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RESEARCH PAPER

# Diverse Molecular Resistance Mechanisms of *Bacillus megaterium* During Metal Removal Present in a Spent Catalyst

**Andrea Rivas-Castillo, Domancar Orona-Tamayo, Marlenne Gómez-Ramírez, and Norma G. Rojas-Avelizapa**

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**Abstract** *Bacillus megaterium* strain MNSH1-9K-1, isolated from a high-metal content site in Guanajuato, Mexico, has the intrinsic capacity to remove vanadium (V) and nickel (Ni) from a petrochemical spent catalyst, and counteract the toxic effects produced in the cell due to the presence of oxidative stress. Since knowledge of the molecular components involved in the microbial resistance to spent catalysts is scarce, this study aimed to identify the proteins potentially involved in the enhanced resistance of a *B. megaterium* strain, during the removal of metals contained in a spent catalyst. Thus, the current research uses a proteomic approach to investigate and evidence the differences in the molecular resistance mechanisms of two *B. megaterium* strains, one isolated from a mining site and a wild type strain, when both are exposed to a spent catalyst. In addition, we studied their ability to eliminate nickel (Ni), vanadium (V), aluminum (Al) and molybdenum (Mo). The data presented here may contribute to the knowledge of the molecular mechanisms involved in the resistance of *B. megaterium* to high metal content wastes, as well as its potential utilization for the recovery of valuable industrial metals.

**Keywords:** *B. megaterium*, metal resistance, proteins, spent catalyst

Andrea Rivas-Castillo, Marlenne Gómez-Ramírez, Norma G. Rojas-Avelizapa*
Centro de Investigación en Ciencia Aplicada y Tecnología Avanzada del IPN, Cerro Blanco 141, Col. Colinas del Cimatario, Querétaro, Qro 76090, México
Tel: +52-442-220-804; Fax: +52 (442) 229 08 04 Ext. 81002
E-mail: nrojasa@ipn.mx

Domancar Orona-Tamayo
Departamento de Biotecnología y Bioquímica, CINVESTAV-Irapuato, Km. 9.6 Libramiento Norte Carr. Irapuato-León, Irapuato, Gto 36821, México

## 1. Introduction

Supported on porous materials through precipitation or impregnation, diverse metals such as Ni, Mo, cobalt (Co), and platinum (Pt) are widely used as catalysts in the petrochemical industry [1]. After their periodical use, these metals become inactivated due to their poisoning by foreign materials and impurities (*i.e.* Ni, V) [2], and are discarded as hazardous wastes [3], which contain large amounts of heavy metals [4]. The recovery of metals from these residues has been widely studied recently, but the treatments currently available utilize chemical compounds, thus generating more residues [5]. Hence, it is important to develop ecological and economically viable methods to treat and/or recycle such materials, such as the use of microorganisms capable of accumulating or leaching metals.

As has been previously reported, some strains of *B. megaterium* not only present high metal resistance, but also removal capability of metals like mercury (Hg), Ni and V [6,7]. Furthermore, *B. megaterium* MNSH1-9K-1, a strain isolated *in-situ* from a high-metal content site in Guanajuato, Mexico, shows tolerance limits greater than 200 ppm for Ni and V [8], and is capable of removing these metals from a spent catalyst [9,10]. Besides, the genes *nccA* (Ni–Co–Cd resistance), *hant* (high-affinity nickel transporter), *VAN2* (V resistance), and s*mtAB* (metal-binding protein) have recently been identified in MNSH1-9K-1 by a polymerase chain reaction (PCR) approach [11]. The changes in the expression of *nccA* and *smtAB* after exposure to a mixture of 200 ppm of Ni and 200 ppm of V is also reported [12].

Aerobic organisms such as *B. megaterium*, generate reactive oxygen species (ROS) as natural byproducts of their normal metabolism. However, environmental stressors

can lead to an overproduction of ROS, thereby altering the intracellular redox status and producing cell death [13]. Reports indicate that the presence of metals primarly induce the cellular oxidative stress response [14, 15]. It has been also documented that the adaptive response to oxidants in *Bacillus subtilis* is triggered by the action of transcription factors that sense oxidative stress and regulate the expression of appropriate defensive and repair functions, as the regulons controlled by PerR, $\sigma^B$, and OhrR, which includes the expression of superoxide dismutase (SOD), and catalase (CAT), both representing the frontline of antioxidant enzymes [16].

Although the metal removal capability from spent catalysts has already been described for diverse microorganisms, mainly acidophilic bacteria [10,17,18], Syed et al., 2015 showed that the ability of *Bacillus spp*. to remove metals like lead (Pb), copper (Cu) and cadmium (Cd) may be elicited by a biosorption process [19]. Hence, the aim of the present work is to describe the first insight into the molecular mechanisms potentially involved in the resistance to a spent catalyst, comparing the soluble proteome shown by a *B. megaterium* strain isolated from a high metal content site (strain MNSH1-9K-1) to the one present in the type strain QMB1551. In addition, the metal removal capability of both strains *B. megaterium* QMB1551 and *B. megaterium* MNSH1-9K-1 is demonstrated.

## 2. Materials and Methods

### 2.1. Bacterial strains, culture media, and growth conditions

For this study, two *B. megaterium* strains were used: the wild type strain *B. megaterium* QMB1551 (GeneBank accession number CP001983) and the strain *B. megaterium* MNSH1-9K-1 (GenBank accession number KM654562.1), which was isolated from a mining site in Guanajuato, Mexico, and whose isolation has been previously described [20]. Both bacteria were studied in Luria-Bertani (LB) [21] or PHGII medium [22], at 37°C and 200 rpm.

### 2.2. Spent catalyst

The Mexican Petroleum Institute (IMP) provided the spent catalyst (H-oil), which is composed of cylindrical particles of length 3 ~ 4 mm and diameter 1 mm. Metal composition of H-oil (in mg of component / kg of catalyst) was previously reported as: Al 109713.3 ± 3040.9; V 57617.7 ± 1156.4; Mo 32688.6 ± 892.7; Ni 24822.3 ± 550.1; Mg 316.1 ± 18.3; Fe 279.9 ± 21.2; Zn 199.5 ± 0.2; P 92.0 ± 3.0; As 42.3 ± 0.0; Cr 40.7 ± 0.0; and Cd 0.3 ± 0.10 [10]. For experimentation, the catalyst was pulverized using a mortar and pestle, and stored until experimental use.

### 2.3. Evaluation of bacterial spent-catalyst resistance

Cultures of *B. megaterium* strains were grown overnight, and 100 μL of each of these cultures were independently inoculated into 125 mL flasks containing 10 mL of LB or PHGII medium with different concentrations of spent catalyst, in the range 0.20 ~ 0.26% (w/v). As cellular viability controls, both strains were cultured in LB or PHGII medium devoid of spent catalyst. After 24 h, an aliquot of 100 μL was removed from each culture, serially diluted 10-fold diluted in phosphate-buffered saline (PBS) [23], and plated on LB media. Plates were incubated at 37°C, and the colony forming units (CFU) were counted to determine the resistance of the strains to H-oil.

### 2.4. Total protein extraction

*Bacillus megaterium* strains QMB1551 and MNSH1-9K-1 were cultured overnight, and 100 μL of each culture was used to inoculate 125 mL flasks containing 10 mL of LB medium, and 0.217 ± 0.001% (w/v) of the spent catalyst; both strains were also cultured without H-oil, as controls. When cultures reached an $O.D._{600nm}$ = 0.5, cells were harvested, and the protein extraction protocol was modified from Kott et al., 2004 [24]. Briefly, the cells were subjected to three freezing-thawing cycles, using liquid nitrogen. Afterwards, 1.5 mL of buffer {Tris-HCl 10 mM pH 8; dithiothreitol (DTT) 1 mM; ethylenediaminetetraacetic acid (EDTA) 1 mM; NaCl 10 mM; urea 2.5 M; 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate hydrate (CHAPS) 1%; protease inhibitors 5%} was added to each sample. Cells were homogenized with ground glass, and finally subjected to sonication using a Branson Sonifier, for 10 cycles of 30 sec each. To extract proteins, samples were centrifuged at 14,000 × *g* for 5 min at 4°C, and supernatants were collected and stored at –70°C. Protein was precipitated using 80% methanol / 20% trichloroacetic acid (TCA, v/v) (protein extract:TCA = 1:1), and incubated at –20°C for 5 h, after which the samples were centrifuged as before, and supernatants were discarded. The pellets obtained were washed twice with 90% methanol, and the protein content was determined using the BCA protein assay kit (Pierce, Rockford, IL, USA), using bovine serum albumin (BSA) as the standard.

### 2.5. SDS-PAGE

The electrophoretic separation of proteins was performed by 13% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) [25]. Extracts (25 μg of protein per sample) were loaded into the wells, and separated on a vertical dual mini gel electrophoresis device (Bio-Rad, Hercules, CA, USA) at 120 V and 30 μA. Gels were stained with Coomassie brilliant blue.

### 2.6. Two-dimensional gel electrophoresis

In order to investigate the *B. megaterium* proteomes, the protein content was separated by two-dimensional gel-electrophoresis (2-DE). All protein samples (50 μg each) was re-suspended in 125 μL of rehydrating solution {urea 7 M, thiourea 2 M, CHAPS 2%, DTT 65 mM, IPG buffer 2% (pH 3-10)}; the samples were then loaded onto 7 cm IPG dry strip gels, and allowed to rehydrate for 15 h. The isoelectrofocusing (first dimension) of IPG strips was carried out according to the manufacturer's instructions (GE-Amersham, Little Chalfont, Buckinghamshire, UK); proteins were separated by 13% SDS-PAGE, and stained with Coomassie brilliant blue. To compare the differences in protein expression of the samples under study, images were first normalized by a weighted marker Euclidian vector approach, where the Euclidian distance was calculated for the vectors of the protein molecular weight markers of each gel. Afterwards, spots densitometry was determined using the free processing program ImageJ, and differences in protein expression were calculated by dividing the densitometry units of each spot by the Euclidian norm calculated for its corresponding gel.

### 2.7. Mass spectrometry

Protein spots of interest were cut from the gel matrix and tryptically digested [26]. Briefly, proteins were in-gel reduced by 10 mM DTT, and alkylated by 55 mM iodoacetamide. Destained, washed and dehydrated gel pieces were rehydrated at 4°C for 60 min in a 0.5 μM solution of bovine trypsin in 25 mM ammonium bicarbonate buffer, and then digested overnight at 37°C. All experiments were performed on a nanoflow LC system, NanoAcquityI (Waters, Milford, MA, USA), coupled to a linear ion trap LTQ velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany), equipped with a nanoelectrospray ion source [27]. For the NanoAcquityI system, solvent A consisted of 100% $H_2O$ and 0.1% formic acid, and solvent B consisted of 100% acetonitrile and 0.1% formic acid. For Liquid Chromatography Coupled with Tandem Mass Spectrometry (LC-MS/MS) experiments, 3 ~ 10 μL of trypsin digested proteins were trapped using pre-column (Symmetry® C18, 5 μm, 180 μm × 20 mm; Waters, Milford, MA, USA), which was then switched in-line onto a 100 mm × 100 μm BEH-C18 1.7 μm particle size capillary UPLC column [28]. The column was enclosed in a column heater operating at 35°C. After a 1 min loading time, the peptides were separated with a 60 min gradient at a flow rate of 400 nL/min. The gradient was as follows: 3 ~ 50% Solvent B (30 min), 50 ~ 85% B (1 min), 85% B (7 min) and 3% B (22 min). Eluted peptides were directly electro sprayed into the mass spectrometer through a standard coated silica tip (New Objective, Woburn, MA, USA). The LTQ was operated in data-dependent acquisition mode to automatically alternate between a full scan (m/z 400 ~ 2,000), and subsequent Top 5 MS/MS scans in the linear ion trap. Collision-Induced Decomposition (CID) was performed with helium as collision gas, at normalized collision energy of 40% and 10 ms of activation time. Data acquisition was controlled by Xcalibur 2.2.0 software (Thermo Fisher Scientific, Bremen, Germany). Tandem mass spectra, obtained from the LTQ-VELOS, were extracted by Proteome Discoverer version 1.3, and searched by use of Sequest against a (*Bacillus megaterium*) database. All files generated by Sequest were searched with the following parameters: 1.6 Da parent MS ion window, 0.8 Da MS/MS ion window and two missed cleavages allowed.

### 2.8. Metal removal from spent catalyst

Strains were grown in 10 mL of LB medium in 125 mL Erlenmeyer flasks, inoculated with 1% (v/v) of overnight cultures, and containing 0.217 ± 0.001% (w/v) of the spent catalyst. Sets were prepared in duplicate, and two controls without inoculum were also prepared in order to assess abiotic removal of metals. After 24 h of growth, samples were filtered to separate the spent catalyst from the cell culture, and the biologically treated spent catalyst samples and non-treated controls were dried at room temperature for 48 h [20]. Afterwards, Ni, Mo, Al and V residual concentrations were determined, as described below.

### 2.9. Digestion of samples and metal analysis

*B. megaterium* strains MNSH1-9K-1 and QMB1551 were grown for 24 h in the presence of 0.217 ± 0.001% (w/v) of the spent catalyst, with control cultures of each strain grown without H-oil. After acid digestion, 10 mg of dry spent catalyst taken from each sample was subjected to metal analysis using an Inductively Coupled Plasma Optical Emission Spectrometer (ICP-OES Model 710-ES, Varian, Palo Alto, CA, USA). Briefly, catalyst samples were placed in cylindrical vials of silicon carbide, to which acids were added (HCl 6 mL; $HNO_3$ 2 mL). Samples were digested in a Microwave Reactions System: Multiwave PRO (Anton Paar, Ashland, VA, USA) using a rotor HF100. Digestion conditions were: power 800 W, 40 Bar, temperature 210 ~ 240°C, with pRate of 0.3 bar/seg, ramp 15 min, hold 60 min, and cooling 15 min. Afterwards, 20 mL of deionized water was added to the cylindrical vials, and each supernatant was collected in a volumetric flask, where volume was set to 100 mL with deionized water. Analyses of Al, V, Mo and Ni were performed at their respective wavelengths (nm): Ni (231.604), V (292.401), Al (396.152), Mo (202.032). Metal residual concentrations were calculated

based on calibration curve of 0.1 ~ 10 ppm using a commercial standard (Cat. # ICP-200.7-6; High-Purity Standards, Charleston, SC, USA) [10].

### 2.10. Statistical analysis

Basic statistical parameters and analyses of variance (ANOVA) were performed using the commercial statistical software OriginPro 9.0. Differences with $P$ values of $\leq 0.05$ were considered statistically significant.

## 3. Results and Discussion

### 3.1. Effect of spent catalyst concentration on cell growth

Although the resistance of *B. megaterium* to diverse metals such as As, Cd, Cr, Cu, Ni, Pb, Se, and Zn has been previously reported [29-31], the resistance and metal removal capability of this bacteria to a mixture of metals or complex metal recalcitrant residues has been scarcely studied [8-11, 17-19]. To investigate the resistance of the bacterial culture *B. megaterium* MNSH1-9K-1 (isolated from a high metal content site) to the spent catalyst H-oil, and compare it to the resistance of the type strain QMB1551, both strains were cultivated at 37°C in liquid LB medium containing different concentrations of the spent catalyst. After 24 h of exposure to the catalyst, cell viability was determined as described in Materials and Methods, showing that the isolated MNSH1-9K-1 strain presents a clear growth advantage in the presence of H-oil, compared to the resistance shown by the type strain QMB1551 (Fig. 1). Furthermore, to evaluate the effect of the fermentation medium on the spent catalyst growth inhibition of *B. megaterium*, both strains were cultured in PHGII medium in the presence of the residue, under same experimental conditions, considering that this medium has been previously used to elucidate the resistance of diverse microorganisms to spent catalysts [10]. Results showed that in comparison to its growth in LB, strain MNSH1-9K-1 presented a $1.5 \times 10^4$ – fold decrease in cell viability when grown in PHGII containing 0.2 % (w/v) of the spent catalyst; this cell viability loss remained costant for all concentrations tested. Besides, QMB1551 growth was not detected in PHGII when H-oil was added, as represented by the line shown in the lower relative cell number plotted ($10^{-5}$; Fig. 1). Although both media contain yeast extract and peptone [21, 22], LB medium contains 5 and 2.5 times more of these two components, respectively, and the latter contains NaCl and not glucose, as does PHGII.

Hence, the observed higher viability of *B. megaterium* strains in LB medium in the presence of the spent catalyst may be due to the following reasons: 1) the higher amount of protein hydrolysates contained in this medium, since previous reports have shown that these hydrolysates could have diverse biological effects, includying protective bioactivities [32]; and/or 2) the presence of NaCl in LB, as it has been reported that the presence of salts enhances the tolerance to toxic compounds, such as heavy metals and oxyanions [33]. As a result of the heightened cell resistance to the spent catalyst observed in LB, all the subsequent experiments were performed in this culture medium.

To investigate the growth inhibition in the presence of

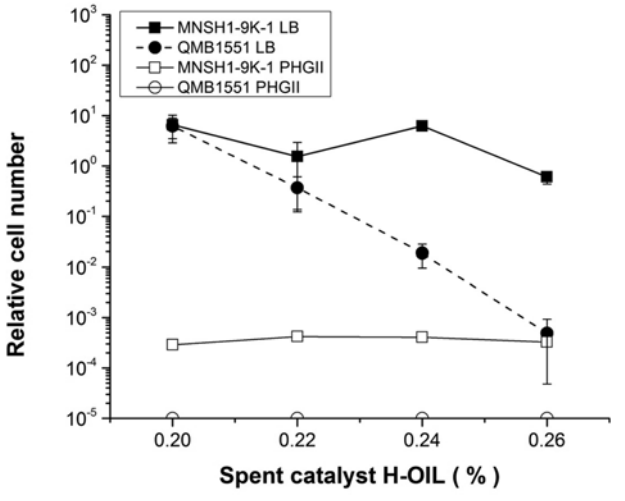


**Fig. 1.** Resistance of *B. megaterium* strains QMB1551 and MNSH1-9K-1 to H-oil. Cultures were cultivated at 37°C for 24 h in LB or PHGII medium, containing varying concentrations of the spent catalyst. Data are shown as the growth measured in each concentration relative to the growth in the control for each medium (without H-oil), and as averages ± standard deviations from duplicate experiments.

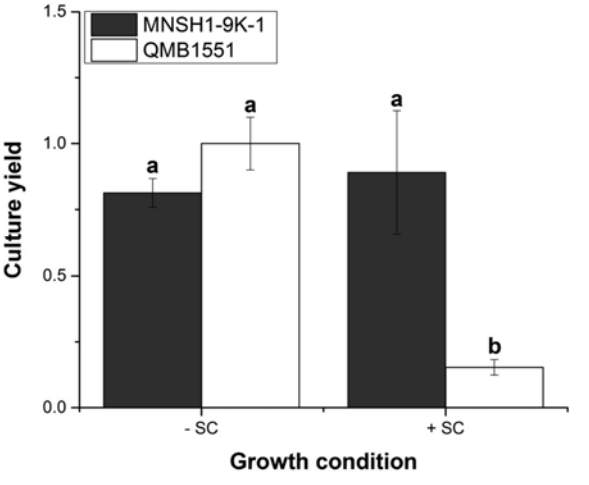


**Fig. 2.** Culture yield of *B. megaterium* strains QMB1551 and MNSH1-9K-1 grown in the presence of H-oil. Cultures were cultivated at 37°C for 24 h in LB medium without (-SC) or with (+SC) the spent catalyst. Results were normalized to culture yield of the type strain QMB1551 grown without H-oil. Data are presented as averages ± standard deviations from duplicate experiments, and different lowercase letters indicate data that were not significantly different by ANOVA ($P > 0.05$).

the spent catalyst in further detail, the median lethal concentration ($LC_{50}$) of *B. megaterium* QMB1551 to H-oil was calculated from the data shown in Fig. 1, and was determined as 0.217 ± 0.005%. *B. megaterium* QMB1551 and MNSH1-9K-1 were both cultivated at 37°C in liquid LB medium, containing 0.217 ± 0.001% of H-oil; both cultures were also grown without the spent catalyst, as reference controls. After 24 h of growth, the viable count of each sample was performed to determine the culture yield (Fig. 2). Strain QMB1551 exhibits a 6-fold decrease when grown in the presence of the spent catalyst, compared to the cell yield presented for the strain MNSH1-9K-1.

### 3.2. SDS-PAGE patterns and 2-DE

Metal resistance mechanisms may appear and be clearly shown during the logaritmic phase of growth [34], and cultures may initiate its differentiation process to sporulate in this phase [35], as compared to the stationary phase, where nutrients are scarce and cells must decide how to use their metabollic program. To investigate proteins that may be implicated in the differentiated resistance of the strain MNSH1-9K-1 to the spent catalyst, as compared to the wild type strain QMB1551, a proteomic approach was developed during exponential growth, where both *B. megaterium* strains were cultured in LB medium containing 0.217 ± 0.005% of H-oil, with controls cultured in medium devoid of the spent catalyst, until they reached an $OD_{600\ nm}$ = 0.5. Cells were then harvested, and total soluble protein content was obtained. As shown in Fig. 3, no difference was observed in an unidimensional SDS-PAGE between the protein expression patterns of the different samples evaluated. However, there are very fine differences related to protein level expression that cannot be displayed by SDS-PAGE, and may be visualized with other important protein techniques, such as two-dimensional gel electrophoresis (2-DE).

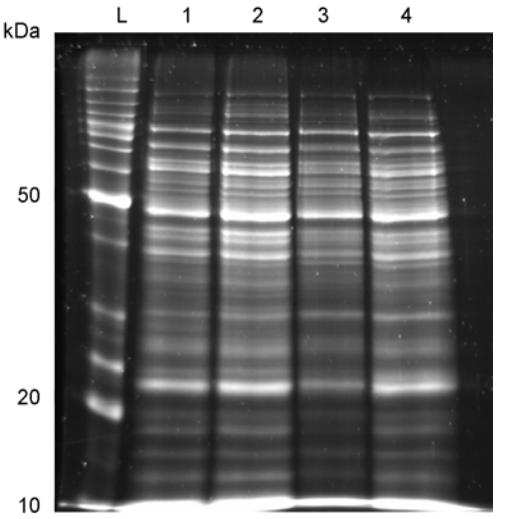


**Fig. 3.** Proteomes obtained *via* SDS-PAGE for *B. megaterium* strain MNSH1-9K-1 grown without (Lane 1), and with H-oil (Lane 2); and for strain QMB1551 grown without (Lane 3), and with the spent catalyst (Lane 4). The result shown is a representative example for $n$ = 3 replicates per sample type. L, size ladder.

Proteomes were further analyzed by 2-DE, and the patterns confirmed the clear differences between the patterns of the two *B. megaterium* strains grown with or without the spent catalyst (Fig. 4). From the proteomes observed in Figs. 4A, 4B, 4C and 4D, 10 proteins were selected and identified by mass spectometry analysis, as presented in Table 1 (For the peptide sequences derived from sequencing analyses, which were used for protein identification, refer to Supplementary Table 1). Additionally, differential protein expression levels were compared using a weighted marker euclidian vector

**Table 1.** Annotation results of *B. megaterium* spots identified under different growth conditions

| Spot number | Description | Accession number | Organism | Mw (kDa) | pI | Peptide Hits | MS Blast Score |
|---|---|---|---|---|---|---|---|
| 1 | Transcriptional repressor of the rex ndh operon | YP_005494780 | *B. megaterium WSH-002* | 16.2 | 4.6 | 5 | 474 |
| 2 | Transcription elongation factor GreA | WP_006637782 | *B. sonorensis* | 17.5 | 4.6 | 9 | 1,390 |
| 3 | FMN-dependent NADH-azoreductase | WP_013058078 | *B. megaterium QM B1551* | 23.2 | 5.4 | 11 | 1,452 |
| 4 | NAD(P)H nitroreductase | WP_013056019 | *B. megaterium QM B1551* | 22.4 | 5.1 | 16 | 2,253 |
| 5 | Superoxide dismutase | WP_013059198 | *B. megaterium QM B1551* | 22.5 | 5.4 | 9 | 1,484 |
| 6 | Thioredoxin | WP_003199933.1 | *Bacillus sp.* | 20.5 | 4.7 | 5 | 56 |
| 7 | Chemical-damaging agent resistance protein C | WP_008342133.1 | *B. pumilus* | 20.6 | 4.5 | 3 | 514 |
| 8 | Arginase | WP_006836969 | *B. sp. SG-1* | 32.2 | 5.3 | 10 | 1,166 |
| 9 | 2-oxoisovalerate dehydrogenase subunit beta | WP_013056055 | *Bacillus sp.* | 35.3 | 4.7 | 16 | 2,319 |
| 10 | Dihydrolipoamide succinyltransferase | WP_013057593 | *B. megaterium* | 47.1 | 5.4 | 4 | 522 |

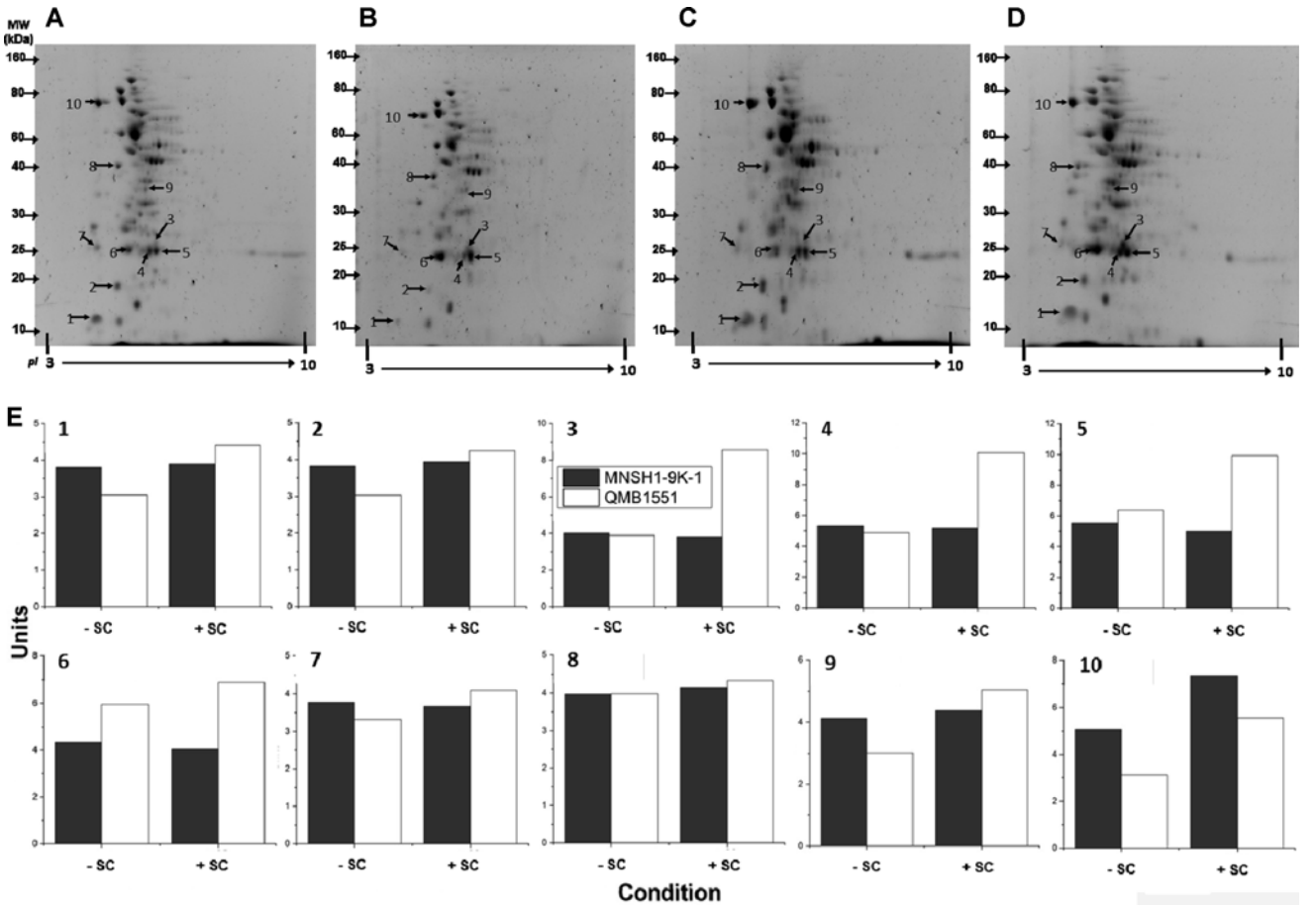


**Fig. 4.** 2-DE patterns of *B. megaterium* under different growth conditions. (A) MNSH1-9K-1, and (B) QMB1551 grown without H-oil; (C) MNS-H1-9K-1 and (D) QMB1551 grown in the presence of 0.217 ± 0.005% of the spent catalyst. Proteins idientified in this study are indicated by arrows and numbers (see Table 1 for protein identity and description). Total protein contents (50 μg of protein from each condition) were separated in a 13% gel concentration and stained with Comassie blue. (E) Comparison of the spot densitometry corresponding to each of the ten proteins evaluated in the four conditions tested. – SC without or + SC with the spent catalyst.

approach, and results are shown in Fig. 4E.

The type strain QMB1551 presented an induction in the expression levels of various proteins when grown in the presence of the spent catalyst: the transcriptional repressor of the *rex ndh* operon (Fig. 4 (E1); 1.45-fold), the transcription elongation factor GreA (Fig. 4 (E2); 1.4-fold), a superoxide dismutase (Fig. 4 (E5); 1.55-fold), and the 2-oxoisovalerate dehydrogenase subunit beta (Fig. 4 (E9); 1.68-fold). An even higher induction was observed in QMB1551 for the proteins FMN-dependent NADH-azoreductase (Fig. 4 (E3); 2-fold), and the NAD (P) H nitroreductase (Fig. 4 (E4); 2.2-fold), in contrast to the undetectable change in the expression of these 6 proteins in the strain MNSH1-9K-1.

It has been suggested that GreA, in addition to its role in transcription elongation [36], may be involved in the adaptive response of *Enterococcus faecalis* to Cu exposure [37], and in acid, salt, and cold stress responses in *Streptococcus mutans* [38], *Sinorhizobium meliloti* [39], *Rhizobium tropici* [40], and *Shewanella livingstonensis* [41]. Also, GreA has been associated with the heat shock and oxidative stress response of *Salmonella enterica* as part of its $\sigma^E$ regulon [42], and may be implicated in resistance to high temperatures [43]. It was also demonstrated that *E. coli* GreA has chaperone activity, and when overexpressed, GreA enhances the resistance of host cells to environmental perturbations [44].

Conversely, it has been widely reported, from prokaryotes to humans, that (SODs) constitute one of the major defense mechanisms of cells against oxidative stress, by catalyzing the disproportionation of superoxide anion into hydrogen peroxide and molecular oxygen [45-47]. In *Escherichia coli*, the expression of the SOD genes is markedly increased by exposure to diverse stress factors, such as redox-cycling agents [48], heat shock [49], high salt concentrations [50], and also by metals [51, 52-54]. Likewise, the SODs of different *Bacillus* species have been identified as important components of the detoxifying systems [55, 56]. It was recently reported that SOD activity is enhanced by salt stress in *B. licheniformis* [57], by the presence of Cd in *B. cereus*

cells [58], and by As in *B. subtilis* and *B. thuringiensis* [59], which may be the case similar to this study, due to the high metal content present in H-oil.

Also, 2-oxoisovalerate dehydrogenase, which is known to be involved in the assimilation of valine, interacts with SOD to promote the thermoadaptation of *Thermus thermophiles* [60]. In addition, this enzyme presented changes in its expression when *Pseudomonas putida* was grown at 10°C instead of 30°C [61], and a proteomic approach in *B. anthracis* showed changes in its expression when cells were treated with 0.3 mM $H_2O_2$ [62], indicating that the latter protein may have some implication in the oxidative stress response.

Although there is less knowledge about the function of the transcriptional repressor of the *rex ndh* operon (*yjlC*), its operon partner, *ndh*, encodes the major NADH dehydrogenase reported in *B. subtilis,* which together with Rex form a "regulatory loop" that maintains the NADH/NAD ratio in vegetative cells, generating a proton motive force utilized to produce ATP for cellular metabolism. It has also been reported that some unknown regulation generates a change in the expression level of *yjlC-ndh* operon under anaerobic conditions [63]. Furthermore, it has been established that the presence of Al has the ability to promote anaerobiosis in hepatocytes [64]. As Al was found to be one of the main components of the spent catalyst H-oil, comprising the 48% of the detected metal content in this catalyst [10], it is possible that Al initiates this detected protein expression change, as well as stimulate other undetected metabolic changes when *B. megaterium* QMB1551 is grown in the presence of H-oil.

Two proteins that present the highest changes of expression in QMB1551 have both been implicated in bacterial detoxification. Azo proteins are a varied family of enzymes identified in almost all species, that possess the ability to reduce a wide variety of both endogenous and exogenous compounds [65], and are mainly involved in the reduction of azo dyes [66]; nevertheless, AzoR from *E. coli* shows a moderate sequence homology in its active site to NQO1, a mammalian FAD-containing protein which plays an important role in detoxification and has a protective effect against mutagenicity, carcinogenicity and other toxicities, by utilizing its reduction activity [67]. The azoreductase of *B. anthracis* revealed different expressions when cells were treated with $H_2O_2$ 0.3 mM [62]. Along with the possible oxidative stress implication of azoreductase, some studies also suggest the potential involvement of nitroreductases in the oxidative stress response, as previously shown in *E. coli*, and recently demonstrated by the Fmr2 nitroreductase deletion mutant in *Saccharomyces cerevisiae* [68, 69]. In this regard, the *E. coli nfsA* gene, which encodes the nitroreductase NfsA, is part of the SoxRS regulon and is strongly induced by paraquat, a well-known superoxide generator [70]**.** The genes *snrA* in *Salmonella typhimurium* and *nprA* in *Rhodobacter capsulatos* are also induced by paraquat [71]. Recently, de Oliveira *et al.* (2010) presented that the Frm2p and Hbn1p nitroreductase-like proteins of the yeast *S. cerevisiae* influence the response to oxidative stress in this organism by modulating the reduced glutathione contents and antioxidant enzymatic activities [72]. Additionally, the DrgA protein of the cyanobacterium *Synechocystis sp.* and NfsB in *E.coli* show ferric reductase activities that potentially play a role in iron metabolism, and can catalyze the reaction by which iron and hydrogen peroxide react, generating the hydroxyl radical (Fenton reaction) [73]. In the other hand, Roldán *et al.* (2008) mentioned the adaptive advantage of nitroreductase, suggesting that the primitive physiological function of these enzymes could have been lost or modified to allow the reduction of different nitroaromatic and nitroheterocyclic compounds [74]. As only the heavy petroleum fraction may remain in the H-oil, it is expected that the quantity of heterocyclic aromatic components is low in the spent catalyst, and the induction of the nitroreductase could probably be due to the oxidative stress emerging in the cell, and not because its nitroheterocyclic reduction function is required.

The protein identified as dihydrolipoamide succinyl-transferase (Fig. 4 (E10)) showed a 1.78-fold increase when *B. megaterium* QMB1551 was grown in the presence of the spent catalyst, and also a 1.45-fold increase was observed when the strain MNSH1-9K-1 was exposed to H-oil. Even more, the expression of this protein was found very similar in MNSH1-9K-1 culture without H-oil and the QMB1551 culture grown with the spent catalyst.

Dihydrolipoamide succinyl-transferase, codified by *sucB*, is an enzymatic component of the 2-oxoglutarate dehydrogenase complex, which catalyzes the overall conversion of 2-oxoglutarate to succinyl-CoA and $CO_2$ in the tricarboxylic acid cycle (TCAC), providing a key step in the regulation of energy production [75]. The enzyme complex also links TCAC to protein synthesis and breakdown, and sugar metabolism to amino acid metabolism [76-78]. A study made by Mishra *et al.* (2012) demonstrated that the dihydrolipoamide succinyltransferase is important for the regulation of various metabolic pathways in *Pseudomonas putida*, and for the degradation of toxic allelochemicals produced by *Parthenium* [79].

Besides, the expression of *sucB* showed 1 to 1.8-fold increase in *rpoS E. coli* mutant colonies after 1 and 7 days of growth, respectively [80]. It is known that *rpoS* encodes a sigma factor, which is a central regulator of the general stress response, allowing the cells to survive environmental challenges and preparing the cell for subsequent stresses [80]. Saint-Ruf *et al.* (2004) suggested a compensatory

transcription in *rpoS* colonies by the expression of genes belonging to the TCAC, involved in energy salvage, to attenuate the lack of various RpoS-dependent functions, especially in aging colonies [80]. It is possible that the higher expression of this enzyme in *B. megaterium* MNSH1-9K-1 during exponential phase, even without subjecting the microorganism to an exogenous stressor agent, and its overexpression when grown in the presence of the spent catalyst, may confer this strain with an advantageous higher resistance capability to the spent catalyst. Even more, as suggested for *E. coli* [81], dihydrolipoamide succinyltransferase may be part of a cell response in *B. megaterium*, different from the general stress response, used by this microorganism to counteract the harmful effect of toxic compounds such as metals.

When QMB1551 was cultured in the presence of H-oil, a visible increment was observed in the expression of Thioredoxin (Fig. 4 (E6)), chemical-damaging agent resistance protein C (Fig. 4 (E7)), and Arginase (Fig. 4 (E8)). It has been previously reported that these proteins are involved in the defense against oxidative stress [82-85], reactive oxygen species [86, 87], chemical response, and general stress response [88,89], and further experiments are needed to elucidate the significant induction and importance of these proteins in the resistance of *B. megaterium* to spent catalysts.

### 3.3. Assessment of Al, V, Mo and Ni removal capability of *B. megaterium*

To analyze the ability of *Bacillus megaterium* strains MNSH1-9K-1 and QMB1551 to remove Al, V, Mo, and Ni, which are the most abundant elements found in the spent catalyst H-oil [10], cultures of both strains were grown for 24 h in the presence of 0.217 ± 0.005% of the spent catalyst, with corresponding controls cultivated without H-oil. Assays were performed to quantify the amount of Al, V, Mo and Ni removed from the spent catalyst.

Results showed that after 24 h of exposure to H-oil, *B. megaterium* QMB1551 is capable of removing V (13,822.48 ± 979.50 mg/kg), and Mo (6488.69 ± 611.28 mg/kg), corresponding to 23.99 ± 1.70% of V and 19.85 ± 1.87% of Mo. In contrast, strain MNSH1-9K-1, besides presenting the ability to remove V (26,734.61 ± 806.65 mg/kg), and Mo (1961.32 ± 947.97 mg/kg), also eliminated Ni (5609.83 ± 1365.22 mg/kg), corresponding to 46.46 ± 1.41% , 6.00 ± 2.93%, and 22.65 ± 5.52% of V, Mo, and Ni, respectively (Fig. 5). The data shown are the percentage results of the subtraction of the concentration (mg/kg) of each element in the spent catalyst minus the residual concentration (mg/kg) of the elements measured in each condition tested. In addition, the percentage eliminated in the abiotic control was subtracted from the results.

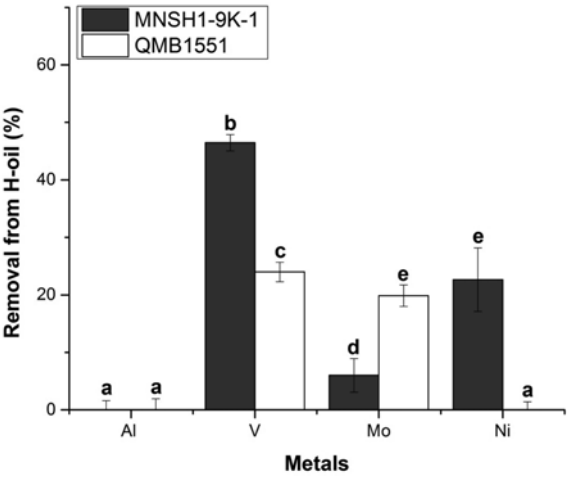


**Fig. 5.** Removal of Al, V, Mo, and Ni from the spent catalyst H-oil. *B. megaterium* strains MNSH1-9K-1 and QMB1551 were grown for 24 h in liquid LB at 37°C and 200 rpm, with 0.217 ± 0.005% of the spent catalyst. Data are presented as averages ± standard deviations ($n$ = 2), and lowercase letters indicate groups of data that were not significantly different by ANOVA ($P > 0.05$).

The data shows that although the type strain QMB1551 has the ability to remove higher amounts of Mo, *B. megaterium* strain MNSH1-9K-1 has higher removal capability of V than the type strain QMB1551. Additionally, MNSH1-9K-1 is capable of removing Ni from the spent catalyst, in contrast to QMB1551. As it has been reported that Ni and V may be more toxic for biological systems than Al and Mo [90], and Ni is particularly considered as a hazardous metal [91], being implicated in alterations in the DNA repair mechanisms, epigenetic effects, and carcinogenesis [92]. It is possible that the Ni removal capability observed in MNSH1-9K-1 conferred this strain with a physiological advantage to counteract the toxicity of this metal, and as a result, this capability also confers the strain an advantageous resistance to the spent catalyst.

Adaptations of cells to grow by generating metabolic changes that help them cope with stress provide bacteria with a selective advantage to survive, especially in polluted environments. It has been extensively reported that the *Bacillus* species possess a wide range of resistance mechanisms that confer this genus with the ability to adapt and counteract adverse environmental conditions [reviewed in 93], including metal resistance [94, 95].

The results obtained in this study reflect the fact that metals induce oxidative stress in the cell, as many of the identified proteins are repeatedly involved in the oxidative stress response of various organisms, thereby acknowledging that ROS may cause DNA, RNA, proteins and lipids damage [reviewed in 96]. The latter is sustained by the fact that previous studies have shown that heavy metals produce reactive oxygen species and induce an increased

synthesis of SODs [14,97]. Also, it has been demonstrated that *E. coli* SODs are involved in the defense against oxidative stress mediated by Cd, Co and Ni [15].

The *B. megaterium* reported genomes contain predicted open reading frames (ORFs) of genes involved in diverse stress responses, and also genes specifically involved in protection from ROS [98]. It has been documented that the *B. megaterium* genome contains predicted open reading frames of 46 genes potentially involved in oxidative stress, 26 for osmotic stress, 14 for heat shock, 2 for cold shock, and 1 for detoxification [99], as well as resistance genes that encode proton efflux/influx pumps [98], similar to the genes *nccA* and *hant* identified in the MNSH1-9K-1 genome [11]. Besides, it has been widely reported that some metals, such as V, Mo and Ni, may be used as enzyme cofactors in prokaryotes, similar to *B. megaterium*, participating in diverse chemical processes, structures, and cell functions [100,101].

It is possible that since the *B. megaterium* strain MNSH1-9K-1 was isolated from a high-metal content site and therefore continuously exposed to metals, it developed forceful mechanisms to counteract metal toxicity, thus providing this strain with a significant advantage to survive, probably by making a convenient use of metals conducting processes of efficient metal storage in its proteins, similarly as the ones proposed earlier for yeasts [102,103], and bacterial SODs [104]. Developing efficient resistance and uptake mechanisms would confer this strain with the molecular potential to be employed in the treatment of residues containing elevated levels of metals. However, further analyses are necessary to identify the membrane associated proteins that confer this *B. megaterium* strain with its high metal removal capability, as the proteomic approach used in the current study only includes the soluble protein fraction. Also, supplementary studies are required to characterize in detail the overall cell uptake systems, and the possible molecular targets that may potentiate MNSH1-9K-1 capacity to be used for the recovery of metals from spent catalysts and other high metal content wastes.

## 4. Conclusion

The results presented in this study show evidence of a differential set of molecular mechanisms that may provide *B. megaterium* strain MNSH1-9K-1 with its clear advantage to survive metal stress, and its ability to eliminate V and Ni from a spent catalyst.

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