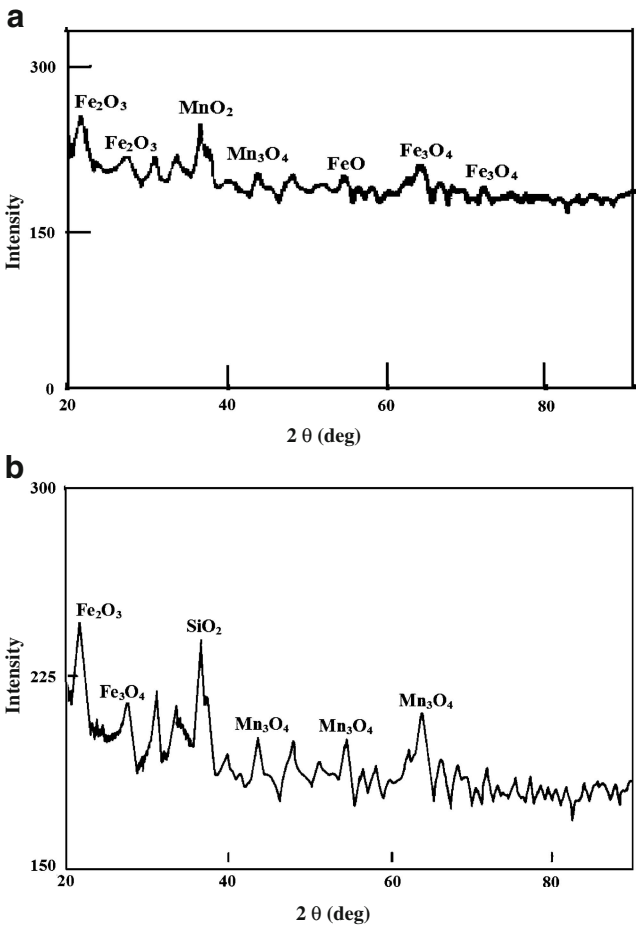

Fig. 4 XRD of the corrosion products. **a** Diesel pipeline; **b** naphtha pipeline

(certain subspecies of *Bacillus* sp.) and iron bacteria such as *P. aeruginosa* AI1 seems to be vital. The manganese deposited by these bacteria was used for scavenging the toxic hydrogen peroxide.

Most of the isolates (AR1, AR2, AR3, and AR4) deposited the ferric hydroxide precipitate which formed as a rust-red color (citrate test); this indicates that the isolates oxidize ferrous iron to the ferric state which precipitated as ferric hydroxide around the cells (Eaton et al. 1995). All the biochemical tests performed in the present study reveal the formation and presence of alcohols and organic acids as intermediate products in the dioxygenase reaction which is again evident from the results of methyl red test and Voges–Proskauer test (Table 1). The presence of ferric and related inorganic chemical species, evident from the XRD analyses (Fig. 4a), indicates that the bacteria accelerated the formation of ferric and manganese complex products.

The microbial distribution was found to comprise *S. marcescens* AR1 (20%), *B. pumilus* AR2 (20%), *B. carboniphilus* AR3 (20%), *B. megaterium* AR4 (20%), and *B. cereus* AR5 (20%), with both Gram-positive and Gram-negative bacteria, with the former dominating (i.e., 80% of the total bacterial population present in the naphtha pipeline). The presence of sulfate in the water is likely to be due to the oxidation of sulfur in naphtha by the acid producers. It is surprising that, although sulfate was present, SRB was not detected in the naphtha pipeline also. Oxidation of elemental sulfur results in the formation of sulfate, and hydrogen ions and sulfur oxidation characteristically result in the lowering of pH. pH range for growth of SRB is 6.5 to 8.5, with optimum being 7.2 to 7.5. The broad ecological classes of sulfur-oxidizing bacteria (acid producers) can be discerned, among those at neutral pH and acidic pH (Buchanan and Gibbons 1974). In the present study, the pH of the sludge was 6.8. In the interface between naphtha and water, pH was between 5.5 and 6.0. The absence of SRB may be due to the low pH.

The presence of ferric oxide and manganese oxide indicates the role of iron- and manganese-oxidizing bacteria in the formation of corrosion product/sludge in the naphtha pipeline. XRD analysis (Fig. 4b) revealed the presence of ferrous and ferric sulfate in the sludge, which indicated the role of iron and manganese bacteria, while acid producers caused the formation of sulfate in the water and sludge. On the inner wall of the tank, severe corrosion was evident where there was no paint or where it had peeled off. The corrosion may be due to the abrasion caused by the movement of the floating roof, followed by exposure of the surface to the atmosphere. It can be concluded that Fe^{++} originated from the storage tank/pipes. Together with products from organic compound degraded by heterotrophic bacteria, Fe^{2+} is oxidized to Fe^{3+} by autotrophs such as *B. megaterium* AR4 and *S. marcescens* AR1, as confirmed in XRD analysis (Rajasekar et al. 2007a). XRD indicates the role of bacteria on manganese/iron deposition in the corrosion product formation and the MIC in the pipeline (Fig. 4b). The redox stain (LBB) turns blue in the presence of *B. carboniphilus* AR3 and *B. megaterium* AR4, which

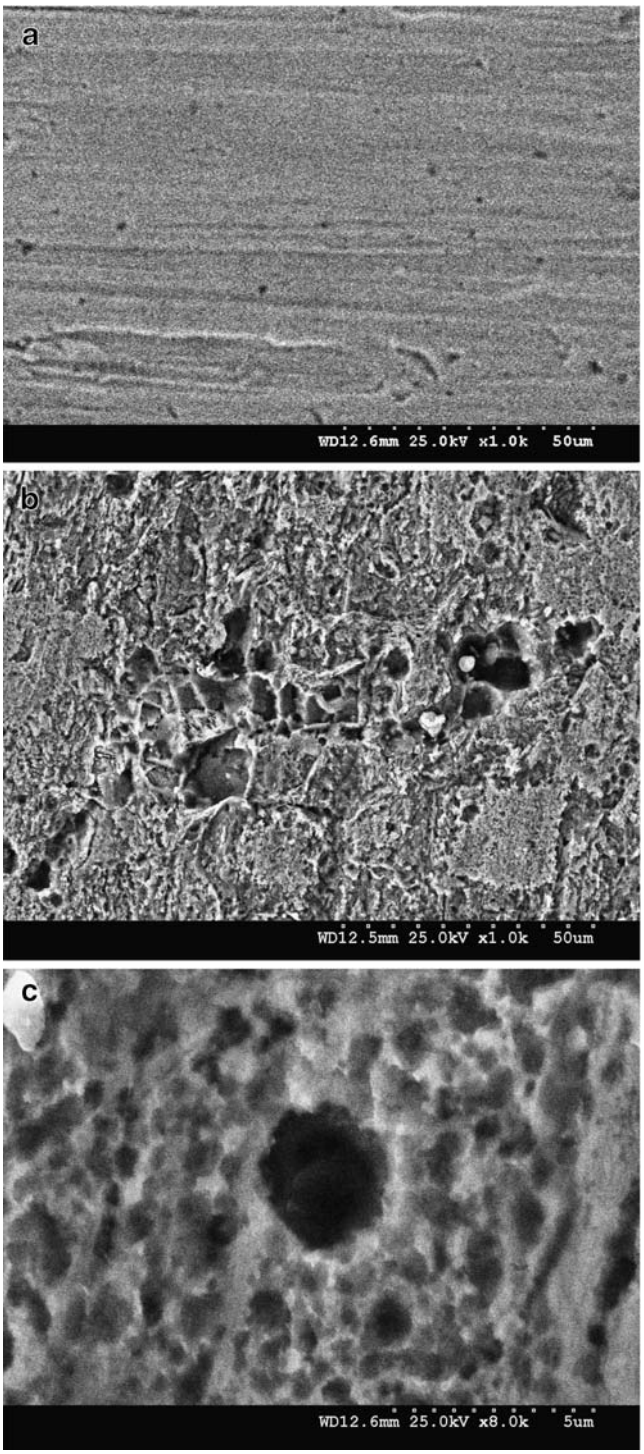

Fig. 5 SEM micrograph of API 5 L-X60 steel. **a** Metal exposed to the medium without bacteria; **b** metal exposed to the medium with bacteria; **c** close view of pit exposed to the medium with bacteria

indicates that these are manganese oxidizer and are able to accumulate and convert the manganese to manganese oxides. Naphtha-degrading bacteria converts ferric complex to ferric oxides by the inclusion of oxygen from the degraded products and forms organic complex. Since ferric/manganese has high affinity for oxygen, it removes oxygen from the degraded product and enhances the formation of ferric/manganese oxides and accelerates corrosion (Rajasekar et al. 2007a). It can be concluded that the degraded organic compounds in naphtha encourage the growth of bacteria and enhance the formation of corrosion products. Ferric oxide formation in the corrosion product was also noted due to the iron oxidizer in the naphtha pipeline. Carbon from naphtha serves as a nutrient and increases the proliferation of bacteria on the steel and influences the extent of corrosion in the petroleum product pipeline.

Although previous microbiological studies have suggested that SRB play a key role in MIC (Graves and Sullivan 1996; Pope and Pope 1998; Drysdale et al. 1999), no SRB was not detected in the genetic analysis of corrosion product samples obtained from petroleum product pipelines. It should be noted that Zhu et al. (2003) had reported that SRB was not detected in biofilm samples obtained from a corroded metal coupon in liquid samples in a gas pipeline. In our study, among the 15 bacterial isolates in petroleum pipeline, ten bacterial isolates were Gram-positive bacteria (Table 3); the dominant bacterial species in diesel and naphtha pipeline were identified as *B. cereus* and *S. marcescens.* Catalase and oxidase were detected in all the bacterial strains. The naphtha and diesel degraders were able to grow at 20°C, 30°C, and 40°C.

Phylogenetic relationship was analyzed between the 16S rDNA sequences of the bacterial phyla Firmicutes (Fig. 1) and Proteobacteria in the three phylogenetic trees (Figs. 2 and 3). The phylogenetic analysis of the isolates showed the dominance of *Bacillus* sp.; in particular, *B. cereus* could be seen as dominant among other species of genus *Bacillus*. *Serratia* sp., *Pseudomonas* sp., and *Klebsiella* sp. were present among phylum Gamma proteobacteria (Table 3).

The characteristically high flow of the petroleum product pipelines ensures an aerobic environment. As a result, the continuous exposure of biofilms to dissolved oxygen from the water (as a contamination at about 2–11%) is likely to have limited the growth of SRB and other anaerobe development. Weight loss and the corrosion rate were also monitored in the presence or absence of the mixed consortia. In the control (uninoculated) system, weight loss of about 11.12 mg occurred over 7 days, compared to 23.55 mg in the presence of the mixed consortia. The corrosion rate was 0.039 and 0.050 mm/year, respectively, and showed that the mixed consortia accelerated the pitting corrosion of API 5LX steel. SEM photomicrograph revealed the extent of pitting corrosion (Fig. 5b, c) compared to the control (Fig. 5a). The partial biochemical characterization of the diesel and naphtha isolates is presented in Tables 1 and 2. The present study indicates that *Bacillus* sp. is the dominant species in the pipeline, followed by *S. marcescens*. *Bacillus* sp. is a spore former and is Gram positive. Both bacteria utilize the hydrocarbon diesel/naphtha as a sole carbon source (Rajasekar et al. 2007a, b). Our previous results (Maruthamuthu et al. 2005) show that the addition of corrosion inhibitor/biocide (which is ester based or toxic inhibitors) by the petroleum product distributors has led to the formation, and indeed dominance, of the resistant spore-forming *Bacillus* sp. In the petroleum-transporting pipeline, many corrosion inhibitors used are degraded by *Bacillus* (Muthukumar et al. 2007). Hence, control measures, including the selection of biocides, are important aspects for the petroleum industry. Bacterial response to biocides is determined essentially by the nature of the chemical agent and the type of organism involved. Bacterial spores of the genera *Bacillus* sp. and *Clostridium* sp. are invariably the most resistant among the different types of bacteria to biocides (Bloomfield and Arthur 1994; Russell 1995). Although many biocides may effectively kill nonsporulating bacteria (but not bacterial spores), high concentrations are often needed to achieve this effect.

This study characterized and examined the relation between different bacterial species in a corrosive bacterial community in a pipeline in a tropical country. *B. cereus* ACE4 and *S. marcescens* ACE2 were the more dominant bacteria among the culturable organisms isolated from corrosion products collected from petroleum-product-transporting pipeline. The presence of manganese and ferric oxides in the corrosion products indicated that the bacterial consortia is capable of converting ferric and manganese on the metal to the metal oxides and accelerated severe pitting attack on the surface of steel API 5 L-X60. Even though these bacteria may be useful in the bioremediation of a diesel-polluted habitat, their presence in diesel pipeline and transportation facilities would lead to the reduction in the quality of diesel and in turn economic loss. This work expands the knowledge of bacterial biofilm communities associated to steel pipelines and recognizes the corrosive potential bacteria of the Enterobacteriaceae family (*K. oxytoca* and *S. marcescens*).

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