

Unveiling the Chromate Stress Response in the Marine Bacterium *Bacillus enclensis* AGM_Cr8: A Multifaceted Investigation

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Abstract

In this study, we introduce *Bacillus enclensis* AGM_Cr8, a gram-positive marine bacterium isolated from the chronically polluted Versova Creek in Mumbai, India. AGM_Cr8 exhibits robust tolerance to chromate stress, thriving in marine agar media containing up to 3200 mg/l of hexavalent chromium [Cr(VI)], with the Minimum Inhibitory Concentration (MIC) established at 5000 mg/l. Notably, AGM_Cr8 also displays tolerance to other heavy metals, including Lead [Pb (II)] (1200 mg/l), Arsenic [As (III)] (400 mg/l), Cadmium [Cd(II)] (100 mg/l), and Nickel [Ni(II)] (100 mg/l). Scanning Electron Microscopy (SEM) reveals the presence of Cr(VI) on the bacterial surface, accompanied by the secretion of extracellular polymeric substances (EPSs) facilitating Cr(VI) sequestration. This observation is validated through Energy Dispersive Spectroscopy (EDS). Transmission Electron Microscopy (TEM) and Scanning Transmission Electron Microscopy–Energy Dispersive Spectroscopy (STEM-EDS) confirm internal bioaccumulation of Cr(VI). X-ray photoelectron spectroscopy (XPS) identifies distinct peaks around 579 eV and 576 eV, indicating the coexistence of Cr(VI) and Cr(III), implying a bioreduction mechanism. *De novo* genome sequencing identifies twenty-two chromate-responsive genes, including putative chromate transporters (*srpC1* and *srpC2*), suggesting an efflux mechanism. Other identified genes encode NAD(P)H-dependent FMN-containing oxidoreductase, NADH quinone reductase, ornithine aminotransferase, transporter genes (*natA*, *natB*, *ytrB*), and genes related to DNA replication and repair (*recF*), DNA mismatch repair (*mutH*), and superoxide dismutase. We therefore, propose a chromate detoxification pathway that involves an interplay of chromate transporters, enzymatic reduction of Cr(VI) to Cr(III), DNA repair and role of antioxidants in response to chromate stress. We have highlighted the potential of AGM_Cr8 for bioremediation in chromium-contaminated environments, given its robust tolerance and elucidated molecular mechanisms for detoxification.

Keywords: *Bacillus enclensis* AGM_Cr8, Versova creek, Genes, Chromium, Bioaccumulation, Bioreduction

1. Introduction

The widespread global concern of environmental pollution, a significant contributor to climate change and detrimental to diverse ecosystems, underlines the challenges posed by a continuous rise in pollutants, particularly Heavy Metals (HMs) (Singh and Christina et al., 2022). Chromium (Cr) compounds and their toxicity is widespread, and they are most commonly found in hexavalent [Cr(VI)] and trivalent [Cr(III)] oxidation states (Monga et al., 2023). They can accumulate in diverse environmental conditions including acidic, warm, cold, alkaline, hypersaline and anoxia (Jroundi et al., 2020). Cr(VI) due to its high permeability through cellular membranes, is known for its toxicity and carcinogenicity as compared to Cr (III) which is generally regarded less toxic and even necessary for human health in small quantities. Prolonged exposure to Cr(VI) can lead to various detrimental health effects, including respiratory disorders, liver and kidney damage, skin problems like dermatitis, and an increased likelihood of developing lung cancer (Genchi et al., 2021; Monga et al., 2022a). In aquatic environments, Cr(VI) can bioaccumulate in organisms, disturbing the delicate ecological balance. Cr(VI) has been designated as a toxic substance by the United States Environmental Protection Agency (Vega Cuellar et al., 2022).

Traditional Cr remediation methods, such as chemical precipitation and ion exchange, have limitations in terms of cost, efficiency, and the potential generation of secondary pollutants (Jobby et al., 2018). So, there is a growing interest in sustainable and environmentally friendly strategies for Cr(VI) mitigation. Unique microorganisms, including bacteria, fungi, and microalgae, have exhibited remarkable resistance and hold promising prospects for environmental and biotechnological applications in extreme conditions (Rahman et al., 2023). Marine sediments are not only are hotspots for biogeochemical cycling and nutrients, but also act as a reservoir of long-term stubborn pollutants like HMs (Chen et al., 2019), especially in anthropogenically impacted areas such as Versova Creek of Mumbai. Indigenous bacteria inhabiting these extreme environments contribute to metal accumulation through various mechanisms, including reduction, biomineralization, and biosorption in biofilm populations (Jroundi et al., 2020). Several studies have identified bacteria employing diverse mechanisms for their survival in response to Cr stress (Banerjee et al. 2019; Baldiris et al. 2018; Li et al. 2021; Elahi et al. 2022; John and Rajan 2022; Kookhaee et al. 2022; Yakasai et al. 2022). These mechanisms include biotransformation, enzymatic reduction, biosorption, bioaccumulation, efflux, precipitation, and cytosolic binding, either individually or in combination, to counteract the toxicity of Cr(VI) (Bhargava and Mishra, 2018; Banerjee et al., 2019). The conversion of Cr(VI) to Cr(III) stands out as a promising approach for remediating Cr(VI)-contaminated sites, offering simplicity, efficiency, and cost-effectiveness. The reduction of Cr(VI) to Cr(III) not only improves its toxicity to

microorganisms but also facilitates its physical removal in the bioremediation processes through precipitation at neutral pH, primarily as Cr(OH)₃ (Cheung and Gu, 2003).

In the specific context of Versova Creek in Mumbai, it receives pollution from a variety of sources. We have highlighted Cr(VI) contamination levels and depleted dissolved oxygen (DO) levels in sediments of Versova Creek in our previous report (Monga et al., 2022b). However, a knowledge gap exists, regarding bacterial strains isolated from extreme marine environments, especially those existing in oxygen-depleted sediments. We hypothesized that indigenous bacteria inhabiting such extreme conditions are likely to develop HM tolerance and are likely to have evolved genomic elements that makes them invaluable for tailor made innovative HM remediation approaches. Our primary goal was to investigate the molecular mechanisms underlying Cr(VI) detoxification in the native *Bacillus enclensis* AGM_Cr8 strain isolated from polluted sediments of Versova Creek. *Bacillus* species, are well known for their Cr tolerance and metal-reducing capabilities, particularly in marine ecosystems (Alotaibi et al., 2021). We used an integrated approach of advanced molecular imaging techniques and genomic profiling, to enhance our understanding of Cr(VI) detoxification mechanisms in AGM_Cr8, identify key genes, and elucidate possible pathways involved in Cr(VI) bioremediation. The outcomes of this research contribute to the potential applications of AGM_Cr8 in sustainable cleanup strategies for chromium-contaminated environments.

2. Materials and Methods

2.1 Sample Procurement and Media Preparation

Nine sediment samples were collected systematically from three stations with varied anthropogenic impact along the transect of Versova Creek, Mumbai. About 3 cm of the top sediment layer was collected in triplicates using a Van Veen grab sampler. For the isolation of metal tolerant bacteria, samples were pooled and stored at 4°C until further processing. The Cr(VI) levels in sediments of the creek have been published elsewhere (Monga et al., 2022b). In context of sediment pollution, determining HM concentrations is a crucial step, but the values do not directly imply its potential toxic effects to the microbial communities present in that ecosystem (di Cesare et al., 2020). To predict the impact of HMs on the native biota in an ecosystem, National Oceanic and Atmospheric Administration (NOAA) devised Sediment Quality Guidelines (SQGs) i.e. Effects Range Low (ERL) and Effects Range Median (ERM) (Burton, 2002). Cr levels in all the samples were at least two folds higher than the acceptable limits of ERL, indicating a moderate to high level of Cr contamination in the sediments (Monga et al., 2022b). Zobell Marine Agar (ZMA) from Hi-Media was used as our primary medium for isolation of Cr(VI) tolerant bacteria from the marine sediment samples. A stock solution of 10,000

mg/l Cr(VI) was prepared, filtered and stored at 4 °C. The source of Cr(VI) was potassium chromate (K₂CrO₄) salt. The salt was procured from Sigma-Aldrich, India. All chemicals used were of analytical grade and commercially available.

2.2 Isolation and Characterization of Bacteria with the Ability to Tolerate Cr(VI)

The isolation of Cr(VI) tolerant bacteria with enrichment culture technique using K₂CrO₄ as the source of Cr(VI) was previously described (Monga et al., 2022b). Briefly, the sediments were serially diluted with 0.8% saline solution up to 10⁻³, and 0.2 ml of all the dilutions prepared were spread plated on pH 7.0 ZMA plates. Chromate concentrations ranged from 12.5 to 250 mg/l initially. Plates were incubated at 37°C for 48 h. Several morphologically distinct colonies were selected and further tested on ZMA plates amended with chromate concentrations of 400 to 3200 mg/l. Isolate AGM_Cr8 showed the highest tolerance level and was thus selected for subsequent investigation.

For identification of the isolate, first DNA was isolated using Qiagen DNeasy Blood and Tissue kit following the manufacturer's protocol for cultured cells with slight modifications. Universal primers 27F and 149R were procured from Sigma and were used for PCR amplification of the 16S rRNA gene. Amplified sequences were sequenced at AgriGenome Labs Pvt Ltd. (Kochi, India) for partial 16S rRNA sequencing and were subsequently submitted to NCBI. A phylogenetic tree was then constructed using neighbour joining method in MEGA v11.0 with other known chromate resistant bacterial sequences that were retrieved from GenBank.

2.3 Optimization of Cultivation Conditions for AGM_Cr8, Evaluation of Minimum Inhibitory Concentration for Cr(VI) and Tolerance to Other Toxic Metals

The cultivation conditions for AGM_Cr8 were essentially optimised for NaCl (%), pH and Temperature (°C) to be used in all further experiments. Test and Control Zobell Marine Broth (ZMB) (Hi-Media) media was inoculated with 24h old cultures of AGM_Cr8 and varying parameters. Growth was tested at Optical Density (O.D.) 600 nm in a UV spectrophotometer (Thermo-Scientific). The growth was tested with NaCl concentrations ranging from 2% (control, unamended media) to 15% (w/v) with pH 7.0, 37°C, and 130 rpm rotation in incubator. To assess the effect of pH on bacterial growth, AGM_Cr8 cultures were incubated at 2% NaCl, 37°C, and 130 rpm in ZMB with varying pH levels (pH 5, 6, 7, 8, and 9), adjusted using 0.1N HCl and NaOH. For optimal temperature, growth was investigated by varying the incubation temperatures (20°C, 30°C, 37°C, 40°C, and 60°C), while maintaining pH at 7.0, 2% NaCl, and continuous agitation at 130 rpm.

The Minimum Inhibitory Concentration (MIC) of the bacterial isolate was described as the concentration of chromate at which no observable growth. It was tested with broth dilution method

with chromate concentrations ranging from 1250 to 5000 mg/l were prepared in ZMB (pH 7.0). Overnight grown cultures of AGM_Cr8 were inoculated in test media and incubated at 30°C for 5 days.

To evaluate the multi-metal tolerance of AGM_Cr8, a broth dilution method was employed with various metal salts: PbNO₃ (Lead nitrate), CdN₂O₆.4H₂O (Cadmium nitrate tetrahydrate), As₂O₃ (Arsenic trioxide), HgCl₂ (mercuric chloride), and NiCl₂.6H₂O (Nickel chloride). Broth media with varying test metal concentrations were incubated at 30°C for 5 days, and the MIC for each metal was determined as the concentration with no observable growth.

2.4 Characterization of AGM_Cr8 with Bio-analytical and Instrumental Methods

For Scanning electron Microscopy (SEM), samples were prepared following the methodology published by Mohapatra et al., (2019). AGM_Cr8 was cultured in ZMB media with 400 mg/l and 1000 mg/l chromate for 7 days under optimized culture conditions. Culture incubated in media without any metal was used as positive control. Negative control was media with metal but no bacterial culture. The bacterial biomass was then separated by centrifugation at 10,000 rpm for 10 minutes (at 4°C). The collected biomass was washed thrice with phosphate buffer (PBS, pH 7.4) procured from Sigma-Aldrich in order to remove the media bound to cells. Subsequently, the bacteria were pre-fixed with 2.5% glutaraldehyde for 3 hours, followed by three post-fixation PBS washes. The cells were then dehydrated with sequential treatment with 30%, 70%, and 100% (v/v) absolute ethanol and were air-dried at room temperature. The cells were mounted on carbon conductive adhesive tapes on the sample holder of the instrument and platinum coated. SEM (FEI NOVA NANOSEM 450 FESEM) and EDS facility at CSIR-National Chemical Laboratory, Pune, India, were used to analyse cellular surface morphology and elemental composition.

Furthermore, to explore cellular tolerance mechanisms the bacterial sample cultivated in 400 mg/l chromate was cut into ultrathin sections using a microtome (Leica Ultracut - UCT) for examination under a TEM (TECNAI 200 Kv TEM, Fei, Electron Optics) and STEM facility at AIIMS, New Delhi. The analysis aimed to obtain surface morphology data at 2 kV and elemental maps at 200 kV.

For the chemical speciation of Cr, X-ray photoelectron spectroscopy was carried out at CSIR-NCL, Pune, India, using the Thermo Fisher Scientific Instruments X-ray Photo Spectrometer and UPS Model equipped with K ALPHA. The instrument was calibrated using Ag3d-352eV, with a sample spot size of 400 µm and a reference for Binding energy-C1s at 284.8eV.

2.5 Effect of Cr(VI) Concentration on Bacterial Growth and Evaluation of Metal Processing Ability

To examine the effect of initial Cr(VI) concentration on growth of AGM_Cr8, we followed the methodology published by Li et al., (2021) with slight modifications. Three Cr(VI) concentrations (100 mg/l, 400 mg/l, and 1000 mg/l) and a metal free medium (control) were prepared in 250 ml Erlenmeyer flasks. All the flasks were inoculated with 1% (v/v) overnight grown bacterial culture (~OD 0.5) and were incubated for 8 days at 30°C, 130 rpm continuous shaking. At specific intervals of 2, 4, 6, and 8 days, 1 ml sample was pipetted from each flask for OD measurement at 600 nm to monitor the bacterial growth under various Cr(VI) stress conditions. The experiment was performed in triplicates.

Next, to evaluate the Cr(VI) processing ability of AGM_Cr8, we quantified Cr(VI) concentrations using a chromogenic reaction with 1,5-Diphenylcarbazide (DPC) following EPA METHOD 7196A CHROMIUM protocol. Autoclaved media in 250 ml Erlenmeyer flasks were inoculated with 1% (v/v) of an overnight-grown bacterial culture and supplemented with 50 mg/l of Cr(VI) at pH 7.0. The flasks were incubated at 30°C and 130 rpm for 8 days. After the chromogenic reaction with DPC from Tianjin Komiou Chemical Reagent Co., Ltd., the Cr(VI) removal rate was measured at 540 nm using a visible spectrophotometer, with reference to an established standard curve ($R^2 = 0.9957$). Removal percentage was calculated using the formula = $[C1 - C2]/C1 * 100\%$, where C1 and C2 represent initial and final Cr(VI) concentrations after batch culture treatment, respectively. The experiment was conducted in triplicate.

2.6 Genomic DNA Extraction, De-novo Whole-Genome Sequencing, Assembly, and Annotation

From a freshly sub-cultured ZMA plate, AGM_Cr8 colonies were picked and was cultivated overnight in ZMB at optimised growth conditions with continuous shaking. These cells were centrifuged and used for genomic DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen), following the manufacturer's protocol with slight modifications. The Nanodrop® ND-1000 Spectrophotometer (Thermo-Fisher) was used to assess the purity and concentration of the extracted DNA.

De-Novo whole-genome sequencing of the genomic DNA was carried out by D&B Genomics Pvt Ltd. (Maharashtra) using the Illumina NovaSeq 6000 platform. Library preparation was performed with the NEBNext Ultra II DNA lib preparation for Illumina Kit. Then the final library quantification was done using a Qubit 4.0 fluorometer with the DNA HS assay kit, revealing an insert size of 284. Quality checks were conducted on the paired-end whole genome sequences using FastQC (version v0.12.1). The Cutadapt 3.5 program with Python 3.10.6 was employed to remove Illumina Universal adapter

sequences. Subsequently, de novo whole-genome assembly of the clean reads was performed using SPAdes bioinformatics tool. The contigs and scaffolds generated were utilized to reconstruct the complete genome sequence. Next, to evaluate the accuracy and completeness of the reconstructed genome, QCAST (Quality Assessment Tool for Genome Assemblies) (Version: 5.0.2) was used. Finally, genome annotation was carried out using PROKKA 1.14.6.

The classification of AGM_Cr8 as a distinct species was conducted using the in-silico Type Strain Genome Server (TYGS). It analysed the genetic information of the strain for a comprehensive taxonomic assessment based on whole-genome analysis. The results of this analysis were received from TYGS on June 23rd, 2023.

2.7 Functional Annotation and Analysis of Genes Involved in Cr(VI) Resistance

The protein sequences generated through PROKKA were aligned with the Uniprot protein sequence database keeping a 65% sequence similarity criterion. Subsequently, Gene Ontology (GO) analysis was conducted to annotate and interpret gene functions, covering molecular functions, biological processes, and cellular components. Metabolic pathways were categorized based on KEGG Ortholog results obtained from the Kyoto Encyclopedia of Genes and Genomes (KEGG). There is a lack of a dedicated gene database for bacterial Cr(VI) detoxification mechanisms. Therefore, genes related to Cr resistance, typically regulated under HM stress conditions, were identified, and analysed based on AGM_Cr8 genome-encoded proteins (Chi et al., 2021). A total of 22 chromate resistance-related genes were identified.

3. Results and Discussion

3.1 Phylogenetic Analysis, Genetic Relatedness and Adaptability

The phylogenetic analysis (Fig. 1) showed a close genetic affiliation between AGM_Cr8 and other Cr(VI)-tolerant marine bacteria, including *Rossellomorea arthrocnemi* EAR8, *Bacillus cereus* b-525k, and *Bacillus haikouensis* signifies a shared evolutionary lineage among these bacteria. AGM_Cr8 exhibited a 98% genetic match with *Rossellomorea arthrocnemi* EAR8, a plant bacterium known for its metal remediation capabilities (Navarro-Torre et al., 2021). AGM_Cr8 also showed similarities to Cr(VI)-tolerant *Bacillus* sp. AKVCr04 and AKVCr05 from Versova Creek, known for their high flocculation activity in response to Cr(VI) stress in our previous research (Monga et al., 2022b). AGM_Cr8 was closely related to *Bacillus cereus* b-525k, a strain recently reported for removing 99% of Cr(VI) in a pilot study (Elahi et al., 2022). Furthermore, AGM_Cr8 displayed a 100% match in 16S rRNA with *Bacillus haikouensis*. The 16S rRNA sequences of AGM_Cr8 was submitted in NCBI as *Bacillus* sp. AGM_Cr8 under the accession number OR458310.1. The close phylogenetic

relatedness of AGM_Cr8 with other known metal-tolerant strains implies a potential conservation of functional genes related to metal tolerance.

3.2 Optimal Growth Conditions and Multi-Metal Tolerance in AGM_Cr8

Furthermore, strain AGM_Cr8 demonstrated best growth with NaCl at 2%, pH at 7.0, and incubation temperature at 30°C. Moreover, AGM_Cr8 exhibited tolerance not only to Cr(VI) but also to other HMs tested, including Pb (II) (1200 mg/l), As (III) (400 mg/l), Cd (II) (100 mg/l), and Ni (II) (100 mg/l). This enhances the practical applicability of AGM_Cr8 in future bioremediation purposes of sites contaminated with multi metals. These findings encourage for a deeper exploration of the bioremediation potential of AGM_Cr8 and its molecular detoxification mechanisms that are discussed in subsequent sections.

3.3 Cr(VI) Removal Mechanism of *Bacillus* sp. AGM_Cr8: Analytical Insights

We used SEM to visualize the impact of Cr(VI) treatment on the cellular morphology of AGM_Cr8. The untreated (control) cells (Fig 2A) displayed smooth texture without any significant deformities. On the other hand, cells treated with 400 mg/l and 1000 mg/l chromate stress (Fig. 2B and 2C) displayed partially altered and textured surfaces, deformations as compared to the normally smooth rod-shaped bacilli as seen in Fig. 2A. These irregularities could be attributed to adsorption of Cr(VI) or precipitation of reduced Cr(III) on bacterial cell surface as shown in previous studies (Liu et al. 2006; Dhal et al. 2010; Kumari et al. 2016; Bharagava and Mishra 2018). Huang et al. (2013) and Mohapatra et al. (2019) have reported similar observations likely due to diminished mechanical forces resulting from the new interactions that take place between the functional groups on the bacterial surface and metal cations.

Bacteria tolerant to HMs employ a variety of detoxification molecular mechanism in response to Cr(VI) stress (Monga et al., 2023). One of the mechanisms frequently reported involves the production of extra polymeric substances (EPSs). These matrices can sequester HMs ions on the bacterial surface as one of the primary responses of bacterial defence (Zeng et al. 2020). SEM graphs of Cr(VI) treated cells (Fig. 2C) revealed the increased secretion of EPS, that were seen as like mesh like structures and a thick layer around the cells (Fig. 2B-a and 2C-a) that could be attributed to the increased production of extracellular matrix in response to Cr(VI) stress. Sequestration of Cr(VI) ions within the bacterial extracellular structures is a defence strategy to counteract Cr(VI) and is widely reported (Sharma et al., 2022). To confirm the presence the presence of chromate ions on the surface of AGM_Cr8, we conducted EDS analysis and element mapping (Fig. S1) on selected regions. Signals corresponding to -C, -N, -O, -Na, -P, and -S were detected in both the control and treated samples (Fig. 2-a, b and c). Cr

signals were only detected in the treated samples (Fig. 2-b and 2-c; Fig. S1). Furthermore, the concentration of Cr detected in cells treated with 400 mg/l (Fig. 2-C and c) and 1000 mg/l (Fig. 2-B and b) of Cr(VI) was 0.2% weight percentage (Wt%) and 0.5% (Wt%), respectively, along with 0.06% atomic percentage (At%) and 0.14% (At%), respectively. The changes in the Wt% and At% of 'C', 'N', and 'O' on the bacterial surface following Cr(VI) treatment indicate the binding of Cr(VI) ions to functional groups and the likely bioprecipitation or adsorption/ sequestration of Cr(VI) ions on the bacterial cell surface. This elemental information obtained from EDS analysis is a crucial validation of the structural changes that were observed in SEM micrographs. Bacterial cell wall is the primary point of contact with the Cr(VI) cations before the subsequent biosorption of the metal or extracellular reduction (Wang and Chen 2009; Gutiérrez-Corona et al. 2016). Li et al. (2021) reported the isolation of a novel bacterium, *Stenotrophomonas acidaminiphila* 4-1, which secreted EPS under Cr (VI) stress, and TEM demonstrated that these EPS acted as binding sites for the adsorption of Cr (VI) on the cell surface. The adsorption process was primarily attributed on electrostatic or complexing bonds, as reported in various studies (Latha et al. 2015; Hussein et al., 2019).

Fig. 3 depicts the results from X-ray photoelectron spectroscopy that was performed to examine the valence state of Cr ions on the bacterial surface. It was performed using an Esca Lab 250Xi spectrometer (Thermo Fisher, USA), equipped with an Al anode (Al-K α excitation) as the X-ray source following the methodology outlined by Shao et al. (2019). The XPS spectra were analysed to determine the valence states of the Cr (VI) reduction products. The survey spectra (Fig. 3a) did not reveal significant Cr peaks, likely due to the relatively low concentration of metal ions in the extracellular space. The dominant peaks observed in the survey spectrum corresponded to O 1s (531.98 eV), N 1s (399.95 eV), and C 1s (285.54 eV), consistent with previous XPS survey spectra of *A. flavithermus* ABF1 (Yang et al. 2022). The high-resolution Cr 2p spectrum was deconvoluted into two distinct peaks at approximately 585.16 eV (Cr 2p 1/2) and 577.31 eV (Cr 2p 3/2), indicating the presence of Cr (III) ionic species (Shao et al., 2019). peaks at approximately 579 eV and 576 eV were attributed to Cr 2p 3/2, signifying the coexistence of Cr (VI) and Cr (III) species. This observation could be attributed to extracellular Cr (VI) reduction facilitated by living cells or reductases released from dead cells, suggesting the existence of unreacted Cr (VI) outside the cells (Fig. 3B) which has been reported previously by Li et al. 2021.

Fig. 4 illustrates the results from TEM analysis that was conducted on cells treated with 400 mg/l Cr(VI) and dense round spots were clearly observed in the cytoplasm of AGM_Cr8. This bioaccumulation within the cells could be either as Cr(VI) or after being bio reduced to Cr(III). Cr(VI) ions can pass through the cellular membranes through active sulphate channels due to their structural

similarity to sulphate anions. Cr(VI) that gets bioreduced (enzymatically or non-enzymatically) to Cr(III) then gets bioaccumulated in the cytoplasm (Karthik et al., 2017; Pushkar et al., 2021; Monga et al., 2023). Similar dense spots were reported in *Stenotrophomonas acidaminiphila* strain 4-1 (Li et al. 2021) and *Cellulosimicrobium funkei* strain AR6 (Karthik et al. 2017) after the Cr (VI) treatment.

The bioaccumulation of Cr was further confirmed by STEM analysis of a selected region on the grid (Fig 5 B, C) and element overlay analysis (Fig. 5-a, b, c). As viewed with High angle annular dark field imaging (HAADF), the distribution pattern of Cr observed in Fig. 5-a, -b, and -c closely matched that of the bacteria. These were valuable insights to understand the potential of AGM_Cr8 to internalize and bioaccumulate Cr from the external environment.

3.4 Assessment of the Growth Response of AGM_Cr8 to Cr(VI) Concentrations and Its Metal Processing Ability

The growth of strain AGM_Cr8 remained unaffected at 100 mg/l Cr(VI) but declined with increasing the concentrations to 400 mg/l and 1000 mg/l (Fig. 6). Nonetheless, the bacteria showed high tolerance to chromate stress. Additionally, we assessed the metal removal capacity of strain AGM_Cr8 using the DPC chromogenic method with a 50 mg/l Cr(VI) initial concentration. AGM_Cr8 could remove 90.50% of Cr(VI) after 5 days of incubation and Cr(VI) was not detected in the supernatant on the 8th day. Above results necessitated further investigation on the genes that were responsible for the Cr(VI) tolerance or detoxification molecular mechanisms.

3.5 De-Novo sequence analysis and Functional annotation of Cr(VI) responsive genes

The *De-Novo* sequence analysis of AGM_Cr8 revealed and constructed a genome size of ~10.2 Mb, distributed in 11,292 contigs, with an average GC content of 46.28% (Fig. 7A). We identified 8375 putative coding sequences (CDSs), 137 tRNA genes, and 22 scattered ribosomal RNA genes [Submitted under BioProject number PRJNA1052949].

The pairwise comparisons to establish the taxonomy classification of AGM_Cr8 using TYGS revealed a digital DNA-DNA hybridization (dDDH) similarity of 79.5% to *Bacillus enclensis* NCIM 5450, with a confidence interval ranging from 76.7% to 82.2%. The G+C content difference between AGM_Cr8 and *Bacillus enclensis* NCIM 5450 was 0.07%. The criteria for accurate taxonomic assignment using dDDH require a threshold of $\geq 70\%$, and the G+C content difference within a species should not exceed 1% for correct species classification. Consequently, we concluded that isolate AGM_Cr8 can be classified as a member of the species *Bacillus enclensis*. Twenty-Two chromate responsive genes were identified in the genome of AGM_Cr8. We propose the following molecular mechanisms/

pathway that could be employed by AGM_Cr8 Cr(VI) in the detoxification and tolerance against chromate stress (Fig. 7B):

a) **Chromate Transmembrane efflux**

srpc1 and *srpc2* are genes encoding putative chromate transporter proteins SrpC1 and SrpC2 identified in the genome of AGM_Cr8. These transporters are likely to be involved in efflux or expelling Cr(VI) ions from the cytoplasm preventing intracellular accumulation (Nicholson and Laudenbach 1995; Nies et al. 1998; Aguilar-Barajas et al. 2011; Walter et al., 2020). The discovery of the *srpC* gene, a homolog of the CHR chromate ion transporter superfamily, remained unseen until 2011 (Aguilar-Barajas et al., 2011). Intriguingly, *srpC* was found to be situated on a plasmid-coded island enriched with sulphur-related genes regulated by sulphate. Members of the CHR superfamily have been shown to confer chromate resistance by acting as chromate transporters (Ramírez-Díaz et al., 2008). Plasmid DNA-encoded efflux systems play a vital role in expelling intracellular chromates from the bacterial cell, thereby contributing to resistance against chromate toxicity (Ahemad et al., 2015).

b) **Chromate Reduction**

Cr contamination is primarily due to anthropogenic inputs, thus the chromate reduction mechanisms in microbes may not have evolved specifically for chromate, but more likely a part of generalized electron transport systems or enzymes with other metabolic functions (Ramírez-Díaz et al., 2008; Bhattacharya et al., 2015). The genome analysis of AGM_Cr8 identified a putative NAD(P)H-dependent FMN-containing oxidoreductase (*ywqN*), NADH quinone reductase, and ornithine aminotransferase (*rocD1*, *rocD2*). Protein YwqN exhibited amino acid similarity to chromate reductases YhdA, ChrR, and YieF, and demonstrated azoreductase activity. However, in the study on *Bacillus subtilis*, YwqN did not catalyse chromate reduction (Valenzuela-García et al., 2020). The authors proposed the involvement of multiple NADPH-dependent two-electron transfer oxidoreductases to counter the oxidative stress resulting from subsequent enzymatic reactions generating semiquinone intermediates. In the presence of O₂, chromate reduction becomes a two- to three step process with the formation of transient species such as Cr(V) and/or Cr(IV) in between before getting reduced to a stable end product Cr(III). In this scenario, electron donors are NADH, NADPH and those from the endogenous reserves (Monga et al., 2023). Aerobic chromate reduction typically takes in cytoplasm and is mostly carried out by soluble enzymes such as flavoprotein, dehydrogenase, NADH-dependent nitroreductase, and azoreductase (Chai et al., 2019; Monga et al., 2023). In the absence O₂, Cr(VI) acts as a terminal electron acceptor in the respiratory chain for a wide range of electron donors such as proteins, fats, carbohydrates, hydrogen, and NAD(P)H (Cheung and

Gu, 2007; Monga et al., 2023). By utilizing alternative electron acceptors, AGM_Cr8 may sustain redox reactions crucial for chromate detoxification under anoxic conditions.

c) DNA Repair and Stress Response

DNA repair protein encoding genes *recF* and *recN* identified in the genome of AGM_Cr8 could be crucial components against chromate stress response. RecF is associated with DNA replication and repair, while RecN is involved in DNA repair processes. These genes were overexpressed in metal tolerant *Deinococcus indicus* in response to HM stress (Dhanapal et al., 2021). *rocD*, an ornithine aminotransferase encoding gene, is typically involved in amino acid metabolism. In the context of chromate detoxification, its presence might indicate a link between amino acid metabolism and cellular homeostasis under chromate stress (Ginguay et al., 2017). Additionally, genes involved in DNA mismatch repair (*mutH*), *gltA2* (encoding citrate synthase), superoxide dismutase, ABC transporter genes (*natA*, *natB*, *ytrB*, and their homologs), transporter permease *natB*, and inner membrane permease proteins *ycdV* and *yeyB* were also found in the genome of AGM_Cr8. HMs can participate in Fenton-like reactions, generating ROS and leading to oxidative stress (Genchi et al., 2021). Superoxide dismutase (SOD) with antioxidant abilities can directly mitigate oxidative stress and chromium toxicity (Elahi et al., 2019). The gene *gltA2*, encoding an alternative citrate synthase, was upregulated, and reported for the first time in association with chromate resistance in *Klebsiella* sp., along with genes encoding stress response, DNA repair and replication, sulphur starvation, and energy metabolism (Wei et al., 2018).

4. Conclusion

In conclusion, we comprehensively investigated into *Bacillus enclensis* AGM_Cr8, a robust indigenous bacterium with exceptional tolerance to Cr(VI). The study focussed on the phylogenetic analysis and the close relationship of AGM_Cr8 to other known Cr(VI) tolerant marine bacteria, optimising growth conditions, multi-metal tolerance and chromate processing ability. With advanced and sophisticated techniques such as SEM-EDS, XPS, TEM and STEM-HAADF, we elucidated the structural alterations, elemental composition, EPS mediated responses and intracellular accumulation of Cr within the cells cultivated under Cr(VI) stress. The previously unrecognized ability of *Bacillus enclensis* AGM_Cr8 to coexist with both Cr (VI) and Cr (III) species suggests the presence of a potential bioreduction mechanism within a multifaceted approach that includes, bioaccumulation, bioreduction and chromate efflux. From twenty-two Cr responsive genes that we identified in the genome of AGM_Cr8, we proposed a chromate detoxification pathway that involves an interplay of chromate transporters, enzymatic reduction of Cr(VI) to Cr(III), DNA repair and role of antioxidants

in response to chromate stress. We highlighted the adaptability of AGM_Cr8 in the context of anoxic conditions and potential evolution of molecular mechanisms to fight chromate stress under depleted oxygen conditions. Our integrated approach, combining advanced analytical studies with genomic profiling, offers a holistic understanding of AGM_Cr8 for its adaptation to chromate stress. This approach bridges the gap between phenotype and genotype, providing valuable insights into metal-microbe interactions.

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Availability of data and material

Sequence data for 16S rRNA sequencing for AGM_Cr8 is openly available in GenBank under accession number OR458310.1. The whole-genome sequencing data of *Bacillus enclensis* AGM_Cr8 is available under the NCBI BioProject number PRJNA1052949.

Competing Interest Declaration

No, the authors have no competing interests as defined by Springer, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

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Figure Captions

Fig. 1: Phylogenetic analysis of AGM_Cr8, isolated from Versova Creek sediments, compared to known marine Cr(VI)-tolerant bacteria.

Fig. 2: SEM micrographs of AGM_Cr8 under varying Cr(VI) stress conditions: (A) absence of metal treatment (control), (B) exposure to 1000 mg/l Cr(VI), and (C) exposure to 400 mg/l Cr(VI). Additionally, Energy Dispersive Spectroscopy (EDS) analysis is shown for each condition.

Fig. 3: Left: X-Ray photoelectron spectroscopy (XPS) survey, and Right: Cr 2p XPS Spectrum of AGM_Cr8 treated with 400 mg/l Cr(VI) for 5 days.

Fig. 4: Transmission Electron Microscopy (TEM) analysis of AGM_Cr8 treated with 400 mg/l Cr(VI).

Fig. 5: Scanning-Transmission Electron Microscopy (STEM-HAADF) analysis of selected areas in strain AGM_Cr8 cultivated in the presence of 400 mg/l Cr(VI) and Element Overlay Analysis.

Fig. 6: The effect of various initial Cr(VI) concentrations on the growth of AGM_Cr8.

Fig. 7(A): Genome circular map representing the GC skew, GC content and location of the genes involved in bacterial Cr(VI) detoxification process; **(B)** Possible Cr(VI) detoxification mechanisms of *Bacillus enclensis* AGM_Cr8 as follows: (1) Enhanced secretion of extracellular polymeric substances (EPSs) under Cr(VI) stress facilitates the binding of chromate ions; (2) Chromosome-encoded sulphate ion channels facilitate the entry and bioaccumulation (3) of Cr(VI) ions. (4) Plasmid-encoded chromate transporter proteins, SrpC1 and SrpC2, induce the efflux of Cr(VI) ions from the cell. (5) Intracellular and (6) and extracellular reduction of Cr(VI) to Cr(III) is accomplished via NADH-dependent quinone reductases; (7) Internalized Cr(VI) ions lead to the generation of reactive oxygen species (ROS) causing DNA damage; (8) ROS generation induces oxidative stress, prompting the expression of antioxidants, such as Superoxide dismutase (9), in response to oxidative stress; (10) in addition to induced expression of DNA replication and repair genes.

Fig. S1: EDS Element mapping showing the presence of Cr on the surface of AGM_Cr8 cells treated under 400 mg/l Cr(VI) stress.

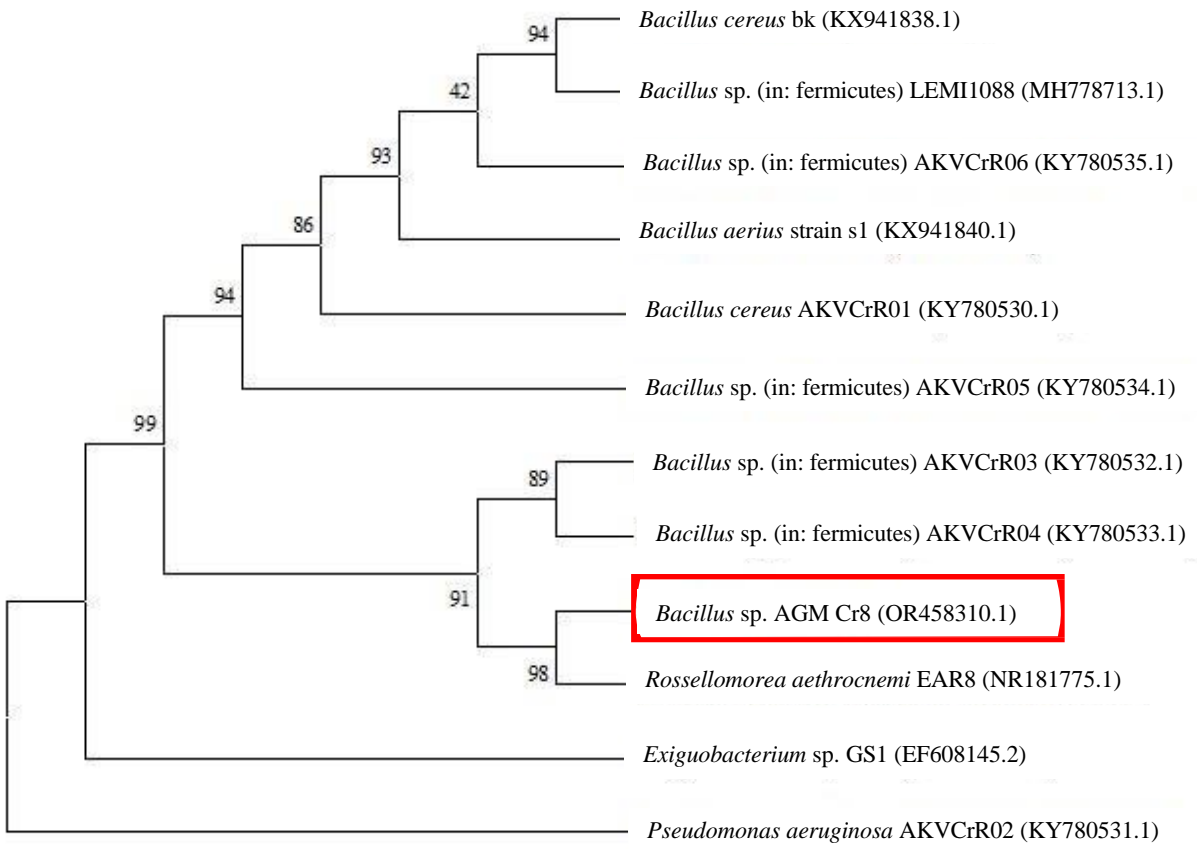


Fig. 1

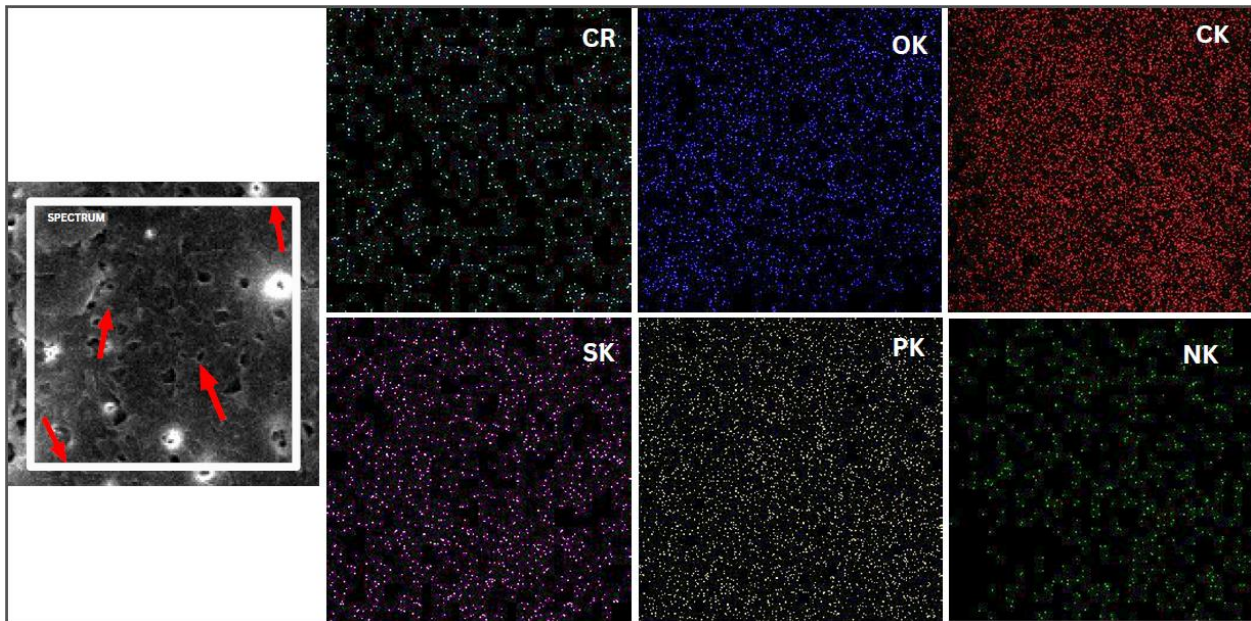


Fig. 2

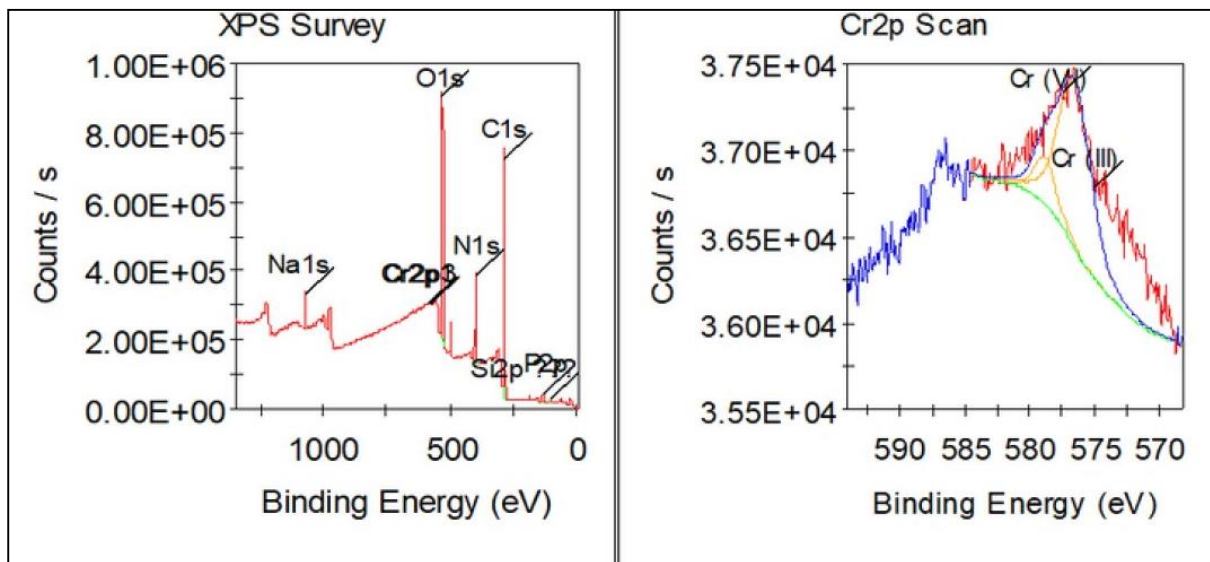


Fig. 3

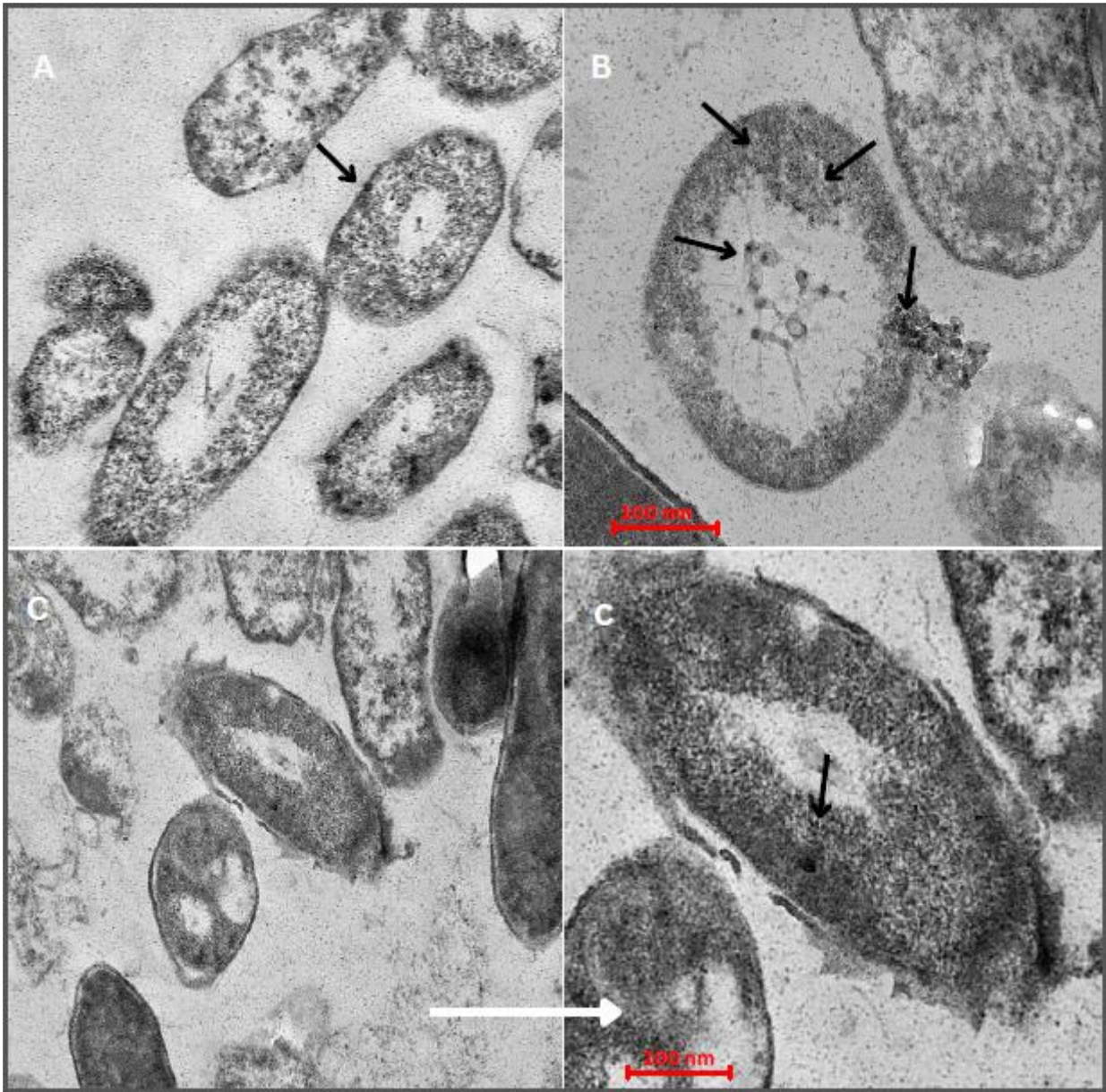
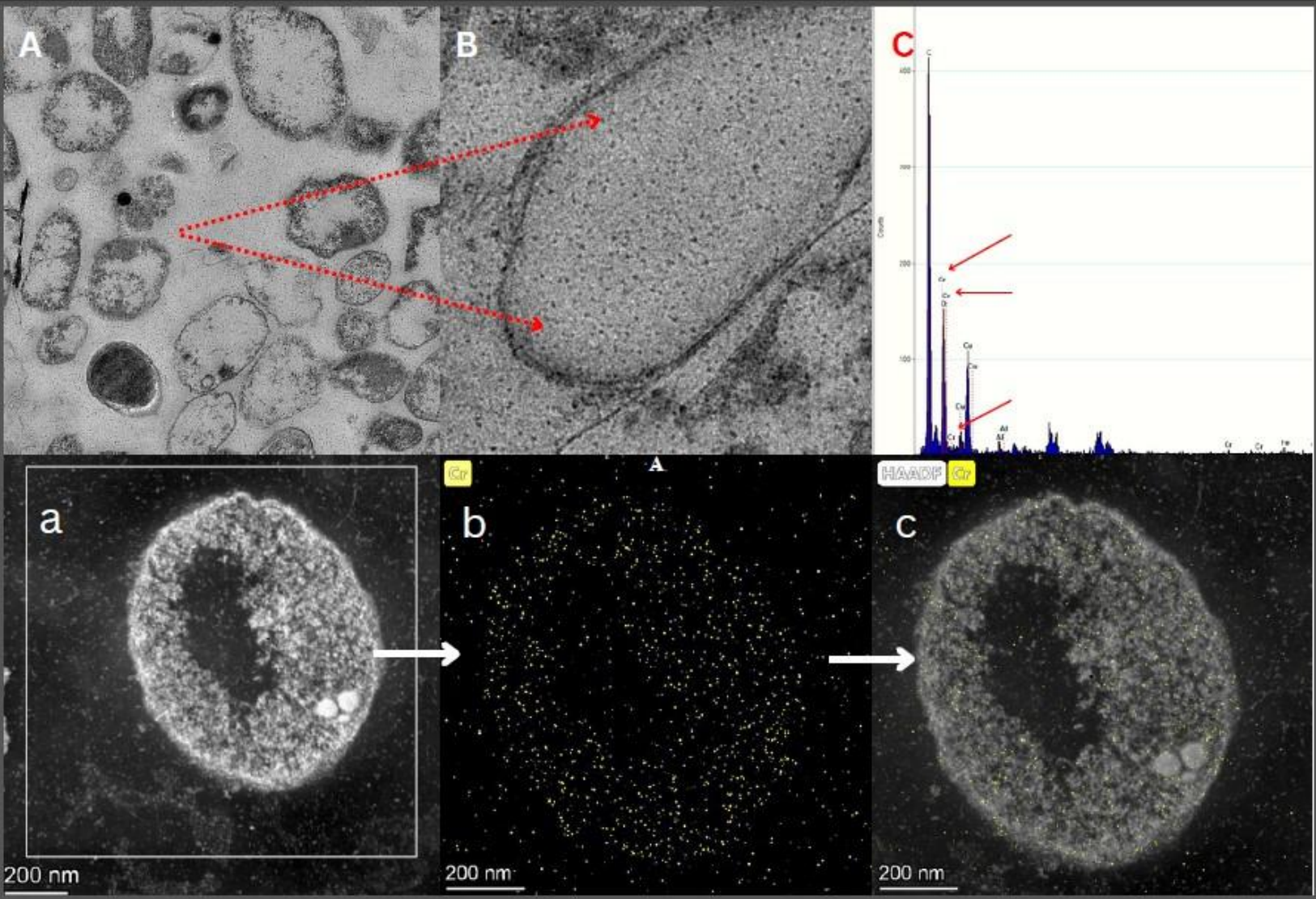


Fig 4

FIG. 5



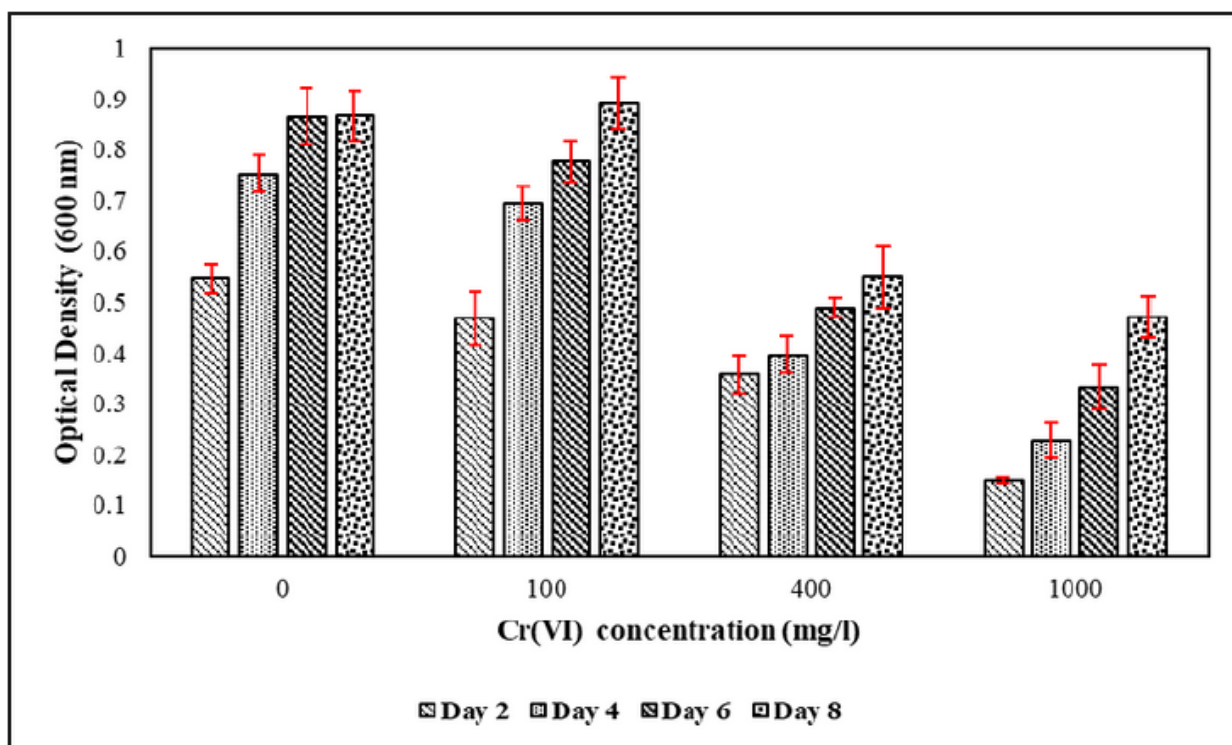


Fig. 6

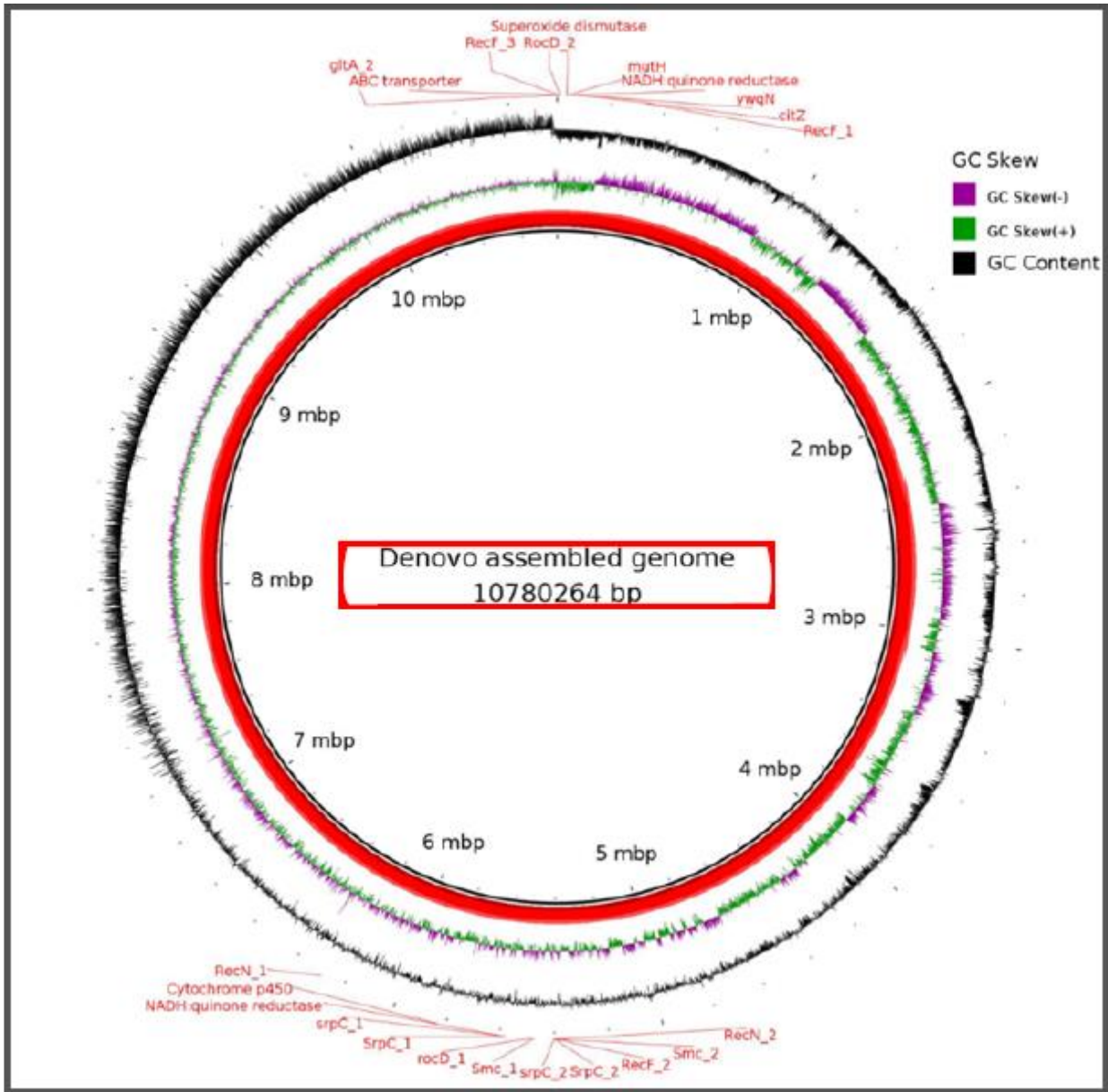


Fig. 7A

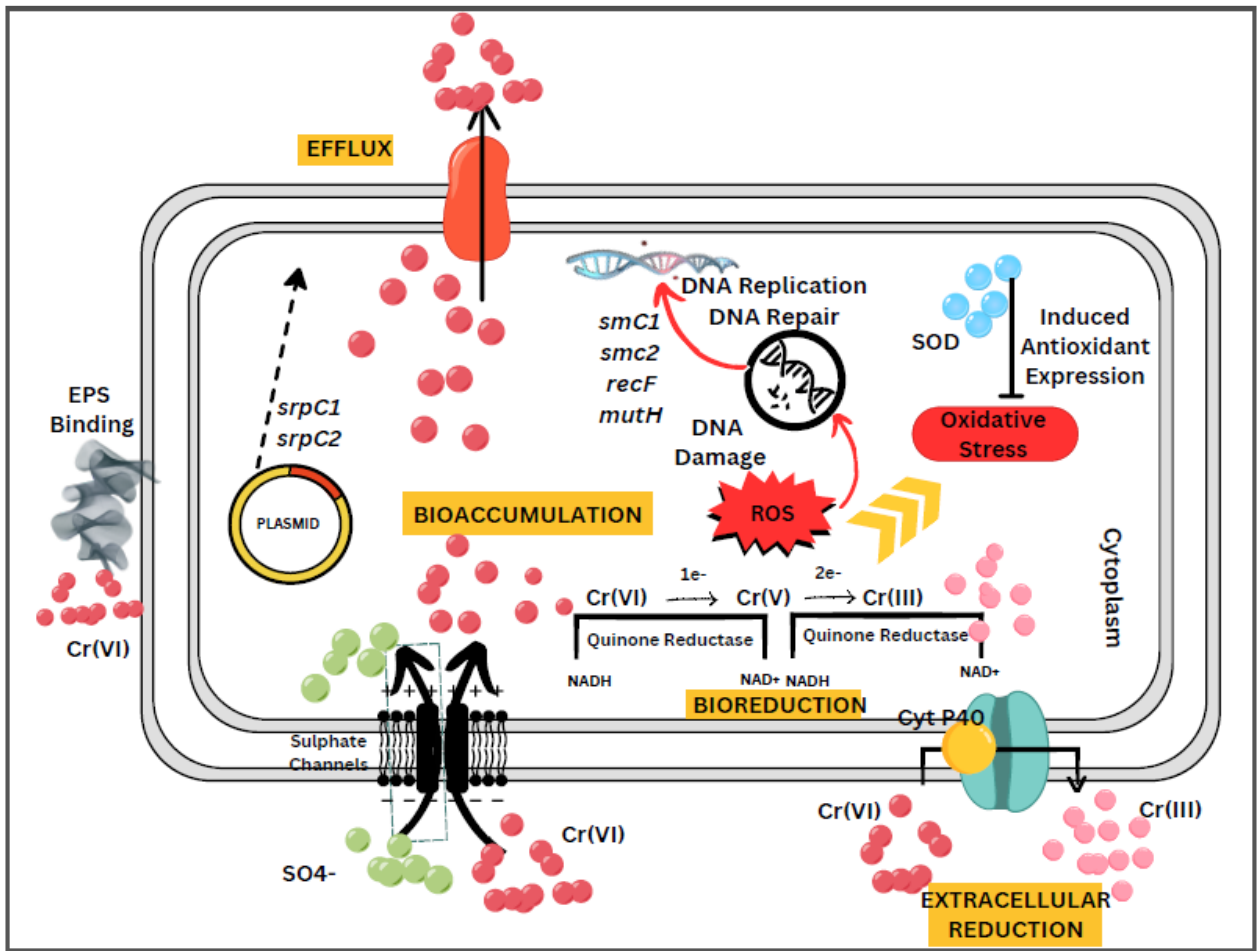


Fig. 7B

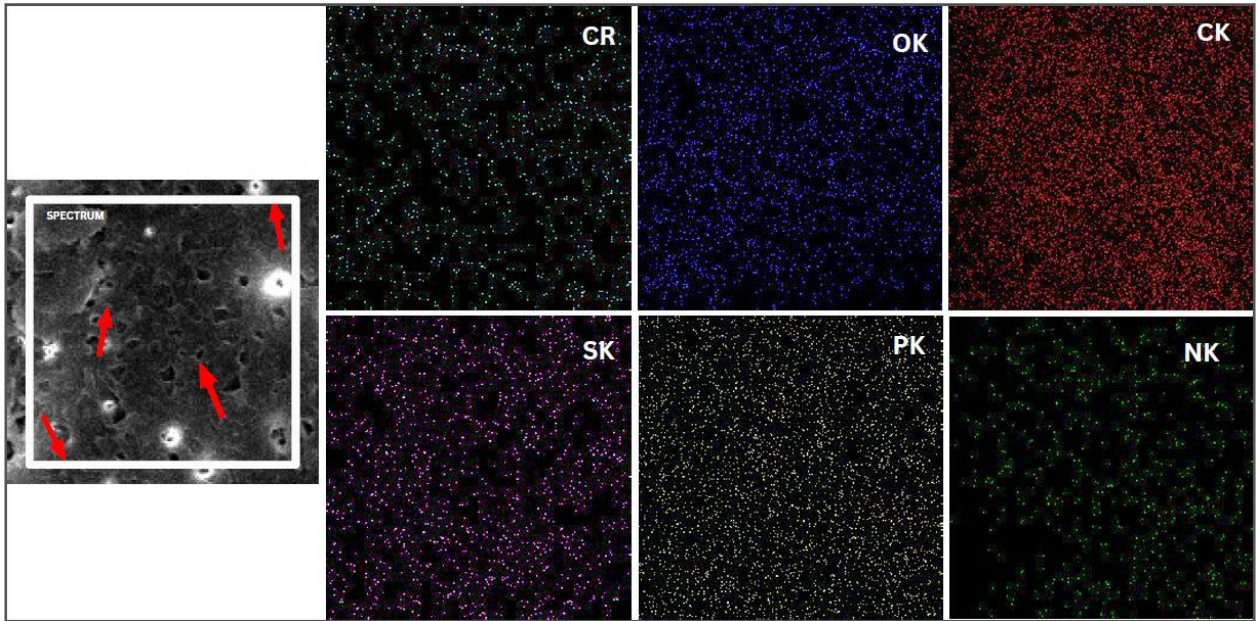


Fig. S1