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Isolation and identification of *Ktedonobacteria* using 16S rRNA gene sequences data

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Abstract. The aims of this study were to isolate and identify the members of *Ktedonobacteria* from soil samples in Cisolok geysers area, West Java, Indonesia. To isolate the bacteria, sample was spread on 10-fold-diluted Reasoner's 2A medium and the plates were incubated at 30 °C until the *Ktedonobacteria*-like colonies appeared. *Ktedonobacteria*-like colonies showed the characteristics of a firm pale orange colony. The colonies were purified and colony PCR was performed using *Ktedonobacteria* specific and universal *eubacterial* primers for 16S rRNA gene. Almost full-length of 16S rRNA gene sequences were obtained in order to know the identity of the taxonomic affiliation of the isolates. Three *Ktedonobacteria*-like isolates, designated S3.2.1.5, S3.2.1.6 (isolated from soil under Bamboo tree) and S3.2.2.5 (isolated from soil in decayed Bamboo stems) were obtained. The detection using *Ktedonobacteria* specific primers showed positive results, which confirmed that all of three isolates belong to class *Ktedonobacteria*. The sequence homology search using EzBioCloud showed that these isolates (S3.2.1.5, S3.2.1.6, and S3.2.2.5) were belonged to the family *Dictyobacteraceae* and closely related to *Bacterium* SOSP1-79 (99.20%, 98.83%, 99.34%, respectively) and *Dictyobacter aurantiacus* S-27^T (97.41%, 97.41%, 98.00%, respectively). The low percentage of sequence homology to type strains indicates that they could be a candidate of novel taxa of the genus *Dictyobacter*. Further characterizations such as morphology, physiology, and chemotaxonomy are needed in order to clarify the taxonomic position of these potential novel species of *Dictyobacter*.

1. Introduction

The class *Ktedonobacteria* was proposed by Cavaletti *et al.* in 2006 for actinomycete-like bacteria and placed in the phylum *Chloroflexi*, which known as a deep branching lineage in the domain *Bacteria* [1]. This class consists of the environmental 16S rRNA gene clones, uncultured, and cultivated isolates with a valid names and cultivated isolates which have not been proposed yet [2]. To date, this class contains



of two orders, three families, four genera [3], and twelve formally proposed species [3-5]. *Ktedonobacter racemifer* SOSP1-21^T, which isolated from Italian soil, is the first proposed species that representing class *Ktedonobacteria*. This species was mistakenly identified as an actinomycete due to the producing of substrate and aerial mycelia [1].

The *Ktedonobacteria* comprises of Gram positive, aerobic, chemolithoorganoheterotrophic, and mesophilic or thermophilic bacteria [2], with low G+C content in their genomic DNA [3]. Members of this class forms branched substrate mycelia and aerial mycelia harbouring multiple exospores produced by budding [6] or spores produced from sporangia with short stalk [7]. The members of class *Ktedonobacteria* were detected from numerous kind of terrestrial environments, such as from common soil to extreme environments, as a garden soil, alpine tundra soil, forest soil, compost, geothermal soil, volcanic ice cave, Hawaiian volcanic deposits, and uranium contaminated soil [8].

The species from class *Ktedonobacteria* have a variety of genome size, ranging from 5.54 to 13.66 Mb [3]. The second largest bacteria genome is reported as a member from this class, namely *Ktedonobacter racemifer* SOSP1-21^T, which harbouring 13.66 Mb genome size that encodes 11,453 putative proteins and 87 RNA genes [9]. The large genome size has potential to encode multiple secondary metabolism gene clusters. The finding of large genome size and complex life cycle are suggesting *Ktedonobacteria* as a potential producer of various bioactive compounds [3].

The study of *Ktedonobacteria* community in Indonesia is still limited. To date, only one study of *Ktedonobacteria* in Indonesia that was reported by Yabe *et al.* [10]. Yabe *et al.* [10] has proposed a new species of *Ktedonobacteria*, namely *Dictyobacter aurantiacus* S-27^T sp. nov., which isolated from paddy soil collected from waterlogged rice paddy field on the mountainside of Gunung Salak, West Java, Indonesia. Thus, the exploration of *Ktedonobacteria* community from different sources of isolation in Indonesia is needed in order to reveal the diversity of this class. The objectives of this study were to isolate and identify the members of *Ktedonobacteria* from soil samples collected from forest around Cisolok geysers area, West Java, Indonesia.

2. Materials and Methods

2.1. Isolation

The sampling was conducted in November 2018 at forest around Cisolok geysers, West Java, Indonesia. Two samples, designated S3.2.1 and S3.2.2 (06°55'991"S, 106°27'187"E), were collected from soil under bamboo tree and soil in decayed bamboo stems in forest around Cisolok geysers, respectively. The Reasoner's 2A medium (1:10 dilution) with the addition of gellan gum as a solidifying agent was used for isolation medium of *Ktedonobacteria* [10]. A soil sample was spread directly on the surface of the medium. The plates were incubated at 30 °C until the *Ktedonobacteria*-like colony appeared, which showed a firm pale orange colony [4]. The *Ktedonobacteria*-like colony was purified at least three times to obtain pure cultures.

2.2. Detection of *Ktedonobacteria*

One of firm pale orange colony was picked and crude DNA extraction was performed using method as described by Yabe *et al.* [10]. The colony PCR was performed with ExTaq DNA polymerase [TaKaRa] under the condition according to Yabe *et al.* [11], with some modifications. The *Ktedonobacteria* specific, KTED161F (5'-ATACCGGBGMGAAAKYGYCGAC-3') and GNSB941R (5'-AAACCACACGCTCCGCT-3') [8] and the universal *eubacterial* primers for 16S rRNA gene (9F and 536R) were used to detect the *Ktedonobacteria*. The positive results of colony PCR indicated by the visualization of the amplified products of both 16S rRNA gene and *Ktedonobacteria* DNA fragments by performing gel electrophoresis. The detection of morphological features for *Ktedonobacteria* were observed under a digital microscope [KH-8700; Hirox].

2.3. The construction of phylogenetic trees based on 16S rRNA gene sequences

The extraction of genomic DNA for the pure cultures that showed *Ktedonobacteria*-like characters was conducted according to Yabe *et al.* [12] by using Puregene Yeast/Bact. Kit [Qiagen]. Amplification of

16S rRNA gene sequences was performed by using 9F and 1510R *eubacterial* primers and PCR condition was carried out according to Yabe *et al.* [11], with some modifications. The purified PCR products were sequenced using MacroGen Sequencing Service in Japan [MacroGen Japan Corp.; <https://dna.macrogen.com/eng/index.jsp>] using 9F (5'-GAGTTTGATCCTGGCTCAG-3'), 536R (5'-GTATTACCGCGGCTGCTG-3'), 802R (5'-TACCAGGGTATCTAATCC-3'), 907F (5'-AAACTCAAAGGAATTGACGG-3'), and 1510R (5'-GGCTACCTTGTTACGA-3') as universal *eubacterial* primers for 16S rRNA gene [13-15]. The sequence similarity search of 16S rRNA gene sequences was carried out using EzBioCloud (<https://www.ezbiocloud.net>) [16]. Multiple alignments of 16S rRNA gene sequences were performed using MEGA ver 7.0.26 [17] with all gap positions were excluded. A phylogenetic tree was constructed with neighbor-joining (NJ) [18], minimum evolution (ME) [19], and maximum likelihood (ML) [20] methods with bootstrap values based on 1000 resamplings [21]. The evolutionary distances were estimated using Kimura two-parameter [22].

3. Results and Discussion

The firm pale orange colonies were observed in plates after incubation at 30 °C for a week. Three isolates designated S3.2.1.5, S3.2.1.6, and S3.2.2.5 as shown in Figure 1, were obtained from soil samples under bamboo tree and in decayed bamboo stems from forest around Cisolok geysers. As shown in Figure 1, three isolates were forming branched substrate mycelia with aerial mycelia borne directly from substrate mycelia. The aerial mycelia were harbouring a sporangium-like structures (Figure 1). The result showed that these three isolates shared similar morphological features to *Ktedonobacteria*, such as forming firm pale orange colonies, branched substrate mycelia, and aerial mycelia with sporangium-like structure. Based on the previous study, the *Ktedonobacteria* showed a firm pale orange colony [4], branched vegetative mycelia with budding [6] or sporangium-like structures in their aerial mycelia [7]. The use of 1/10 R2A gellan gum as isolation medium was demonstrated by the previous study of Yabe *et al.* [10] for isolation of *Ktedonobacteria*.

Three *Ktedonobacteria*-like isolates showed an amplified 16S rRNA gene fragments after the amplification using *Ktedonobacteria*-specific primers by colony PCR method (data not shown). The amplification of 16S rRNA gene by colony PCR for three *Ktedonobacteria*-like isolates in this study showed PCR bands after amplification using both *Ktedonobacteria* specific and universal *eubacteria* 16S rRNA gene primers, indicating that these isolates are members of the class *Ktedonobacteria*. According to Yabe *et al.* [8], the KTED161F is proposed as *Ktedonobacteria* specific primer due to the dissimilarities to the 3' end of non-target sequences in each class within the phylum *Chloroflexi*, while the GNSB941R is a *Chloroflexi*-specific primer. Yabe *et al.* [8] was successfully demonstrated the use of primer set of KTED161F and GNSB941R which shows high specificity for detecting class *Ktedonobacteria* within the employed samples.

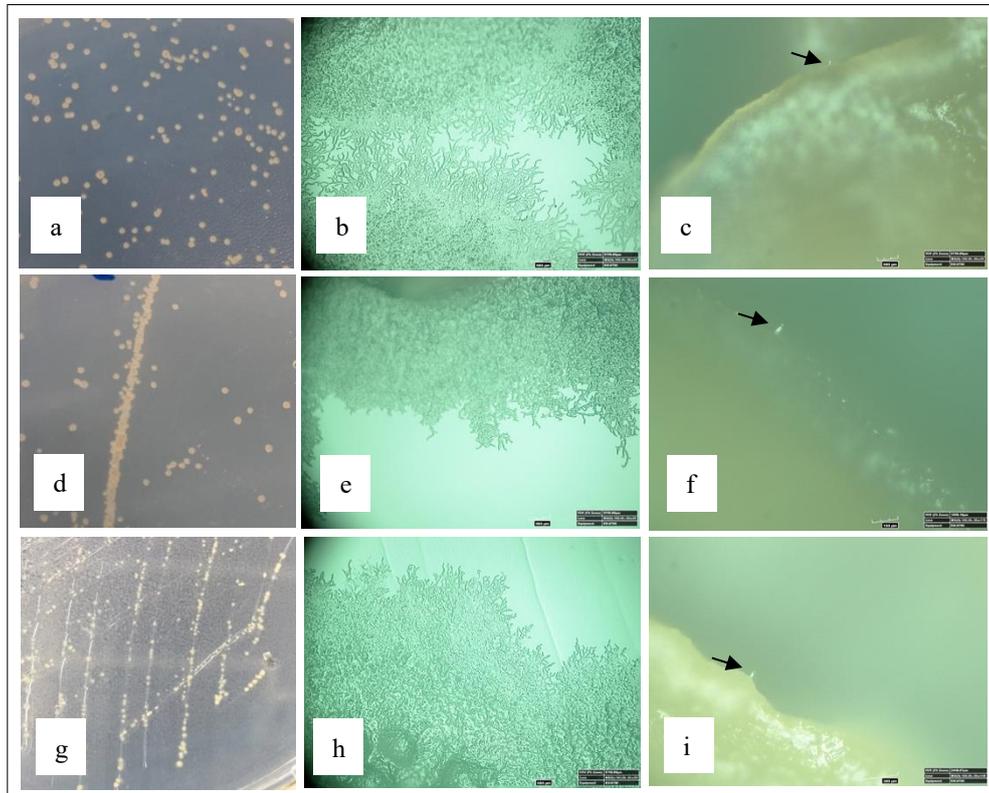


Figure 1. The morphological features of three *Ktedonobacteria*-like isolates: S3.2.1.5 (a-c); S3.2.1.6 (d-f); and S3.2.2.5 (g-i). The formation of pale orange colonies (a,d,g), branched substrate mycelia (b,e,h), and aerial mycelia with the sporangium-like structures (c,f,i) from three isolates.

Table 1. The result of sequence similarity search on EzBioCloud for three *Ktedonobacteria*-like isolates used in this study (S3.2.1.5, S3.2.1.6, and S3.2.2.5).

Closely related species	Isolate		
	S3.2.1.5	S3.2.1.6	S3.2.2.5
SOSP1-79 (AM180160)	99.20% (1357/1368 nt)	98.83% (1352/1368 nt)	99.34% (1358/1367 nt)
<i>Dictyobacter aurantiacus</i> S-27 ^T (LC210808)	97.41% (1317/1352 nt)	97.41% (1317/1352 nt)	98.00% (1324/1351 nt)
<i>Dictyobacter kobayashii</i> Uno11 ^T (LC278466)	96.21% (1319/1371 nt)	95.99% (1316/1371 nt)	96.57% (1323/1370 nt)
SOSP1-9 (AM180155)	96.13% (1318/1371 nt)	95.92% (1315/1371 nt)	95.35% (1320/1370 nt)
<i>Dictyobacter alpinus</i> Uno16 ^T (LC278467)	95.42% (1312/1375 nt)	95.13% (1308/1375 nt)	95.78% (1316/1374 nt)
<i>Tengunoibacter tsumagoiensis</i> Uno3 ^T (LC278465)	91.26% (1253/1373 nt)	91.11% (1251/1373 nt)	91.48% (1256/1373 nt)
SOSP1-142 (AM180162)	91.04% (1250/1373 nt)	90.90% (1248/1373 nt)	91.55% (1256/1372 nt)

The DNA sequencing of 16S rRNA gene sequences then was performed for three isolates (S3.2.1.5, S3.2.1.6, and S3.2.2.5) in order to clarify their taxonomic position in the class *Ktedonobacteria*. Table

1 showed the result of sequence similarity search on EzBioCloud for three *Ktedonobacteria*-like isolates. The result confirmed that these three isolates are belong to members of class *Ktedonobacteria*. They showed low similarity values to their closely related taxa within the class *Ktedonobacteria*, e.g. bacterium SOSP1-79 (98.83-99.34%), and *Dictyobacter aurantiacus* S-27^T (97.41-98.00%), respectively. According to Cavaletti *et al.* [1], the bacterium SOSP1-79 is a *Ktedonobacteria* isolates from soil collected in cereal field in Egypt. Their closest related species is *Dictyobacter aurantiacus* S-27^T sp. nov. As reported by Yabe *et al.* [10], this type strain was isolated from paddy soil collected from waterlogged rice paddy field on the mountainside of Gunung Salak, West Java, Indonesia. This study confirmed that the use of *Ktedonobacteria* specific primers as designed by Yabe *et al.* [11], was able to successfully and accurately detect the *Ktedonobacteria* by colony PCR method. Yabe *et al.* [8] reported the numerous members of *Ktedonobacteria* were detected from forest soil by using *Ktedonobacteria* specific primers, with most of the representative sequences were belonged to unknown *Ktedonobacteriales* group.

As shown in Figure 2, the 16S rRNA gene sequence-based NJ phylogenetic tree (as supported by ME and ML trees) depicted that the three isolates were belonged to the genus *Dictyobacter* within family *Dictyobacteraceae* within the class *Ktedonobacteria*, as supported by 90% of bootstrap value. The phylogenetic tree showed that these isolates were clustering together with bacterium SOSP1-79 with 99% of bootstrap value, and they were formed a distinct lineage from their closest related species *Dictyobacter aurantiacus* S-27^T as supported by 76% bootstrap values. The tree indicated that most of the members of *Dictyobacteraceae* belong to undescribed taxa. These undescribed taxa are challenging and interesting for taxonomic study and prospecting. Further analysis, such as the analysis of morphological, physiological, and biochemical are required in order to determine the identity of these three isolates. The previous study [4] showed that the family *Dictyobacteraceae* consists of two genera, namely *Dictyobacter* and *Tengunoibacter*, four valid species name, and 13 cultivated isolates which have not been proposed yet.

The three *Ktedonobacteria*-like isolates found in this study shared similar colony character to bacterium SOSP1-79 strain, such as the formation of orange colour of colony, which indicated the SOSP1-79 strain was the closely-related taxon to these three isolates. According to Cavaletti *et al.* [1], the colony of SOSP1-79 bacterium on ISP 3 agar after 3 weeks of incubation showed orange colour. Additionally, Cavaletti *et al.* [1] reported the antibiotic resistance profiles of strain SOSP1-79, which showed that the bacterium was sensitive to 5 µg/ml of novobiocin or ramoplanin and 20 mg/ml of apramycin and glycopeptide A40926, meanwhile resistant to 20 µg/ml of rifampin. Further study is needed to elucidate the antibiotic profiles of the three *Ktedonobacteria*-like isolates (S3.2.1.5, S3.2.1.6, and S3.2.2.5) obtained in this study.

4. Conclusions

Three bacterial isolates that shared similar morphological features to *Ktedonobacteria* were obtained from soil samples collected under bamboo tree and in decayed bamboo stems in forest around Cisolok geysers, West Java, Indonesia. Three *Ktedonobacteria*-like isolates showed an amplified 16S rRNA gene fragments by using *Ktedonobacteria*-specific primers that performed based on colony PCR method. Based on phylogenetic tree analyses, three isolates were affiliated with the genus *Dictyobacter* within the family *Dictyobacteraceae*, with *Dictyobacter aurantiacus* as the closest related species. Interestingly, they showed low homology percentage (97.41-98.00%) to *Dictyobacter aurantiacus* and they were formed a distinct lineage from this species. Therefore, the full characterization of morphological, physiological, biochemical, and whole genome analysis for these isolates are needed to elucidate the taxonomic position of these potential novel species. The results of this study confirmed that colony PCR method is useful method for the detection of the members of class *Ktedonobacteria*. This study also indicated that the forest soil around Cisolok geysers is a potential source for isolating novel taxa of *Ktedonobacteria*.

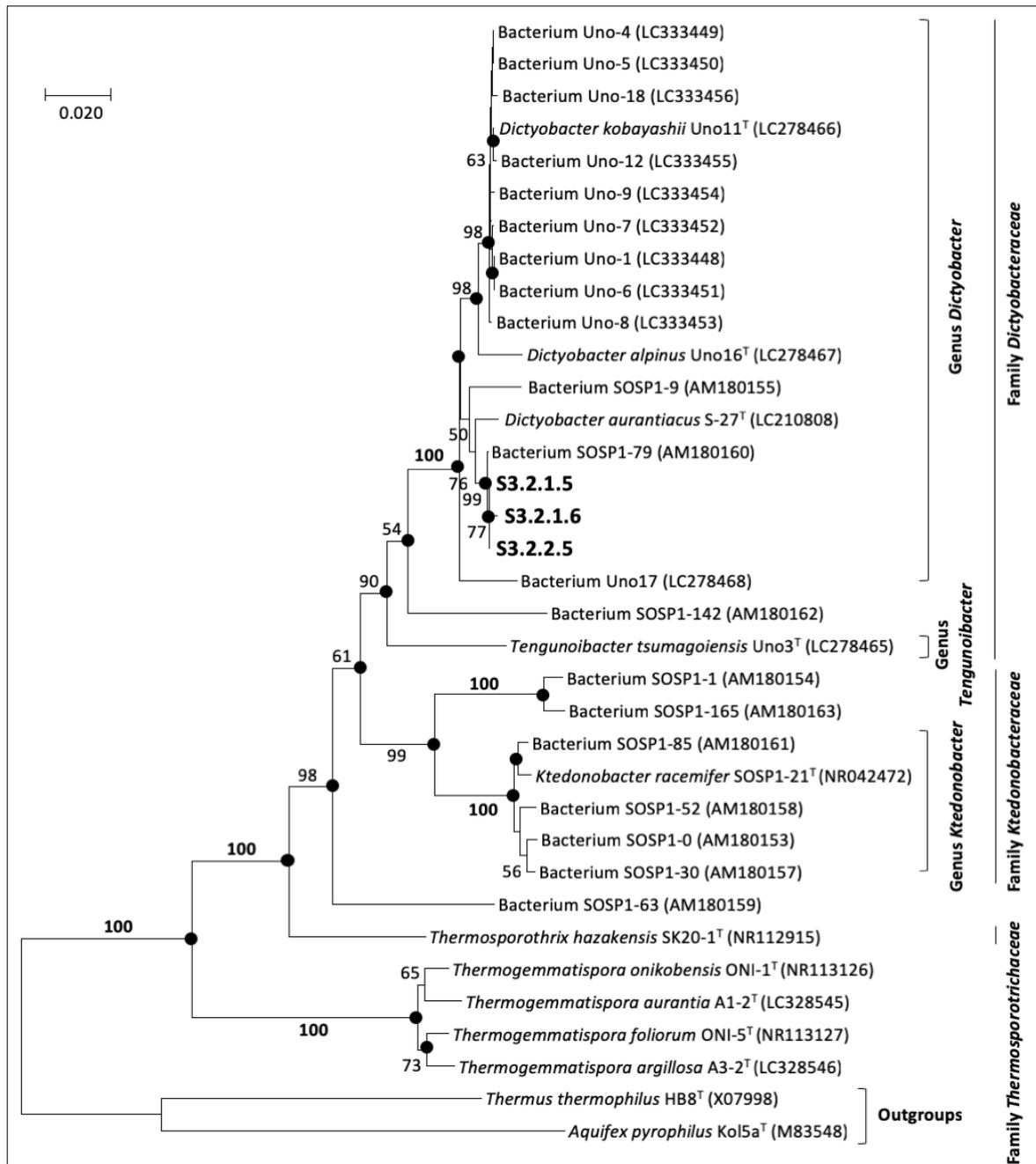


Figure 2. The neighbor joining tree of three *Ktedonobacteria* isolates (S3.2.1.5; S3.2.1.6; and S3.2.2.5) based on 1228 aligned position of 16S rRNA gene sequences. *Thermus thermophilus* HB8^T and *Aquifex pyrophilus* Kol5a^T were represented as outgroups. Closed circles indicated branches of the tree were found using minimum evolution and maximum likelihood trees. Number at the branched nodes indicated as percentage of bootstrap values with 1000 replicates and only more than 50% of bootstrap values are shown.

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