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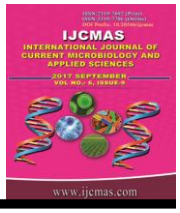

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In silico Analysis to Identify Synteny in Bacterial Signatures of Iron Nanoparticles Synthesizing Bacteria Contributing to Big Data

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A B S T R A C T

16S rrna gene sequencing technology is widely used to determine the microbial diversity which was earlier not possible with traditional methods. It is the most common housekeeping genetic marker which is used to study bacterial phylogeny and taxonomy. In this experiment iron nanoparticles synthesizing bacteria were isolated and characterized by morphological and biochemical test and confirmed by molecular approach using 16S rrna gene sequencing technology. The amplified gene sequence was compared to various bacterial genomes using various computational biology and bioinformatics tools in NCBI sequence database. Phylogenetic relationships and DNA sequencing identified three bacterial isolates as *Stenotrophomonas maltophilia* KBS 2.4, *Bacillus cereus* MSS 2.8 and *Bacillus cereus* MJS 3.0 of 1500bp after carrying BLASTn (NCBI Gene Bank Accession No: MF155656, MF155655, MF155656). Phylogenitic analysis and pairwise alignment revealed its close synteny with related species. A total of 100 bootstrap replications were performed to represent the evolutionary history of the taxa analysed. Using 16S rrna gene sequences, numerous bacterial genera and species have been classified 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. Characterization of iron nanoparticles synthesizing bacteria would be helpful in defining wide variety of applications in near future. Further, in the last decade sequencing of various bacterial genomes and comparison between genomes has confirmed the representativeness of the 16S rrna gene in bacterial phylogeny.

Introduction

Isolation of bacteria from new sources has been initiated and different types of bacteria have been reported from various types of metal mines, extreme environments, ocean beds, amorphous solids, effluents etc (Francois *et al.,* 2012). Various bacterial species such as *Actinobacter* sp., *Escherichia coli, Klebsiella pneumonia, Lactobacillus* spp., *Bacillus cereus, Corynebacterium* sp., *and Pseudomonas* sp. undergoes intracellular and extracellular mechanism to synthesize metallic nanoparticles (Mohanpuria *et al.,* 2008; Tollamadugu *et al.,* 2011). It is very interesting to add that various metal nanoparticles synthesizing bacteria has been added to list with respect to iron, gold, silver, copper, selenium, titanium etc (Li *et al.,* 2011). Traditional method of classification was carried out 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, 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. Molecular characterization technology is now widely used for wide range of bacterial species (Petti *et al.,* 2007). The analysis of DNA has been used in a large number of studies on bacterial taxonomy and bacterial typing and as well to further understand the basic mechanisms of evolution. The availability of DNA sequences for the analysis 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, 1998). Invention of PCR and automated DNA sequencing three 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/rdna 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 these differ between organisms of other genera and species (Woo *et al.,* 2008). 16S rrna gene sequencing has played a pivotal role in bacterial classification and discovery of novel bacteria (Woo *et al.,* 2003) and it depends on significant inter-species differences and small intra-species differences in 16S rrna gene sequences (Stackebrandt *et al.,* 1994).

To calculate similarity coefficients among organisms, a detailed analysis of 16S rRNA oligonucleotide catalogues or complete sequences revealed that there are both variable and highly conserved regions of the molecule called signature sequences which leads to determination of both close and distant relationships. 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 discovery and classification of novel bacterial species has been facilitated (Mignard *et al.,* 2006).

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 (Spiegelman *et al.,* 2005).

Advances in computational biology and bioinformatics has been remarkable in the last few decades, that established large scale sequencing, structure and function determination, gene prediction and specific landmarks on the genome as well as proteome analysis on strong foundations. Several services such as NCBI and EMBL are used when homologous sequences are to be compared and are essential precursors to numerous further analyses. Use of these tools in multiple sequence alignment, computational phylogenetic studies and proteomics has been carried out.

Keeping in view above considerations, the present study was framed for isolation, biochemical characterization and molecular identification of iron nanoparticles synthesizing bacteria by use of 16S rRNA technology to determine evolutionary rates of different bacterial isolates and close synteny between them by using various bioinformatics tools.

Materials and Methods

Isolation of iron nanoparticles synthesizing bacteria

A survey was conducted for selection of various sites of Himachal Pradesh for the collection of samples from Kangra and Mandi districts which were far flung and at high altitudes. Total of two subsites each form two districts were selected for present study. Different samples such as soil, pebbles and rock matting were collected from each selected site in sterilized screw capped tubes and jars which were brought to the laboratory and kept at 4^{o}C in refrigerator till further processing.

Isolation and screening of iron nanoparticles synthesizing bacteria was carried out using nutrient agar enriched with 2mM $FeSO_4$ solution using standard pour plate method (David *et al.*, 2014) by incubation at 37º C for 24 hrs. Iron nanoparticles synthesizing bacterial isolates were characterized morphologically and biochemically and confirmed using PCR- 16S rDNA technology.

Bacterial DNA isolation and PCR amplification of rrna gene

Total genomic DNA of selected bacterial isolates was extracted using Genomic DNA extraction Mini kit (Real Genomics) used as template for amplification of the 16S rrna gene (Fig. 1) using universal primers for 16S *rrna* gene of bacteria. Amplifications were performed using thermal cycler (Biorad) and with a temperature profile standardized for 16S *rrna* gene amplification.

The PCR amplification was carried out in 0.2 ml PCR tubes with 20 µl reaction volume containing Taq DNA polymerase (5U/reaction), PCR buffer (10x) with $MgCl_2$ (1.5 mM), primers (10 nmol/reaction), deoxynucleotide triphosphate (dNTPs) (0.5 mM) of Genei, Bangalore, India and template DNA. Initial cycle of 3 min at 95°C followed by 35 cycles of 30 sec at 95°C, annealing temperature of 50°C for 30 sec, elongation step of 2 min at 72°C and a final extension step of 10 min at 72°C followed by a 4^{0}C soak until recovery.

Agarose Gel Electrophoresis

PCR products were analysed by electrophoresis on 1% agarose (GeNei, Bangalore, India) in 1X TAE buffer containing (10 mg/ml) ethidium bromide and images were taken through Gel Documentation Unit (Syngene, UK). Size of the amplified products was determined by electrophoresis of 100bp standard molecular weight markers (GeNei, Bangalore, India). The selected bacterial isolates were further characterized using 16S rrna gene technology and genomic DNA extracted from these isolates were selectively amplified using PCR technology. Universal primers B27F and U1492R for 16S rrna gene were used.

DNA sequencing of 16S rrna gene fragment

The 16S rRNA purified PCR products (100ng concentration) were subjected to sequencing using the chain termination method developed by Sanger and his coworkers in 1977 (Applied Biosystem Inc).Sequencing of 16S rrna gene fragments of selected bacterial isolates was done from both forward and reverse directions. The selected sequences obtained were subjected to BLASTn search leading to identification of bacterial species.

The percentages of sequence matching were also analyzed and the sequences were submitted to NCBI-Gen Bank and accession numbers were obtained for the same.

Computational analysis (BLAST) and identification of bacterial species

Basic Local Alignment Search Tool (BLAST) uses an algorithm of Altshul *et al.,* (1997) 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 (Sacchi *et al.,* 2002).

Results and Discussion

Genotypic methods of bacterial identification are more accurate as compared to the traditional techniques including phenotypic and metabolic characteristics. In recent times comparison of the bacterial 16S rrna gene sequence which are conserved in nature has emerged as a preferred genetic technique. 112 total bacterial isolates were obtained initially out of which only 30 were quantified positive (Fig. 2) for synthesis of iron nanoparticles when assessed morphologically and biochemically (Fig. 3) followed by spectrophotometric analysis. Three maximum iron nanoparticles synthesizing bacteria MJS 3.0, MSS 2.8 and KBS 2.4 with 0.667, 0.645 and 0.618 activity were selected for further studies. Total genomic DNA of three selected bacterial isolates was extracted successfully using Genomic DNA extraction Mini kit (Real Genomics).Genomic DNA extracted from these isolates was selectively amplified using PCR technology and were characterized using 16S rrna gene technology. After 35 cycles of PCR amplification, universal primers for 16S rrna gene were able to successfully amplify 16S rrna gene and produced an amplicon of expected size i.e. 1500 bp for three bacterial isolates KBS 2.4, MJS 3.0 and MSS2.8 (Fig. 4). On the basis of results obtained from 16S rrna gene analysis and in addition to G+C content analysis, (Tables 1, 2, 3) the selected bacterial isolates were found to belong to genera *Stenotrophomonas* and *Bacillus*. Further *in silico* analysis pertaining to the sequence, so obtained, was carried out using various bioinformatics tools available online.

Analysis of 16S rrna gene of the selected bacterial isolates revealed homology with various other 16S rrna gene sequences.

BLASTn

BLASTn search of selected bacterial 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 (Marchler– Bauer *et al.,* 2000; Pruitt *et al.,* 2005). The percentages of sequence matching were also analyzed. The 16S rrna gene sequence analysis showed 93%, 98%, 99% similarity with *Stenotrophomonas maltophilia* strain HII-I and *Bacillus cereus* strain MX-5 and *Bacillus sp.* strain *cereus* 16S ribosomal RNA, complete sequence, respectively (Table 4, 5, 6).

Multiple sequence alignment

Multiple sequence alignment of test nucleotide sequence of the selected nucleotide sequences was performed using CLUSTAL W program (Higgins *et al.,* 1992) available online at European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/) and the CLUSTAL W output was then used in (MEGA 6.0 software) bioinformatics tool for constructing phylogenetic tree.

Phylogenetic analysis

To trace out the evolutionary pattern of the selected bacterial isolates, and assess its relationship with other selected sequences at

NCBI, phylogenetic tree was constructed using MEGA 6.0 Neighbour-Joining (NJ) method of mathematical averages (UPGMA) among 16S rrna gene sequence and corresponding gene sequences. The selected bacterial isolates were united with quite high statistical support by the bootstrap method estimates for 100 replications and values inferred greater than 50 percent, were presented (Fig. 5, 6, 7). Phylogenetic tree also verified bacterial isolates as *Bacillus cereus* and *Stenotrophomonas maltophilia* as it clustered closely with *Bacillus cereus* MX-5 and *Bacillus cereus*, *Stenotrophomonas maltophilia* HII-I 16S ribosomal RNA, complete sequence. Thus, based on molecular characterization, isolates has been submitted to NCBI as under *Bacillus cereus* MJS, *Bacillus cereus* MSS, *Stenotrophomonas maltophilia* KBS with accession number MF155657, MF155655, MF155656, respectively.

Fig.1 Isolated DNA of three selected bacterial isolates

Fig.2 Thirty selected iron nanoparticles synthesizing bacterial isolates

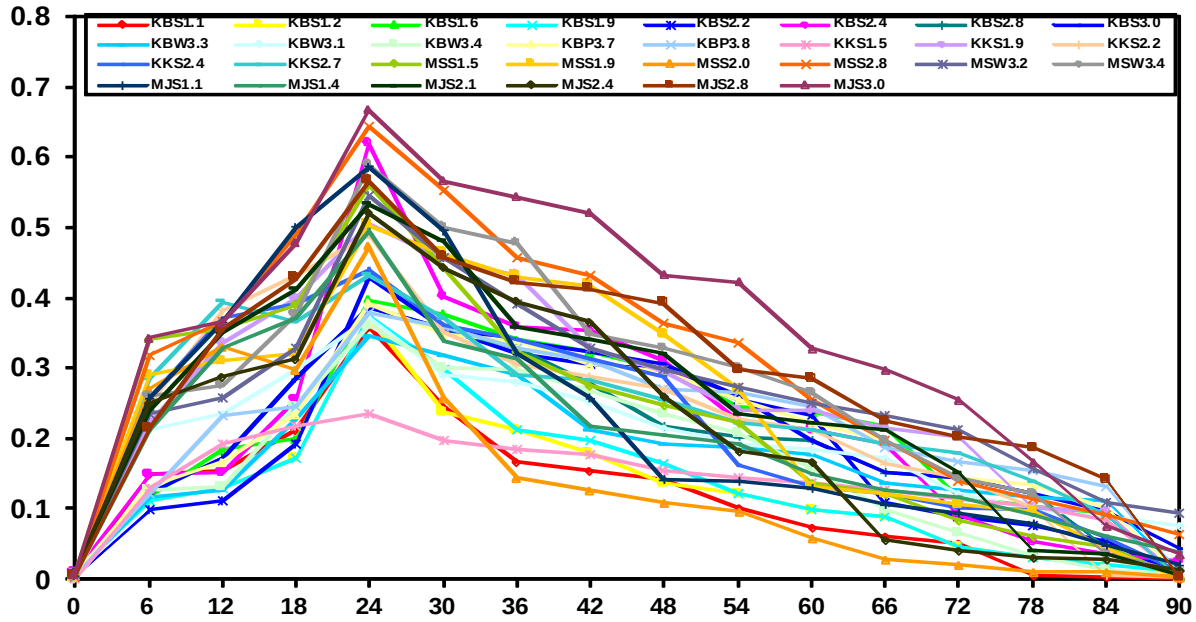


Fig.3 Dendrogram of selected thirty isolates based on biochemical characterstics

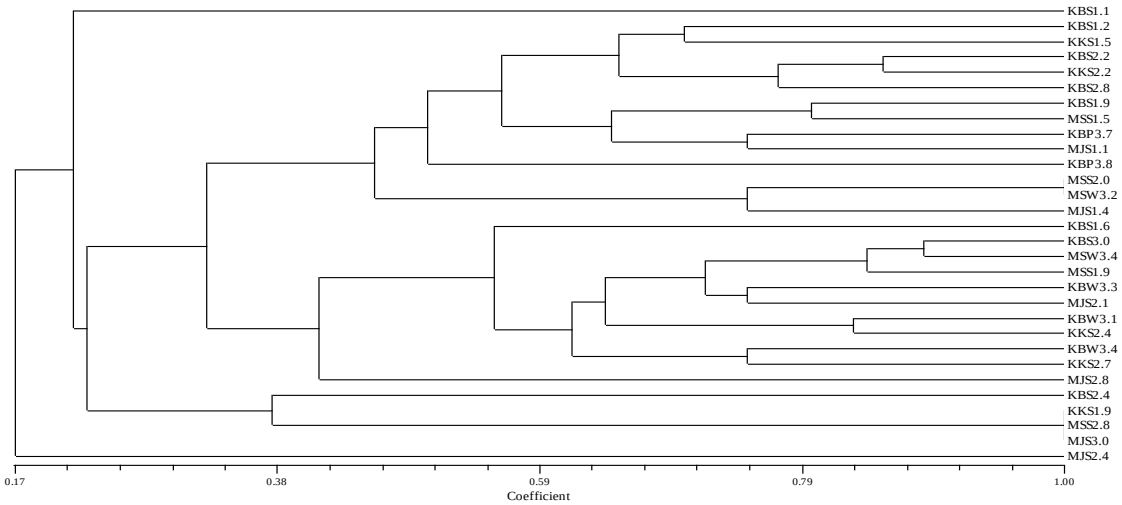
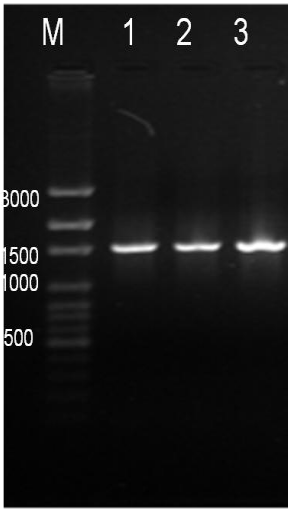


Fig.4 Amplified product of 1500 bp produced by 16S rRNA PCR amplification



1. KBS 2.4; 2. MSS 2.8; 3. MJS 3.0

Fig.5 Phylogenetic tree of KBS 2.4 isolate

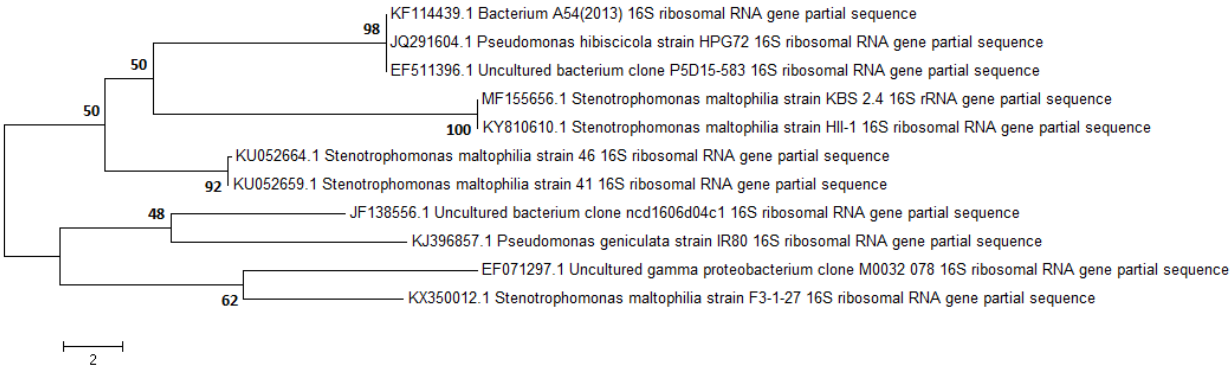


Fig.6 Phylogenetic tree of MJS 3.0 isolate

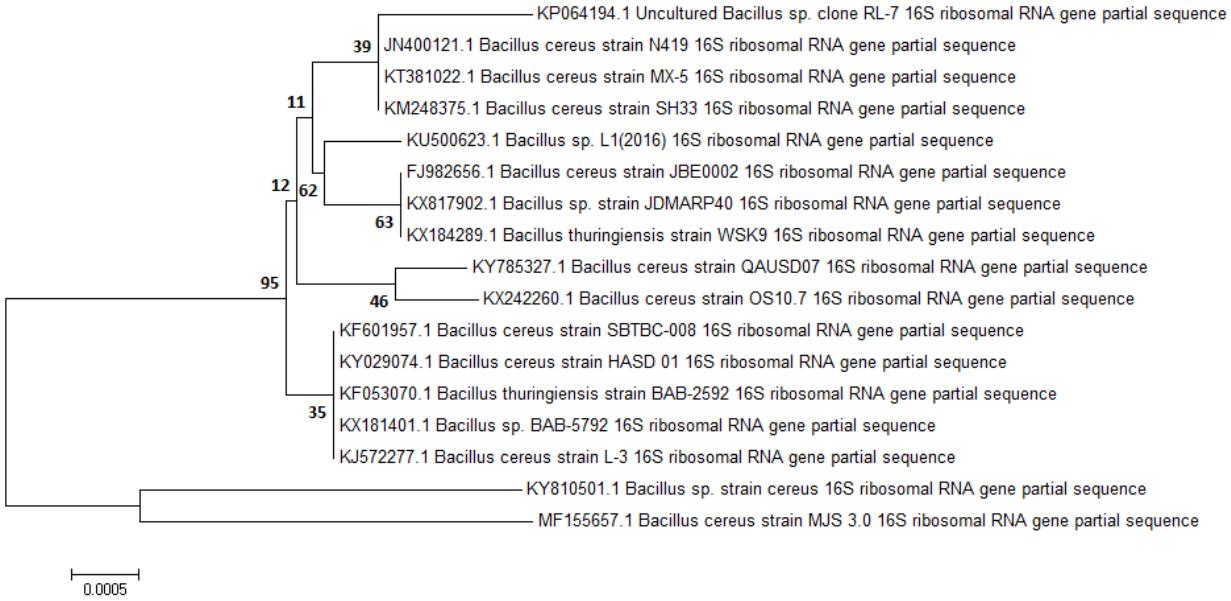


Fig.7 Phylogenetic tree of MSS 2.8 isolate

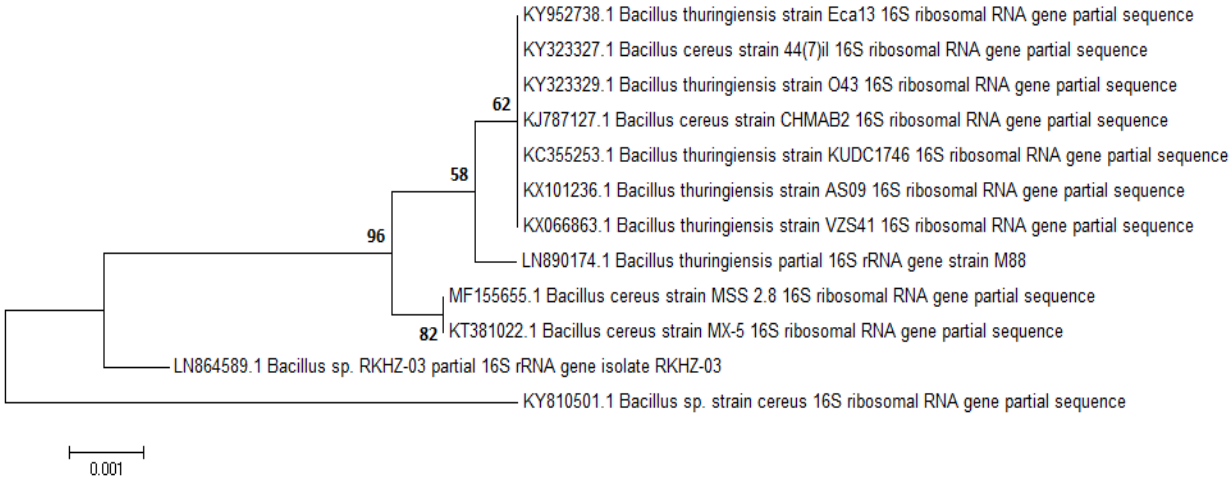


Table.1 Nucleotide base composition in the query sequence KBS 2.4 isolate (1606bp)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	360	22
Thymine (T)	407	27
Cytosine (C)	361	22
Guanine (G)	478	29
G+C	839	52.25
A+T	767	47.75

Table.2 Nucleotide base composition in the query sequence MJS 3.0 isolate (1588bp)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	363	22
Thymine (T)	384	26
Cytosine (C)	396	24
Guanine (G)	445	28
G+C	841	52.96
A+T	747	47.04

Table.3 Nucleotide base composition in the query sequence MSS2.8 isolate (1585bp)

Nitrogenous Base	Nucleotide Count	
	Total	Percentage (%)
Adenine (A)	362	22
Thymine (T)	384	26
Cytosine (C)	394	24
Guanine (G)	445	28
G+C	839	52.94
A+T	746	47.06

Table.4 Percent homology of 16S rrna gene sequence MJS 3.0 isolate with other nucleotide sequences present in the database using BLASTn analysis

Accession Number	Closest match	Length (bp)	Per cent Similarity
KY810501.1	Bacillus sp. strain cereus	939	99%
KF601957.1	Bacillus cereus strain SBTBC 008	1557	99%
KM248375.1	Bacillus cereus strain SH33	1553	99%
KP064194.1	Uncultured Bacillus sp. clone RL7	1574	99%
KU500623.1	Bacillus sp.L1(2016)	1537	98%
KT381022.1	Bacillus cereus strain MX5	1500	98%
JN400121.1	Bacillus cereus strain N419	1484	98%
FJ982656.1	Bacillus cereus strain JBE0002	1470	98%
KY029074.1	Bacillus cereus strain HASD 01	1586	97%
KF053070.1	Bacillus thuringiensis strain BAB 2592	1533	97%
KY785327.1	Bacillus cereus strain QAUSD07	1523	97%
KX817902.1	Bacillus sp. strain JDMARP40	1498	97%
KX242260.1	Bacillus cereus strain OS10.7	1497	97%
KX181401.1	Bacillus sp. BAB 5792	1487	97%
KJ572277.1	Bacillus cereus strain L3	1481	97%
KX184289.1	Bacillus thuringiensis strain WSK9	1436	97%
KY880974.1	Bacillus cereus strain G5	890	97%

Table.5 Percent homology of 16S rrna gene sequence isolate KBS 2.4 with other nucleotide sequences present in the database using BLASTn analysis

Accession Number	Closest match	Length (bp)	Per cent Similarity
KY810610.1	Stenotrophomonas maltophilia strain HII-1	1497	93%
AB680480.1	Stenotrophomonas maltophilia strain: NBRC 13692	1470	93%
EU048328.1	Stenotrophomonas maltophilia strain CMG3098	1272	93%
KY595447.1	Stenotrophomonas maltophilia strain M58	1458	93%
KX672778.1	Uncultured Stenotrophomonas bacterium clone S8_P1	1394	93%
EF071297.1	Uncultured gamma proteobacterium clone M0032	887	93%
FJ605176.1	Pseudomonas sp. CTN-7	1430	93%
JF138556.1	Uncultured bacterium clone ncd1606d04c1	1366	93%
EF511509.1	Uncultured bacterium clone P5D15-445	1486	93%
JQ433923.1	Pseudomonas sp. ZR3	1469	93%
JQ746029.1	Uncultured bacterium clone 1710643_4	876	93%
KF114439.1	Bacterium A54(2013)	1537	93%
JQ291604.1	Pseudomonas hibiscicola strain HPG72	1507	93%
EF511396.1	Uncultured bacterium clone P5D15-583	1486	93%
KJ396857.1	[Pseudomonas] geniculata strain IR80	1414	90%
KT154923.1	Stenotrophomonas sp. FHS12	1407	90%
KU052664.1	Stenotrophomonas maltophilia strain 46	906	89%
KU052659.1	Stenotrophomonas maltophilia strain 41	906	89%
KX350012.1	Stenotrophomonas maltophilia strain F3-1-27	1446	89%
KU756576.1	Stenotrophomonas maltophilia strain SsT2	1412	89%

Table.6 Percent homology of 16S rrna gene sequence isolate MSS2.8 with other nucleotide sequences present in the database using BLASTn analysis

Accession Number	Closest match	Length (bp)	Per cent Similarity
KT381022.1	Bacillus cereus strain MX-5	1500	98%
KY810501.1	Bacillus sp. strain cereus	939	98%
KC414685.1	Bacillus thuringiensis strain KUDC1705	1538	97%
KX101236.1	Bacillus thuringiensis strain AS09	1503	97%
KJ787127.1	Bacillus cereus strain CHMAB2	1554	97%
KY323326.1	Bacillus thuringiensis strain 41(7)il	1516	97%
LN890196.1	Bacillus thuringiensis strain B20	1679	97%
KX066863.1	Bacillus thuringiensis strain VZS41	1482	97%
KY323329.1	Bacillus thuringiensis strain O43	1525	97%
LN890174.1	Bacillus thuringiensis strain M88	1680	97%
KY323327.1	Bacillus cereus strain 44(7)il	1423	97%
KU198624.1	Bacillus cereus strain FSL H8-0488	1540	97%
KY952738.1	Bacillus thuringiensis strain Eca13	1427	97%
KC414723.1	Bacillus thuringiensis strain KUDC1744	1481	97%
LN864589.1	Bacillus sp. RKHZ-03 isolate RKHZ-03	1476	97%
KC414685.1	Bacillus thuringiensis strain KUDC1705	1538	97%

Although conventional phenotypic methods are relatively inexpensive and allow identification of most common bacteria but certain groups of bacteria have been difficult to identify and specified equipment/expertise may be required. Molecular characterization using 16S rrna gene technology lead to rapid and accurate identification of bacterial isolates upto species level. Carl Woese (1987) developed this state of art technology to analyse the sequence of 16S rrna gene technology to analyse the sequence of 16S rrna gene of various bacteria. The inventions of PCR and automated DNA sequence two decades ago followed by subsequent research on 16S rRNA sequencing of bacteria has led to accumulation of vast amount of sequence data on the rrna gene of smaller subunit of ribosomes. Comparison of these sequences has revealed that rrna gene sequences are highly conserved within living organisms of the same genus and species and differ between organisms of other genera and species. In addition to highly conserved primer binding sites, 16S rrna gene sequences contain hypervariable regions that can provide species-specific signature sequences useful for bacterial identification.

In the present study, universal primers B27F 5́ -AGAGTTTGATCCTGGCTCAG-3́ and U1492R 5`-GGTTACCTTGTTACGACTT-3́ with an annealing temperature of 50^{o}C produced an amplified product of 1500 bp of 16S rrna gene of three selected bacterial isolates.

In other studies also universal primers have been reported to amplify 16S rrna gene. In similar context several *Bacillus* species were reclassified based on 16S rDNA and separated into different phylogenetically distinct clusters (Dong and Jeancharles, 2003). On the basis of 16S rrna gene technology, bacterial isolates were identified as *Stenotrophomonas maltophilia* KBS2.4, *Bacillus cereus* MJS3.0 and *Bacillus cereus* MSS 2.8 in the present study. Similarly, 16S rrna gene technology along with *in silico* analysis have been successfully adopted for sequence of 16S rDNA of strain OS4 and was submitted to Gen-Bank (entry code: JN247637). The information obtained by the BLAST program indicated a close genetic relatedness of strain OS4 with the rDNA sequence of *S. maltophilia* in NCBI database (Oves *et al.,* 2013). Such a higher identical value confirmed the strain OS4 to be *Stenotrophomonas maltophilia.*

In conclusion, the present study reported use of 16S rrna gene sequencing for definitive identification of iron nanoparticles synthesizing bacteria for harmonious interpretation of sequence data. These bacteria has the ability to synthesize iron nanoparticles extracellularly at a much faster rate and are cost effective, free from any toxic chemicals and thus are safely used in wide variety of applications such as bioremediation, biocontrol agents, antimicrobial agent, pesticide degradation, textile dye decolourization etc. The appropriate use of such technology requires the adoption of standards similar to those previously defined for DNA-DNA hybridization (Darland *et al.,* 1971; Deinhard *et al.,* 1987) as the adaptation of 16S rrna gene sequencing as a tool in species identification is still a relatively new phenomenon and will continue to evolve over time. Furthermore, use of microarray based technologies with 16S or other housekeeping gene targets in the future may provide a much more sensitive and definitive platform for molecular species identification in the future.

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