Iran. J. Biotechnol. October 2022;20(4): e2818 DOI: 10.30498/ijb.2022.240607.2818

Research Article

A Cross-section Metagenomics and 16S Ribosomal DNA Based Evaluation of the Bacterial and Archaeal Communities Resident in the Forumad Chromite Mine, Northeastern of Iran

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Background: The Forumad chromite area from Sabzevar ophiolite belt, Northeastern Iran, is an environment with high concentration of heavy metals, particularly chromite and magnesite minerals, containing chromium and magnesium.

Objectives: In this study for the first time, we analyzed and report the diversity of microbial (bacterial and archaeal) community inhabiting in Forumad chromite mine environment using metagenomics approach.

Materials and Methods: Samples were obtained from different areas of the mine, and total DNA was extracted from water and soil samples. 16S rDNA was amplified using universal primers and the PCR products were cloned in pTz57R/T plasmid. Then, 43% of the positive clones were randomly sequenced. BLAST program in NCBI and EzTaxon databases were used to identify similar 16S rDNA sequences. Phylogenetic analysis was performed using the MEGA5 software and multiple alignments of sequences.

Results: In the phylogenetic analyses, *proteobacteria,* which contains many heavy metals tolerant bacteria especially chromium, were the dominant population in bacterial libraries with *Rheinheimera* and *Cedecae*as the most abundant genuses. Other phyla were *Bacteroidetes, Firmicutes, Verrucomicrobia, Chloroflexi, Actinobacteria, Acidobacteria, Cyanobacteria, Gemmatimonadetes,* and *Planctomycetes.* In the archaeal clone library, all the sequences were related to the phylum *Thaumarchaeota*. Further, 68.6% of the sequences had less than 98.7% similarity with the recorded strains which could represent new taxons.

Conclusions: The results showed that there was a high microbial diversity in the Forumad chromite area. These results can be used for detoxification and bioremediation of regions contaminated with heavy metals, although more studies are needed.

Keywords: 16S rRNA, Forumad chromite mine, Metagenomics, Microbial diversity

1. Background

Biological diversity, commonly known as biodiversity, is variety of life on the Earth. Biodiversity is recognized as the main factor affecting ecosystem performance. Microbial diversity is related to material cycling, biogeochemical processes, ecosystem stability and productivity. Ecosystem biodiversity is critical for its sustainability and better exploitation of ecosystem potentials (1). Therefore, it is necessary to realize how microbial diversity is related to the community structure and its function (2-4). Microorganisms that live in the soil are of particular importance because represent the

largest pool of biodiversity on Earth (4, 5). Bacteria are the source of many different biological processes and metabolites that can significantly affect the ecosystem. Variety in these processes and products confirm bacterial genetic diversity. Therefore, the systematic study of bacteria can make significant progress in understanding of the metabolic processes which can be used to improve environmental and living conditions (6). In this regard, metal mines, due to their extreme environmental conditions, are generally considered as attractive resources for microbial diversity studies. Mine indigenous microorganisms, which are alive under these conditions, may have suitable applications in biotechnology and biological processes such as bioremediation. Since the Forumad area is an extreme environment with high concentrations of chromite and magnesite and alkaline pH; it seems the microbial population of this ecosystem can tolerate heavy metals, and may be a suitable source for identifying microorganisms that can be used for environmental applications.

In diversity analysis, since many environmental microorganisms are non-cultivable, metagenomics-based approaches, as a combination of genomic and bioinformatics technologies, are developed for detailed elaboration on the genetic diversity of microbial community of soil, sediments and aquatic environments (7) which used to assess cultivable and non-cultivable microorganisms (8, 9). The *16S rRNA* gene is a relatively short conserved DNA segment to identify bacteria and thus, serves as a more time and cost-effective strategy, as compared to many other unique bacterial genes, to predict phylogenetic relationships (10, 11). According to this, the strains with about 98.5% ribosomal RNA gene similarity or less are unlikely to have more than 60 to 70% genomic DNA similarity and therefore, are categorized as different species. However, the opposite is not always true, and if the *16S rRNA* gene sequences similarity is higher than 98.5%, yet they may be introduced as different or the same species (12, 13)

2. Objectives

In this study, microbial diversity of an ore mine in Iran, the Forumad chromite area, was evaluated using a metagenomics-based approach and the evolutionary relationship of the identified strains was compared with those recorded in biological databases. Since the *16S rRNA* gene-based assay provides a rapid and broad-spectrum analysis platform to reliably identify the microbial diversity, we used this approach for identification of the unexplored microbial diversity in this mine located at Sabzevar ophiolitic belt, Northeastern Iran.

3. Materials and Methods

3.1. Site Description and Sample Collection

The Forumad chromite deposit is located within the Sabzevar ophiolitic complex (SOC) at 1,500 meters above the sea level with a long time mining activities. The mean concentrations of Cr (5837.5 ppm) and Ni (570.7 ppm) in the nearby environment are significantly high. The mean concentrations of other heavy metals existing in the region such as As, Cd, Co, Cu, Pb, and V are also close to the geological background values (14). The samples were collected from eight different sites of the Forumad chromite area (effluent water, mine's soil and soil around mines) at spring and autumn of 2011. Classical sampling methods were performed in sterile bottles and the samples were transported to the laboratory on ice as soon as possible.

3.2. DNA Extraction

In diversity studies based on metagenomics approaches, preparation of enough high quality DNA, especially from soil and other samples containing humic acid or other contaminants, is critical. Therefore, in this study, several DNA extraction kits and manual methods were used. The highest amount of DNA was gained through a combination of Zhou's manual method (15) and MOBIO Kit. High amounts of crude DNA were extracted from 5 g of each sample by Zhou's method and purified by MOBIO Kit according to the manufacturer`s instruction

3.3. 16S rRNA Gene Amplification and Library Construction

Pure metagenoms were PCR amplified for bacterial and archaeal *16S rRNA* genes using the universal primers (**Table 1, Supplementary data**). One hundred Nano gram of DNA was used in a PCR reaction mixture (final volume of 50 µL) containing 1.5 mM $MgCl_2$, 1X Reaction buffer, 0.2 mM dNTP, 5 pmoL of each primer and 2.5 U Taq DNA polymerase. PCR was performed with an initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 60 s (denaturation), 50-54 °C for 60 s (annealing), 72 °C for 1.5 min (extension) and 72 °C for 10 min (final extension). The PCR products

were visualized on 1% agarose gel in TAE buffer and then purified using the Roche High pure PCR purification kit.

The amplicons were ligated into pTz57 R/T vector, according to the Fermentas's protocol. Ligation products were transformed into *E. coli* DH5α cells by heat shock transformation method (16) and screened on LB/Ampicillin/IPTG/X-Gal plates in 37 °C for 16 h. The positive clones were selected based on the blue-white screening method; accordingly, white colonies were considered as recombinant clones and confirmed by PCR using vector specific primers M13F (5′-GTAAAACGACGGCCAG-3′) and M13R (5′-CAGGAAACAGCTATGAC-3′) (17). Then, plasmids were extracted from positive clones by plasmid extraction Kit (Roche, Germany) for sequencing (Sanger method, Macrogen, South Korea).

3.4. Phylogenetic Analysis

The sequences were edited using Chromas Pro program (Technelysium Pty Ltd, Australia) and checked by Bellerphon program (18) for chimers in amplified fragments. BLAST program in NCBI and EzTaxon databases (19) were used to identify and compare similar 16S rDNA sequences. Phylogenetic analysis was performed using the MEGA5 software (20) after multiple alignments of sequences available from EzTaxon database by CLUSTAL X (21). Pairwise evolutionary distances were computed using the correction method and clustering was performed using the neighbor-joining method (22). Bootstrap analysis was used to evaluate the tree topology by means of 100 alternative trees.

4. Results

4.1. Physicochemical Parameters of the Samples

The geographical location of the sampling sites is presented in **Table 2 in Supplementary data**. The samples pH was around 9. The type and amount of ionic compounds available in the Forumad chromite mine are shown in **Table 3 in Supplementary data**. The maximum amount of compounds belongs to the chromite oxide (41%) which indicates the presence of large amounts of chromium in this area.

4.2. 16S rDNA Library

Two *16S rRNA* gene libraries from soil samples (Bacterial-Archaeal) and one library from water samples (Bacterial) were constructed in *E. coli* DH5α. No archaea PCR products was obtained from the water samples. Total of 248 white colonies were maintained at the libraries of the soil and water samples, from which 56 bacterial colons of the water samples (Prefix FMW-Bac), 32 bacterial colons of the soil samples (Prefix FMS-Bac) and 19 archaeal colons of the soil samples (Prefix FMS-Arc) were randomly sequenced. Four identical sequences and also 17 bacterial incomplete sequences of the water samples were excluded from the study. The 83 sequences (39 Bacteria from water samples, 29 Bacteria from soil samples and 15 Archaea from soil samples) were deposited in GenBank with accession numbers KF975505-KF975587 (**Tables 1, 2 and 3**). Accordingly, frequency of the *Gammaproteobacterial* strains in water libraries and *Alphaproteobacterial* and *Gammaproteobacterial* strains in soil libraries were dominant, respectively.

4.3. Phylogenetic Analysis of Bacteria in the Water Samples

The *16S rRNA* gene sequences of the bacterial library from the water samples were classified in 40 Operational Taxonomic Units (OTUs) falling into five phylogenetic phyla: *Proteobacteria* (30 OTUs - 75% of total bacterial colonies), *Bacteroidetes* (6 OTUs - 15%), *Firmicutes* (1 OTU - 2.5%), *Verrucomicrobia* (1 OTU - 2.5%) and uncultured (2 OTUs - 5%). The *Proteobacteria* group contains the largest section of the OTUs, the majority of which showed more similarity with *Cedecea* and *Rheinheimera* species (**Fig. 1**).

4.4. Phylogenetic Analysis of Bacteria in the Soil Samples

The *16S rRNA* gene sequences of the bacterial library from the soil samples were classified in 31 OTUs, falling into nine phylogenetic phyla: *Proteobacteria* (14 OTUs - 45% of total colonies), *Cyanobacterria* (2 OTUs - 6.45%), *Gemmatimonadetes* (1 OTUs - 3.22%), *Bacteroidetes* (2 OTUs - 6.45%), *Actinobacteria* (1 OTUs - 3.22%), *Acidobacteria* (1 OTUs - 3.22%), *Firmicutes* (1 OTUs - 3.22%), *Chloroflexi* (1 OTUs - 3.22%), *Planctomycetes* (1 OTUs - 3.22%) and uncultured (7 OTUs - 22.5%). The *Proteobacteria* constitute the largest group of this library. Phylogenetic tree was constructed with the above mentioned and related strains from the Eztaxon database (**Fig. 2**).

Table 1. Bacterial clones obtained from water samples.

Clone Library Bacteria of water samples	Accession No.	Sequene length bp	Closest sequence match in NCBI	Similarity %	Closest sequence match in EzTaxon	Similarity %
FMWB1	KF975505	1496	*Enterobacter sp. Bn-12 JX456175.1*	99	*Cedeceaneteri GTC1717(T) AB086230*	99.56
FMWB2	KF975506	1496	*Enterobacter sp. Bn-12 JX456175.1*	99	*Cedeceaneteri GTC1717(T) AB086230*	99.48
FMWB6	KF975510	1533	*Enterobacter sp. Bn-12 JX456175.1*	99	*Cedeceaneteri GTC1717(T) AB086230*	99.21
FMWB9	KF975513	1496	*Enterobacter sp. Bn-12 JX456175.1*	99	*Cedeceaneteri GTC1717(T) AB086230*	99.12
FMWB10	KF975514	1359	*Enterobacter sp. Bn-12 JX456175.1*	99	*Cedeceaneteri GTC1717(T) AB086230*	99.11
FMWB17	KF975521	1497	*Enterobacter sp. Bn-12 JX456175.1*	99	*Cedeceaneteri GTC1717(T) AB086230*	98.89
FMWB18	KF975522	1496	*Enterobacter sp. Bn-12 JX456175.1*	99	*Cedeceaneteri GTC1717(T) AB086230*	98.82
FMWB21	KF975525	1500	*Enterobacter sp. Bn-12 JX456175.1*	99	*Cedeceaneteri GTC1717(T) AB086230*	98.75
FMWB3	KF975507	1525	*Delftia acidovoransSPH1 strain SPH-1 NR074691.1*	99	*Delftia lacustrisDSM 21246 (T) EU888 308*	99.33
FMWB4	KF975508	1448	*Acidovorax sp. Asd MW-A3 FM955883.1*	99	*Acidovorax radicisN35(T) AFBG0100003*	99.31
FMWB5	KF975509	1530	*Pseudomonas anguilliseptica strain BI AF439803.1*	99	*Pseudomonas peli R-20805(T)AM114534*	99.25
FMWB7	KF975511	1514	*Rheinheimera soli* strain BD-d46NR 044294.1	99	*Rheinheimera soli* BD-d46(T)EF575565	99.19
FMWB8	KF975512	1517	*Rheinheimera soli*strain BD-d46NR 044294.1	99	*Rheinheimera soli* BD-d46(T)EF575565	99.19
FMWB16	KF975520	1489	*Rheinheimera soli*strain BD-d46NR 044294.1	99	*Rheinheimera soli* BD-d46(T)EF575565	98.92
FMWB19	KF975523	1517	*Rheinheimera soli*strain BD-d46NR 044294.1	99	*Rheinheimera soli* BD-d46(T)EF575565	98.79
FMWB24	KF975528	1518	*Rheinheimera sp. BZ19 GQ240227.1*	99	*Rheinheimera soli* BD-d46(T)EF575565	98.58
FMWB31	KF975535	1517	*Rheinheimera sp. BZ19 GQ240227.1*	98	*Rheinheimera soli* BD-d46(T)EF575565	98.52
FMWB33	KF975537	1518	*Rheinheimera sp. BZ19 GQ240227.1*	97	*Rheinheimera* chironomiK19414(T) DQ 298025	97.97
FMWB12	KF975516	1487	*Limnobacter thiooxidans strain HLSB 157 FJ999570.1*	99	*Limnobacter thiooxidans CS-K2(T) AJ 289885*	99.10
FMWB14	KF975518	1487	*Limnobacter sp. e8(2011) HQ652592.1*	99	*Limnobacter thiooxidans CS-K2(T) AJ 289885*	99.03
FMWB22	KF975526	1486	*Limnobacter sp. e8(2011) HQ652592.1*	99	*Limnobacter thiooxidans CS-K2(T) AJ 289885*	98.62
FMWB23	KF975527	1526	*Hydrogenophaga sp. CL3 DQ986320.1*	99	*Hydrogenophaga taeniospiralisATCC 49743 (T) AF078768*	98.59
FMWB27	KF975531	1462	*Runella sp. NBRC 15128 AB680774.1*	99	*Runella slithyformis DSM 19594(T) CP 002859*	98.04
FMWB11	KF975515	1529	*Uncultured JQ824901.1*	99	*Uncultured EF540413*	99.11
FMWB13	KF975517	1494	*Uncultured AF523040.1*	99	*Polaromonas jejuensisJS12-13(T) EU 030285*	99.08
FMWB15	KF975519	1501	*Uncultured JN392908.1*	99	*Pseudomonas peliR20805(T) AM114534*	98.92
FMWB20	KF975524	1445	*Uncultured KC683142.1*	99	*Bradyrhizobium lablabiCCBAU23086(T) GU433448*	98.75
FMWB25	KF975529	1521	*Uncultured JN685475.1*	99	*Aquabacterium parvum B6(T) AF035052*	98.51
FMWB26	KF975530	1497	*Uncultured AB583905.1*	99	*Hydrogenophaga taeniospiralis ATCC 49743 (T) AF078768*	98.32
FMWB28	KF975532	1488	*Uncultured AF445684.1*	99	*Algoriphagus boritolerans T-22(T) AB 197852*	97.82

FMWB29	KF975533	1527	*Uncultured EF632936.1*	99	*Curvibacter delicates LMG 4328 (T) AF 078756*	97.44
FMWB30	KF975534	1478	*Uncultured FJ801195.1*	99	*Flavobacterium chungangense CJ(T)EU 924275*	97.18
FMWB32	KF975536	1519	*Uncultured JN869095.1*	98	*Rheinheimera chironomiK19414(T) DQ 298025*	98.44
FMWB34	KF975538	1544	*Uncultured JN178248.1*	97	*Anaerobacillus macyae JMM-4 (T) AY 032601*	97.19
FMWB35	-	1529	*Uncultured JN392908.1*	96	*Pseudomonas peliR20805(T) AM114534*	95.39
FMWB36	KF975539	1549	*Uncultured Opitutales AB479055.1*	94	*Uncultured AB479055*	94.13
FMWB37	KF975540	1489	*Uncultured JN488684.1*	93	*Uncultured DQ329894*	88.68
FMWB38	KF975541	1502	*Uncultured Sphingobacteria EF 520608.1*	91	*Uncultured EU753655*	87.50
FMWB39	KF975542	1507	*Uncultured Sphingobacteria EF 520608.1*	91	*Uncultured AB369173*	87.36
FMWB40	KF975543	1507	*Uncultured Sphingobacteria EF 520608.1*	89	*Uncultured EU328009*	85.02

Table 2. Bacterial clones obtained from soil samples.

Clone Library **Bacteria of Soil samples**	**Accession No.**	**Sequene length bp**	**Closest sequence match with NCBI**	**Similarity %**	**Closest sequence match with EzTaxon**	**Similarity %**
FMSB1	KF975544	1530	*Ralstonia pickettii12J strain 12J NR 102967.1*	99	*Ralstonia pickettii ATCC 27511 (T) AY7 41342*	99.8
FMSB9	KF975552	1497	*Ralstonia sp. NT80 AB740040.1*	98	*Ralstonia insidiosa AU2944(T) AF488 779*	98.39
FMSB2	KF975545	1529	*Pseudomonas sp. MBR EU307111.2*	99	*Pseudomonas toyotomiensisHT-3(T) AB 453701*	99.8
FMSB3	KF975546	1529	*Pseudomonas sp. MBR EU307111.2*	99	*Pseudomonas toyotomiensisHT-3(T) AB 453701*	99.7
FMSB4	KF975547	1528	*Pseudomonas sp. MBR EU307111.2*	99	*Pseudomonas toyotomiensisHT-3(T) AB 453701*	99.46
FMSB5	KF975548	1516	*Arthrobacter sp. EM5 FJ517625.1*	99	*Arthrobacter scleromaeYH-2001 AF330 692*	98.87
FMSB13	KF975556	1478	*Pseudanabaena sp. Sai011 GU 935357.1*	98	*Oscillatoria limnetica MR1 AJ007908*	97.72
FMSB6	KF975549	1495	*Uncultured KF511881.1*	99	*Silanimonas lenta25-4(T) AY557615*	97.47
FMSB7	KF975550	1537	*Uncultured HQ119931.1*	99	*Pseudoxanthomonas sacheonensisBD-c54(T) EF575564*	97.41
FMSB11	KF975554	1500	*Uncultured AF467297.1*	98	*Pseudoxanthomonas yeongjuensis GR12 -1(T) DQ438977*	98.07
FMSB8	KF975551	1519	*Uncultured Acidobacteria FR 749746.1*	98	*Uncultured FR749746*	98.52
FMSB14	KF975557	1548	*Uncultured Acidobacteria HQ 597972.1*	98	*uncultured HM438150*	97.69
FMSB10	KF975553	1440	*Uncultured AY957902.1*	98	*Erythromicrobium ramosumDSM 8510 (T) AF465837*	98.37
FMSB12	KF975555	1530	*Uncultured JQ769882.1*	98	*uncultured DQ378223*	97.86
FMSB15	KF975558	1516	*Uncultured AF445684.1*	98	*Algoriphagus boritoleransT-22(T) AB 197852*	97.34
FMSB16	KF975559	1445	*Uncultured KC683122.1*	98	*Bosea minatitlanensis AMX51(T), AF 273081*	93.75

FMSB17	KF975560	1443	*Uncultured KC683122.1*	98	*Bosea massiliensis63287(T) AF288309*	93.70
FMSB21	KF975564	1445	*Uncultured KC683122.1*	97	*Bosea massiliensis63287(T) AF288309*	93.56
FMSB22	KF975565	1445	*Uncultured KC683122.1*	97	*Bosea minatitlanensis AMX51(T), AF 273081*	93.46
FMSB23	KF975566	1506	*Uncultured FJ592715.1*	97	*Uncultured HQ327283*	91.30
FMSB24	KF975567	1506	*Uncultured JQ711705.1*	97	*Uncultured GQ116319*	91
FMSB25	KF975568	1441	*Uncultured AB757744.1*	97	*Thermosynechococcus elongatusBP-1 BA000039*	90.19
FMSB26	KF975569	1532	*Uncultured JN178820.1*	97	*Gemmatimonas aurantiacaT-27(T) AP009153*	83.20
FMSB27	-	1421	*Uncultured Rhodobacteraceae FJ516816.1*	96	*Rubrimonas shengliensis SL014B-28A2 (T) GU125651*	92.81
FMSB18	KF975561	1549	*Uncultured KC011114.1*	97	*Uncultured GQ472363*	97.42
FMSB28	KF975570	1548	*Uncultured JQ978959.1*	94	*Uncultured GQ472363*	94.5
FMSB29	-	1489	*Uncultured JX225716.1*	93	*Uncultured AY79604*	91.25
FMSB30	KF975571	1478	*Uncultured Planctomycetales JN825575.1*	92	*Phycisphaer amikurensisNBRC 102666 (T), AP012338*	81.40
FMSB19	KF975562	1515	*Uncultured JF449956.1*	97	*Blastocatella fastidiosa A2-16(T) JQ 309130*	95.56
FMSB20	KF975563	1515	*Uncultured Hymenobacter JN367223.1*	97	*Adhaeribacter aquaticusMBRG1.5(T) AJ 626894*	95
FMSB31	KF975572	1529	*Uncultured HQ397151.1*	89	*Sphaerobacter thermophilus DSM 20745 (T), CP001824*	79.41

Table 3. Archaeal clones obtained from soil samples.

Clone Library **Archaea of Soil samples**	**Accession No.**	**Sequene length bp**	**Closest sequence match with NCBI**	**Similarity %**	**Closest sequence match with EzTaxon**	**Similarity %**
FMSA1	KF975573	1442	*Uncultured FJ784315.1*	99	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	97.84
FMSA2	KF975574	1442	*Uncultured FJ784315.1*	99	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	97.49
FMSA3	KF975575	1442	*Uncultured FJ784315.1*	99	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	97.28
FMSA4	KF975576	1441	*Uncultured FJ790536.1*	99	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	95.5
FMSA5	KF975577	1441	*Uncultured FJ790536.1*	99	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	95.3
FMSA6	KF975578	1441	*Uncultured EF690622.1*	99	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	94.4
FMSA7	KF975579	1441	*Uncultured EF690622.1*	99	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	94.3
FMSA8	KF975580	1441	*Uncultured EF690622.1*	99	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	94.27
FMSA9	KF975581	1441	*Uncultured EF690622.1*	99	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	94
FMSA10	KF975582	1441	*Uncultured FJ784309.1*	99	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	93.85
FMSA11	KF975583	1441	*Uncultured EF690622.1*	98	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	95.67

FMSA12	KF975584	1441	*Uncultured U62812.1*	98	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	95.67
FMSA13	KF975585	1441	*Uncultured EF690622.1*	98	*Nitrososphaeragargensisenrichment culture Ga9.2 GU797786*	95
FMSA14	KF975586	1441	*Uncultured EF690622.1*	98	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	94.83
FMSA15	KF975587	1442	*Candidatus Nitrososphaera gargensis Ga9.2 NR102916.1*	97	*Nitrososphaera gargensis enrichment culture Ga9.2 GU797786*	97.28

According to our results, although bacterial abundance of the soil library was less than that of the water library, the soil library was more diverse. On the other hand, 83% of its OTUs had less than 98.7% similarity to the recorded strains, meaning that they may represent new species.

4.5. Phylogenetic Analysis of Archaea in the Soil Samples

Strains of the archaea library were classified in *Nitrososphaera,* belonging to the *Thaumarchaeota* phylum with 15 OTUs. Comparison of the sequences together and with the closest recognized strains in terms of similarity (**Fig. 3**) shows that 68.6% of the sequences had less than 98.7% similarity with the recorded strains. These clones are often different, known as uncultured archaea, and probably represent new species.

5. Discussion

Generally, heavy metals, being toxic in nature, have detrimental effects on activities and performance of microorganisms. Such effects inhibit microbial nutrients recycling processes such as nitrogen mineralization in soil and thus, the soil respiration rate and microbial biomass are decreased (23). In study of microbiome of the heavy metal (chromite) mine, our analysis led to the identification of 86 independed OTUs in 107 clones of a *16S rRNA* gene library consisting of 248 clones (less than 50% of the library) with only 4 identical sequences. Accordingly, it can be concluded that the Forumad area has limited microbial community (as biomass) with high diversity, which confirms the previous studies affirming the significant negative impact of heavy metals on microbial community (24-26). Low identical OTUs could be due to the fact that most microorganisms are unable to cope with harsh conditions such as low nutrients, drought, high sunlight (25), and/or the existence of toxic heavy metals for multiplication. Some microbial communities may tolerate high concentrations of heavy metals which could be attributed to the precipitation, adsorption, or biotransformation of heavy metals (23, 27) or other resistance mechanisms. For instance, non-specific interactions with proteins or secondary metabolites, being capable of donating electrons to Cr (VI) and converting it into Cr (III), which may resulted in tolerance to chromium (25). Therefore, identification of these microbial communities with heavy metals bio-detoxification and bioremediation properties is an ecologically attractive finding with probable application in cleanup of the pollutants.

Accordingly, many studies have been conducted on bacterial diversity in the environments containing chromium that shown the *Proteobacteria* as dominant population (25, 28). As expressed in these studies, tolerance to arsenic and chromium is widespread in *Proteobacteria.* Moreover, *Alphaproteobacteria* and *Gammaproteobacteria* were abundant in all these areas (25).

Also, another similar study on gold mine have confirmed that *Proteobacteria* constitute the biggest part of the clone libraries (29). Dhal *et al*. have compared two uranium-contaminated and non-uranium-contaminated regions. *Proteobacteria* were found in both areas; however, these bacteria were dominant in the uranium contaminated region (30). In addition, in a study of bacterial, fungal, and archaeal communities in a uranium mine, located in Eastern Finland, a total of 814 bacterial, 54 archaeal and 167 fungal genera were identified, in which *Proteobacteria*, *Euryarchaeota*, and *Mortiriella* were dominant bacterial, archaeal and fungal phyla, respectively (31). In the present study, the frequency of the *Proteobacteria* strains in both bacterial libraries related to water (the dominance of *Gammaproteobacteria*) and soil (the dominance of *Alphaproteobacteria* and *Gammaproteobacteria*) is in agreement with findings by Pradhan *et al*. that investigated diversity of bacterial community in chromite mine of Sukinda Valley, India (32).

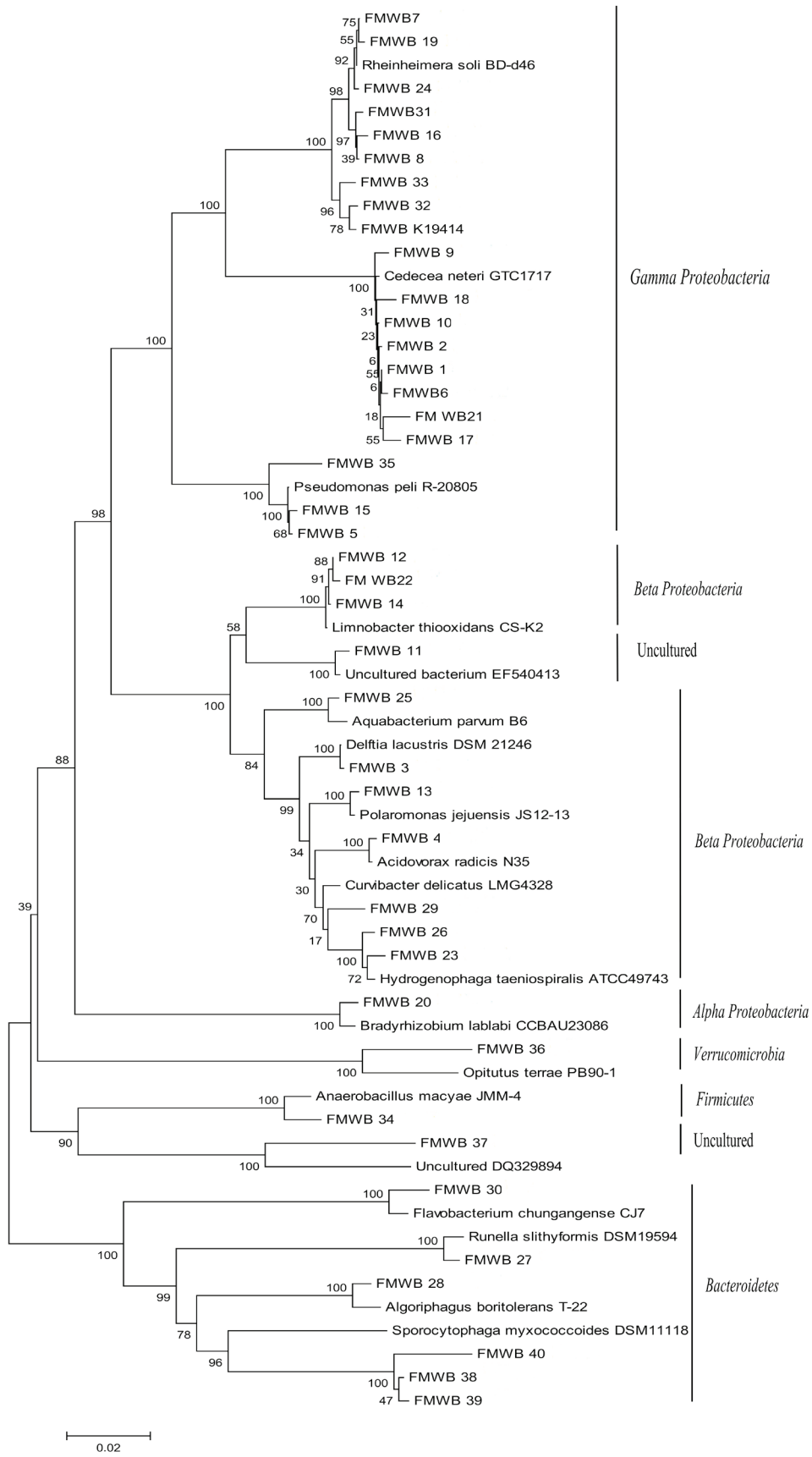

Figure 1. Phylogenetic tree of bacterial OTUs (soil samples) to show the phylogenetic relationships by Neibour-Joining algorithm and bootstrap analysis 100.

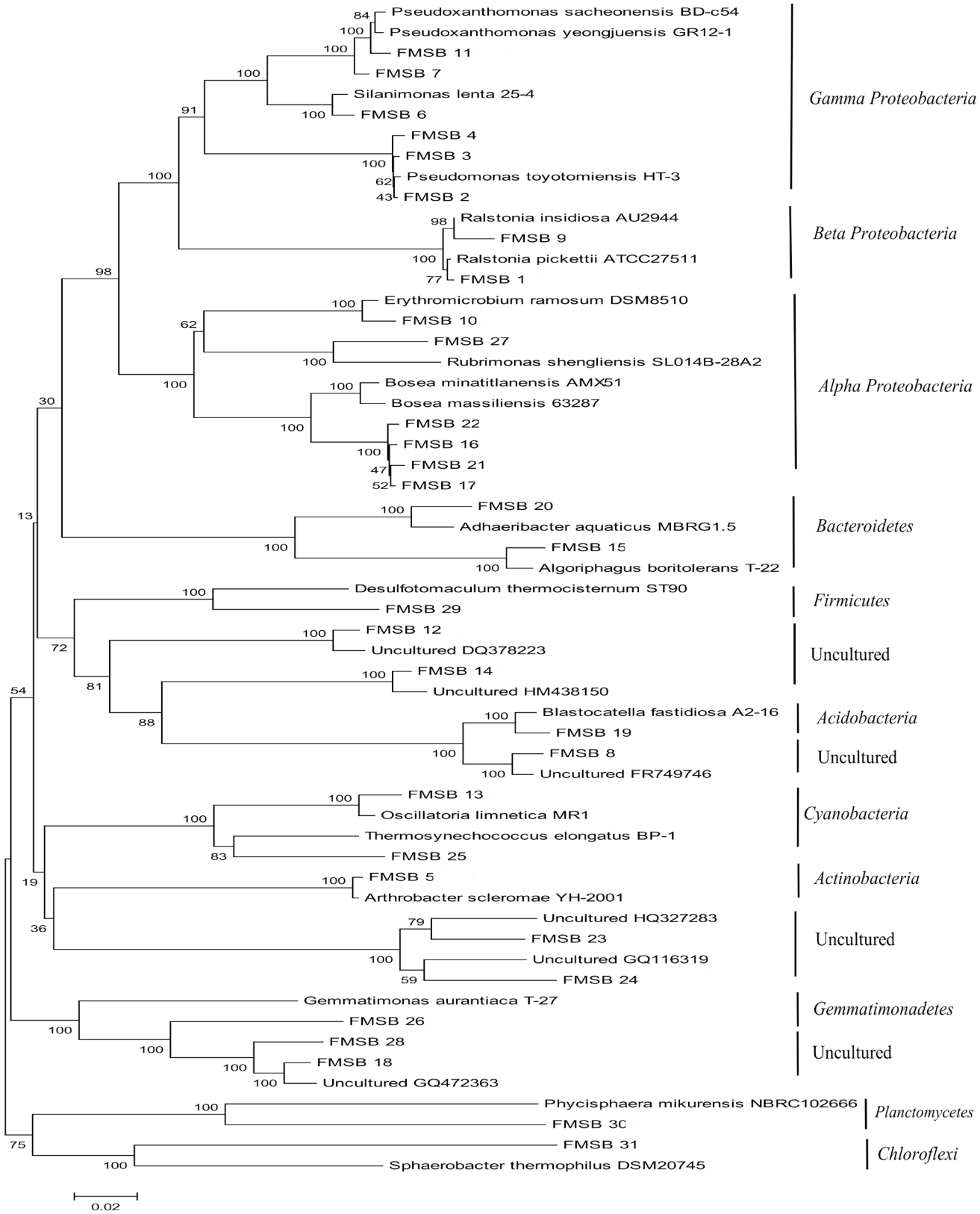

Figure 2. Phylogenetic tree of bacterial OTUs (water samples) to show the phylogenetic relationships by Neibour-Joining algorithm and bootstrap analysis 100.

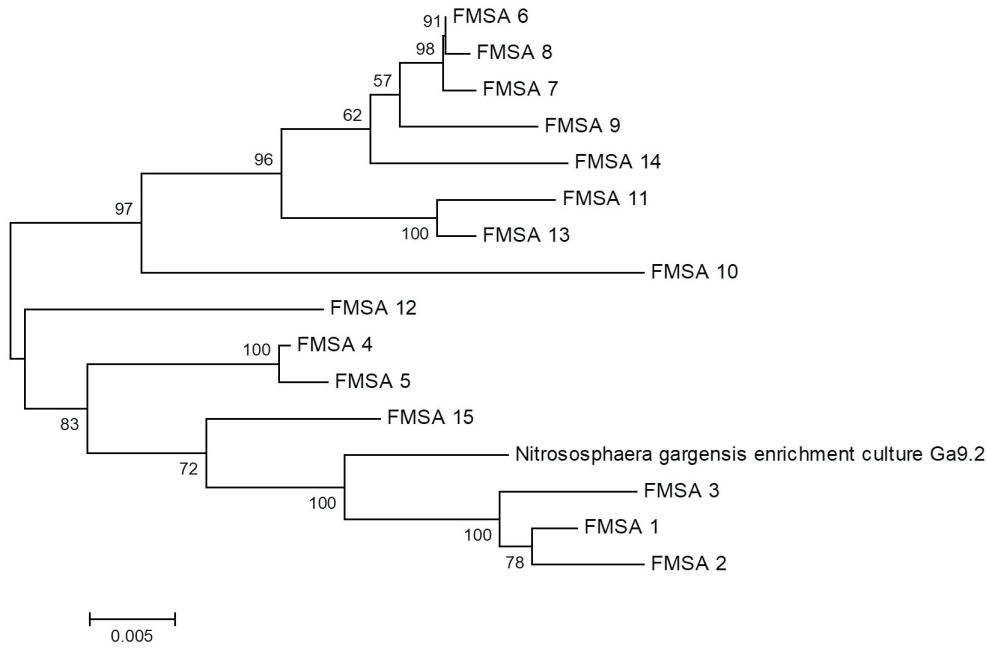

Figure 3. Phylogenetic tree of archaeal OTUs (soil samples) to show the phylogenetic relationships by Neibour-Joining algorithm and bootstrap analysis 100.

The reason for dominance of the *Proteobacteria* strains in high heavy metal contained environments with limited nutrients might be due to their resistance mechanisms against such metals (29, 33). Bacteria tolerant to chromium and capable of chromium or arsenic conversion, mainly belong to the *Proteobacteria*, *Actinobacteria* and *Firmicutes* phyla (25). In addition, similar to report by Katsaveli *et al*. the members of the *Pseudomonadales* and *Enterobacteriales* orders were identified in the microbial ecosystem of the Forumad area (28). Since these strains belonging to *Proteobacteria* are inhabitant in environment containing high amounts of heavy metals, it can be concluded that they are probably tolerance to chromium and capable to convert or remove it. Accordingly, comparing the finding in this area with those in other mine areas revealed significant similarities at phylum level. However, some differences also exist in the orders of each phylum, mostly arising from various chemical and physical properties such as metal composition and pH in these areas. Most of the studied areas have a neutral or acidic pH, while the Forumad area has an alkaline pH. According to phylogenetic trees, in the Forumad area, the microbial diversity is high, whereas the microbial biomass and OTUs frequency is low.

In this study, the similarity level of 59 OTUs (68.6% of sequences) with the type strains was less than 98.7%, showing a high percentage of unknown bacteria in this environment. These OTUs may represent new bacteria which their physiological role is unclear. Therefore, more investigations and identifications are required to obtain complete microbial feature of the Forumad area. Further, cultivated members in phyla such as *Acidobacteria* and *Verrucomicrobia* are limited in number (29).

According to our results, in the bacterial library related to the water samples, 20% of the sequences belonged to *Cedecea* (with similarity greater than 98%) and also, 20% belonged to *Rheinheimera* (with similarity greater than 98%) while these genera did not exist in the soil samples. *Rheinheimera* have been reported as the dominant population in the copper mine wastewater tanks (17).

In the bacterial library of the soil samples, two OTUs had 98% to 99% similarity to *Ralstonia*. This genus was identified in arsenic-contaminated systems and soils contaminated with radioactive materials, chromate and chromite (28). These microorganisms are oligotrophe and can be found in humid environments such as soil, river and lake. Some strains of this genus are capable to live in low nutrient environments and use various sources as energy and carbon sources. They also have

the ability to degrade many toxic substrates and are able to tolerate chromate concentration up to248 $g.L^{-1}$ in pH 1.3 (28, 34). Several strains of *Ralstonia* have been reported to live in environments contaminated with heavy metals such as copper, nickel, iron and zinc (35). According to EzTaxon database, there were OTUs in this library with 98% similarity to *Erythromicrobium*. This genus belongs to *Sphingomonadales* and has high tolerance to heavy metal oxides and the ability to reduce such toxic compounds. Also, species of *Erythromicrobium* are capable of reducing soluble tellurium (IV), which is highly toxic for microbes and other organisms (27). It may be said that these microorganisms are important for bioremediation of environment and have potential industrial and biotechnological applications.

In addition, the FMWB13 and FMWB27 OTUs showed 99% similarity to *Polaromonas* and *Runella*, respectively. Previous researches have shown that these species can contribute to bioremediation of aromatic hydrocarbon (such as naphthalene) contaminated sites (36, 37). On the other hand, several OTUs of the water bacterial library had 95%, 98% and 99% similarity to *Pseudomonas*. Also, some OTUs in the soil bacterial library were 99% similar to *Pseudomonas*. This genus has been shown to have the ability for bioremediation of various contaminants (38, 39).

Archaea are initially viewed as extremophiles living in harsh environments, such as hot springs and salt lakes (40). However, little information is available about the effects of heavy metals on them. Based on our findings, in the archaeal clone library, all the sequences were related to the phylum *Thaumarchaeota.* This phylum was proposed in 2008, distinguishing mesophilic ammonia-oxidizing archaeal (AOA) lineages from hyperthermophilic *Crenarchaeota* lineages (41). According to recent studies, the phylum *Thaumarchaeota* has been estimated to represent up to 20% and 5% of all prokaryotes in marine and terrestrial environments, respectively (42). Our results showed that the identified archaea communities (from the soil samples) belonged to *Nitrososphaera*as, a genus of ammonia oxidizing archaeans in the phylum *Thaumarchaeota.* Based on analyses by the EzTaxon database, all the archaeal colonies were 99% similar to *Candidatus Nitrososphaera gargensis* Ga 9.2 adapted to environments contaminated with heavy metals. This archaea contained a heavy metal tolerance gene which responds to environmental stresses (43). Based on the findings by BLAST program of NCBI, the archaeal sequences were placed in an uncultured group and likely to present new species.

According to the studies on microbial diversity, the microbial abundance and diversity in the environment is changeable depending on environmental conditions, method and sampling time. More realistic results can be achieved by increasing the amount and number of samples. As an example, the investigation of microbial diversity in Antarctica and northern Victoria Land soils by Niederberger *et al*. showed the effect of soil fertility on microbial population's diversity and frequency (44). They investigated the microbial diversity of two soil types with high and low productivity. In soils with high productivity, *Proteobacteria* (84%) were dominant while in soils with low productivity *Acidobacteria* (68%) and *Gemmatimonas* (55%) were dominant. In other studies, the depth-dependency of archaea distribution has been mentioned and thus, *Crenarchaeota* were observed to be abundant in deeper soil layers and increased by increasing the soil depth. According to these studies, there is a relationship between increased number of *Crenarchaeota* and decreased nutrient and oxygen concentration in deep soil layers (43). Finally, based on results in this study and similar studies, composition of heavy metals in different environments can be effective on their microbial population and diversity. generally, for environmental contamination specially in the case of chromium, *Proteobacteria* are dominant and have important role in the bioremediation due to their certain mechanisms.

6. Conclusion

In this study, construction of clone library, among metagenomics approaches, was used to investigate the diversity of the microbial community of a chromite mine; in hope to find microbial strains may tolerant to heavy metals for application in bioremediation programs.

Our findings showed that the Forumad area can be a significant source of heavy metal resistant microorganisms which can be used for detoxification and bioremediation of regions contaminated with heavy metals, although more studies are needed. During this study and afterward, progress in NGS and bioinformatics analysis tools resulted in deeper insight and more microbial diversity information in different

environments but clone library analysis gave a total and nearly complete feature of microbial diversity of a mine (at that time) in Iran and opened a new way for investigation in environmental microbiology in this field.

Future investigation of toxic substance decomposition mechanisms in these environments using NGS and metagenomics analysis can lead to identification of more heavy metal tolerant microorganisms and related genes. These genes would serve as a potential source for environmental and industrial biotechnology which is in the way.

Acknowledgments

This research was funded by the Iran National Science Foundation (INSF) with research project no. 87041055. Analyses were performed in the Industrial and Environmental Biotechnology Laboratory of the National Institute of Genetic Engineering and Biotechnology (NIGEB).

Conflicts of Interest

The authors confirm that this article content has no conflicts of interest.

Abbreviations

SOC: Sabzevar ophiolitic complex. OTUs: Operational Taxonomic Units. Cr: Chromium. Ni: Nickel. As: Arsenic. Cd: Cadmium. Co: Cobalt. Cu: Copper. Pb: Lead. V: Vanadium.

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