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Full Length Research Paper

Computational approach for comparative phylogenetic analysis of isolated chromium resistant strain *Brevibacterium casei*

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The isolated (Cr [VI]) resistant bacterial strain was identified as *Brevibacterium casei* by 16S rRNA sequencing (Genbank Accession Number: EU781952). The generated sequenced data was used for construction of Phylogenetic tree (MEGA 3.1and clustalw2) and predict the two dimensional alignment of the highly conserved regions. Computational comparative 16S rRNA sequence analysis of the isolated strain with different chromium resistant strains was carried out .The result shows that the compared sequence differs from the other especially in the region of 210 to 242, 440 to 480 nucleotides and had higher resemblance from 245 to 440, 500 to 724 in nucleotide base pair (in the *Escherichia coli* numbering system). From the overall study it can be predicted that the conserved region in the nucleotide base pair in all Cr (VI) resistant species may play an important role in chromium resistance.

Key words: Hexavalent chromium, 16S rRNA sequence, *Brevibacterium casei*, computational approach, phylogenetic analysis.

INTRODUCTION

RNA functions as an information carrier, catalyst and regulatory element, perhaps reflecting its importance in the earliest stages of evolution (Gesteland et al., 2005). The 16s rDNA sequence has hypervariable regions, where sequences have diverged over evolutionary time. These are often flanked by strongly-conserved regions. Primers are designed to bind to conserved regions and amplify variable regions. The DNA sequence of the16S rDNA gene has been determined for an extremely large number of species. In fact, there is no other gene that has been as well characterized in as many species. Sequences from tens of thousands of clinical and environmental isolates are available over the internet through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and the Ribosomal Database Project (www.cme.msu.edu/RDP/html/index.html)(Newman et al.,1999).These sites also provide search algorithms to compare new sequences to their database. The structures of RNAs provide insight into the mechanisms behind these functions. Determining sequence is the first step in determining structure. The second step is determining secondary structure and relatively few classes of RNAs currently have known secondary structures (Chen et al., 1999). Even fewer classes of RNAs have known three-dimensional structures. In principle, computational methods can predict both secondary and three-dimensional structure. Secondary structure prediction provides a foundation for the prediction of three-dimensional structure. The accuracy of structure prediction is significantly improved by predicting a secondary structure common to multiple sequences. Several software programs that use multiple sequences were recently benchmarked for speed and accuracy with the same data set. Three computational approaches might be taken to find a secondary structure common to two or more sequences. The most rigorous and computationally expensive approach is to simultaneously align and fold the sequences, with either dynamic programming or genetic algorithms. The least computationally expensive approach is to find the secondary structure of sequences that have been previously aligned. The accuracy of this latter approach is

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limited by the inherent limitations of sequence alignment and, therefore, user intervention is often required to refine predictions. A middle road has also been explored, whereby a set of secondary structures is determined for each sequence separately and these are post-processed to find the best scoring structure common to all sequences. The program RNAcast (Reeder et al., 2005) fits into this category and builds on recent work defining 'abstract shapes' (Giegerich et al., 2004; Gardner et al., 2004). An abstract shape is a representation of RNA secondary structure that displays the branching pattern of the helices (Sankoff et al., 1985). The main objective of our work is to correlate the sequence similarity among the chromium resistance strains and to predict their secondary RNA structures.

MATERIALS AND METHODS

Bacterial strains isolation and growth conditions

Hexavalent chromium resistant bacterial culture isolated from a chromium contaminated soil of sukinda, was selected for its high ability to both tolerate and reduce hexavalent chromium Cr(VI) to less-toxic trivalent chromium Cr (III). Four strains used in this study were originally isolated by Mr. A.P. Das from sukind chromite mines, jajpur, India. Bacterial strains, resistant to Cr (VI), were isolated from the soil using the serial dilution technique in PYE medium (peptone, yeast extract). Agar supplemented with 100 g Cr (VI)/ml as $K_2Cr_2O_7$ and 0.5% (wt/vol) dextrose served as carbon source. The pH was maintained at 7±0.2 by using HCl or NaOH. The isolates are tested for their chromate tolerance at different concentrations (12.5, 25, 50, 75, 100 µl/ml) of hexavalent chromium supplemented as $K_2Cr_2O_7$. Significant growth of the specific bacterial species in the presence of 100 mg Cr(VI)/l in PYE medium during two-day incubation at 30°C, were considered as Cr(VI) resistant. A single strain was capable of growing at a maximum concentration of 500 ppm, optimum temperature and pH being 30°C and 7 respectively for maximum chromium reduction and was selected for further experiments. The results of the study indicated removal of more than 97% chromium by the selected species determined by diphenylcarbazide colorimetric assay.

Cr(VI) analysis

Chromate-reducing activity was estimated as the decrease in chromate concentration in supernatant with time using the Cr(VI)-specific colorimetric reagent 1,5-diphenylcarbazide (DPC), prepared in acetone/H_2SO_4 to minimize deterioration (Urone, 1955) as follows: DPC (0.025 g) was dissolved in 9.67 ml acetone (AR) and 330 µl of 3 M H_2SO_4 was added. The reaction mixture was set up in an Eppendorf tube containing the following:

200 µl sample or standard sodium chromate solution, 400 µl 20 mM MOPS-NaOH buffer pH 7.0, 33 µl 3 M H_2SO_4, 40 µl 0.25% (w/v) DPC, and 327 µl distilled water.

Spectrophotometric measurements were made immediately at 540 nm.

Preliminary screening for Cr (VI)-reducing activity

Strain was grown aerobically in PYE at 30°C overnight. For anaerobic Cr(VI) reduction tests, 45 ml of PYE in 50 ml serum bottles was degassed with O_2-free N_2 (10 to 20 min). Each bottle was inoculated using a syringe with 10% (v/v) of the starter culture and incubated statically at 30°C. Potassium dichromate (100 µM) was added after 2 h. Samples (1 ml) were withdrawn periodically and the bacterial density was determined as OD 600 prior to harvest by centrifugation at room temperature. The cell pellet was suspended in 1 ml of isotonic saline [0.85% (w/v) NaCl] for protein assay and the supernatant was assayed for residual Cr(VI).

Identification of selected strain

Gram staining of bacterial strain was carried out using established methods (Collins and Patricia, 1984). For PCR amplification, a small amount of a bacterial colony was resuspended in 100 µl of sterile deionised water (SDW), mixed and lysed at 70°C (10 min). Crude lysate (0.2 µl) was added to 19.8 µl SDW and used as a PCR template. Universal bacterial 16S rDNA gene primers pA (5′-AGAGTTTGATCCTGGCTCAG-3′) and pH′ (5′-AAGGAGGTGATCCAGCCGCA- 3′) were used to amplify the ~1.5 kb 16S rDNA gene fragment (Edwards et al., 1989). Sequence data was aligned and analyzed for finding the closest homology for the microbe.

Taxon sampling

Partial nucleotide sequences were obtained from prior phylogenetic submission of chromium resistant strains (GenBank Accession Nos. EU275362 (*Staphylococcus* sp.), EU275361 (*Shewanella* sp.), EU275360 (*Rahnella* sp.), EU275357 (*Pantoea agglomerans* strain), EF692531 (*Stenotrophomonas*)

Comparison of rRNAs from various species

A phylogenetic comparison is made of the 16S-like rRNAs of our isolated species *Brevibacterium casei* (GenBank Accession Number: EU781952), to prior phylogenetic submission of chromium resistant strains (GenBank Accession Nos. EU275362 (*Staphylococcus* sp.), EU275361 (*Shewanella* sp.), EU275360 (*Rahnella* sp.), EU275357 (*Pantoea agglomerans* strain), EF692531 (*Stenotrophomonas* sp.)..

RESULTS AND DISCUSSION

Cr(VI)-reducing activity of isolates

The Cr(VI)-reducing activity of the isolate was investigated. Strains APD15, was selected for further study, with a maxi-mum removal of 97% of the Cr(VI). The bacterium was Gram-positive, non spore-forming rods. On PYE agar, colonies were 1 to 3 mm in diameter, circular, low convex with an entire margin, opaque, and moist.

Biomass growth and Cr(VI) reduction by strain APD15

The inoculum of the bacterial strains cultured overnight was used for this experiment. Culture flasks (150 ml) with

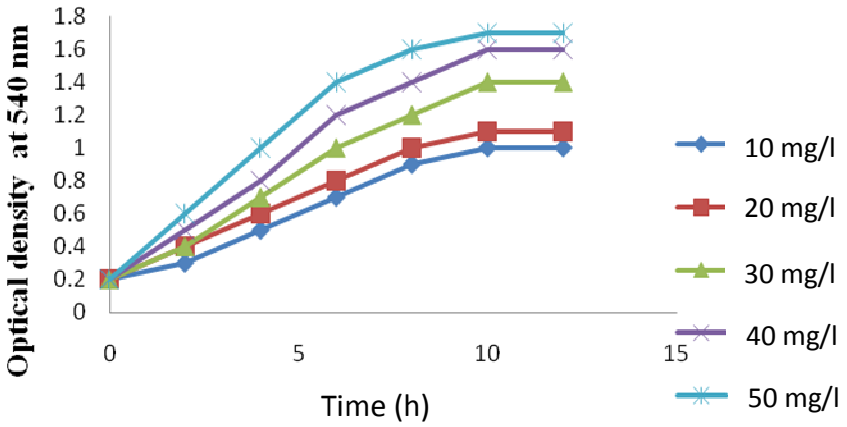

Figure 1. Biomass growth at varying Cr (VI) concentration using optimum conditions: initial pH-7, temp-300℃, agitation-200rpm, inoculum volume-1.5 ml, inoculum age-12 h.

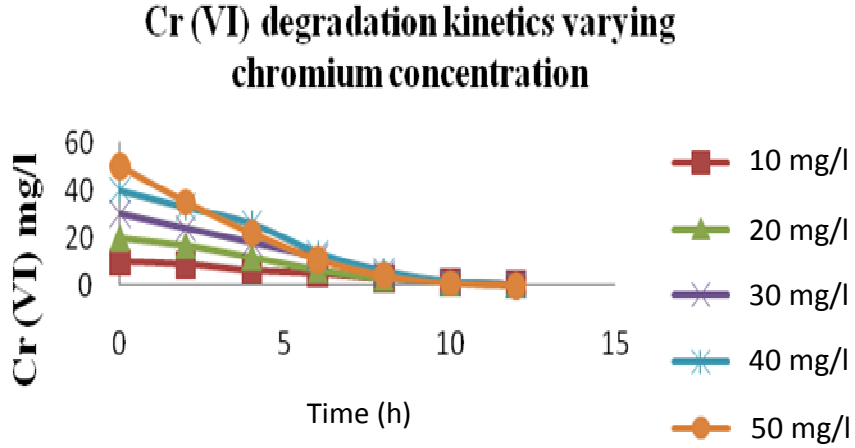

Figure 2. Cr(VI) reduction kinetics at varying Cr(VI) concentration using optimum conditions: Initial pH-7, temp-300℃, agitation-200 rpm, inoculum volume-1.5 ml, inoculum age-12 h.

a final volume of 100 ml supplemented with (10 to 50 mg/L) of Cr(VI) were inoculated with 2 ml of inoculums for 24 h. The growth kinetics of bacteria is characterized as initial lag phase, second exponential phase, stationary phase and death phase. In this experiment it is observed that lag phase is increasing with increased initial Cr(VI) concentration (Figures 1 and 2). It is basically due to inhibitory effect of higher chromium concentration on the growth of the organism. Each organism has a specific resistance at a specified growth condition. As the initial age of the inoculum was fixed at 24 h the acclimatization period at varying chromium concentration will not remain same. Hence the following behavior is observed. The chromium-resistant bacteria isolate exhibited reduced bioaccumulation when cells were in stationary phase. At higher concentrations the growth of the bacteria is inhibited due to fixed amount of inoculum for all the different concentration of Cr(VI) considered in the experiment.

16SrRNA based identification

By the use of internal primers, 1.5 kb sequence of amplified 16S rRNA gene fragment was determined. Analysis of the 16S rRNA from the isolate revealed that it was *B. casei* (GenBank Accession Number: EU781952), with nearest homolog species was found to be *Brevibacterium sanguinis*. A phylogenetic tree was made in Mega 3.1 software.

The 16S rDNA nucleotide sequences from NCBI genbank were compared with known sequences in the EMBL database using ClustalW2 to identify the most similar sequence and Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 Parameter) (Figure 3).

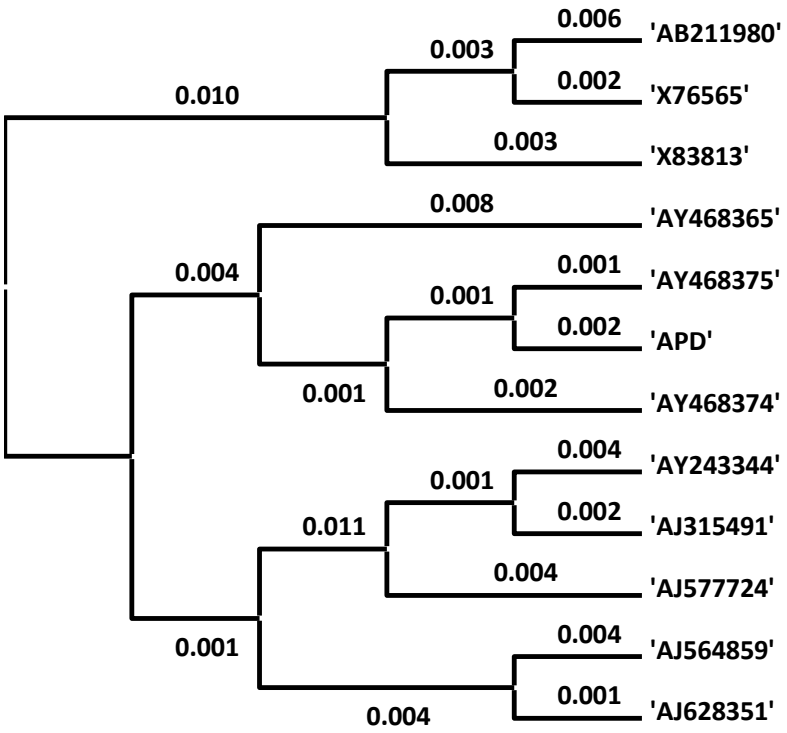

Figure 3. Phylogenetic Tree made in MEGA 3.1 software using Neighbour Joining method.

Secondary structural characterization

We use Insilco based programming software's CLC RNA Workbench to determine "secondary structure annotation, atomic composition, nucleotide distribution and counts of dinucleotide" of our bacterial sequence. 16SrRNA of *B. casei* contains bulge-11,hairpin loop-15,interior loop-12,multi loop-11,region-10,stem-26 and atomic composition phosphorous(p)-689(0.031), carbon(c)-6.736(0.299), nitrogen(N)-2.735(0.121), oxygen(O)-3.978(0.176), hydrogen(H) 8.404(0.373) (Figure 6).

Multiple alignments

The reconstruction of phylogenetic history is predicated on being able to accurately establish hypotheses of character homology, which involves sequence alignment for studies based on molecular sequence data. In an empirical study investigating nucleotide sequence alignment, Mathews et al. (2002) and Gorodkin et al. (1997) inferred phylogenetic trees for 20 species *Enterobacter amnigenus (E*U275356*), Comamonas* sp. *(E*U275355*), Bacillus pumilu (E*U275354*), Bacillus cereus (E*U275353*), Acinetobacter johnsonii (E*U275352), *Achromobacter xylosoxidans (E*U275351*), Bacillus* sp. *(E*U236673*), Bacillus* sp. *(E*U236676*), Bacillus* sp. *(E*U236677*), Bacillus* sp. *(E*U236675*), Bacillus* sp. *(F*J178872*), "B. casei (E*U781952*)"*, *Staphylococcus* sp. *(E*U275362*), Shewanella* sp. *(E*U275361*), Rahnella* sp. *(E*U275360*), Pantoea agglomerans* sp. *(E*U275357*), Stenotrophomonas* sp. *(E*F692531*), Delftia* sp. *(E*F692532*), Pseudomonas stutzeri (E*U275359*), Pseudomonasstutzeri (E*U275358), *Stenotrophomonas* sp. *(E*F692531*)* based on complete small-subunit rDNA sequences, using a single multiple-alignment procedure: manual alignment based on the secondary structure of the 16s rRNA molecule, and automated similarity-based alignment algorithms using the ClustalW computer programme (Figures 4 and 5).

Secondary structure alignment

The secondary structure alignment from 230 to 427 and 509 to 628, was done on CastP based on the template *B. casei* (Accession Number: EU781952**)** with five highly similar sequence taken from NCBI (http://bibiserv.techfak.uni-bielefeld.de/rnacast/).

DISCUSSION

In this study, a newly isolated Cr(VI) reducing bacterium was identified as a *Brevibacterium* sp. This bacterium reduced Cr(VI) anaerobically at the expense of peptone and yeast extract as the source for growth. However, *Brevibacterium* sp. APD15 is unusual because it is

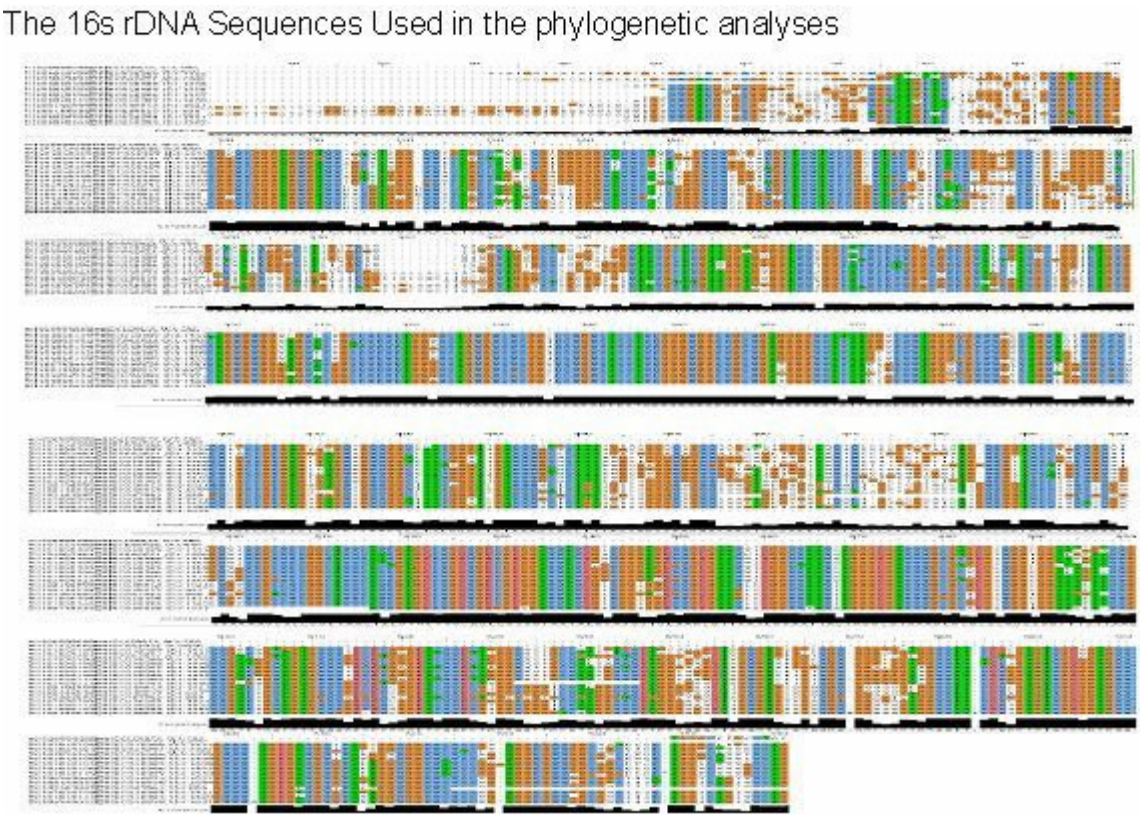

Figure 4. Comparison of *Brevibacterium casei* 16S rRNA nucleotide sequence with those of other identical nucleotide with that of *B. casei.* Using clustalw2 (BLOSOOM Parameter).

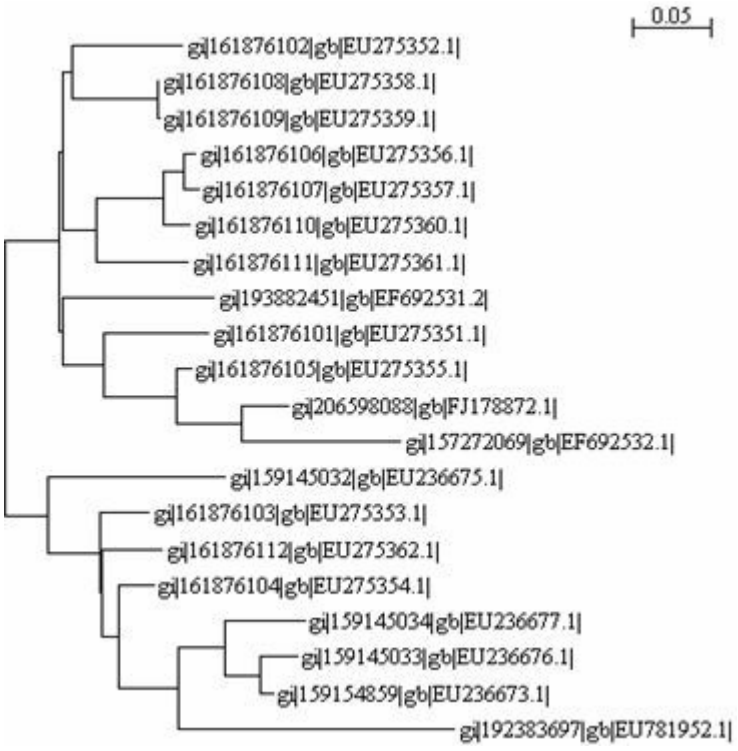

Figure 5. Phylogenetic tree of *B.casei* with similar sequence from NCBI genbank.

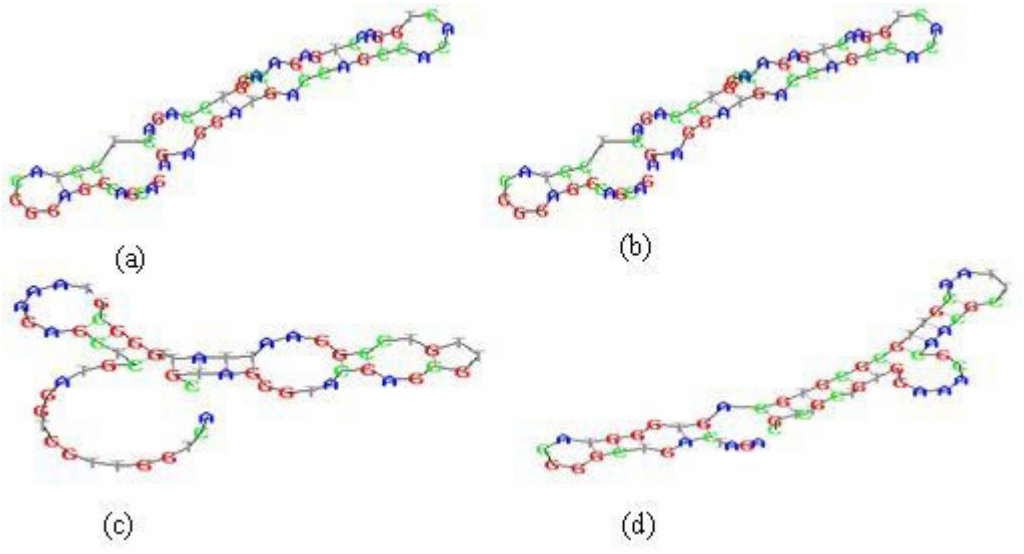

Figure 6. (a)Secondary structural alignment of region from 230- 290 based on our sequence, (b) Secondary structural alignment of region from 365- 427 based on our sequence, (c) Secondary structural alignment of region from 509-568 based on our sequence, (d) Secondary structural Alignment of region from 568-628 based on our sequence.

resistant to Cr(VI) during growth while being able to reduce Cr(VI). A striking similarity in secondary structure emerges. Remarkably, these secondary structures are similar despite the fact that the nucleotide sequences of these rRNAs themselves exhibit a low degree of similarity. Apparently, evolution is acting at the level of rRNA secondary structure, not rRNA nucleotide sequence. Similar conserved folding patterns are seen for the 23S-like and 5S-like rRNAs that reside in the large ribosomal subunits of various species. An insightful conclusion may be drawn regarding the persistence of such strong secondary structure conservation despite the millennia that have passed since these organisms diverged. These experiments concluded that the cells have an elevated ability to reduce Cr(VI) up to 97% coupled with resistance of the Putative metal reductase to toxic chromate and the Cr(III) product. These findings are potentially useful because this bacterium could be harnessed to the detoxification of chromate-contaminated industrial and mining waste by growth in the waste solution aerobically followed by an anaerobic reductive step, with the potential for biomass regeneration in a second aerobic cycle. This potential, and the resistance mechanism, are the subject of ongoing studies.

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