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Method

Development of PCR primer systems for amplification of 16S-rDNA to detect of *Thiobacillus* spp

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Abstract: *Thiobacillus* is a genus of Gram-negative, rod-shaped and autotrophic *Betaproteobacteria*. They catalyze the dissimilatory oxidation of elemental sulfur and reduced inorganic sulfur compounds. Whereas more than 30 species have been known in this genus, most were never reliably or effectively published. The rest were either reclassified into *Thiomonas*, *Paracoccus*, *Starkeya*, *Sulfuriferula*, *Halothiobacillus*, *Thermithiobacillus* or *Acidithiobacillus*, were lost from culture. Most of *Thiobacillus* species are obligate autotrophs via elementary sulfur, thiosulfate or polythionates as energy sources. Based on 16S ribosomal RNA sequence analysis, many members of *Thiobacillus* have been reclassified. A system was developed for the detection of *Thiobacillus* bacteria by the amplification of specific 16S ribosomal RNA sequence gene (16S rDNA) fragments with PCR. Primer sequences were designed for the amplification of fragments of 16S rDNA.

Key words: 16S rDNA; Primer; PCR; *Thiobacillus* spp.

Introduction

The *Thiobacillus* is a genus of autotrophic acidophilus bacteria that oxidizes iron or sulfur. When they have an appropriate environment for growth, they obtain their energy and food requirements from the oxidation of sulfur and iron (1-3).

So far 20 species of the genus *Thiobacillus* bacteria have been identified. They are the most important oxidizing the sulfur in the soil. These bacteria oxidize the sulfur and supply the required sulfate to the plant. In addition, by reducing the acidity of the soil around the roots, they will increase the solubility of micronutrients in the soil (4, 5) and may effect on soil properties (6).

Due to oxidation ability, *Thiobacillus* can be used in industrial extraction sulfide metals such as copper and gold. This process is called bioleaching (2, 7).

At heavy alkaline and calcareous lands, the availability of plant to nutrients, especially phosphorus, iron and zinc significantly reduced. In the case of sulfur in the soil and the presence of sulfur-oxidizing microorganisms (*Thiobacillus*), in the appropriate humidity and the temperature sulfur is dioxide. Then sulfuric acid is produced and some parts of soil will be acidic and above elements will be accessible (8, 9).

As a result of 16S ribosomal RNA sequence analysis, many members of *Thiobacillus* have been reclassified as *Thiobacillus versutus* to *Paracoccus*, *T. acidophilus* to *Acidiphilium*, *T. intermedius*, *T. perometabolis*, *T. thermosulfatus*and *T. cuprinus* to *Thiomonas, T. thiooxidans*, *T. ferrooxidans*, *T. caldus* and *T. albertensis* to *Acidithiobacillus*, *T. neapolitanus*, *T. halophilus* and *T hydrothermalis* to *Halothiobacillus* and *T. tepidarius* to *Thermithiobacillus* (10-13)

Lane et al., 1985 determined the 5S rRNA nucleotide sequences from *T. neapolitanus*, *T. ferrooxidans*, *T. thiooxidans*, *T. intermedius*, *T. perometabolis*, *T. thioparus*, *T. versutus*, *T. novellus*, *T. acidophilus*, *T. pelophila*, *T.* sp. strain L-12, and *Acidiphilium cryptum* (14).

A highly reliable method for fast detection and taxonomical classification of thiobacilli is accessible. The method includes RFLP analysis of PCR amplified 16S rDNA, 23S rDNA, and intergenic spacer rDNA between the 16S and the 23S rRNA-genes (amplified ribosomal DNA restriction enzyme analysis – ARDREA), as well as genomic fingerprinting using random primers (RAPD) and repetitive primers (Rep-APD) (15-17).

The taxonomic and discriminatory power of ARDREA, RAPD and Rep-APD was compared by Selenska-Pobell et al., 1998. It was showed that the RAPD and Rep-APD methods provide taxonomic results that are in agreement with the classification based on the RFLP analysis of the highly conservative ribosomal RNA operons of the strains studied (15).

Several hundreds of Acidithiobacillus strains have been isolated from all over the world (18) and a large number of 16S rRNA sequence clones have been obtained from environmental studies (19).

The detection of acidophilic bacteria culture methods is time-consuming and unreliable. In current study, several PCR approaches were developed to amplify small-subunit rRNA sequences from the DNA of eight Thiobacillus ssp.

Materials and Methods

This research was carried out to PCR-mediated and molecular specific detection of some species of the ge-

nus Thiobacillus based on 16S-rRNA gene sequence.

The studied Thiobacillus is composed of eight validated species, described between 1922 and 2017 that are included *T. halophilus, T. hydrothermalis, T. thermosulfatus, T. delicates, T. aquaesulis, T. thiophilus, T. denitrificans* and *T. thioparus*. The characterizations of each species will be explained in the following.

T. halophilus

Type strain: (see also StrainInfo.net) ATCC 49870= DSM 6132.

Sequence accession no. (16S rRNA gene) for the type strain: U58020.

Etymology: Gr. n. *hals halos*, salt; N.L. masc. adj. *philus* (from Gr. masc. adj. *philos*), friend, loving; N.L. masc. adj. *halophilus*, salt-loving (20).

T. hydrothermalis

Type strain: (see also StrainInfo.net) R3 = ATCC 51453= DSM 7121.

Sequence accession no. (16S rRNA gene) for the type strain: M90662.

Etymology: N.L. masc. adj. *hydrothermalis*, hydrothermal, pertaining to a hydrothermal vent in the North Fiji basin (21).

T. thermosulfatus

Type strain: (see also StrainInfo.net) ATCC 51520.

Sequence accession no. (16S rRNA gene) for the type strain: U27839.

Etymology: Gr. n. *thermê*, heat; N.L. n. *sulfas -atis*, sulfate; N.L. masc. adj. *thermosulfatus*, intended to mean organism that produces sulfate and grows at high temperatures (22).

T. delicatus

Type strain: (see also StrainInfo.net) THI 091 = DSM 17897 = IFO (now NBRC) 14566.

Sequence accession no. (16S rRNA gene) for the type strain: AB245481.

Etymology: L. masc. adj. *delicatus*, delicate (23).

T. aquaesulis

Type strain: (see also StrainInfo.net) ATCC 43788= DSM 4255.

Sequence accession no. (16S rRNA gene) for the type strain: U58019. Etymology: L. pl. n. *aquae*, waters; L. n. *Sulis -is*, Sulis (a goddess), here the Temple of Sulis Minerva (Minerva, the Roman goddess of wisdom); N.L. gen. n. *aquaesulis*, of/from the waters of Sulis Minerva (24).

T. thiophilus

Type strain: (see also StrainInfo.net) D24TN = DSM 19892 = JCM 15047.

Sequence accession no. (16S rRNA gene) for the type strain: EU685841.

Etymology: Gr. n. *theion* (Latin transliteration *thium*), sulfur; N.L. adj. *philus -a -um* (from Gr. adj. *philos -ê -on*), friend, loving; N.L. masc. adj. *thiophilus*, sulfur-loving (25).

T. denitrificans

Type strain: (see also StrainInfo.net) AB7 = ATCC 23644= CIP 104767 = DSM 12475 = JCM 3870 = NCIMB 9548.

Sequence accession no. (16S rRNA gene) for the type strain: AJ243144.

Etymology: N.L. part. adj. *denitrificans*, denitrifying (26).

T. thioparus

Type strain: (see also StrainInfo.net) Starkey = ATCC 8158= CIP 104484 = DSM 505 = JCM 3859 = NBRC 103402.

Sequence accession no. (16S rRNA gene) for the type strain: HM173629.

Etymology: Gr. n. *theion* (Latin transliteration *thium*), sulfur; N.L. masc. adj. *parus* (from L. v. *paro*, to furnish, provide); N.L. masc. adj. *thioparus*, sulfur-producing (27).

Primers based on 16S rRNA sequences were designed to be specific for bacterial above groups. The 16S rDNA sequence data was obtained from GenBank databases.

To evaluate similarities and differences in of 16S-rDNA sequences for these species, they were aligned by the software CLUSTAL O (1.2.1). The primers were designed based on dissimilar areas.

In addition, the designed primers were used for amplification of a part rRNA gene of three *Thiobacillus* species including *T. thioparus*, *T. denitrificans* and *T. novellus*. The PCR products were loaded in the electrophoresis agarose gel.

Results

In this study, 16S-rDNA sequence of 8 species for the genus *Thiobacillus* was investigated. First, to assess similarities and differences in of sequences for this species, they were aligned by the software CLUSTAL O (1.2.1). The obtained results and designed primers based on dissimilar areas as follows.

The phylogram of 8 studied *Thiobacillus* species was designed by sequence alignment (Figure 1) to eva-

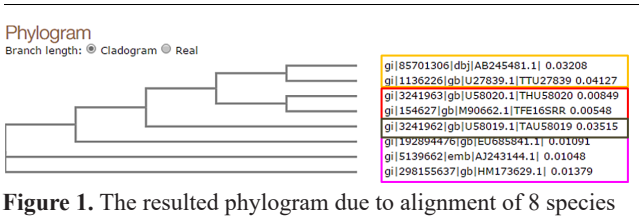

Figure 1. The resulted phylogram due to alignment of 8 species of the genus Thiobacillus.

Table 1. Primer parameters of *T. aquaesulis* (U58019) (gi|3241962|gb|U58019.1|TAU58019).

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	AATACGGGTGGTGGATGACG	Plus	20	452	471	59.82	55.00
Reverse primer	ACCTGTGTTCGCATTCCCTT	Minus	20	1039	1020	59.89	50.00
Product length		500					

Table 2. Primer parameters of *T. halophilus* (U58020.1) (gi|3241963|gb|U58020.1|THU58020).

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	CTAGTTGGTAGGGTAAAG	Plus	18	241	258	48.06	44.44
Reverse primer	AACGTATTCACCGCAGAC	Minus	18	1375	1358	55.10	50.00
Product length		1135					

Table 3. Primer parameters of *T. hydrothermali* (M90662.1) (gi|154627|gb|M90662.1|TFE16SRR).

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	CTAGTTGGTGGGGTAAGA	Plus	18	241	258	52.25	50.00
Reverse primer	AACGTATTCACCGCAGCA	Minus	18	1375	1358	57.31	50.00
Product length		1135					

Table 4. Primer parameters of *T. delicatus* (AB245481) (gi|85701306|dbj|AB245481.1|).

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	GATAGATGTGAAAGCCCT	Plus	18	564	581	50.79	44.44
Reverse primer	AAATCTCTTCGGGATTCCA	Minus	19	985	967	53.36	42.11
Product length		422					

luate the differences between the sequences.

Studies showed that in general these 8 species could be assigned to 4 groups (Figure 1). The species that are marked with the same color, showed very high sequence similarity and are assigned to same group.

The place of designed primer for any species is known based on its corresponding color. According to results, the species *T. aquaesulis* (U58019) can be placed alone in one group. So based on dissimilar areas with other species, the primers were designed (Table 1).

In order to design appropriate primers to identify and differentiate closely related species than another, the same-grouped species were re-aligned.

According to alignment, there is a high similarity between the two species *T. halophilus* and *T. hydrothermali*. Therefore, the primers were designed in a way that different single nucleotide located exactly at the 3 end (Tables 2 and 3).

According to alignment results, there is a high similarity between the three species *T. thioparus*, *T. thiophilus* and *T. denitrificans*. Therefore, the primers were designed in a way that different single nucleotide located exactly at the 3 end (Tables 6-8). In *T. denitrificans* two pairs primers have been designed (Table 6).

We design the specific primers for *T. novellus* (Starkeya novella) that their characterizations have been shown in Table 9.

The primers were used for amplification of a part of rRNA gene in three *Thiobacillus* species including *T. thioparus*, *T. denitrificans* and *T. novellus*. The results

Table 5. Primer parameters of *T. thermosulfata* (U27839.1) (gi|1136226|gb|U27839.1|TTU27839).

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	TCGCAAGACCTCACGTCATA	Plus	20	167	186	58.83	50.00
Reverse primer	CATGCTCTGGCGAGTTCCT	Minus	19	982	964	59.78	57.89
Product length	816						

Table 6. Primers parameters of *T. denitrificans* (AJ243144.1) (gi|192894476|gb|EU685841.1|). Two pairs primers have been designed.

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	ACGAAACGGTACGCGCTAACATC	Plus	23	427	449	63.83	52.17
Reverse primer	TCCAATTCCCTTTCGGGCACC	Minus	21	1023	1003	62.96	57.14
Product length		597					
Forward primer	CTAGTTGGTGGGGTAATGGCTC	Plus	22	227	248	60.42	54.55
Reverse primer	TCCAATTCCCTTTCGGGCACC	Minus	21	1023	1003	62.96	57.14
Product length		597					

Table 7. Primer parameters of *T. thiophilus* (EU685841.1) (gi|192894476|gb|EU685841.1|).

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	CAGGGGATCGCAAGACCTTG	Plus	20	188	207	60.75	60.00
Reverse primer	AATTCCACCCCCCTCTGACA	Minus	20	667	648	60.48	55.00
Product length		480					

Table 8. Primer parameters of *T. thioparus* (HM173629.1) (gi|298155637|gb|HM173629.1|).

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	GAAACGGTACGCTCTAACATAGC	Plus	23	381	403	59.51	47.83
Reverse primer	AGCACCTGTGTTCCGGTTCT	Minus	20	986	967	61.70	55.00
Product length		606					

Table 9. Primer parameters of *T. novellus* (DSM 506) (TFEIAM121A).

	Sequence (5'->3')	Template strand	Length	Start	Stop	Tm	GC%
Forward primer	TTATCGCCATTGGATGAACCCG	Plus	22	154	175	61.07	50.00
Reverse primer	AGTATCAAGGGCAGTTCTGGA	Minus	21	558	538	58.45	47.62
Product length		405					

of electrophoresis agarose gel have been shown in Figure 2.

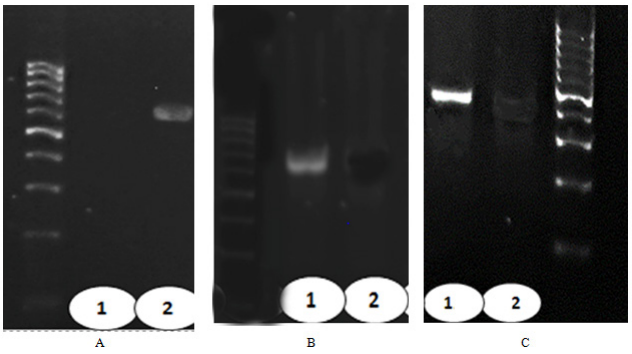

Figure 2. The amplification of a part of rRNA gene in three *Thiobacillus* species on electrophoresis of agarose gel. A: *T. thioparus*, 1: negative control, 2: PCR product B: *T. denitrificans*, 1: PCR product, 2: negative control C: *T. novellus*, 1: PCR product, 2: negative control. The size marker was 100 bp marker in the all samples.

Discussion

The Polymerase Chain Reaction (PCR) has been known as one of the most important inventions of the twentieth century in molecular genetics. Small quantities of the genetic material can now be amplified to be able to identify, manipulate DNA, identify infectious organisms, detect genetic variations, including mutations, in human genes and numerous other applications (28-30).

Single nucleotide polymorphism (SNP) genotyping is a common technique applied in relationship with investigations on polymorphism. The polymerase chain reaction experiments via confronting two-pair primers (PCR-CTPP) is a time- and cost-effective technique for SNP (single nucleotide polymorphism) genotyping. In the past decades, many SNPs were genotyped successfully using this technique (28, 31).

Proper primer designing is essential for successful PCR and a key to specific amplification with high yield. The 3' end stability is the maximum ΔG value of the five bases from the 3' end. An unstable 3' end (less negative ΔG) will result in less false priming. The ability to precisely predict amplification from mismatched primers is critically important in many applications of PCR (31, 32).

The *Taq* DNA polymerase is sensitive to 3' mismatches because it has not 3' to 5' exonuclease proofreading activity (31).

The acidithiobacilli are sulfur-oxidizing acidophilic bacteria that thrive in both natural and anthropogenic low pH (acidic) environments. They take part in some processes that lead to the generation of acid rock drainage in some different geoclimatic contexts. Their properties have long been harnessed for the biological processing of minerals (1, 18).

In this research, we have surveyed a PCR-based method for detect single nucleotide polymorphism (SNP) of the 16S-rDNA gene in some *Thiobacillus* species by the specific primer.

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