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ORIGINAL PAPER

Antagonistic activity of *Bacillus* sp. obtained from an Algerian oilfield and chemical biocide THPS against sulfate-reducing bacteria consortium inducing corrosion in the oil industry

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Abstract The present study enlightens the role of the antagonistic potential of nonpathogenic strain B21 against sulfate-reducing bacteria (SRB) consortium. The inhibitor effects of strain B21 were compared with those of the chemical biocide tetrakishydroxymethylphosphonium sulfate (THPS), generally used in the petroleum industry. The biological inhibitor exhibited much better and effective performance. Growth of SRB in coculture with bacteria strain B21 antagonist exhibited decline in SRB growth, reduction in production of sulfides, with consumption of sulfate. The observed effect seems more important in comparison with the effect caused by the tested biocide (THPS). Strain B21, a dominant facultative aerobic species, has salt growth requirement always above 5% (w/v) salts with optimal concentration of 10–15%. Phylogenetic analysis based on partial 16S rRNA gene sequences showed that strain B21 is a member of the genus *Bacillus*, being most closely related to *Bacillus qingdaonensis* DQ115802 (94.0% sequence similarity), *Bacillus aidingensis* DQ504377 (94.0%), and *Bacillus salarius* AY667494 (92.2%). Comparative analysis of partial 16S rRNA gene sequence data plus physiological, biochemical, and phenotypic features of the novel isolate and related species of *Bacillus* indicated that strain B21 may represent a novel species within the genus *Bacillus*, named *Bacillus* sp. (EMBL, FR671419). The results of this study indicate the application potential of *Bacillus* strain B21 as a biocontrol agent to fight corrosion in the oil industry.

Keywords *Bacillus* · Antagonist · SRB consortium · Injection water · Biocide

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Introduction

Souring is one of the main problems facing the petroleum industry, due to toxicity of sulfides (H_2S) and corrosiveness caused by sulfate-reducing bacteria (SRB). SRB are a ubiquitous group of anaerobes that reduce oxidized sulfur compounds, such as sulfate, sulfite, and thiosulfate, as well as sulfur, to H_2S. Although SRB are strictly anaerobic (obligate anaerobes), some genera tolerate oxygen and are even able to grow at low oxygen concentrations [6]. SRB utilize organic compounds or hydrogen as electron donor in sulfate reduction to sulfide according to Eq. (1) [62].

$$SO_4^{2-} + 8e^- + 4H_2O \rightarrow S^{2-} + 8OH^- \quad (1)$$

Beside sulfide production through sulfate reduction, SRB are also known to reduce thiosulfate to sulfide. This latter process, performed not only by SRB, was reported as

a major risk factor increasing biocorrosion processes [15, 21, 31]. SRB are a subject of concern and interest in the oil industry not only because of sulfide production, but also probably due to the ability of some of them to oxidize hydrogen and use O_2 and Fe^{3+} [22]. These bacteria could be responsible for H_2S production, which causes "souring" of crude oil, microbiologically influenced corrosion (MIC), and increased solids loading in water injection systems (iron sulfides, etc.), and can cause plugging of producer and injection wells with sulfide scale or biofilm proliferation.

In addition, souring lowers the economic value of produced oil and induces safety hazards [39, 52]. A number of methods for controlling sulfide production in different oil production facilities have been proposed to reduce microbial activity, including use of oxidizing (such as halogen and ozone) or nonoxidizing biocides (such as formaldehyde, glutaraldehyde, isothiazolones, and quaternary ammonia compounds) [73] and recently tetrakishydroxymethylphosphonium sulfate (THPS) [18, 36, 46]. To prevent SRB growth, the Algerian oil industry spends up to US $30 million annually on chemical products such as biocides. Among the oilfields worldwide that maintain their pressure by water injection are cited those located in the southern Algerian Sahara operated by Sonatrach Oil Company. These injection waters were treated with THPS biocide from start-up in 1999. The water injection pipeline system consists partly of carbon steel, and the amount of water injected varies between 13,000 and 15,000 m^3/day. Although biocide treatments are widely used to decrease biofouling and MIC in steel pipes [32, 35] and in closed systems [34], the results are far from satisfactory. This is because MIC occurs under adherent biofilms [20], and biocides are much less effective against sessile organisms within biofilms compared with against planktonic population [38] due to biofilm's dramatically enhanced resistance to antimicrobial agents [25]. Besides the risk to human health, the efficacy of these biocides is questioned. In addition, microbial resistance to some of these products has been reported [36], where bacteria can change and modify their genetic features by undergoing periodic mutation and, after a period of time, become resistant to a given biocide. Al-Hashem et al. [1] differentiate between adaptation and developing resistance to a biocide from an antibiotic. It may be observed that bacteria would need to alter the structure of every protein in the cell to be able to resist a biocide. This would require the bacteria to undergo a large number of mutations at the same time, or in a short time, to become resistant to the biocide. Such patterns of mutations, however, must occur over time spans much longer than what is normally available in an industrial system. For these reasons, the search for biological methods has recently been started, based on two approaches. The first approach makes use of antagonistic interactions involving release of bactericidal substances (antibiotics, lytic enzymes) by antagonist microorganisms [9, 69]. However, the success of such biocontrol and the yield increase achieved depend on the nature of the antagonistic properties and on the organism's mechanisms of action. The modes of action vary widely and can include, for instance, nutrient competition and production of secondary metabolites [51]. For the second approach, the literature describes the antagonistic effects of several microorganisms, often referred to as potential biocontrol agents, against a variety of microorganisms [47]. Among microorganisms producing metabolites with antifungal activity, bacteria of the genera *Pseudomonas*, *Streptomyces*, and *Bacillus* have been shown to be effective against wood blue-stain fungi [33, 68]. Secondary metabolites produced by certain species and strains of *Bacillus* show antifungal and antimicrobial activity against phytopathogenic microorganisms [61].

Many *Bacillus* species produce bacteriocins [79]. Bacilli grow quickly on nutrient-rich cultivation media such as tryptic soy agar (TSA), plate count agar, or nutrient agar at 37°C. Some physicochemical conditions are used for bacterial isolation in many studies [17, 71]. Some authors have suggested that the use of such strains or species, or their metabolites, may be an alternative to agrochemical plant protection [9, 47]. Other metabolites, such as biosurfactants and enzymes that degrade the cell wall, are also produced by *Bacillus* species [47]. Antibiotics produced by *Pseudomonas* and *Streptomyces* species have also been described; Benko [7] tested more than 200 bacteria of different genera for antibiosis and observed *Pseudomonas* and *Streptomyces* spp. to be the most active.

Another promising way of using microorganisms for this purpose employs protective biofilms formed by some microorganisms. It was shown that biofilms produced by *Escherichia coli* and *Pseudomonas fragi* reduced corrosion losses by a factor of 4 to 40 [42]. Examination of 42 organochemotrophic bacterial strains isolated from biofilms and water of industrial cooling systems demonstrated a decrease in metal corrosion in the presence of most of these microorganisms [63].

Furthermore, Rodin et al. [64] demonstrated that cocultivation of destructor microorganisms and their antagonists decreases the corrosion loss of carbon steel by 20% to 80%. It was found that a microorganism can either accelerate or inhibit corrosion, depending on the nutrients present. The magnitude of the effect on corrosion depends on the ability of the microorganism to respond to changes in the nutrient medium composition by releasing acidic or alkaline metabolites [64].

Moreover, Jayaraman et al. [43] studied the ability of genetically engineered *Bacillus* strains to inhibit SRB growth by secreting antimicrobials, characterized by using concentrated supernatant phases of the recombinant strains to inhibit exponential growth of SRB in suspension cultures

and by measuring the reduction of populations consisting of representative SRB (*Desulfovibrio vulgaris* and *D. gigas*) in biofilms, finding significantly reduced corrosion rates under continuous culture conditions. Jayaraman et al. [43] showed that antimicrobial peptides produced by the gramicidin-S-overproducing *Brevibacillus brevis* 18 strain inhibited formation of a corrosive biofilm on steel by sulfate-reducing *Desulfovibrio vulgaris* in modified Baar's medium. The same authors [43] suggested the production of a protective biofilm by an antimicrobial-producing primary colonizer (*B. brevis* 18) as an attractive alternative to conventional biocides for corrosion control.

Zuo [80] presents a review and summarizes recent progress using this novel strategy of corrosion control using beneficial bacterial biofilms. He explains that the possible mechanisms may involve: removal of corrosive agents (such as oxygen) by bacterial physiological activities (e.g., aerobic respiration), growth inhibition of corrosion-causing bacteria by antimicrobials generated within biofilms [e.g., inhibition of corrosion by sulfate-reducing bacteria (SRB) due to gramicidin-S-producing *Bacillus brevis* biofilm], and generation of protective biofilm layers (e.g., *Bacillus licheniformis* biofilm produces a sticky protective layer of γ-polyglutamate on aluminum surface). Zuo [80] confirmed that successful use of this novel strategy relies on advances in study at the interface between corrosion engineering and biofilm biology.

Generally, antagonist microorganisms have been successfully used in agriculture. This provides the basis for using this approach in other fields, such as combating MIC. In this study, we attempted to evaluate the potential for use of antagonists to reduce the activity of sulfate-reducing bacteria (SRB) and the corrosiveness of the resulting sulfides (H_2S) to protect metals from biocorrosion. The objectives of this study are to assess the feasibility of using microbial antagonists in the fight against biocorrosion, and to compare the results achieved with those of the effect of the last generation of THPS biocide on sulfate-reducing bacteria and thus to demonstrate importance and relevance of the biological treatment.

Materials and methods

Injected water samples and chemical analysis

Four water samples were collected from four different oilfields located in the southern Algerian Sahara: Hassi Berkine (sample 1), Hassi Messaoud (sample 2), Tin Fuin Tabankort (sample 3), and In-Amenas (sample 4). The injection water samples were collected in sterile glass bottles, which were filled completely to prevent contact with air, then sealed with rubber stoppers. They were transported by ice box to the laboratory and stored at −20°C. The analytical method used in this study was based in detail on 4500-S^{-2} F standard methods, as described elsewhere [2].

Water injection for flooding and pressure support is a key element of oilfield operation. Formation (reservoir) waters in most oil production fields are typically hypersaline chloride-type waters that contain alkaline cations such as calcium, strontium, and barium. Potable water injection, although an expensive option for onshore fields, could damage reservoir formation (incompatible salinity). Table 1 presents the chemical composition of injection and aquifer water samples from the Algerian Sahara oilfields.

Isolation of bacteria, media composition, and culture condition

Culture of the sulfate-reducing bacteria

For this purpose we used an SRB consortium, previously obtained in a modified Postgate's SRB medium, from sample 4. This was chosen because it contains the highest sulfate concentration; moreover, In-Amenas oilfield was more affected by a microbiological charge induced by the

Table 1 Chemical composition of injection water samples collected from four sites of oil areas in the southern Algerian Sahara

	Sample 1	Sample 2	Sample 3	Sample 4
Analyte (mg/L)				
Barium	0	0	0	0
Calcium	267.6	197.5	187.3	834
Iron	0.03	0	0.1	0
Magnesium	58.8	62.5	163.4	311
Potassium	68	52	39	65
Sodium	760	830	300	7,250
Strontium	3.1	4.3	2.1	/
Bicarbonate	164.1	178.1	165.9	198
Phosphorous	3.0	2.3	4.3	3.5
Ammonia nitrogen	11.5	12	11	12.75
Nitrate	41	23.2	33.5	45.5
Nitrite	14.3	12.3	11.65	15.5
Carbonate	0	0	0	0
Propionate	<5	<5	<5	<5
Chloride	1,428.2	926.1	670.9	11,275
Sulfate	100	1,200	600	2,200
pH in situ	6.90	7.00	7.30	7.00
Temperature in situ (°C)	45	46	50	35
Salinity (g/L)	3.54	0.92	2.51	2.07

SRB than other oilfields. It will promote optimal growth of these bacteria. Organisms issued from the fields were isolated in Postgate's medium and then diluted to extinction in a most probable number (MPN)-type serial dilution using the same medium. After 28 days of incubation at 37°C, the last positive vial in this initial dilution series was then used to inoculate a second MPN-type series of the same medium. This technique allowed isolation of the most dominant SRB species (able to grow in the medium) in the original sample.

Isolation and identification of antagonist bacteria

Complex medium (CM) [67] at pH 7.0 and containing 12% (w/v) NaCl was used for bacterial enrichment and growth. A reservoir water aliquot from the In-Amenas production oil reservoir was first inoculated into the liquid medium and cultured on a shaker (120 strokes min^{-1}) at 37°C in the dark until turbid, then streaked onto solid medium to obtain single colonies. The streaking was repeated several times to obtain pure single colonies. Morphologically dissimilar colonies were selected randomly from all plates, and isolated colonies were purified using appropriate medium by streaking methods. The pure cultures were maintained in slants for further analysis. Three genera were isolated and identified from In-Amenas injection water by biochemical tests: *Bacillus* sp. (strain B21), *Halomonas* sp., and *Vibrio* sp. Strain B21 was selected for further study and was identified by 16S rDNA gene analysis as *Bacillus* sp.

The purified strain B21 was cultured and maintained in liquid or solid American Type Culture Collection (ATCC) 213 medium [37] at pH 7.0, with 12% (w/v) NaCl, in the dark. Cell morphology was determined by using phase-contrast microscopy. Gram staining was performed according to Dussault [27], and electron microscopy was performed with negative staining according to Bouchotroch et al. [13]. Bacterial growth was determined by measuring absorbance at 600 nm at certain intervals during liquid culture. The effects of different NaCl concentrations on strain B21 growth were tested in liquid ATCC 213 medium by measuring absorbance at 600 nm after 7 days of incubation. Susceptibility of strain B21 to different antibiotics, including ampicillin, tetracycline, hygromycin, kanamycin, streptomycin, rifampicin, bacitracin, penicillin, chloramphenicol, neomycin, and erythromycin (Sigma), was tested on solid ATCC 213 medium after 3 days of incubation at 37°C.

Physiological and biochemical tests were performed as described by Buchanan and Gibbons [14] and Dong and Cai [23]. Strain B21 anaerobic growth with nitrate as electron acceptor was tested according to the method of Mancinelli and Hochstein [50]. Growth with dimethyl sulfoxide (DMSO) and by L-arginine fermentation as alternative electron acceptors was tested as described by Oren et al. [57] and Oren and Trüper [56] using closed tubes fully filled with the growth medium and then kept in the dark for 3 weeks before comparison of growth with that found on medium lacking the test compounds.

Amplification, cloning, and sequencing of 16S rRNA gene

Genomic DNA was extracted according to Ausubel et al. [5]. Amplification of gene encoding for small subunit ribosomal RNA was done using eubacterial 16S rDNA primers [forward primer 5′AGAGTTTGATCCTGGCTC AG3′ (*Escherichia coli* positions 8–27), reverse primer 5′ACGGCTACCTTGTTACGACTT3′ (*E. coli* positions 1494–1513)] [76]. Polymerase chain reaction (PCR) was performed with a 50 µl reaction mixture containing 2 µl (10 ng) DNA as the template, each primer at concentration of 0.5 µM, 1.5 mM $MgCl_2$, and each deoxynucleoside triphosphate at concentration of 50 µM, as well as 1 µl Taq polymerase and buffer as recommended by the manufacturer (MBI Fermentas). PCR was carried out using a Mastercycler Personal (Eppendorf, Germany) with the following program: initial denaturation at 95°C for 1 min; 40 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C), and extension (2 min at 72°C); followed by 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 Luria-Bertani (LB) medium containing ampicillin (100 µg/ml) and X-gal (80 µg/ml).

Phylogenetic analysis was performed using the software package BioNumerics (Applied Maths, Belgium) after including the sequence as received in an alignment of small ribosomal subunit sequences collected from the international nucleotide sequence library EMBL. This alignment was calculated pairwise using an open gap penalty of 100% and a unit gap penalty of 0%. Similarity matrix was created by homology calculation with a gap penalty of 0% and after discarding unknown bases. A resulting tree was constructed.

Biological inhibition assay (coculture) and chemical inhibition

Preparation of different biocide concentrations

In the present study, commercially available inhibitor THPS, generally used to protect petroleum facilities, was

evaluated to determine the nature of inhibition. Tetrakishydroxymethylphosphonium sulfate (THPS) is a quaternary phosphonium; it disrupts cell membrane with a rapid uptake mechanism through cell wall and inhibits lactate dehydrogenate activity [24, 34]. The biocide was obtained from Sigma (St. Louis, MO). From pure THPS solution, a stock solution of 10^4 mg/L was prepared. From this stock solution, serial dilutions were prepared to obtain THPS concentrations of 5, 10, 20, and 50 mg/L (v/v), which were used to perform the inhibition tests (chemical treatment).

Coculture tests

To compare difference between biological and chemical treatments, seven sets were conducted in Erlenmeyer flasks containing 100 ml injection water, each sample of the fourth contains respectively, 100, 600, 1,200, and 2,200 mg/L sulfate. These samples were inoculated with SRB. In each test the optical density of the first SRB inoculate was 0.055 at 600 nm (initial load $\sim 1.9 \times 10^5$ cells ml^{-1}). These preparations were further inoculated with strain B21 at different concentrations and secondly by biocide THPS at different concentrations as well.

The antagonist cultures of strain B21 inoculate were prepared by growth on medium M4, containing (w/v): 10.3% NaCl, 1.8% $MgSO_4.7H_2O$, 0.025% $CaCl_2{\cdot}2H_2O$, 0.019 KCl, and 0.05% peptone (Difco). pH was adjusted to 7.2. Cultures were incubated at 37°C in an orbital shaker (New Brunswick Scientific) at 200 strokes min^{-1}. After reaching late exponential phase, which lasts about 18 h, cells were harvested under sterile conditions by centrifugation at 6,000 g, washed, and resuspended in small amounts of sterile peptone (0.05%, w/v) in water. The optical density of each strain B21 inoculate was 0.050 at 600 nm (initial load $\sim 1.8 \times 10^5$ cells ml^{-1}). After inoculating each medium with 2 ml cell suspension (2% concentration), the inoculants were added to the corresponding flasks (50 ml medium, each taken in 100-ml conical flasks). Subsequently, we followed the growth kinetics of SRB consortium with addition of the biological inhibitory (strain B21) at different inoculum concentrations of 2%, 5%, and 6%. In the second assay, we controlled the growth kinetics of SRB consortium by adding the chemical inhibitory THPS at different concentrations of 5, 10, 20, and 50 mg/L. All the Erlenmeyer flask medium sets (with and without inhibitor) were transferred to an anaerobic hood under 5% H_2, 10% CO_2, 85% N_2 atmosphere. Cultures (in triplicate) were incubated at 37°C on a rotary shaker (100 strokes min^{-1}). The SRB growth kinetics was followed for 12 days. At required intervals, the flasks from each test were removed and various analyses were carried out immediately. For each realized test, a control was also used.

Quantitative determinations

Enumeration of sulfate reducers and analytical procedures

SRB microbial counts were performed (following most experiments) using the five-tube most probable number (MPN) method [41] using mCSB. Medium was prepared, autoclaved, distributed into 10-ml open-capped glass tubes, inoculated, and transferred to an anaerobic hood (Coy Laboratory Products) under 5% H_2, 10% CO_2, 85% N_2 atmosphere. The tube sets were inoculated with liquid samples taken from the sets of cocultures with and without antagonist strain B21 or biocide (THPS). Following 3 weeks of incubation, tubes that became turbid were scored positive for growth. SRB enumeration by plate-counting used the same medium with 15 g/L agar. Following pouring, plates were air-dried and then transferred to the anaerobic hood. Plates were inoculated in the anaerobic hood by spreading 100 µl from each of the freshly prepared MPN dilutions onto the plates. Colonies were counted following 3 weeks of incubation.

Sulfide and sulfate concentrations were determined by spectrophotometry as described elsewhere [2, 40, 53]. A culture sample was collected daily from vials containing cocultures with and without biological and chemical antagonists.

Statistical analysis

Data are presented as arithmetic averages of at least three replicates, and error bars indicate standard deviation. Analyses were carried out using Microsoft Excel software, SMBios (version 2.4).

Results

Characterization of isolate

Sample 4 was used to enable isolation of antagonistic bacteria belonging to genus *Bacillus* (strain B21). This strain grew at salt concentrations in the range 5–25% (w/v) NaCl; optimum growth occurred on media containing 10–15% (w/v) NaCl. No growth was observed in the absence of NaCl. Colonies obtained were cream in color, smooth, low-convex, and circular/slightly irregular on the modified nutrient agar medium containing 15% (w/v) NaCl. Strain B21 grew at pH values in the range of 6.5–7.5 in 15% (w/v) NaCl-containing tryptic soy broth; optimal growth was observed at pH 7.1. Morphological and phenotypic characteristics suggested that the isolate is a halophilic member of the *Bacillus* genus. Growth was observed at temperatures between 25°C and 45°C; optimum growth was at 37°C. Cells of the isolate

were Gram-positive, slender, short rods, 0.3–0.5 mm wide, and 1.3–1.9 mm long, and were facultatively anaerobic. Spherical terminal endospores were produced. The strain is oxidase negative and catalase positive, and negative for hydrolysis of soluble starch, gelatin, Tweens 20 and 80, production of H_2S, and indole. It is positive for hydrolysis of urea, arginine, and for nitrate reduction. It utilizes, and produces acid, from sucrose, D-glucose, D-fructose, D-galactose, cellobiose, D-mannose, D-mannitol, D-galactose, lactose, D-xylose, and dextran as sole carbon source. Voges–Proskauer and methyl red reactions were negative. Growth of strain B21 could be inhibited by the antibiotics rifampicin, penicillin, hygromycin, ampicillin, and bacitracin. No inhibition was observed when the strain was grown in the presence of kanamycin, erythromycin, streptomycin, tetracycline, chloramphenicol or neomycin. Comparison of the phenotypic and physiological properties of strain B21 with those of the type strains of three closely related *Bacillus* species is shown in Table 2.

A partial (529 bp) 16S rRNA gene sequence (EMBL, FR671419) was obtained for strain B21 and was used for initial BLAST searches in GenBank and for phylogenetic analysis. The phylogenetic tree was constructed using neighbor-joining [65] and revealed that strain B21 formed a phyletic group with *Bacillus qingdaonensis* DQ115802 (94.4% sequence similarity), *Bacillus aidingensis* DQ504377 (94.0%), *Bacillus salarius* AY667494 (92.2%), and *Bacillus chagannorensis* AM492159 (89.1%) (Fig. 1). Strain B21 shared very low 16S rRNA gene sequence similarities (<95%) with the closely related type strains of *Bacillus* species; this is sufficient to allow the classification of this strain as a different species [70].

Kinetic growth of SRB consortium with addition of biological and chemical inhibitor

All experiments were conducted and performed in culture medium consisting of injection waters collected as mentioned in oil areas in the south of Algeria. In our study, waters present a wide sulfate concentration, and SRB growth and biological activity depend on sulfate. Also, a variability of sulfate concentration depends on the nature and origin of water injection. In general, SRB tend to synthesize one or more metabolic products. Biochemically, SRB transform sulfate to sulfide, catalyzed by adenosine phosphosulfate reductase. According to Postgate [62], these bacteria produce sulfides, after carbohydrate substrate degradation and internal cellular energy release.

The SRB consortium growth is presented in Fig. 2, with and without addition of biological and chemical inhibitors.

Table 2 Characteristics of strain B21 and related species

Characteristic	1	2	3	4	5
Cell morphology	Short rods	Rods	Short rods	Short-rod-shaped	Short rods
Colony color	Cream	Cream white to yellow	Cream white	Cream	Yellow–orange
Spore formation	+	−	+	+	+
Oxidase	−	−	−	+	−
Catalase	+	+	+	+	+
NaCl concentrations for growth (% w/v)	5–25	2.5–20	8–33	3–20	3–20
Minimum	5	2.5	8	3	3
Optimum	10–15	12	12	10–12	7
Maximum	25	20	33	20	20
Optimum temperature for growth (°C)	37	37	37	30	37
Nitrate reduction	+	+	+	−	+
H_2S production	−	−	+	ND	−
pH range	6.5–7.5	6.5–10.5	6.0–9.5	6.8–9.5	5.8–11.0
Optimum pH	7.0	9.0	7.2	8.0	8.5
Hydrolysis of					
Gelatin	−	−	+	ND	−
Urea	+	+	−	ND	ND
Tween 20	−	−	−	ND	ND
Tween 80	−	−	−	+	−

Strains: 1, B21; 2, *Bacillus qingdaonensis* DQ115802; 3, *Bacillus aidingensis* DQ504377; 4, *Bacillus salarius* AY667494; 5, *Bacillus chagannorensis* AM492159

Data from Wang et al. [75], Xue et al. [77], Lim et al. [48], Nielsen et al. [54], and this study. +, positive; −, negative; *ND* no data

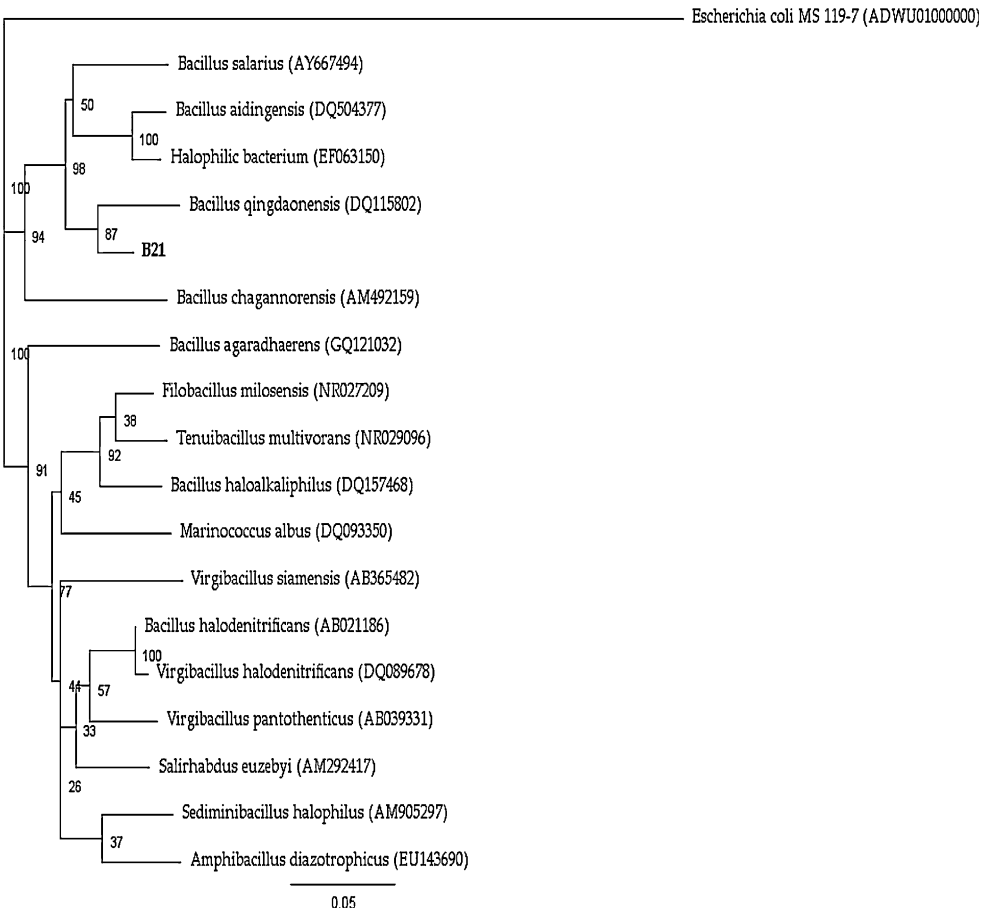

Fig. 1 Neighbor-joining phylogenetic tree, based on 16S rRNA gene sequences, showing the relationships among strain B21 (*Bacillus* sp.) and related species. Bootstrap values are shown as percentages of 100 replicates. *Escherichia coli* MS 119-7 was used as an outgroup

The cell density evaluation revealed bacterial growth varying with time and for different injection water samples containing 100 mg/L (S1), 600 mg/L (S2), 1,200 mg/L (S3), and 2,200 mg/L (S4) sulfate. These waters were inoculated with the same concentration of SRB consortium (initial load $\sim 1.9 \times 10^5$ cells ml^{-1}). The curves in Fig. 2 reveal significant differences in cell growth behavior.

We also noticed that, during the incubation time, the cell density increased progressively in all injection waters and for each sulfate concentration considered. The lag phase was absent, having occurred during preculture. However, it is worth noting that growth was relatively dependent on the sulfate ion concentration present in the media, being largest (1.2×10^6 cells ml^{-1}) for the negative control medium containing 2,200 mg/L sulfate. Also, we noticed that, as the sulfate concentration decreased (from S4 to S1), the cell density decreased, reaching a minimum value (10^6 cells ml^{-1}) in all control tests, after 12 days of incubation.

During the cell density monitoring in the presence of biological and chemical inhibitors, we noticed that the cell density of the negative controls was higher than for cultures treated by either the bacterial antagonist or the biocide. We observed this phenomenon in all experiments. Thus, the cultures inoculated with the SRB showed higher cell density compared with culture containing SRB and THPS. We found, also, that cell density values of waters treated by the biocide were much higher than those obtained in the cocultures containing SRB and strain B21, highlighting the inhibitor effect on the SRB consortium. The inhibition was remarkably strong. In the presence of biocide and in all experiments, SRB growth was reduced during the first 9 days, restarting thereafter. In the oil industry, the inhibition duration is generally considered important for optimization of THPS efficiency in injection waters contaminated by SRB [24].

On the other hand, in the presence of antagonistic bacteria cocultured with SRB, we noticed a significant decrease in cell density in all injection waters. We can explain this by the fact that the effect caused by the bacterial antagonist is of either bactericidal or bacteriostatic type as long as it is undergoing exponential growth. Furthermore, and after an incubation period exceeding just 3 days of coculture, we saw a decrease in SRB cell density in all tests of culture containing 2% and also in cocultures containing 5% and 6%. We observed that B21 inoculation reduced SRB growth during 12 days. Compared with THPS inhibition, strain B21 inhibition was very strong. For oilfield site efficiency tests, this is very advantageous.

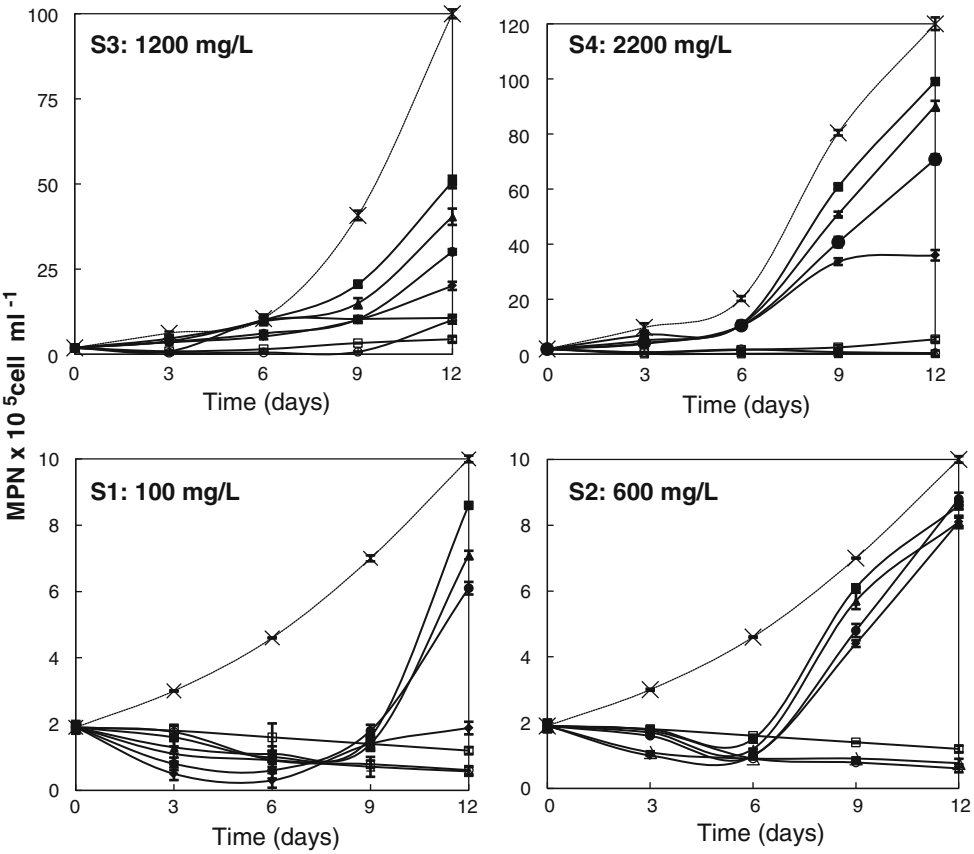

Fig. 2 Growth kinetics of SRB consortium with addition of biological inhibitor (strain B21) at different concentration of inoculum: 2% (*open squares*), 5% (*open triangles*), and 6% (*open circles*). In the second assay, we estimated the growth kinetics of SRB consortium with addition of chemical inhibitor (THPS) at different concentrations: 5 mg/L (*filled squares*), 10 mg/L (*filled triangles*), 20 mg/L (*filled circles*), and 50 mg/L (*filled diamonds*). In all cases, a negative control was used (*crosses*). Each culture was grown at 37°C and 100 rpm in medium (sample of water) at different concentrations of sulfate (100, 600, 1,200, and 2,200 mg/L). Reported values are averages of three replicates

Moreover, Fig. 3 shows that the SRB reduced sulfate to sulfide, using ion sulfate as final electron acceptor. They are the only bacteria reducing sulfate ions present in waters. In the presence of the ions sulfates, the SRB synthesized ATP for their metabolism, according to the dissimilation of sulfate biochemical reaction. Thus, the enzyme ATP sulfurylase induced generation of hydrogen sulfide (H_2S).

In agreement with Fig. 2, it was observed that the sulfate concentration evolution versus SRB consortium incubation time when cultivated without inhibitor in the four water media exhibited the same tendency. We also noticed that the sulfate concentration decreased with incubation time because it was used by SRB. Indeed, the amount of sulfate used was higher in water dosed at concentrations of 2,200 mg/L (S4) and 1,200 mg/L (S3) than at 600 mg/L (S2) and 100 mg/L (S1).

As shown in Fig. 3, the SRB degrade sulfate better in the presence of inhibitor, whatever its nature. In the biological treatment experiment, strain B21 probably tended to inhibit cellular activity by acting either on the wall or on the cytoplasmic membrane. There is competition for nutrients between the different microbial species in coculture. Figure 4 shows measurements of sulfide during the incubation of cocultures and controls to highlight the evolution of the sulfides in the four injection water samples. The rate of this phenomenon varies with time and depends on the sulfate concentration in the medium. Adenosine phosphosulfate reductase is induced by SRB, but proportionally. At the end of the incubation time, the amount of sulfide released varied with the sulfate concentration; the highest sulfide concentration (0.66 mg/L) was obtained in the medium containing the highest sulfate concentration (S4, 2,200 mg/L), whereas the lowest (0.142 mg/L) sulfide concentration was founded in the medium containing less sulfate (S1, 100 mg/L). Regarding the other two media, sulfide concentrations were intermediate, corresponding to S2 and S3.

Comparison of the sulfide production kinetics in the injection waters used for SRB cultivation with and without chemical or biological inhibitor showed that SRB became less corrosive at low sulfide concentrations. In general, the inhibitor concentration has a direct influence on sulfide production. Indeed, the sulfide concentration decreased significantly with increasing inhibitor concentration. The presence of sulfate in the injection waters presents a number of problems due to the activity and growth of SRB. SRB use sulfate as terminal electron acceptor during organic matter oxidation, resulting in production of hydrogen sulfide. The presence of H_2S produces a wide range of biological and physicochemical problems, including precipitation of non-alkali metals, odor, and corrosion of pumps and pipes. In all cases, gradual consumption of sulfate by SRB was noted.

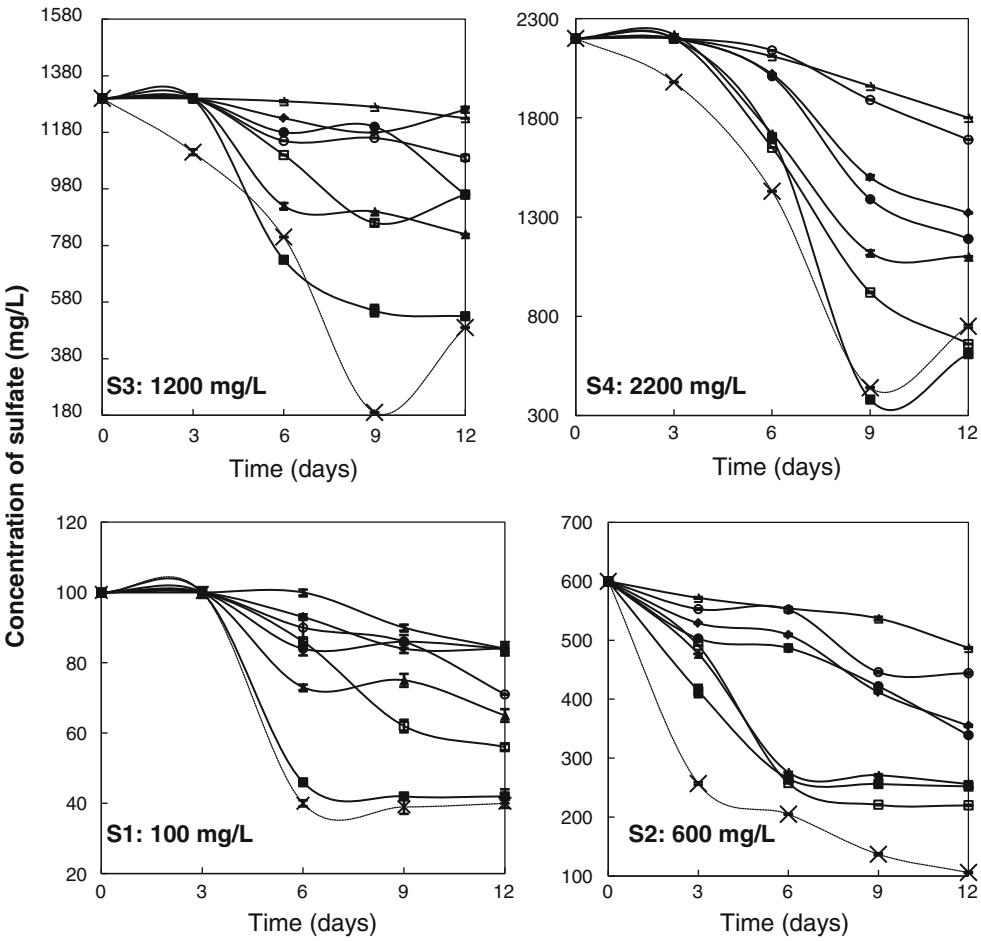

Fig. 3 Kinetics of consumption of sulfate by SRB consortium with addition of biological inhibitor (strain B21) at different concentration of inoculum: 2% (*open squares*), 5% (*open triangles*), and 6% (*open circles*), or addition of chemical inhibitory THPS at different concentrations: 5 mg/L (*filled squares*), 10 mg/L (*filled triangles*), 20 mg/L (*filled circles*), and 50 mg/L (*filled diamonds*). In all cases, a negative control was used (*crosses*). Each culture was grown at 37°C and 100 rpm in medium as sample of water at different concentration of sulfate (100, 600, 1,200, and 2,200 mg/L). Reported values are averages of three replicates

For instance, after 12 days of growth in medium S1, the residual sulfate reached 40 mg/L in control. Using 2% inoculum of strain B21 resulted in consumption of sulfate by SRB, with the sulfate concentration decreasing from 100 to 56 mg/L (Fig. 3). Similarly, when we used 5 mg/L THPS, we observed a decrease in this concentration from 100 to 42 mg/L. This shows the inhibitory effect of strain B21 on sulfate consumption. The same reasoning can be adopted with the other media (S1, S2, S3, and S4). On the other hand, Fig. 4 shows that the sulfide production inhibition follows an opposite argument to the inhibition of sulfate consumption. Sulfide production during metabolism of SRB was slower in all tested waters compared with the control. This decreased rate of sulfide production was more pronounced in the presence of strain B21 compared with the biocide THPS, for all medium (S1, S2, S3, and S4).

Discussion

Production of oil from subsurface reservoirs requires water or gas injection to maintain reservoir pressure, and water is usually injected at onshore Algerian sites. The combination of abundant electron donors (selected oil components) and electron acceptors (sulfate) can lead to significant sulfide production in the subsurface through the action of resident or injected sulfate-reducing bacteria (SRB). However, identification of SRB is not easy, because of their diversity. Actually, SRB form a complex ecophysiological group of prokaryotes with the common property of using sulfate as the main electron acceptor during anaerobic metabolism [29, 55]. They are adapted to a great variety of environments and contribute to the mineralization of 50% of organic matter. SRB form an assemblage of more than 200 species with great variety of morphology and metabolism, including Gram-negative mesophilic SRB, Gram-positive spore-forming SRB, thermophilic bacterial SRB, and thermophilic archaeal SRB [16]. However, SRB are not the only bacteria found, as it has been shown [10, 30] that many bacteria isolated from marine biofilms are phylogenetically close to some type strain species that are members of the *Enterobacteriaceae* family. These isolated bacteria are able to produce sulfide ions in anaerobic conditions by sulfate reduction, so it is important to discriminate all bacteria able to reduce sulfate. Sulfide is produced by SRB, presumably in

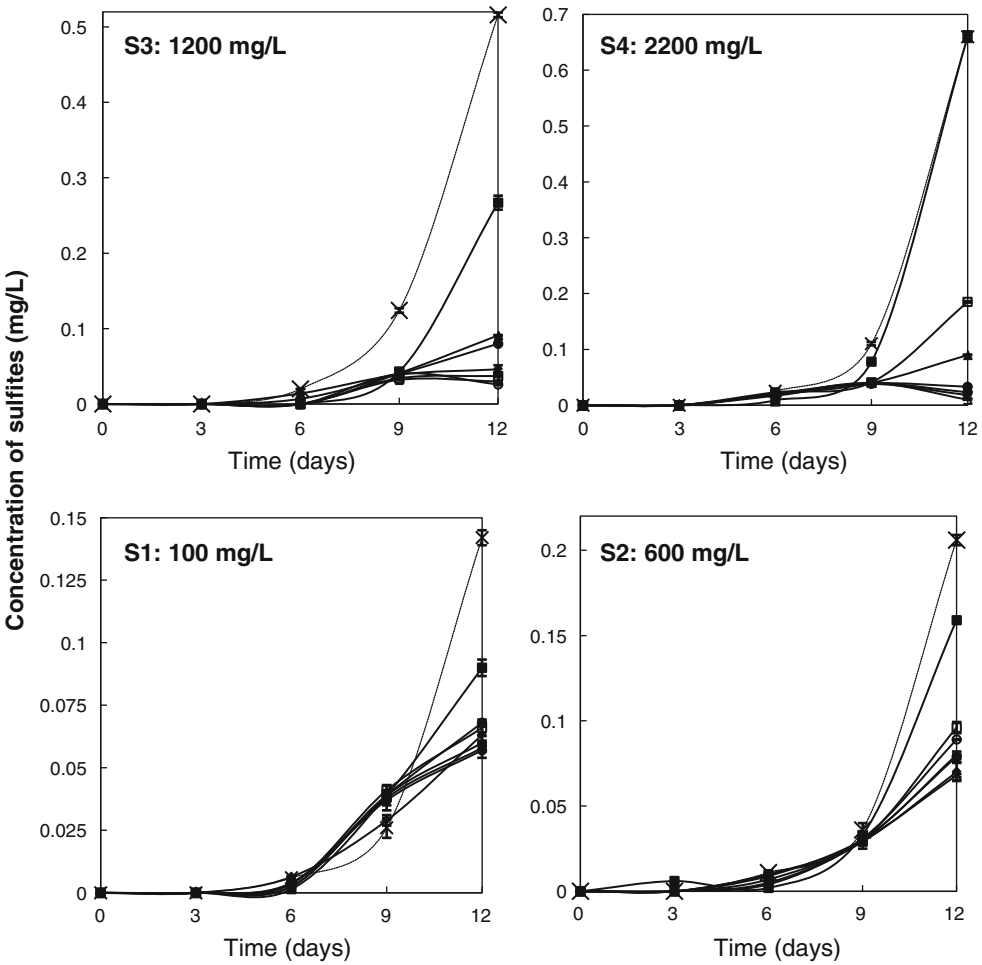

Fig. 4 Production kinetics of sulfite by SRB consortium with addition of biological inhibitor (strain B21) at different concentration of inoculum: 2% (*open squares*), 5% (*open triangles*), and 6% (*open circles*), or addition of chemical inhibitory (THPS) at different concentrations: 5 mg/L (*filled squares*), 10 mg/L (*filled triangles*), 20 mg/L (*filled circles*), and 50 mg/L (*filled diamonds*). In all cases, a negative control was used (*crosses*). Each culture was grown at 37°C and 100 rpm in medium as sample of water at different concentration of sulfate (100, 600, 1,200, and 2,200 mg/L). Reported values are averages of three replicates

the zone where sulfate-containing injected water mixes with organic-containing formation water.

Biogenic production of H_2S in oil reservoirs subjected to water flooding (souring) is a serious concern in the oil industry, with the toxicity of H_2S, accelerated corrosion of pipelines, production, and processing equipment, decreased secondary oil recovery efficiency due to plugging of oil-bearing strata by biomass, and precipitation of metal sulfides being some of the associated problems. Furthermore, the necessity for H_2S removal prior to oil and gas use and before recycling of produced water increases production costs. Sulfate-reducing bacteria (SRB) are believed to be major players in souring of oil reservoirs. Our study has shown that, whatever the concentration of sulfate present in the culture media, growth of SRB is always optimal and favorable. This growth was strongly inhibited by the bacterial antagonist strain B21, identified as an extremely halophilic Gram-positive bacterium isolated from injection water collected in In-Amenas locality, Algeria.

Biocides are synthetic antimicrobial compounds that are used to protect industrial systems such as water-treatment or metal-working-fluid systems [12]. So, in the Algerian petroleum industry, the chemical biocide THPS at concentrations of 5–50 mg/L exhibited excellent activity against problems caused by microorganisms. In particular, it is extremely effective against SRB, a major cause of hydrogen sulfide souring and corrosion. Formulations containing THPS may be used in many applications where microbiological contamination is present, and they have extensive use in enhanced oil recovery injection water systems, topside recovery systems, pipeline protection, and storage. However, these expensive products are toxic and not environmentally friendly. The mechanisms by which biocides and preservatives work have generally been classified as either membrane active or electrophilic [66]. This biocide acts on the bacterial cytoplasmic membrane, a very delicate organelle that is highly active metabolically and which acts mainly as a selective permeability barrier between the cytosol and the cell's external environment [24]. Any membrane-active agent can induce damage by action upon membrane potentials, bound enzymes or permeability. The results of some studies indicate that

pyrithione biocides are able to disrupt membrane function of Gram-negative bacteria. In addition to and simultaneously with this disruption, these compounds can also chelate potassium ions from the medium [24]. A combination of these two events may explain some of the inhibitory effects of this biocide upon bacterial cells [24].

Use of bacterial antagonists is rarely mentioned. From some reports, it is known that some SRB, such as *Desulfovibrio desulfuricans* [28] and *Desulfovibrio gracillis* [26], are capable of reducing nitrate. Some methods have been proposed and exercised to use nitrate-reducing bacteria (NRB) against SRB. Two examples of these methods are biocompetitive exclusion and bio-augmentation [49]. Little et al. reported successful trials of biocompetitive exclusion as exercised on oil platforms, where corrosion rates were reduced by at least 50% [49]. On the other hand, with respect to bio-augmentation, although researchers such as Hubert et al. [39] have reported failures regarding introduction of bacteria into natural mixed cultures, Zhu et al. [78] reported simultaneous application of nitrate and denitrifying bacteria as "the most effective way" for controlling MIC induced by SRB. However, research in this area remains incomplete. Use of *Bacillus* species is regarded very favorably, for example, in paper products, where they are frequently the dominant microbial contaminant [60] because their heat-resistant spores survive the hot drying process. Mechanisms by which the bacilli are retained in the industrial environment are not known. Some *Bacillus* species are resistant to several industrial biocides [58], and some form biofilms that offer protection against antimicrobial agents [19]. The biosurfactants produced by *Bacillus* genus are lipoproteins or lipopeptides, such as surfactin and subtilisin produced by *Bacillus subtilis* [11]. *Bacillus subtilis* excretes biosurfactant in both liquid- as well as solid-state fermentation systems [45]. The microbial surfactant produced by these organisms also shows properties of a good cleansing agent for dislodging pesticides from used containers, mixing tanks, cargo docks, etc. Attempts have also been made to standardize parameters for biosurfactant production in both liquid- and solid-state fermentations. Strain B21 has been mentioned as producing biosurfactants, although the nature of these biosurfactants has not been elucidated [44].

The strain B21 part of the moderately halophilic bacteria that grow in media containing 3–15% (w/v) salts are widely distributed in hypersaline habitats. These organisms are of considerable interest because of their biotechnological potential for production of compatible solutes and/or hydrolytic enzymes [72]. Moderate halophiles constitute a highly heterogeneous physiological group that includes both Gram-positive and Gram-negative microorganisms. Aerobic, spore-forming, moderately halophilic, and Gram-positive rods are also taxonomically diverse and have been isolated from saline environments such as soils and aquatic habitats [4]. The introduction of molecular methods, especially the use of 16S rRNA gene sequencing, has had a major impact on *Bacillus* taxonomy and has resulted in a splitting up of the genus. Based on their activity against SRB in suspension cultures, the biological inhibitor strain B21 tested in this study seems to be more potent at comparable (inocula) concentrations than commercially available antibiotics (e.g., kanamycin, nalidixic acid, tetracycline) and about as effective as the tested biocide [3]. This inhibition, which may be due to the production of antimicrobial substances, was neither suspected nor sought. The genus *Bacillus* is known for its production of antibiotics and/or hydrolytic enzymes. Secretion of enzymes and antibiotics may interact synergistically in this process [8]. Thus, Postgate [62] compiled lists of antimicrobials which are inhibitory to various SRB, which include the peptide polymyxin B (that inhibits *Desulfovibrio vulgaris* at 100 mg ml^{-1}).

Moreover, it is also worth mentioning another antagonism class bonded to the substrate. In a natural environment, such as soil and water, which is inhabited by a mixed microbiological population, numerous relations of association and antagonism occur. All organisms inhabiting such a medium are affected, directly or indirectly, by one or more of the other constituent members of this population. These relationships were at first visualized as due primarily to competition for nutrients, as well expressed by Pfeffer [59]. Waksman [74] suggested that this is a problem of food competition. When two organisms are capable of utilizing the same nutrients (in this case, sulfate), but are differently affected by environmental conditions, such as reaction, air supply, and temperature, the one that finds conditions more suitable for its development will grow more rapidly, and in time be able to suppress the other. However, it soon became clear that antagonism among microorganisms embraces phenomena other than mere competition due to exhaustion of nutrients. So, we suggested that there exists a typical constant number of living cells capable of living in a given space. When this concentration is reached, multiplication comes to a standstill without the nutrients being exhausted or toxic substances being produced. The same is believed to hold true when two bacteria live together [74]. If the limiting concentrations of the two organisms are different, the one with a higher M value will repress the other; the weaker species may check the stronger one when present in sufficient excess [74]. It has been suggested that "biological activity" and "competitive capacity" must also be taken into consideration [74]. Antagonism may be either one-sided or two-sided, namely, when only one bacterium represses another, which is not antagonistic to it, or when each organism represses the other [74]. Therefore, the assay

showed promising results for use of strain B21 as an antagonistic agent against SRB responsible for MIC. Strain *Bacillus* sp. was selected for one trait often associated with biocontrol agents and for the probable ability to produce extracellular metabolites active against other microorganisms.

Conclusions

The present paper focuses on the antagonistic potential of a nonpathogenic *Bacillus* sp. strain against SRB consortium. Our study is the first such report from the petroleum industry; generally, the antagonist is used in the agriculture industry. *Bacillus* was chosen as the model target organism, because it is one of the important contaminants of injection water and has significant saline resistance and antagonism. The effects of the antagonism were studied at 37°C in four samples of injection water taken from four different oilfields in southern Algerian. The inhibitor effects of strain B21 were compared with those of the chemical biocide THPS, generally used in the petroleum industry. The biological inhibitor showed much better and effective performance.

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