Thiosulphate oxidation by *Thiobacillus thioparus* and *Halothiobacillus neapolitanus* strains isolated from the petrochemical industry

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Abstract Sulphur Oxidizing Bacteria (SOB) is a group of microorganisms widely used for the biofiltration of Total Reduced Sulphur compounds (TRS). TRS are bad smelling compounds with neurotoxic activity which are produced by different industries (cellulose, petrochemical). *Thiobacillus thioparus* has the capability to oxidize organic TRS, and strains of this bacterium are commonly used for TRS biofiltration technology. In this study, two thiosulphate oxidizing strains were isolated from a petrochemical plant (ENAP BioBio, Chile). They were subjected to molecular analysis by real time PCR using specific primers for *T. thioparus*. rDNA16S were sequenced using universal primers and their corresponding thiosulphate activities were compared with the reference strain *T. thioparus* ATCC 10801 in batch standard conditions. Real time PCR and 16S rDNA sequencing showed that one of the isolated strains belonged to the *Thiobacillus* branch. This strain degrades thiosulphate with a similar activity profile to that shown by the ATCC 10801 strain, but with less growth, making it useful in biofiltration.

Keywords: biofiltration, dimethyldisulphide, dimethylsulphide, *Thiobacillus*, thiosulphate

INTRODUCTION

Petrochemical industries, cellulose factories, slaughterhouses, food industries, liquid effluents and solid waste treatment plants worldwide produce Total Reduced Sulphur compounds (TRS), such as hydrogen sulphide (H_2S), dimethylsulphide (DMS), dimethyldisulphide (DMDS) and methyl mercapthane (MM). These compounds have toxic effects in humans (Truong et al. 2006) at concentrations over 320 (ppmv), a bad smell at very low concentrations (Bordado and Gomes, 2001; Barbosa et al. 2006; Aroca et al. 2007), produce concrete corrosion (Okabe et al. 2007) and play an important role in the global warming, acid precipitation, and the sulfur cycle (Lomans et al. 2002). Emissions containing TRS can be treated by scrubbing or adsorption, but this process is technically and economically not practical in the majority of cases, where it is necessary to treat the high gas flows and low contaminant concentrations characteristic of industrial TRS emissions.

The treatment of a gaseous current by biofiltration technology has been developed as a solution. In this process the gaseous emissions pass through a solid matrix containing biofilm (a microorganism complex, marked by the excretion of an adhesive matrix or protective exopolysaccharide) which is able to degrade the contaminants in the gas and has been shown to be a practical solution for high gas flows and low contaminant concentrations (Oyarzún et al. 2003). TRS oxidation has been reported in different microorganisms and consortia, in particular Sulphur Oxidizing Bacteria (SOB). SOB form a diverse group of bacteria, with representatives in the alpha-, beta- and gamma-*proteobacteria* classes. These microorganisms, mainly mesophylls (Kelly and Wood, 2000; Friedrich et al. 2001) and neutrophylls, use reduced sulphur compounds and other chemical species like thiosulphate as electron donors, and oxygen as an electron acceptor (Maestre et al. 2010). Different carbon sources are utilized

within the group. SOB species that are able to use CO_2 as their sole carbon source (chemolithotrophs) are best, because they do not require feeding with organic compounds, making them economically attractive to maintain. New SOB strains need to be researched and tested in order to design improved biofilms and more efficient biofiltration systems.

In this study, we made a comparison between the thiosulphate oxidation capacity of two SOB isolated from a petrochemical industry at Concepcion, Chile, and the *Thiobacillus thioparus* ATCC 10801 strain. We analyzed their activity against temperature profiles and evaluated their potential for use in TRS biofiltration technology.

MATERIALS AND METHODS

Samples

Samples were taken from ENAP BIOBIO Petroleum refinery (Concepcion, Chile). Sludge (10 g wet weight, taken using a sterile steel spoon), and water (30 ml, taken using a sterile plastic 50 ml syringe) were taken from an API separator (American Petroleum Institute design) and transported to the laboratory in sterile hermetic flasks (4°C, in darkness).

Standard bacterial strains

Thiobacillus thioparus ATCC 10801 was used as standard positive control in thiosulphate degradation and for molecular characterization experiments.
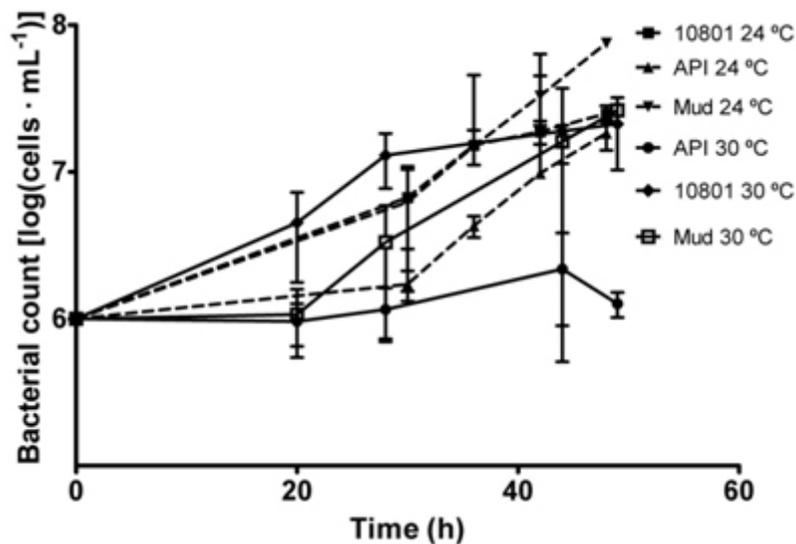

Fig. 1 Growth curves of strains isolated in this study and the *T. thioparus* ATCC 10801 strain at 24ºC and 30ºC. 10801, ATCC 10801 strain; API, water separator strain; MUD, sludge strain.

Isolation

Five ml of each sample was cultured in hermetically closed 250 ml glass flasks, made up to 100 ml with culture medium. The flasks were incubated on a rotary shaker at 180 rpm at 24°C for 4 days. Prior to this, 20 ml inoculum was taken and placed in hermetically closed 500 ml flasks, made up to 200 ml with medium for repeating the same enrichment procedure again. Solid cultures were developed by transferring small aliquots to solid plates (1.2% agar), in a sterile chamber, using the same 290 culture medium with 15 g l^{-1} agar. A sample of the *T. thioparus* ATCC 10801 strain was cultured as described

above as a control. Incubation was done for at least 7 days at 24ºC including a negative control (no TRS oxidative strain). The strains obtained (single colonies) were enriched in hermetically closed 250 ml flasks, made up to 100 ml with 290 liquid culture medium.

Culture medium and incubation conditions

Cultures (Strain isolates and controls) were performed in 500 ml Erlenmeyer flasks with hermetic stopper, using ATCC 290 culture medium (according to the instructions provided), and incubated on a shaker (180 rpm) at 25ºC or 30ºC. The cultures were transferred to new flasks with fresh medium, three times, to avoid carry-over.

Growth and thiosulphate concentration curves

Strains were enriched in volumes of 20 ml before being transferred to hermetically closed 250 ml flasks, with 100 ml of liquid culture volume, ensuring a final bacterial concentration of 10^6 bacteria ml^{-1}. Cultures were incubated at 24ºC and 30ºC on an orbital shaker at 220 rpm. Periodically, 1 ml of each culture was taken for microbial growth estimation by spectrophotometry at 605 nm, and 3 ml was taken for thiosulphate determination by iodometric titration by excess of iodine (Kiryushov and Skvortsova, 2005).

DNA extraction

A small number of pellets were collected from 1.6 ml by centrifuging in Eppendorf tubes at 8,000 rpm for 3 min. The supernatant was eliminated and each pellet was re-suspended in 310 µl HTE buffer (HTE: 50 mM Tris-HCl, 20 mM EDTA, pH = 8). 350 µl 2% SDS in HTE buffer was added and mixed by inversion 7 times. 5 µl Rnase A was added and the mix was incubated at 37 ºC for 15 min. The mix was incubated with 35 µl Proteinase K at 50 ºC for 1 hr and then shaken for 2 min in vortex. Under extractor hood, 700 µl phenol:chloroform:isoamilic alcohol was added, mixed briefly in vortex and then centrifuged at 11,000 rpm for 3 min. The aqueous phase was taken (superior) and transferred into new tubes. The phenol:chloroform:isoamilic alcohol step was done twice. 3 M sodium acetate at 1/10 of the final volume was added and mixed. The mix was refrigerated at -20ºC overnight. Each tube was then centrifuged at 11,000 rpm for 10 min. The supernatant was eliminated, and the DNA pellet was washed with 70% ethanol stored previously at -20ºC. Finally, without mixing, the final extract was centrifuged at 10,000 rpm for 10 min, the supernatant was eliminated and the washed DNA pellet was re-suspended with 30-50 µl distilled water. All water used in the extraction procedure was treated with Diethyl Pyrocarbonate (DEPC) for nuclease inhibition.

DGGE characterization

DNA extraction of isolated colonies was done as described above, from plates inoculated with the control strain (*T. thioparus* ATCC 10801), API and MUD. The isolated DNA was amplified by traditional Polymerase chain reaction (PCR) using the general 16S rDNA primers:

341F-GC: 5'CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG-CCTACGGGAGGCAGCAG 3' (Muyzer et al. 1993) and 907F: 5' CCGTCAATTCCTTTRAGTTT3' (Muyzer et al. 1995). The amplified fragment was separated and analysed by DGGE with 25% and 65% denaturation (Biorad Dcode Universal Mutation Detection System). The DGGE was stained using ethidium bromide and photographed with digital camera (Kodak DC290).

Table 1. Melting temperatures of real time PCR for DNA extracted from strains isolated in this study and the *T. thioparus* standard strain at 24ºC, using *T. thioparus* specific primers. 10801) *T. thioparus* ATCC 10801 strain; API) API water separator strain; MUD) Sludge strain.

Strain	Tm (ºC)
API	85.98
MUD	84.95
10801	85.59

Real time PCR melting curves

The DNA was quantified by ND-1000 (Nanodrop) spectrophotometer. The corresponding dilution of each DNA sample (extracted from cultures at 25ºC) was amplified in a Lyght Cycler 2.0 (Roche) thermocycler using the *T. thioparus* specific primers TT-F:5' CCAGCAGCCGCGAATACGTA3' and TT-R: 5'TCGCCATTGGTGTTCCCTCC3' (Sercu et al. 2006). Melting curves were developed (not shown) and melting temperatures T_m were displayed.

DNA sequencing and phylogenetic analysis

The DNA fragments obtained by DGGE (Vissers et al. 2009; Wang et al. 2009) was cut and re-suspended in 50 µl of DEPC treated water and amplified again with primers 341F: 5' CCTACGGGAGGCAGCAG 3' (Muyzer et al. 1993) and 907F: 5' CCGTCAATTCCTTTRAGTTT3' (Muyzer et al. 1995). The fragments obtained were sequenced in Macrogen, Inc. sequencing service, Korea, for DNA. A BLAST search was done relating the API and MUD sequences as the query sequence (separately), using Megablast on the non-redundant database. The parameters for DNA sequence search were set as follows: match/mismatch scores were set to 1/-3, gap penalty opening/extension was set to linear (default), expected threshold t was set to 10, and word size k was set to 20. There was no need to relax the E value, since the BLAST output resulted in above 95% identity for at least 20 sequences. Sequences whose identities with the query sequence were above 95% were selected, and subjected to multiple alignment using ClustalW (Mount, 2004). The default match/mismatch scoring system was used. The affine gap penalization scheme was used with parameters 15/6.66, while the delay divergent cut-off was set to 50 (default) (Jopia et al. 2010). The evolutionary history was inferred using the neighbour-joining method, which was validated by utilizing the bootstrap consensus tree inferred from 500 replications (Jopia et al. 2010) and displaying a scale of 0.01 nucleotide substitution per sequence position. The evolutionary distances were computed using the maximum composite likelihood method (Tamura et al. 2004). All positions containing gaps and missing data were eliminated from the dataset (by means of the "complete deletion" option). Phylogenetic analyses were conducted using the MEGA4 software (Tamura et al. 2007). The phylogenetic tree included 9 beta-proteobacteria sequences, *Halothiobacillus neapolitanus* (gamma-proteobacteria), and was rooted with gamma-proteobacteria *Acidithiobacillus ferroxidans*.

The API and MUD sequences were classified using the classifier tool from Ribosomal database Project II (RDP II), set at 95% confidence threshold. The complete sequence of the *T. thioparus* 16S ribosomal RNA gene used for sequence analysis is in GenBank under accession number AF005628.
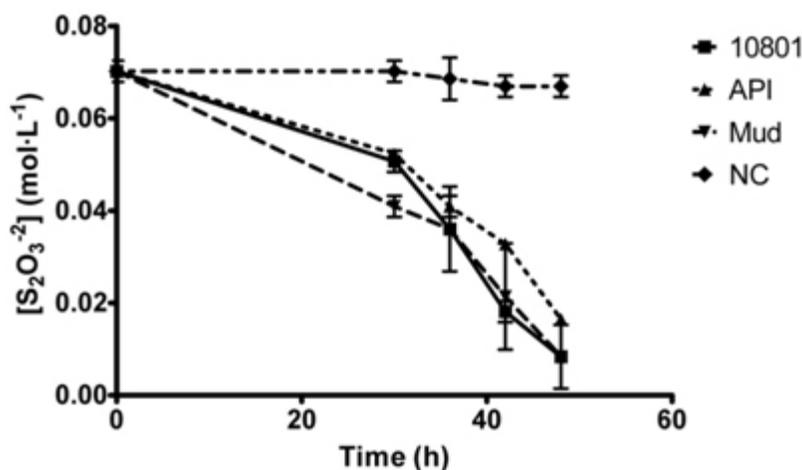

Fig. 2 Thiosulphate consumption (mol L^{-1}) vs Time (hrs) of strains isolated in this study and the *T. thioparus* ATCC 10801 strain at 24ºC. 10801, ATCC 10801 strain; API, water separator strain; MUD, sludge strain; NC, Negative Control (non inoculated). [$S_2O_3^{-2}$]: Thiosulphate concentration.

RESULTS

Isolation

One type of colony was obtained for each type of sample. The strains were called MUD (from the ENAP BIOBIO sludge sample) and API (from the ENAP BIOBIO API separator water sample). The colonies were similar to ATCC 10801 colonies in form and appearance: very small (1 mm aprox.), white in colour and circular. All bacterial strains in this study were Gram negative rod-shaped cells. The liquid cultures of API, MUD, and the standard strain developed a thin visible film on the surface of the medium. At the same time, the medium in each case itself became cloudy white after 48 hrs. In electronic microscopy of the API and MUD strains it appears as a rod-shaped cell, approximately 1 x 2 µm.
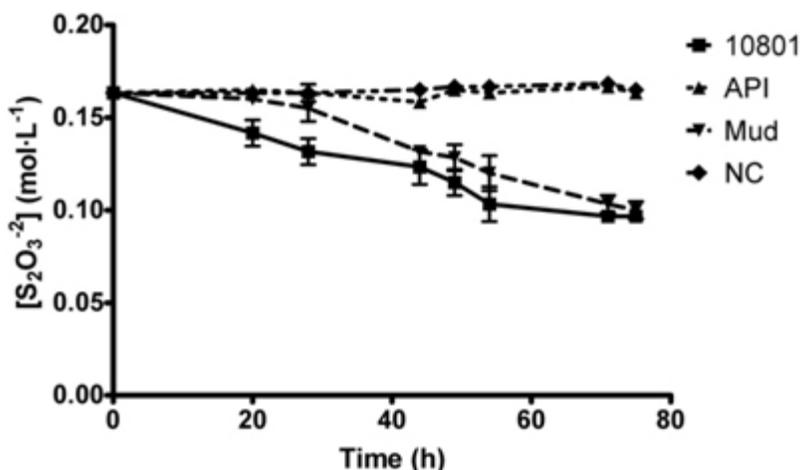

Fig. 3 Thiosulphate consumption (mol L^{-1}) vs Time (hrs) of strains isolated in this study and the *T. thioparus* ATCC 10801 strain at 30ºC. 10801, ATCC 10801 strain; API, water separator strain; MUD, sludge strain; NC, Negative Control (non inoculated). [S$_2$O$_3$$^{-2}$]: Thiosulphate concentration.

Growth curve

The bacterial growth curves at 24ºC and 30ºC are shown in Figure 1. At 24ºC, there are significant differences in the growth rate between the API and MUD strains, (API presents a lower growth rate until 36 hrs of incubation). At 30ºC, the API strain did not grow, while the MUD strain does not show significant differences from the growth rate of the ATCC strain. The specific growth speed at 24ºC for *T. Thioparus* ATCC 10801 strain, API strain and MUD strain are shown in Figure 4.

Thiosulphate concentration vs time

Thiosulphate concentration values vs time at 24ºC and 30ºC are shown in Figure 2 and Figure 3 respectively. There were no significant differences between the three strains for thiosulphate concentrations over time.

Real time PCR, DGGE and sequencing

The melting temperatures (T$_m$) obtained by the melting curve of real time PCR with *T. thioparus* specific primers and the DGGE done with the 341F-GC and 907R primers are displayed in Table 1 and Figure 5 respectively. The T$_m$ for API and MUD strains showed differences of 0.39ºC and 0.64ºC with respect to the melting point for the ATCC 10801 strain. In DGGE only the MUD strain 16S rDNA has a different denaturation point to the *T. thioparus* strain. This is consistent with BLAST identity searches made with the sequences of 16S rDNA obtained from DGGE fragments, where the API strain has 98%

identity with *T. thioparus*, while the MUD strain has a 100% identity with *Halothiobacillus neapolitanus*. On the other hand, the strain roots provided by the RDP classifier tool (Table 2) show that API belongs to the genus *Thiobacillus,* while MUD belongs to the genus *Halothiobacillus*.
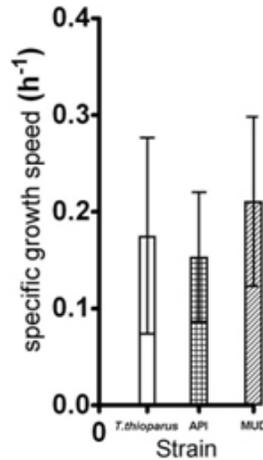

Fig. 4 Specific growth speed at 24ºC, reached by: *T. thioparus*, ATCC 10801 strain; API, water separator strain; MUD, sludge strain.

A phylogenetic tree (Figure 6) shows that the API strain sequence fits with the *T. thioparus* branch and the MUD strain (sludge strain) sequence alignment fits better with *Halothiobacillus neapolitanus.* The sequences obtained for the API and MUD strains are indexed in GenBank under the accession numbers EU591536 and EU591537 respectively.

DISCUSSION

The isolation experiments gave unique strains by sample, and between the two strains isolated; the API strain was more similar to *T. thioparus* than the MUD strain. In both API and MUD, as well as in *T. thioparus* ATCC 10801 cultures, a thin visible film formed on the culture medium surface and the medium itself became cloudy white after 2 days. This film and the size of the bacteria in this study are in agreement with the data previously reported for *T. thioparus* LV43, but the film developed after a period of 5 days (Vlasceanu et al. 1997).

Fig. 5 Picture of Denaturing Gradient Gel Electrophoresis (DGGE). 1, API strain; 2, MUD strain; 3, *T thioparus* ATCC 10801 strain*.*

The growth experiments (Figure 1, Figure 2 and Figure 3) showed that the cell concentration reached by the API strain after 36 hrs was smaller than that of the *T. thioparus* ATCC 10801 strain (1.52×10^7 bacteria ml^{-1} for *T. thioparus* ATCC 10801 strain and 4.29×10^6 bacteria ml^{-1} for API). API effected thiosulphate oxidation at a similar rate to that achieved by the ATCC strain, which would help to reduce the accumulation of sludge in the biofilter due to excessive biomass. This is good for any type of

biofilter (water treatment or gas treatment) because excessive accumulation of sludge prevents continuous biofilter performance, requiring purification cycles in order to discharge it. Also, excessive biomass accumulation causes the clogging that often occurs in the inlet sections of biofilters, leading to a considerable increase in the total pressure drop and shortening the life span of the bed material (Yang et al. 2010). However, the specific growth speed was not significative different between the three strain in this study (Figure 4). This is explained by the growth behavior in the lag phase (Figure 1).

Table 2. RDP classification generated using the 16S rDNA sequences resulting from DGGE.

	API strain	**MUD strain**
Domain	Bacteria	Bacteria
Phylum	Proteobacteria	Proteobacteria
Class	Betaproteobacteria	Gammaproteobacteria
Order	Hydrogenophylales	Chromatiales
Family	Hydrogenophilaceae	Halothiobacillaceae
Genus	*Thiobacillus*	*Halothiobacillus*

For the exploration of strain identity, real-time PCR was used with a set of specific primers (melting curve) to test the identification and genetic differentiation of the strains. These results (Table 1) showed that API had a T_m closer to the T_m of ATCC than that of MUD, suggesting that API is more likely to belong to the species *T. thioparus* and that MUD is a more distant strain. To verify the previous statement, DGGE was done and the result (Figure 5) was consistent with the melting curves, showing a fragment for the MUD strain with a different denaturation point as compared with *T. thioparus* ATCC 10801, reinforcing the identification suggested by the melting curve. NCBI BLAST alignment against Genbank database of DNA fragments resulting from DGGE, displayed in Figure 5, revealed that the API strain had 98% homology with the *T. thioparus* LV43 strain (Vlasceanu et al. 1997), while the MUD strain has 100% homology with *Halothiobacillus neapolitanus*. We found very similar results when the DNA sequences were introduced into the RDP classifier tool (Table 2), which showed that the API strain belongs to the genus *Thiobacillus*, while the MUD strain is related to the genus *Halothiobacillus*. Finally, the phylogenetic tree (Figure 6), demonstrated that the API strain is closely related to *T. thioparus*, while the MUD strain is highly related to *Halothiobacillus neapolitanus*. The temperature sensitivity of the API strain relative to growth, together with the genetic evidence, suggests that API is a new strain of *T. Thioparus*.
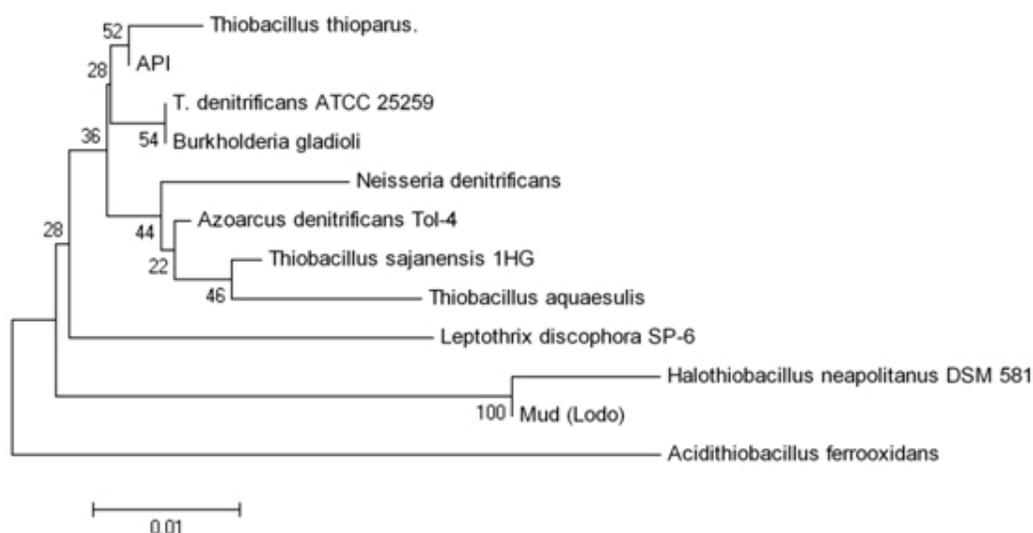

Fig. 6 Phylogenetic tree for API strain, MUD strain, *T. thioparus*, *Halothiobacillus neapolitans*, rooted with the gamma-proteobacteria *Acidithiobacillus thiooxidans*. The scale of 0.01 nucleotide substitution per sequence position corresponds to 1% of difference.

In conclusion we isolated two strains, called API and MUD, which show similar growth and thiosulphate bio-oxidation to *Thiobacillus thioparus* ATCC 10801. The API strain has the advantage of reaching a smaller cell concentration than the *T. thioparus* ATCC 10801 strain at an early stage of culture. This observation can be projected to the oxidation of other sulphur compounds by the API strain, and suggest that it is useful for application as the inoculum in biofilters used to eliminate industrial sulphur compounds. The utility of the MUD strain is related to the same field, but its capacity to use salts as energy source is an advantage. This feature gives the MUD strain a tolerance to high salt concentrations and a potential use in biofiltration under these conditions.

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