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Isolation and characterization of a mesophilic heavy-metals-tolerant sulfate-reducing bacterium *Desulfomicrobium* sp. from an enrichment culture using phosphogypsum as a sulfate source

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Abstract

A sulfate-reducing bacterium, was isolated from a 6 month trained enrichment culture in an anaerobic media containing phosphogypsum as a sulfate source, and, designated strain SA2. Cells of strain SA2 were rod-shaped, did not form spores and stained Gram-negative. Phylogenetic analysis of the 16S rRNA gene sequence of the isolate revealed that it was related to members of the genus *Desulfomicrobium* (average sequence similarity of 98%) with *Desulfomicrobium baculatum* being the most closely related (sequence similarity of 99%). Strain SA2 used thiosulfate, sulfate, sulfite and elemental sulfur as electron acceptors and produced sulfide. Strain SA2 reduced sulfate contained in 1–20 g/L phosphogypsum to sulfide with reduction of sulfate contained in 2 g/L phosphogypsum being the optimum concentration. Strain SA2 grew with metalloid, halogenated and non-metal ions present in phosphogypsum and with added high concentrations of heavy metals (125 ppm Zn and 100 ppm Ni, W, Li and Al). The relative order for the inhibitory metal concentrations, based on the IC_{50} values, was Cu, Te > Cd > Fe, Co, Mn > F, Se > Ni, Al, Li > Zn.

Keywords: Sulfate reduction; Sulfate-reducing bacteria; Phosphogypsum; Heavy-metal tolerance; *Desulfomicrobium*

1. Introduction

The presence of heavy metals in the environment represents a serious threat to the environment and human life. Heavy metals are generally released in the environment as a consequence of anthropogenic activities such as smelting, land disposal of metal-contaminated municipal and industrial solid wastes and wastewaters, and use of pesticides containing metals and metalloids. Biological treatment of metal-containing wastewaters with sulfate-reducing bacteria (SRB) is an attractive technique for the bioremediation of this kind of media. SRB are dissimilatory anaerobes that require strictly anaerobic conditions and a redox potential of less than 200 mV and characterized by their ability to reduce sulfate with the simultaneous oxidation of the organic substrates [1]. Sulfate reduction leads to production of sulfide, which can readily react with metals and form insoluble metal sulfides [2]. Thus, SRB play an important role in the bioremediation of toxic wastes containing heavy metal ions [3]. The use of SRB in bioremediation processes has been widely reported, for example, bioprecipitation of cadmium [4], copper [5] and selenium [6]; reduction of chromium [7] and uranium [8] and biosorption of aluminum [9].

Selection of the most efficient and the most heavy metal resistant SRB requires their long exposure to toxic wastes containing heavy-metal ions. Phosphogypsum is an industrial by-product formed during the production of phosphate fertilizers. It is produced during the process called the "wet process" of phosphoric acid production when $Ca_3(PO_4)_2$ ore is reacted with H_2SO_4. Phosphogypsum is composed mainly of $CaSO_4$ (calcium sulfate) and contains impurities such as Al, P, F, Si, Fe, Mg as well as many trace elements, including the rare earth elements and naturally occurring radioactive materials [10]. The ability of SRB to grow on phosphogypsum has been reported. Rzeczycka et al. [11] demonstrated that sulfate and other biogenic elements

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present in phosphogypsum were good sources for growth of SRB if organic carbon and nitrogen were supplemented to the culture medium. Karnachuk et al. [12] showed that the rate and quantities of sulfate reduction to sulfide in pure gypsum by pure cultures of non spore-forming SRB was comparable to that produced from soluble sulfate salt (Na_2SO_4). In a previous study, we have demonstrated that phosphogypsum was a good source of sulfate for the cultivation of SRB when the culture medium was supplemented with additional carbon sources [13]. The optimal concentration of phosphogypsum leading to the highest sulfide production was defined as 10 g/L. In the present paper, we describe the isolation and characterization of a sulfate-reducing bacterium *Desulfomicrobium* strain SA2, which reduces phosphogypsum to produce high concentrations of sulfide compared to other alternate electron acceptors. In addition, this isolate shows resistance towards various heavy metals and other elements when copper and telluride were the most toxic.

2. Materials and methods

2.1. Phosphogypsum source and composition

Phosphogypsum was collected from a phosphogypsum stack from the vicinity of Sfax, Tunisia. The major components of the phosphogypsum were SO_4 and CaO at 44.3% (w/w) and 33.6% (w/w), respectively and a high number of heavy metals which included Cd (5–20 ppm), Zn (50–100 ppm), Cu (5–18 ppm), Cr (10–30 ppm), V (<5 ppm), Ni (3–5 ppm), Mn (5–7 ppm) and Ti (50–60 ppm). 1 kg of phosphogypsum was collected from the stockpile, homogenized well by shaking and used in growth experiments [13].

2.2. Media, media preparation and growth conditions

All media used in the expermients were prepared anaerobically using the Hungate technique [14–16]. A sulfate free basal medium contained (per liter of deionised water): yeast extract 1 g, NH_4Cl 1 g, $MgCl_2{\cdot}6H_2O$ 0.2 g, $CaCl_2{\cdot}2H_2O$ 0.1 g, KCl 0.1 g, cysteine HCl 0.25 g, resazurin 1 mg, trace element solution [17] 1.5 mL. The pH of the medium was adjusted to 7.2 with 6N NaOH. The medium was boiled then cooled to room temperature under a stream of oxygen-free nitrogen (OFN) gas and 4.5 mL aliquots were dispensed into 15 mL-Hungate tubes. Finally the tubes were autoclaved at 120 °C for 20 min.

Carbon sources, soluble electron acceptors and heavy metals were injected into the basal medium from sterile, anaerobic stock solutions to give the required final concentration. For the insoluble electron acceptors (phosphogypsum and sulfur), basal medium was dispensed under a stream of OFN gas into Hungate tubes containing pre-weighed phosphogypsum or sulfur (1% (w/v) final volume) and the medium was sterilized as described above. For the isolation and routine cultivation of the pure culture of strain SA2 the basal medium (4.5 mL) described above was supplemented with 0.2 mL of 5% (w/v) $NaHCO_3$, 0.2 mL of 1% (w/v) $Na_2S{\cdot}9H_2O$ and 0.1 mL of vitamin solution [18]. These components were injected into the pre-sterilized medium from sterile stock solutions prior to inoculation.

2.3. Isolation of SRB growing on phosphogypsum

The isolation of pure cultures of SRB was performed using the enrichment culture, which had been previously exposed to phosphogypsum for about 6 months [13]. Phosphogypsum (10 g/L) was used as the sulfate source for SRB cultivation. Lactate, formate or pyruvate (10 mM final concentration) was added to the basal medium containing 1% phosphogypsum for the isolation of heterotrophic SRB and for the isolation of autotrophic SRB, the OFN gas phase was replaced with H_2/CO_2 (80/20 (v/v)). All inoculated media were further buffered by injecting 0.2 mL of 5% (w/v) $NaHCO_3$, 0.1 mL of vitamin solution and rendered anaerobic by injecting 0.2 mL of 1% (w/v) $Na_2S{\cdot}9H_2O$. All incubations were at 37 °C for up to 10 days during which time microscopic examination was carried out and sulfide production was measured. Culture tubes that were turbid and had the highest rate of sulfide production were therefore maintained and selected for isolation of pure cultures by using the roll tube method [14]. For this, serial dilutions of the culture was inoculated into basal medium that had been fortified with agar (1.6% (w/v)), KH_2PO_4 0.3 g/L, K_2HPO_4 0.3 g/L and Na_2SO_4, 3 g/L (instead of phosphogypsum) and roll-tubes were made by purging the tubes with H_2/CO_2 (80/20 (v/v)). Single well-isolated colonies that developed were picked and the procedure repeated three times before the cultures were deemed to be pure. Purity of the cultures was checked frequently by microscopic examination and by an absence of growth in anaerobic basal medium containing glucose 5 g/L but lacking sulfate or phosphogypsum.

2.4. Growth studies

Unless indicated, all growth studies were performed in Hungate tubes containing the basal medium described above with 20 mM sodium lactate and 20 mM Na_2SO_4. All experiments were performed in duplicate with an inoculum size of 10% (v/v), which had been subcultured at least once under the same test condition. Unless indicated, all incubations were conducted at 37 °C for up to 5 days during which time the growth of the cultures was examined microscopically, visually for turbidity and by spectrophotometer for sulfide production.

The temperature range for growth, pH studies and sodium chloride requirement of strain SA2 were determined as described by Mechichi et al. [19]. Electron acceptors were injected from freshly prepared sterilized stock solutions to give the final desired concentrations: 10 mM (sulfate, thiosulfate and sulfite) and 5 mM (nitrate, nitrite, and fumarate). Phosphogypsum (2 g/L) and elemental sulfur (10 mM) were weighed directly into Hungate tubes, the basal medium dispensed and the medium autoclaved. The optimum concentration of phosphogypsum that could be utilized was tested at concentrations ranging from 1 to 30 g/L in Hungate tubes containing 20 mM lactate as the carbon source.

2.5. Heavy-metal tolerance study

To study the influence of heavy metal ions on the bacterial growth, pure cultures of strain SA2 were exposed to different

concentrations of each of the following ions: Zn(II), Cu(II), Fe(III), Cd(II), Ni(II), W(II), Mn(II), Co(II), Li(I) and Al(III). F(I) a halogen, Se(II) and Te(II) metalloids were also tested in this study because of their occurrence in phosphogypsum. The metal salts used for the study includes $ZnSO_4{\cdot}7H_2O$ (Merck), $CoSO_4{\cdot}7H_2O$ (Merck), $MnSO_4{\cdot}H_2O$ (Merck), $CuCl_2{\cdot}2H_2O$ (Merck), LiCl (Merck), $KAl(SO_4)_2{\cdot}12H_2O$ (Prolabo), NaF (Merck), $CdCl_2{\cdot}H_2O$ (Merck), $Na_2WO_4{\cdot}2H_2O$ (Sigma), $FeCl_3{\cdot}12H_2O$ (Sigma), Na_2SeO_4 (Sigma) and K_2TeO_3 (Merck). Unless indicated, 20 mM sodium lactate and 20 mM Na_2SO_4 was added to the basal medium described in Section 2.2 was used throughout these experiments. Samples were collected at regular intervals during incubation and used to measure bacterial growth and calculate maximal growth rates. The heavy-metal tolerance of strain SA2 was evaluated by using two parameters: (a) IC_{50} (50% inhibitory concentration) which is defined as the metal concentration which causes a 50% decrease in maximal growth rate and (b) MTC (maximum tolerated concentration), which is defined as the maximum metal concentration above which no bacterial growth is observed.

2.6. Identification

Genomic DNA was prepared using modified method [20], which achromopeptidase was added at a final concentration of 1 mg/mL for cell lysis and RNAase was added at a final concentration of 20 μg/mL to digest RNA [21]. The methods used for 16S rRNA gene amplification and sequencing have been reported previously [22]. Partial sequences generated in this investigation were assembled and the consensus sequence corrected manually for errors using BioEdit v5.0.1 [23]. The most closely related sequences against GenBank and Ribosomal Database Project II were identified using BLAST [24] and Sequence Match program [25], extracted, aligned and manually adjusted according to the 16S rRNA secondary structure using BioEdit. Sequence uncertainties were omitted and phylogenetic reconstruction achieved using TreeCon [26] in which pairwise evolutionary distances were computed from percentage similarities [27] and phylogenetic trees constructed from the evolutionary distances using the Neighbour-Joining method [28]. Tree topology was re-examined by bootstrap method of re-sampling [29] using 1000 bootstraps. Strain SA2 has been deposited in the Deutche Sammlung von Mikroorganismen und Zellkulturen as strain DSM 16234 and the 16S rRNA gene sequence has been deposited in GenBank under accession number AY548759. DNA was isolated, purified and the G + C content determined by using HPLC, as described by Mesbah et al. [30].

2.7. Analytical methods

A phase contrast microscope OLYMPUS BX50 equipped with OLYMPUS DP 70 digital camera was used to determine the morphology and arrangement of cells and photomicrographs were taken by using wet mounts of exponential-phase cultures on agar-coated slides [31]. Cells were washed gently with sterile bi-distilled water to remove sulfide prior to Gram staining. Bacterial growth was assessed by measuring the optical densities in a Shimadzu UV-110-01 spectrophotometer at 600 nm. Sulfide was determined using the method of Cord-Ruwisch [32].

3. Results

3.1. Isolation of strain SA2

All SRB cultures initiated in a basal medium containing 1% (w/v) phosphogypsum as the sulfate source and lactate, formate, pyruvate (10 mM final concentration) or H_2/CO_2 (80/20 (v/v)), as the carbon source became turbid with black precipitates within 2–3 days of incubation at 37 °C. Bacterial reduction of phosphogypsum was confirmed in these cultures by the presence of high concentrations of sulfide. The enrichment tubes with H_2/CO_2 gas phase grew the fastest and produced the highest concentration of sulfide. They showed the presence of bacteria with diverse cellular morphologies and were used for the isolation of pure cultures of SRB using the roll tube technique. Several single, well-isolated brownish and circular colonies, 0.5 mm in diameter appeared after one week of incubation at 37 °C, were picked and a pure culture designated SA2 was selected for further characterization.

3.2. Characterization of strain SA2

Cells of strain SA2 stained Gram-negative, were rod-shaped cells (4–5 μm × 1–1.2 μm) with rounded ends and occurred singly or in short chain (Fig. 1). Spores were not detected. Autotrophic growth was monitored by comparison of bacterial density in tubes containing yeast extract with H_2/CO_2 (80/20 (v/v)) as the gas phase and tubes containing yeast extract with OFN the gas phase. Results showed that SA2 growth was significantly higher with H_2/CO_2 as the gas phase (data not shown). Strain SA2 used formate (10 mM), lactate (10 mM), pyruvate (10 mM), ethanol (10 mM), butanol (10 mM) and vanillin (5 mM). Strain SA2 used sulfate, thiosulfate, sulfite, elemental sulfur and phosphogypsum but not nitrate, nitrite or fumarate as electron acceptors. Strain SA2 did not grow in oxidized medium (oxidation was indicated by the pink color of resazurin in the growth medium) and was deemed to a strict anaerobe. It was

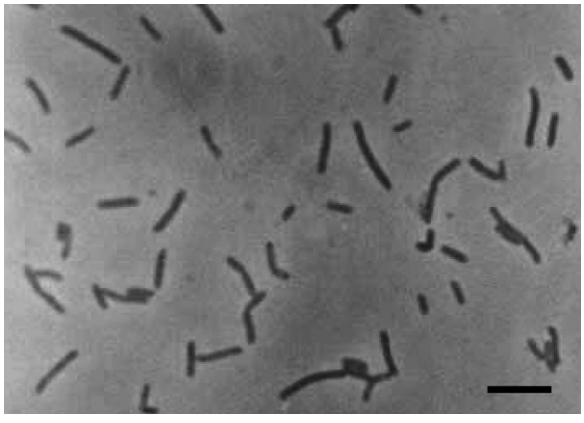

Fig. 1. Phase-contrast photomicrograph of cells of strain SA2 in the exponential growth phase. Bar, 10 μm.

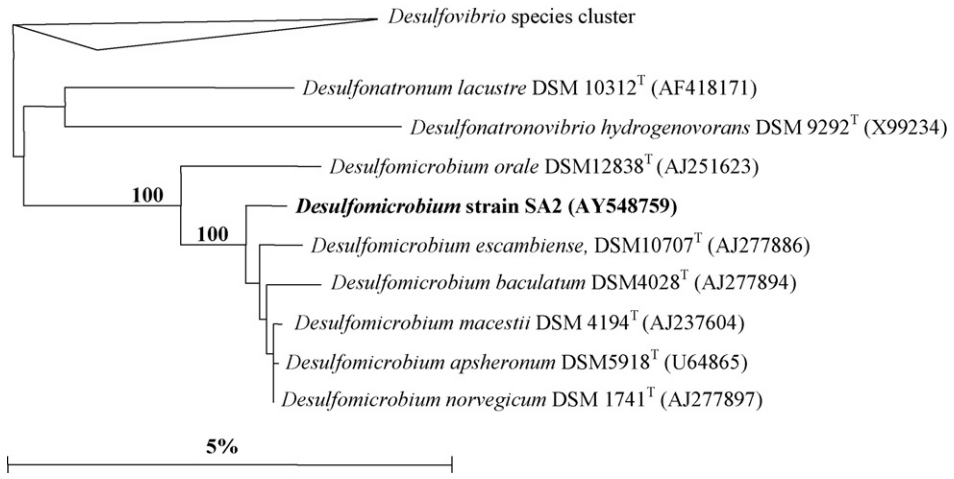

Fig. 2. The position of *Desulfomicrobium* strain SA2 amongst the members of the sulfate reducing bacteria in the delta subdivision of the *Proteobacteria*. The dendrogram was drawn based on 1254 unambiguous nucleotides of the 16S rRNA sequences extracted from GenBank The sequences represented by *Desulfovibrio africanus* DSM2603^T (X99236), *Desulfovibrio mexicanus* DSM13116^T (AF227984), *Desulfovibrio halophilus* DSM5663^T (X99237), *Desulfovibrio senezii* DSM8436^T (AF050100), *Desulfovibrio alcoholovorans* DSM5433^T (AF053751) and *Desulfovibrio longreachensis* ACM 395^T (Z24450) are shown as a triangle. Accession numbers are shown in brackets. The scale bar represents 5 nucleotide substitutions per 100 nucleotides.

mesophile with the optimal temperature for growth at 37 °C and a growth temperature range between 30 and 47 °C. The optimum pH for growth was 7 and growth occurred between pH 6 and 9. The isolate grew in the presence of NaCl added at concentrations ranging from 0 to 30 g/L, with optimum growth occurring in the absence of NaCl. The G + C content of strain SA2 was 60.4 mol% as determined by the HPLC method. Phylogenetic analysis of the 16S rRNA gene sequence of the isolate revealed close similarity to the members of the genus *Desulfomicrobium* with *Desulfomicrobium macestii*, *Desulfomicrobium norvegicum* and *Desulfomicrobium apsheronum*, being almost equidistantly placed (99% similarity), *Desulfomicrobium baculatum* and *Desulfomicrobium escambiense* (97% similarity) and *Desulfomicrobium orale* (94% similarity) and hence it will be tentatively referred to as *Desulfomicrobium* sp. strain SA2 (Fig. 2).

3.3. Phosphogypsum utilization

Fig. 3, reports the effect of phosphogypsum concentration on both growth rate (a) and sulfide production (b) by the strain SA2. This bacterium grew when phosphogypsum concentrations were less than 20 g/L. The best bacterial growth rate (0.065 h^{-1}) was obtained for 2 g/L of phosphogypsum over which growth rate decreased as the phosphogypsum concentration increased. Sulfide measurement confirmed that growth of the isolate was possible for all the cultures grown in media containing phosphogypsum concentrations up to 20 g/L with 2 g/L being the optimal concentration from which 8 mM of sulfide was formed after 5-day incubation. The ability of strain SA2 to reduce the sulfate entity contained into phosphogypsum in comparison with the other electron acceptors, which are sodium sulfate, sodium thiosulfate, sodium sulfite and elemental sulfur was studied. Results showed that sulfide production from phosphogypsum, started slowly compared to the soluble sulfate and thiosulfate but reached almost similar sulfide production rate obtained with sodium sulfate 57 h later (Fig. 4). In the other hand, among the electron acceptors tested, the rate of sulfide formation from elemental sulfur was the slowest.

3.4. Heavy-metal tolerance

The tolerance of strain SA2 to 10 heavy metal ions [Zn(II), Cu(II), Fe(III), Cd(II), Ni(II), W, Mn(II), Co(II), Li(II) and

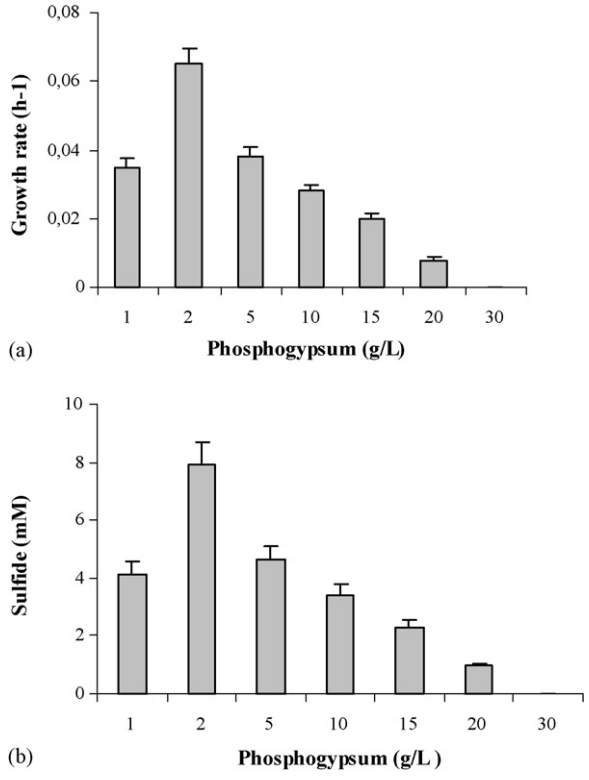

Fig. 3. Growth rate (a) and sulfide formation (b) by strain SA2 with different concentrations of phosphogypsum after 5-day incubation.

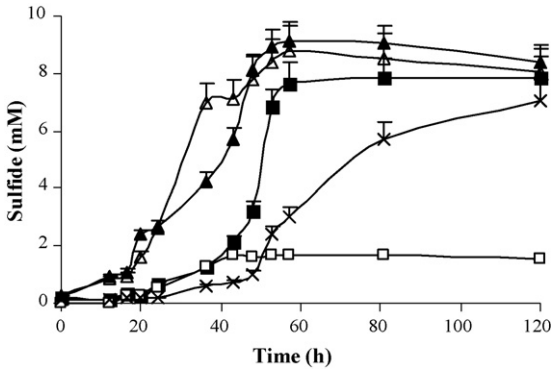

Fig. 4. Sulfide formation by strain SA2 with different sulfur compounds used as electron acceptors. (■): 2 g/L phosphogypsum; (▲): 10 mM sulfate; (△): 10 mM thiosulfate; (×): 10 mM sulfite; (□): 10 mM sulfur.

Al(III)], the halogen F(I) and the metalloids Te(II) and Se(II) were studied. The tolerance study for the isolate SA2 was carried out by performing several series of experiments with different concentrations of each chemical element in tandem with a control culture. Samples were withdrawn at regular intervals during incubation and bacterial growth measured. Concentrations of the different heavy-metals tested ranged from 0 to 100 ppm. The results obtained from the experiments allowed two parameters to be calculated and these were used in the interpretation of the results: inhibitory concentration 50% (IC_{50}), defined as the concentration required for 50% inhibition of bacterial growth and maximum tolerated concentration (MTC), defined as the maximum concentration at which bacterial growth is observed. The values of the two parameters for each ion studied are shown in Table 1. The relative order for inhibitory metal and other elements concentration, based on the IC_{50} values, was Cu, Te > Cd > Fe, Co, Mn > F, Se > Ni, Al, Li > Zn. For Zn, bacterial growth was observed at initial zinc concentration of 100 ppm. Consequently, higher concentrations of zinc (120, 150 and 200 ppm) were tested in order to determinate the MTC value. In general, it was found that bacterial growth is affected by the presence of the metal ions studied and that the lag phase (phase during which cell number remains relatively constant prior to rapid growth) of these cultures increases as the metal concentration increases.

Table 1
IC_{50} and MTC for strain SA2 in the presence of heavy metals studied

Heavy metal	IC_{50} (ppm)	MTC (ppm)
Zn	71	125
Cu	5	10
Fe	41	60
Cd	24	40
F	49	80
Ni	54	100
Se	47	80
W	51	100
Mn	31	60
Te	5	10
Co	32	60
Li	67	100
Al	65	100

4. Discussion

Great attention has been paid to SRB due to their ability to remove heavy metals through sulfides. Selection of the most efficient and the most heavy metal resistant SRB requires their long exposure to toxic wastes containing heavy-metal ions. Phosphogypsum, a residue of the phosphoric acid produced from apatite, seems to be an alternative possibility for enrichment of heavy metal resistant SRB. Phosphogypsum, a waste by-product from phosphoric acid production, accumulates in large stockpiles and occupies vast areas of land in several phosphate producing nations such as USA, Morocco and Tunisia. Contaminants emanating from phosphogypsum stacks can impact the environment including water-bodies. The major constraint for phosphogypsum environmental bioremediation *in situ* is the presence of high concentrations of metals. Phosphogypsum has already been tested as an electron acceptor for SRB cultivation [11,13,33] but this is the first report on the isolation of a pure culture of SRB, which uses phosphogypsum as an electron acceptor. The strain is interesting as it not only produces high concentrations of sulfide from sulfate contained in phosphogypsum but it also tolerates the relatively high concentrations of heavy metals present in phosphogypsum. Initial reduction of sulfate present in phosphogypsum to sulfide starts slowly in comparison to the soluble electron acceptor, sodium sulfate, but the rates improve in late growth phase and almost same sulfide concentrations than their soluble counterparts are produced. The discrepancy could be due to the presence of metal ions in phosphogypsum. Indeed, Sani et al. [34] have reported that metal ions extend the lag-period when SRB grow in batch culture.

When compared to a previous study [13], in which sulfate contained in up to 40 g/L of phosphogypsum was reduced to sulfide by a mixed SRB consortium, our present report shows that sulfate in only up to 20 g/L phophogypsum is reduced to sulfide by strain SA2, an isolate that was derived from the SRB consortium.

The long exposure of SRB to phosphogypsum for around 6 months of incubation at 37 °C allowed the selection of heavy-metal tolerant SRB from which strain SA2 was isolated in pure culture. The relative order for inhibitory metal and other chemical elements tested concentration, based on the IC_{50} values, was Cu, Te > Cd > Fe, Co, Mn > F, Se > Ni, Al, Li > Zn. This order was in agreement with those found by Cabrera et al. [35]. They reported that Cu was the most toxic where Zn was the less toxic heavy metal for both cultures of *Desulfovibrio vulgaris* and *Desulfovibrio* sp. The long exposure of strain SA2 to phosphogypsum could result in its tolerance to various heavy-metals and other chemical elements that occur naturally in phosphogypsum. Alternately, the concentration of the dissolved metal is much lower than the actual expected concentration because the metal ions could have reacted with the sulfide produced during growth, and precipitated reducing consequently the toxicity of the heavy metal. Strain SA2

revealed close similarity, with respect to the 16S rRNA gene sequence, to the members of the genus *Desulfomicrobium* with *D. macestii*, *D. norvegicum* and *D. apsheronum*, being almost equidistantly placed (99% similarity). Strain SA2 differs from *D. macestii* in that it does not ferment fumarate or oxidize propanol, from the type strain of *D. norvegicum* by chemoautotrophic growth on H_2 plus CO_2 and from *D. apsheronum* in that it does not oxidize fumarate or malate or ferment fumarate. However, placing strain SA2 in a new species *Desulfomicrobium* basing only on the phenotypic traits cannot be judicious. DNA–DNA similarities and restriction fragment length analysis should be realized to justify phylogenetic affiliation of strain SA2.

D. norvegicum showed the presence of genetic determinants for metal resistance [36]. Thus, taking into account phylogenetic relatedness between *D. norvegicum* and strain SA2, this latter could also present genetic determinants for metal resistance encoding bacterial metallothioneins and heavy metal-transporting ATPases. Strain SA2 grows autothrophically with H_2/CO_2 (80/20 (v/v)) as an electron donor and sulfate as electron acceptor. Strain SA2 can also grow with formate, lactate, pyruvate, ethanol and vanillic acid to produce sulfide and acetate as a metabolic end product. Strain SA2 did not use carbohydrate nor did it use amino acids.

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