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Rajni Kant Thakur
Department of Biotechnology, Dr. Y.S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Poonam Shirkot
Department of Biotechnology, Dr. Y.S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Correspondence
Rajni Kant Thakur
Department of Biotechnology, Dr. Y.S Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh, India

Molecular identification of gold nanoparticles synthesizing bacteria through *in silico* methods

Rajni Kant Thakur and Poonam Shirkot

Abstract
Classifying individual bacterial through traditional methods is stagnant, which require lot of consultation and efforts. DNA sequencing can provide more definitive taxonomic classification than culture based approaches for many organisms. While providing less time consuming and labour intensive. Molecular characterization technology is now widely used for wide range of bacterial species. We have used blastn, Cluster W, MEGA6.0 software to identify species of bacterial isolates on the basis of 16S rrna gene and then homology searching has been done through Cluster W. Phylogenetic relationship established through MEGA 6.0 software. We have identified *Bacillus thuringiensis* strain GBI-3 with a noble trait such as to synthesize Gold nanoparticles.

Keywords: Blastn, Cluster W, MEGA, Gold nanoparticles

Introduction
16S rRNA gene sequencing is popular alternative to traditional methods and provides several advantages. DNA sequencing can provide more definitive taxonomic classification than culture based approachs for many organisms. While providing less time consuming and labour intensive. Molecular characterization technology is now widely used for wide range of bacterial species. 16S rRNA gene sequencing has played a pivotel role in bacterial classification and discovery of novel bacteria. (Woo *et al.,* 200l). The analysis of DNA has been used in a large number of studies on bacterial taxonomy and bacterial typing, and as well to further our understanding of the basic mechanisms of evolution. The availability of DNA sequences for the complete genome of a number of bacteria has paved the way for the identification of orthologous families of genes within genomes based on DNA sequence and gene function (Eisen, 1998a) [2]. The characterization of orthologous gene families has been termed phylogenomics. Related to phylogenomics is the concept of exaptation defined as the recruitment of DNA or RNA sequences into variant or novel function: RNAs from all categories have been identified that may be exapted as novel genes or regulatory elements (Brosius, 1999; Brosius & Gould, 1992) [3, 4]. Similarly, bacterial morphology provides significant evolutionary insights when mapped onto *rrna* phylogeny since bacterial morphology has been shown to be remarkably consistent with *rrna* phylogeny (Siefert & Fox, 1998) [5]. A phylogenomic approach that includes information about the function of genes, exaptation and bacterial genetics of morphology and or biochemistry in the phylogenetic analysis may in the future be of value in inter-genomic as well as intra-genomic analyses of isolates.
The evolution of bacteria has long been viewed primarily through the perspective of *rrna* sequences (Woese, 1987) [1]. The gene content of the genome (Snel *et al*., 1999), the evolutionary rates of different classes of genes (Jain *et al*., 1999) [7], genome signatures (defined as the ratios between the observed dinucleotide frequencies and the frequencies expected if neighbours were chosen at random). G+C content (Sueoka, 1999) [8] and the occurrence and location of gene duplications, rearrangements, insertions, gene capture (Szpirer *et al*., 1999) [9] and horizontal gene transfer (Lawrence & Hartl, 1992) [10] are all indices of genome organization that vary between species. A much more complete analysis of the taxonomy and evolution of bacteria may be possible with the current rapid DNA analysis techniques if these parameters are used as a framework.
The analysis of DNA sequences has significantly furthered knowledge about the relatedness of different isolates in at least three categories including random whole-genome analysis, specific

gene variation and mobile genetic elements. The numerous studies in these areas has provided considerably more information than traditional phenotypic methods and with this more questions have been raised that were previously not evident. The recent techniques for molecular typing and taxonomy and evolution of bacterial isolates include both single-locus and multi-locus approaches. The single-locus approach uses highly variable genes. In contrast, multi-locus approaches include multi-locus sequence typing (MLST) and the analysis of multigene families such as *rrna* operons and tRNA genes. The latter includes 16S *rrna* variations: intergenic (16S-23S *rrna*) spacer regions (ISR) typing; ISR concerted evolution; ISR tRNA genes and tRNA genes not associated with *rrna* operons. The analysis of small *rrna* gene sequences is another important landmark in the study of evolution and classification of living organisms. Traditionally, living organisms were classified, according to similarities and differences in their phenotypic characteristics, into prokaryotes and eukaryotes, and these were in turn further classified into various kingdoms, phyla, classes, orders, families, genera and species. However, objective taxonomic classification by these methods can be difficult because of variations in phenotypic characteristics. Three decades ago, Carl Woese and others started to analyse and sequence the 16S *rrna* genes of various bacteria, using DNA/RNA sequencing, and used the sequences for phylogenetic studies. The invention of PCR and automated DNA sequencing two decades ago, and subsequent work on 16S *rrna* gene sequencing of bacteria, as well as 18S *rrna* gene sequencing of eukaryotes, has led to the accumulation of a vast amount of sequence data on the *rrna*/r DNA genes of the smaller subunit of the ribosomes in a large number of living organisms. Comparison of these sequences has shown that the *rrna* gene sequences are highly conserved within living organisms of the same genus and species, but that they differ between organisms of other genera and species.

In addition to the calculation of similarity coefficients among organisms, a detailed analysis of 16S *rrna* oligonucleotide catalogues or complete sequences reveals that there are both quite variable and highly conserved regions of the molecule, presumably reflecting different degrees of functional constraint on different parts of the molecule. Separate comparison of the variable and conserved regions allows determination of both close and distant relationships. In addition, careful inspection of the conserved regions has revealed that there are sequences that are characteristic of major groups of bacteria, termed signature sequences. At great phylogenetic distances these signature sequences are important in allowing the determination of relationships. Using these *rrna* gene sequences for phylogenetic studies, three domains of life, Archaea, Bacteria and Eukarya, as opposed to the traditional classification of living organisms into prokaryotes and eukaryotes only, were described. Using 16S *rrna* gene sequences, numerous bacterial genera and species have been reclassified and renamed, classification of uncultivable bacteria has been made possible, phylogenetic relationships have been determined, and the discovery and classification of novel bacterial species has been facilitated. In the last decade, sequencing of various bacterial genomes and comparison between genome and 16S *rrna* gene phylogeny has confirmed the representativeness of the 16S *rrna* gene in bacterial phylogeny.

Material and methods

Forty three samples in form of water, soil, biofilm, pebbles, roof topping/stalagamite and rock matting were collected from gold mine sites. All these samples were kept at 4°C in refrigerator in laboratory till further experimentation. Three different culture media were investigated for isolation of gold nanoparticles synthesizing bacterial isolates *viz.*, Nutrient agar medium, Eosin methylene blue agar medium and Luria bertani medium. One gram soil, Pebbles, stalagmite /1.0 ml water, biofilm samples collected from sampling sites were dissolved in 9.0 ml of sterile water and serial dilution technique was used for isolation of bacterial isolates. The plates were incubated at 37 ºC for 24-48 hrs for bacterial growth. Turbid cultures were streaked on plates of solidified growth medium. Individual colonies were restreaked repeatedly, and the axenic cultures thus obtained were stored at 4 ºC.

Morphological characterization

All the bacterial isolates obtained in previous step were further studied for various morphological characters. Various morphological descriptors of colour, size, optical property and elevation of the colonies and various microscopic characteristics studied were gram reaction, shape, arrangement and spore formation.

Quantitative screening of bacterial isolates for gold nanoparticles synthesis ability

Assessment of all forty three bacterial isolates for their ability to synthesize gold nanoparticles was carried out. One percent concentration of the inoculum (overnight culture) of each bacterial isolate was inoculated into the 50 ml nutrient broth followed by incubation at 37 ºC for 24-48 hrs at 250 rpm. Supernatant of each bacterial culture was collected by centrifugation at 10000 rpm for 12 minutes at 4ºC to study extracellular synthesis of gold nanoparticles. Ten ml of each supernatant was mixed with 10 ml of 1mM solution of $HAuCl_4$ and incubated at 37 ºC for 240 hrs. Formation of gold nanoparticles was studied 0-240 hrs, with an interval of 12 hrs and confirmed by colour change of the solution from light yellow to red wine/purple colour (Figure 1). This formation of gold nanoparticles was also confirmed by the Spectrophotometer (Spectronic 20, Milton Roy Company) at two different wavelengths of 540 and 560 nm.

Biochemical characterization and Molecular characterization

Various biochemical characters were investigated using standard assays. Genomic DNA extraction Mini kit (Real Genomics). Presence of DNA and its quality was checked using 1.0% agarose gel and then was viewed by UV trans-illuminator. After visual confirmation of DNA bands in the gel, photograph of the same gel were taken by gel documentation apparatus, AlphalmagerTM (Alpha Infotech Corporation, USA). The DNA of GBI-3 was selectively amplified using PCR technology. Universal primers B27F (5`-AGAGTTTGATCCTGGCTCAG-3'U1492R) and (5`-GGTTACCTTGTTACGACTT-3') for 16S *rrna* gene were used for the experiment. The eluted and purified DNA of GBI-3 was sequenced. The sequences have been submitted to NCBI with accession number KP 219453. To gain insight of the evolutionary pattern, phylogenetic tree was constructed using MEGA 5.0 bioinformatics tool. Neighbour-Joining (NJ) technique of mathematical averages (UPGMA) was used.

Characterization of bacterial isolates using 16S *rrna* gene technology
The above extracted genomic DNA of selected bacterial isolates were amplified by using universal primers for 16S *rrna* gene of bacteria. The PCR amplification was carried out in 0.2 ml PCR tubes with 20 µl reaction volume with composition as depicted in Table-3. Amplifications were performed using thermal cycler (Biorad) and with a temperature profile standardized for 16S *rrna* gene amplification (Table-4).

Universal Primers
B27F 5`-AGAGTTTGATCCTGGCTCAG-3'
U1492R 5`-GGTTACCTTGTTACGACTT-3'

16S *rrna* gene sequencing
Sequencing is the process of determining the nucleotide (A, T, G and C) order of a given DNA fragment. DNA sequencing has been performed using the chain termination method developed by Sanger and his coworkers in 1977. The PCR products obtained through amplification with universal primers targeting *rrna* gene were sent for sequencing using same upstream and downstream primers to Merck lab, India.

***In silico* analysis of sequences**
In silico is an expression used to mean "performed on computer or via computer simulation". Raw sequence data was obtained as a set of two sequences for each sample one with forward and other with reverse universal primers of sequencing reaction. Analysis of raw sequence data was done using Chromas Lite (version 2.1) of Technelysium Pvt. Ltd. Sequences of the selected bacterial isolates were analyzed using different approaches which have been discussed below briefly.

BLASTn
Basic Local Alignment Search Tool (BLAST) uses an algorithm of Altshul *et al*., (1997) [11] for searching similarities above certain threshold between a query sequence and all other sequences present in a database. 16S *rrna* gene sequence of the selected bacterial isolates were analyzed using BLASTn to align them with corresponding sequences of 16S *rrna* from the database.

Multiple Sequence Alignment
Sequence alignment is done for comparing sequences and this comparison of sequences provides a foundation for usage of bioinformatics tools and hence it becomes easier to study the sequence at genomic and proteomic level. Multiple sequence alignment is the sequence alignment of three or more biological sequences. During present investigation, multiple sequence alignment of nucleotide sequences of the selected isolate and other isolates retrieved from NCBI database, was generated. Multiple sequence alignment was performed using CLUSTAL W program (Thompson *et al*., 1994) [12]. From such alignment studies, inference of sequence homology and phylogenetic analysis has been drawn.

Phylogenetic analysis
Phylogenetic analysis has been used in comparative genomics and proteomics to depict evolutionary relationships. The analysis began with alignment of sequences using tools like Clustal W and after alignment, phylogenetic tree was constructed. During present investigations entire set of nucleotide sequences (nucleotide sequences of selected isolate and rest all sequences from NCBI) were placed in a single notepad in their Fasta format, and these were aligned through EBI tool, CLUSTALW program, integerated with MEGA version 5.0 (Molecular Evolutionary Genetics Analysis; Tamura *et al*., 2007) and the tree was generated using MEGA version 5.0 and analyzed. The evolutionary history/phylogenetic analysis were inferred using the Neighbor-Joining method (Saitou & Nei, 1987) [14]. The bootstrap consensus tree inferred from 100 replicates was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (100 replicates) has been shown above the branch (Felsenstein, 1985) [15].

Results
Isolation of gold nanoparticles synthesizing bacteria
Isolation of gold nanoparticle synthesizing bacteria was carried out from samples using nutrient agar medium at 37 ºC. All the samples were inoculated in 150 ml flasks containing 50 ml nutrient broth and incubated at 37 ºC at 50 rpm for 24 hrs. Turbid cultures were streaked on plates of solidified nutrient agar medium (2% agar). Individual colonies were restreaked repeatedly and the purified colonies were stored at 4ºC in refrigerator till further processing/use. A total of hundred three bacterial isolates were isolated from eighty seven samples. From goldmine a total of 43 bacterial isolates were obtained and rest 42 bacterial isolates were obtained from sample of various hot water springs. Different colony morphotypes have been observed. On the basis of colour, 10.5% morphotypes were found creamish white in colour, rests of them were found white in colour. On the basis of shape 49% isolates showed circular morphotypes, 51% were irregular in shape. On the basis of opacity only 15.3% morphotypes possessed non- opaque colony and rest were opaque. Dendogram was produced from the resultant similarity matrices using the UPGMA method. The dendogram bifurcates into two clusters A and B, B cluster clearly separated isolate GYI-9 from rest of the thundred three bacterial isolates. Cluster A further divided and sub divided the into different sub clusters and sub-sub clusters, and so on dividing the 84 bacterial isolates into 17 groups (Figure 2).

Biochemical characterization of selected bacterial isolates
Out of a total of forty three bacterial isolates from gold mine, eleven were selected on basis of their ability to synthesize maximum gold nanoparticles, for biochemical characterization. Thus 11 bacterial isolates were examined for various biochemical tests *viz*., catalase, oxidase, triple sugar (TSI), indole, methyl red, Voges-Proskauer, citrate and fermentation of sugars i.e. glucose, sucrose, lactose and manitol. Dendrogram was produced from the resultant similarity matrices using the UPGMA method. According to dendrogram constructed using NTSYS-PC version 2.2 (Figure. 3), the first major bifurcation divides the dendrogram into two clusters A and B, cluster B clearly separate the isolate GBI-3 from other ten bacterial isolates and this isolate was also found to possess maximum gold nanoparticles synthesis activity. Cluster A further divided and sub divided the dendrogram into different and sub clusters and sub-sub clusters, which divided the ten bacterial isolates into eight groups.

Molecular characterization of selected bacterial isolates

Only three maximum gold nanoparticles synthesizing bacterial isolates *viz*., GBI-1, GPI-2 and GBI-3 were selected and subjected to molecular characterization using 16S *rrna* gene technology.

Characterization of selected bacterial isolates using 16S *rrna* gene technology

The DNA samples extracted from selected isolates *viz*., GBI-1, GPI-2 and GBI-3 were selectively amplified using PCR technology. Universal primers for 16S *rrna* gene were used for the experiment. After 35 cycles of PCR amplification as described under section 3.8.2, universal primer for 16S *rrna* gene were able to successfully amplify 16S *rrna* gene of selected bacterial isolates and produced amplicons of expected size i.e. 1500 bp (Figure -4).

Sequencing

The eluted and purified DNA of selected bacterial isolates *viz*., GBI-1, GPI-2 and GBI-3 were sequenced. To determine the nucleotide sequence of the PCR products of the 16S *rrna* gene of the selected isolates, sequencing was carried out using same upstream and downstream universal primers which were used for amplification and as a result, the size of the 16S *rrna* gene sequence of the selected bacterial isolates was found to be approximately 1200-1500 base pairs. The sequences have been submitted to NCBI. The nucleotide sequences and base composition of the selected gold nanoparticles synthesizing bacterial isolate *viz*., GBI-3 (Table-1), obtained after sequencing of 16S *rrna* gene has been depicted here as under:

***In silico* analysis of the sequences**

Further *in silico* analysis pertaining to the sequences, so obtained, was carried out using various bioinformatic tools available online. Analysis of 16S *rrna* gene of the selected three isolates revealed their homology with various other 16S *rrna* gene sequences. Various online approaches were followed to assess and predict relationship of the selected isolates with other 16S *rrna* gene sequences.

BLASTn

BLASTn search of these three sequences with the most similar 16S *rrna* gene sequences of the GenBank database (http://www.ncbi.nlm.nih.gov/blast) revealed the closest sequence identities from the sequence database. Characterization of the gold nanoparticles producing bacterial isolates on basis of the 16S *rrna* gene technology revealed that all the three bacterial isolates showed maximum similarity with *Bacillus* species. Sequences similarity between three gold nanoparticles producing bacterial isolates were compared with those from database using FASTA programme. The 16S *rrna* gene sequence analysis of bacterial isolates- GBI-1 bacterial isolate, showed 98% similarity with *Bacillus flexus* strain NBRC 15715, 16S ribosomal RNA, partial sequence; GPI-2 bacterial isolate, showed 99% similarity with *Bacillus licheniformis* strain BCRC 11702, 16S ribosomal RNA, partial sequence. GBI-3 bacterial isolate showed 100% similarity with *Bacillus thuringiensis* strain IAM 12077, 16S ribosomal RNA, partial sequence (Table-2)

Multiple sequence alignment

Multiple sequence alignment of test nucleotide sequence of maximum gold nanoparticles synthesizing *Bacillus thuringiensis* strain GBI3 with that of the selected nucleotide sequences was performed using CLUSTAL W program (Higgins *et al*., 1994) [16] available online at European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/). Pairwise percentage similarity score of fifteen nucleotide sequences obtained from NCBI database with test isolate GBI-3 from goldmine, elucidates that sequence-1 (*Bacillus thuringiensis* strain GBI-3) showed maximum similarity score of 100% (Table-3).

Phylogenetic analysis

To gain insight of the evolutionary pattern, phylogenetic tree was constructed using MEGA 5.0 bioinformatics tool. Neighbour-Joining (NJ) technique of mathematical averages (UPGMA) was used. The results have been presented in the form of phylogenetic tree (Figure 5). By Neighbor-Joining algorithm, the major bifurcation divided the phylogenetic tree into two major clusters, which then divided and sub divided into sub clusters and sub-sub clusters. These sub-sub clusters verified that bacterial isolate GBI-3 as *Bacillus thuringiensis,* as it clustered closely with *Bacillus thuringiensis* strain IAM 12077, 16S ribosomal RNA, partial sequence.

Discussion

It is surprising that microbial systematics and genomics have not yet been reconciled. This might be due to the intrinsic difficulties in inferring reasonable phylogenies from genomic sequences, particularly in the light of the significant amount of lateral gene transfer in prokaryotic genomes. However, recent studies indicate that the species tree and the hierarchical classification based on it are still meaningful concepts, and that state-of-the-art phylogenetic inference methods are able to provide reliable estimates of the species tree to the benefit of taxonomy. Conversely, we suspect that the current lack of completely sequenced genomes for many of the major lineages of prokaryotes and for most type strains is a major obstacle in progress towards a genome-based classification of microorganisms. We conclude that phylogeny-driven microbial genome sequencing projects such as the Genomic Encyclopaedia of Archaea and Bacteria (GEBA) project are likely to rectify this situation.16S ribosomal RNA, partial sequence. GBI-3 bacterial isolate showed 100% similarity with *Bacillus thuringiensis* strain IAM 12077, 16S ribosomal RNA, partial sequence.

Multiple sequence alignment of maximum gold nanoparticle synthesizing *Bacillus thuringiensis* strain GBI-3 nucleotide sequence was found to possess 100% homology with *Bacillus thuringiensis* strain IAM 12077, 16S ribosomal RNA, partial sequence. Phylogenetic analysis based on nucleotide sequences using NJ method was achieved *via* MEGA 5.0 and *Bacillus thuringiensis* strain GBI-3 was closely matched with *Bacillus thuringiensis* strain IAM 12077, 16S ribosomal RNA, and partial sequence with high boot strap value of 86%. Weisburg *et al*., (1991) described 16S ribosomal RNA amplification for phylogenetic study. A set of oligonucleotide primers capable of initiating enzymatic amplification (polymerase chain reaction) on a phylogenetically and taxonomically wide range of bacteria is described along with methods for their use and examples. One pair of primers is capable of amplifying nearly full-length 16S ribosomal RNA from many bacterial genera; the additional primers are useful for various exceptional sequences. Methods for purification of amplified material, direct sequencing, cloning, sequencing, and transcription are outlined. An obligate intracellular parasite of bovine erythrocytes, *Anaplasma marginale,* is used as an example; its 16S *rrna* was amplified, cloned, sequenced, and phylogenetically placed. *Anaplasmas* are related to the

genera *Rickettsia* and *Ehrlichia*. In addition, 16S *rrna* from several species were readily amplified from material found in lyophilized ampules from the American Type Culture Collection. By use of this method, the phylogenetic study of extremely fastidious or highly pathogenic bacterial species can be carried out without the need to culture them. In theory, any gene segment for which polymerase chain reaction primer design is possible can be derived from a readily obtainable lyophilized bacterial culture. Rainey *et al.,* (1997) [17] reported phylogenetic diversity of the *Deinococci* as determined by 16S ribosomal DNA Sequence comparison. 16S *rrna* sequences were determined for the five species of the genus *Deinococcus (Deinococcus erythromym, Deinococcus proteolyticus, Deinococcus rudioduruns, Deinococcus radiophilus,* and *Deinococcus radiopugnuns)* and the single species of the genus *Deinobucter (Deinobucter grandis).* With the exception of *Deinococcus erythromyxa,* the deinococci formed a coherent phylogenetic cluster which was related to the *Themus-Meiothemus* lineage. An analysis of the 16S *rrna* sequence of *Deinococcus erythromyxa* revealed that this organism was an actinomycete and a member of the genus *Kocuriu. Deinobucter grandis* falls within the radiation of the genus *Deinococcus* and phylogenetically can be considered a member of this genus. The results of the phylogenetic analyses were consistent with chemotaxonomic data. On the basis of our data, *Deinobacter grandis* was transferred to the genus *Deinococcus* as *Deinococcus grandis* comb. nov., the description of the genus *Deinococcus* was amended accordingly, and *Deinococcus erythromyxa* was transferred to the genus *Kocuriu* as *Kocuriu erythromym* comb. nov. The description of the family *Deinococcuceae* was amended to include organisms with rod-shaped cells, and a set of 16S *rrna* signature nucleotides was designated for this group. On the basis of the distinct phylogenetic position of the *Deinococcus* lineage and a set of 16S *rrna* signature nucleotides, the order *Deinococcules* ord. nov. was described. Shi *et al*., (1997) [18] reported characterization of viable bacteria from Siberian Permafrost by 16S *rrna* sequencing. Viable bacteria were found in permafrost core samples from the Kolyma-Indigirka lowland of northeast Siberia. The samples were obtained at different depths; the deepest was about 3 million years old. The average temperature of the permafrost was reported to be −10 °C. Twenty-nine bacterial isolates were characterized by 16S *rrna* sequencing and phylogenetic analysis, cell morphology, Gram staining, endospore formation, and growth at 30 °C. The majority of the bacterial isolates were rod shaped and grew well at 30 °C; but two of them did not grow at or above 28 °C, and had optimum growth temperatures around 20 °C. Thirty percent of the isolates could form endospores. Phylogenetic analysis revealed that the isolates fell into four categories: high G+C Gram-positive bacteria, b-proteobacteria, g-proteobacteria, and low G+C Gram-positive bacteria. Most high G+C Gram-positive bacteria and *β-proteobacteria*, and all *γ-proteobacteria*, came from samples with an estimated age of 1.8–3.0 million years. Most low G+C Gram-positive bacteria came from samples with an estimated age of 5,000–8,000 years. Philip *et al*., (2000) [19] proposed phylogeny of microorganisms populating a thick, subaerial, predominantly lithotrophic biofilm at an extreme acid mine drainage site. An unusually thick (1cm) slime developed on a slump of finely disseminated pyrite ore within an extreme acid mine drainage site at Iron Mountain, near Redding, California. The subaerial form of the slime distinguished it from more typical submerged streamers. Phylogenetic analysis of 16S *rrna* genes revealed a diversity of sequences that were mostly novel. Nearest relatives to the majority of sequences came from iron-oxidizing acidophiles, and it appeared that iron oxidation was the predominant metabolic characteristic of the organisms in the slime. The most abundant of the 16S *rrna* genes detected were from organisms related to *Leptospirillum* species. The dominant sequence (71% of clones) represented a new genus. Sequences within the *Archaea* of the *Thermoplasmales* lineage were detected. Most of these were only distantly related to known microorganisms. Also, sequences affiliating with *Acidimicrobium* were detected. Some of these were closely related to "*Ferromicrobium acidophilus*," and others were affiliated with a lineage only represented by environmental clones. Unexpectedly, sequences that affiliated within the delta subdivision of the *Proteobacteria* were detected. The predominant metabolic feature of bacteria of this subdivision is anaerobic sulfate or metal reduction. Thus, microenvironments of low redox potential possibly existed in the predominantly oxidizing environments of the slime. The 16S *rrna* technology has been reported to be used successfully for identification followed by phylogenic and evolution analysis gold nanoparticles synthesizing bacteria by Nangia *et al*., (2009); Sharma *et al*., (2012) [20].

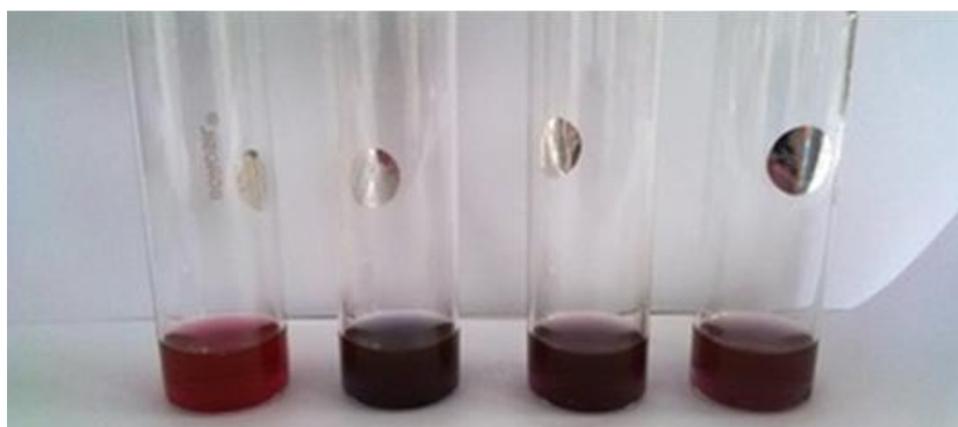

Fig 1: Supernatant of each bacterial culture was collected by centrifugation at 10000 rpm for 12 minutes at 4°C to study extracellular synthesis of gold nanoparticles. Ten ml of each supernatant was mixed with 10 ml of 1mM solution of $HAuCl_4$ and incubated at 37 °C for 240 hrs. Formation of gold nanoparticles was studied 0-240 hrs, with an interval of 12 hrs and confirmed by colour change of the solution from light yellow to red wine/purple colour (Figure 1). This formation of gold nanoparticles was also confirmed by the Spectrophotometer (Spectronic 20, Milton Roy Company) at two different wavelengths of 540 and 560 nm.

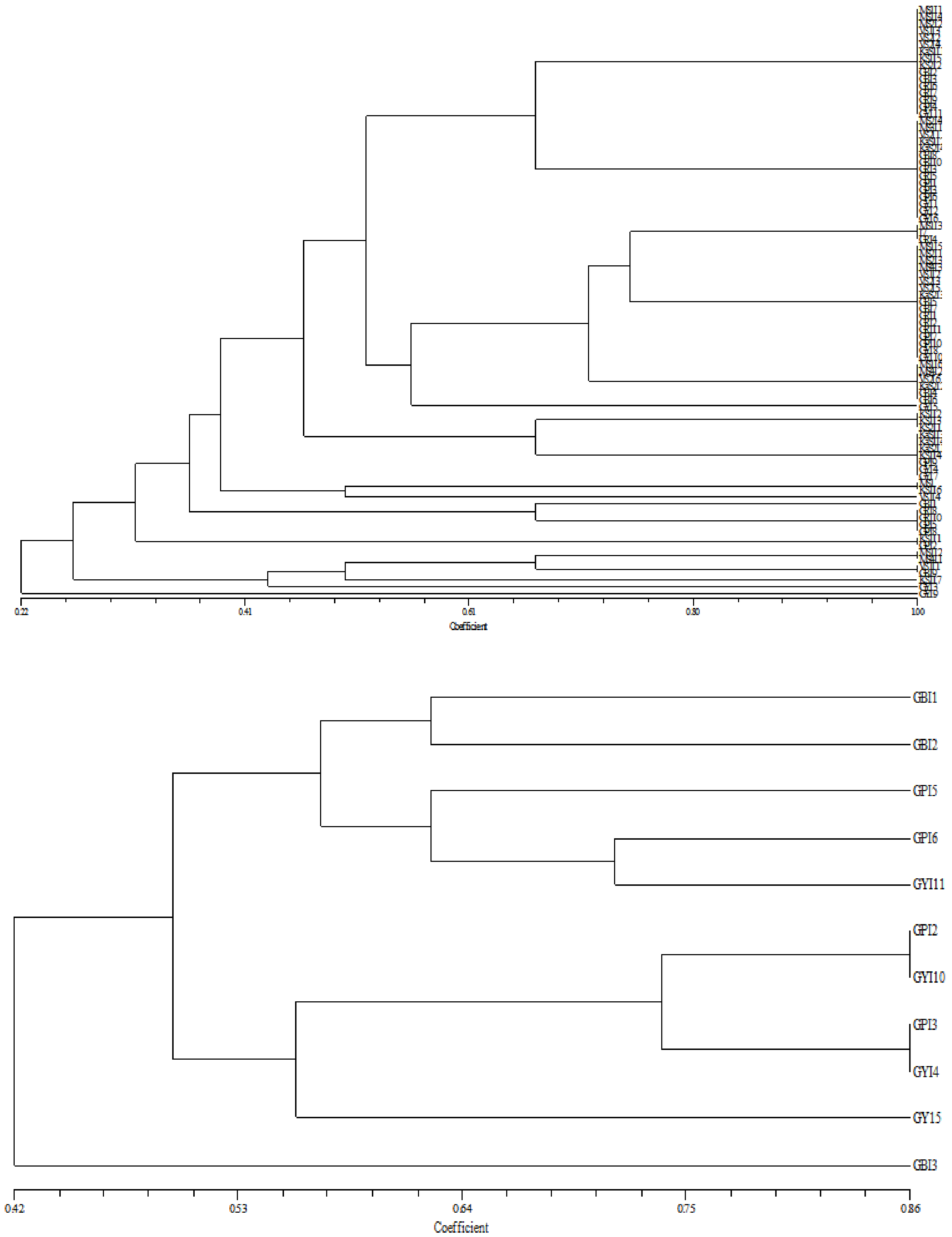

Fig 3: Dendrogram of 11 bacterial isolates based on biochemical characterization

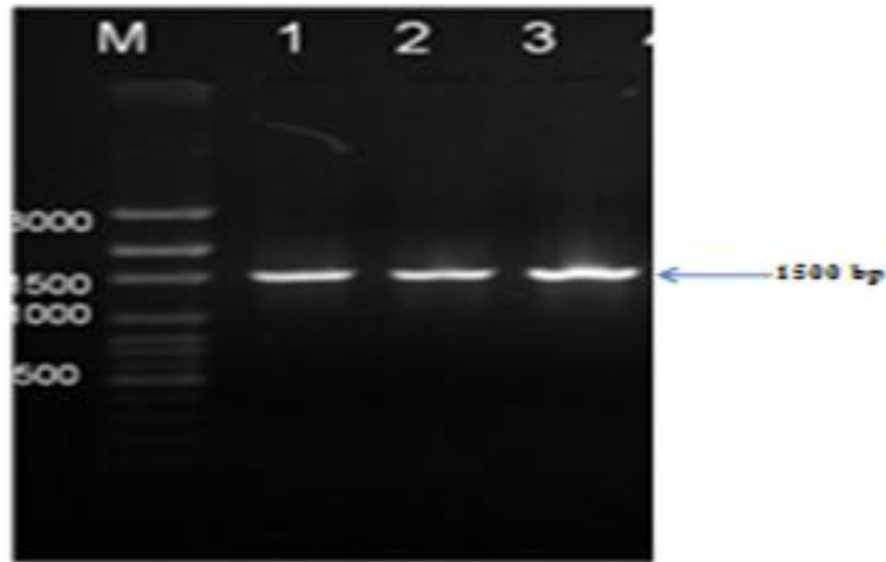

Figure 4 : Gel showing amplified DNA of selected three bacterial isolates
Lane M : 100 bp DNA ruler
Lane 1 : Amplified DNA of strain GBI-1
Lane 2 : Amplified DNA of strain GPI-2
Lane 3 : Amplified DNA of strain GBI-3

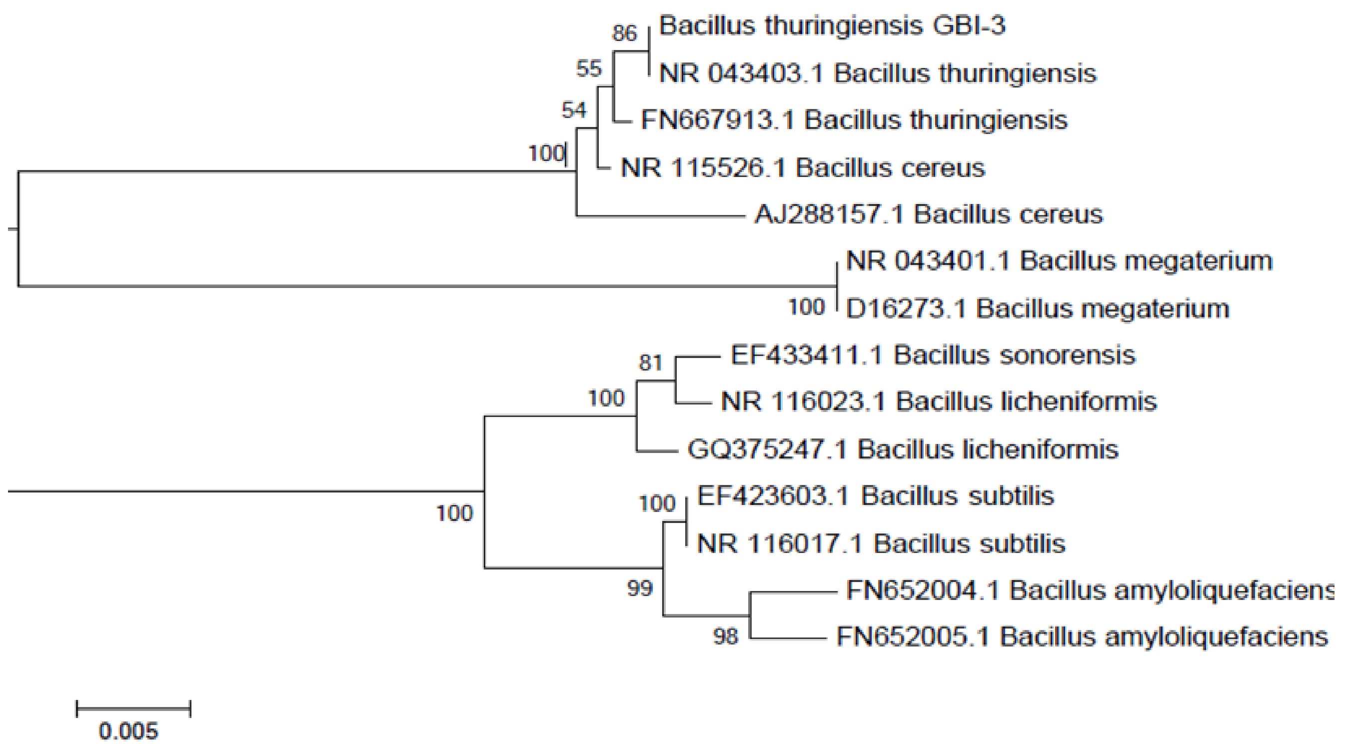

Fig 5: Phylogenetic tree of *Bacillus thuringiensis* strain GBI-3, 16S *rrna* gene. (GenBank accession numbers with name of organisms of the analyzed sequences are shown. A total of 100 bootstrap replicates were performed, and the bootstrap values are indicated at the branching points)

Table 1: Nucleotide base composition in the query sequence (GBI-3 isolate)

Nitrogenous Base	**Nucleotide Count**	
	Total	**Percentage (%)**
Adenine (A)	353	25.78
Thymine (T)	284	20.74
Cytosine (C)	315	23.00
Guanine (G)	417	30.46
G+C	732	**53.46**
A+T	637	46.53

Table 2: Similarity values of 16S *rrna* gene sequences of selected three gold nanoparticles producing bacterial isolates isolated from Khaltunala goldmine samples of H.P.

Bacterial Isolate	Closest Match	Accession Number	Per cent Similarity	G+C content (%)
GBI-3	*Bacillus thuringiensis* strain IAM 12077, 16S ribosomal RNA, partial sequence.	NR 043403.1	100%	53.46

Table 3: Percent homology of nucleotide query sequence of maximum gold nanoparticles synthesizing *Bacillus thuringiensis* strain GBI-3 with other nucleotide sequences present in the database using BLASTn analysis.

Accession No.	Description	Percent Homology
NR 043403.1	*Bacillus thuringiensis* strain IAM 12077, 16S ribosomal RNA, partial sequence	100%
NR 112780.1	*Bacillus thuringiensis* strain NBRC 101235, 16S ribosomal RNA, partial sequence	99%
NR 102506.1	*Bacillus thuringiensis* Bt407, 16S ribosomal RNA, partial sequence	99%
NR 113266.1	*Bacillus cereus* JCM 2152, 16S ribosomal RNA, partial sequence	99%
NR 112630.1	*Bacillus cereus* strain NBRC 15305, 16S ribosomal RNA, partial sequence	99%
NR 074453.1	*Bacillus anthracis* strain Ames, 16S ribosomal RNA, partial sequence	99%
NR 113990.1	*Bacillus mycoides* strain NBRC 101228, 16S ribosomal RNA, partial sequence	99%
NR 115993.1	*Bacillus mycoides* strain ATCC 6462, 16S ribosomal RNA, partial sequence	99%
NR 024697.1	*Bacillus weihenstephanensis* strain DSM 11821, 16S ribosomal RNA, partial sequence	99%
NR 113991.1	*Bacillus pseudomycoides* strain NBRC 101232, 16S ribosomal RNA, partial sequence	99%

Table 4: Pairwise percentage similarity score table of *Bacillus thuringiensis* strain GBI-3

Seq A	Name	Length	Seq B	Name	Length	Score
1	*Bacillus*	1369 bp	2	gi|343202909|ref|NR_043403.1|	1486 bp	100
1	*Bacillus*	1369 bp	3	gi|295027210|emb|FN667913.1|	1355 bp	95.0
1	*Bacillus*	1369 bp	4	gi|7321091|emb|AJ288157.1|	1340 bp	95.0
1	*Bacillus*	1369 bp	5	gi|636559466|ref|NR_115526.1|	1486 bp	99.0
1	*Bacillus*	1369 bp	6	gi|636559963|ref|NR_116023.1|	1468 bp	92.0
1	*Bacillus*	1369 bp	7	gi|256265123|gb|GQ375247.1|	1448 bp	92.0
1	*Bacillus*	1369 bp	8	gi|636559957|ref|NR_116017.1|	1468 bp	92.0
1	*Bacillus*	1369 bp	9	gi|126567887|gb|EF423603.1|	1538 bp	92.0
1	*Bacillus*	1369 bp	10	gi|126653866|gb|EF433411.1|	1468 bp	91.0
1	*Bacillus*	1369 bp	11	gi|115333962|gb|DQ993679.1|	1549 bp	91.0
1	*Bacillus*	1369 bp	12	gi|291088018|emb|FN652005.1|	845 bp	92.0
1	*Bacillus*	1369 bp	13	gi|291088017|emb|FN652004.1|	929 bp	91.0
1	*Bacillus*	1369 bp	14	gi|343202907|ref|NR_043401.1|	1486 bp	93.0
1	*Bacillus*	1369 bp	15	gi|457650|dbj|D16273.1|BAC16SRR08	1486 bp	93.0

Competing interests

The authors declare that they have no competing interests.

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