Isolation and identification of rare actinomycete-like bacteria from soil-based on 16S ribosomal RNA gene sequences

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Abstract. The rare actinomycete-like bacteria are mycelium-forming bacteria other than phylum Actinobacteria that difficult to isolate and cultivate. This group of bacteria was recently speculated by many scientists as a potential new microbial resource for the discovery of novel compounds, as a substitute for actinomycetes. In this study, we isolate and identify rare actinomycete-like bacteria from forest soil collected under bamboo trees, near the Cisolok Geysers, Sukabumi, Indonesia. The isolation of bacteria was performed using Reasoner’s 2A (1:10 dilution) medium with 2% gellan gum instead of agar and incubated at 30 °C for three weeks. The 16S rRNA gene sequences of the isolates were examined to determine their taxonomic position. Four isolates designated K17-1, K17-2, K42, and K44 showed pale oranges colonies and formed mycelia were obtained. The results of 16S rRNA gene sequences of these isolates showed high similarity to members of the genus Dictyobacter in the family Dictyobacteraceae of the class Ktedonobacteria of the phylum Chlorofexi, with values 97.16-98.02%, and most closely related to the species Dictyobacteraurantiacus S-27T (97.16-98.02% similarities). This result suggested that the member of the class Ktedonobacteria, which considered as rare actinomycete-like bacteria, such as Dictyobacter could be found in the forest soil of the geothermal area.

1. Introduction

Actinobacteria are gram-positive bacteria that have a high percentage of guanine and cytosine in their genome [1]. This group morphologically comprises unicellular organisms to mycelium-forming bacteria which called Actinomycetes [1,2]. However, bacteria that have filamentous appearance also could be found in the phylum Chlorofexi. The member of this phylum which has actinomycete-like morphology is present in the four different class namely Chlorofexi, Anaerolineae, Caldilineae and Ktedonobacteria [3]. Among these class, Ktedonobacteria has some obvious morphological features which distinguish themselves from others. The member of Ktedonobacteraeae aerobic organism and forming branched mycelia with spores like actinomycetes [3,4]. Moreover, most validly published strains of Ktedonobacteriabudding their multiple spores per cell on the aerial mycelium which unique among bacteria [5]. All of Ktedonobacteria identified as gram-positive bacteria while almost of the member in phylum Chlorofexi were gram-negative [3,6]. Based on these exceptional characters, class Ktedonobacteria could be included as the rare actinomycete-like bacteria.

Rare actinomycete-like bacteria could provide an alternative for the discovery of new compounds derived from microorganisms because spore formation usually would be followed by the production of secondary metabolites [7,8]. Further analysis of the genomic of nine members of rare actinomycete-like bacteria.
confirmed this possibility because there are still many unknown metabolites in their biosynthetic gene clusters [9,10]. Besides, some Ktedonobacteria members showed a wide spectrum of antimicrobial activity against some pathogenic tested bacteria [10]. Recently, two novel compounds with adipocyte differentiation ability successfully identified from Thermosporothrixhazakensis [11]. Thus, it could be concluded that the current finding of secondary metabolites from rare actinomycete-like bacteria remains unclear and this group of bacteria could become the alternative of secondary metabolites from microorganisms.

Isolation is the first step in bioprospecting for microbial secondary metabolites [12]. The importance of the isolation step is to obtain pure culture for further analysis [12,13]. Most of rare actinomycete-like bacteria members were successfully isolated from soil samples. So far, there are eleven species of rare actinomycete-like bacteria originally from soil [4,6,14,15]. The number of unculturable rare actinomycete-like bacteria found by metagenomic analysis in soil far exceeds the culturable members [5]. The difficulties to isolate these unculturable rare actinomycete-like bacteria perhaps related to the lack of knowledge about the exact nutrient composition in artificial media or other specific conditions such as temperature, pH, and gelling agent [16,17,18]. The current species of rare actinomycete-like bacteria were successfully isolated using a different kind of media and there are still no specific media as well as selective methods to obtain this group [5].

The 16S rRNA genes are one of the representatives of RNA genes which can exhibit evaluatory relatedness among microorganism [19]. The 16S rRNA genes become the foundation for molecular phylogeny in domain eubacteria because these genes are highly conserved, present in all cellular organisms and easy interpretation [20]. The full-length 16S rRNA gene sequences also consist of nine hypervariable regions that are useful to identify phylogenetic characteristics of microorganisms [21]. Another advantage of using 16S rRNA is the ability to determine until the genus or species level [22]. Thus, the use of 16S rRNA in this research is would give representative results about the identity of the rare actinomycete-like bacteria isolates along with the phylogenetic position.

In this study, we aim to isolate and identify rare actinomycete-like bacteria from Cisolok geothermal area in Indonesia. The geothermal area is very interesting in the exploration of the biodiversity of microorganisms because the diverse of geochemical compositions and unique habitat conditions led to the possibility to obtain novel species [23,24]. Previous research showed that some thermophilic bacteria present in Cisolok geothermal area[25,26,27]. Recent research by Ningsih et al. [28] successfully finding several rare-actinomycete bacteria which are a new genus in the phylum Actinobacteria. There are still no mesophilic rare actinomycete-like bacteria which finding in this area. Hence, the result of this study would give the knowledge about the presence of rare actinomycete-like bacteria from the soil in the geothermal area as well as their potency as the source of the novel compound in foreseeable future.

2. Material and methods

2.1 Sampling, isolation and purification

Soil samples under the bamboo tree were collected from Cisolok geothermal area, West Java Indonesia (Coordinate S06°55.99 E106.27.187). Bacterial isolation was carried out using Reasoner’s 2A 10% (pH 5.5) media (Wako Pure Chemical Industries) with gellan gum as a solidified agent. About 10 mg of soil were streaked into media and incubated at 30 °C for 3 weeks in aerobic and anaerobic condition according to Yabe et al. [14]. Orange-colored colonies that have similar morphological characteristics with the rare actinomycete-like bacteria marked as selected colonies. These colonies were purified using the streak plate and spread and streak plate methods according Wang et al. [4].

2.2 DNA extraction and amplification of 16s RNA gene

The DNA extraction was carried out by extracting some single colonies of rare actinomycete-like bacteria using the Qiagen DNA isolation kit (Mo Bio Laboratories, Carlsbad, CA, USA) with the instruction of kit procedures. DNA amplification of the 16S rRNA gene was carried out by a total reaction of 50 μl using MY TAQ HS RED MIX from Bioline. Polymerase Chain Reaction for 35cycles were finished with initial denaturation at 94 °C for 2 minutes; denaturation at 94 °C for 2 minutes, annealing at 55 °C for 30 seconds, and extension at 72 °C for 1 minute; and final extension at 72 °C for 2 minutes according to Yabe et al.,[14] and Wang et al. [4]. The first amplification was to detect members of the rare actinomycete-like bacteria using KTED161F(5’ATACCGBGGAARRKGGYGCAC3’) and GNSB 941R(5’AAACC ACACGCTCCGCT3’) primers as described by Yabe et al. [5]. The strain which detected as rare actinomycete-like bacteria then
amplified using universal primer 9F (5'-GAGTTTGATCCTGGCTCAG-3') and 1510R (5'-GGCTACCTTGTTACGA-3') as described by Nonaka et al.[29] to obtain the nearly-full sequence.

2.3 DNA sequencing
Purification of PCR products and sequencing cycles of amplification products was carried out by Macrogen Japan. the primers used for sequencing were 9F (5'-GAGTTTGATCCTGGCTCAG-3'), 536R (5'-GTATTACCGGCTGCTG-3'), 802R (5'-TACCCAGGTATCTAATCC-3'), 907F (5'-AACCTAAAGGAATTTGACGT-3') and 1510R(5'-GGTACCTTGTTACGA-3') According to Nanoka et al.[29].

2.4 Alignment analysis and phylogenetic analysis
The sequencing of sequencing data is then trimmed and assembled manually using DNA Basser Assembler software according to Nayak et al., 2009 [30]. The sequences then compared with the gene data bank at EzBio Cloud (https://www.ezbiocloud.net/identify)[31]. The sequences of rare actinomycete-like bacteria were aligned with type strains achieved from the DDJBL/EMBL/GenBank databases. phylogenetic analysis was carried out using the Molecular Evolutionary Genetics Analysis version X software package [32]. The phylogenetic tree was arranged using the neighbor-joining method[33], maximum-likelihood [34] and maximum-parsimony[35]in MEGAX software with a bootstrap value based on 1000 replications and distance calculation according to the Kimura two-parameter method[36].

3. Result and discussions
Overall, there are four isolates of rare actinomycete-like bacteria that were obtained in this study based on detection using rare actinomycete-like bacteria-specific primers (data not shown) according to Yabe et al. [5]. The isolates were given name K17-1, K17-2, K42, K44respectively. Further analysis by comparing nearly-full length 16S rRNA gene sequences on databases showed that all isolates had the closest relationship with Dictyobacter aurantiacus S-27Tas type strain. The four isolates have different bases ranging from 27 to 39 base pairs with D. aurantiacus. K17-1 isolates had 97.16% alignment while K17 -2 isolates had 97.24% similarity. Two other isolates from anaerobic incubation namely isolate K42 and K44, each had a similarity of 97.91% and 98.02%. This is the first report of the finding of rare actinomycete-like bacteria from the geothermal region in Indonesia.

Table 1. The result of identification using 16S rRNA using EZbio Cloud and the similarity with the closely related type strain

| No | Isolate code | Bases length | Similarity with D. Aurantiacus |
|----|--------------|--------------|-------------------------------|
| 1  | 17-1         | 1427 bp      | 97.16% (39 bases difference)  |
| 2  | 17-2         | 1434 bp      | 97.24% (38 bases difference)  |
| 3  | K42          | 1425 bp      | 97.97% (28 bases difference)  |
| 4  | K44          | 1391 bp      | 98.02% (27 bases difference)  |

According to Stackebrandt and Eberg [37], two species are different species if they have similarities below 98.7%. This was later updated by Kim et al.[38] which states that bacterial species that have similarity below 98.65% with type strains are likely different bacterial species. Dictyobacter aurantiacus is a mesophilic bacterium that was isolated from paddy soil in Mount Salak, West Java by Yabe et al. [14]. Thus, it could be concluded that the four strains that were successfully obtained from the Cisolok geothermal areahas possibility as different species withDictyobacter aurantiacus.
The result then confirmed based on macroscopic observations, all of the rare actinomycete-like bacteria in this research have a circular colony shape with an entire margin. Colony color is pale orange with firm colony texture. This result was similar to the previous study by Yabe et al. [14]and Wang et al. [4] which showed that rare actinomycete-like bacteria belonging of genus *Dictyobacter* have pale orange colored colonies with firm colony texture. Besides, *D. aurantiacus* which the closely related species with strain in this research is known to have a sporangia formation cycle similar to members of the *Actinoplanes* in the *Actinobacteria* class. Uniquely, *D. aurantiacus* is also the only bacterium that forms sporangia besides members of *Actinobacteria* and *Myxobacteria* that cause this species to belong to rare actinomycete-like bacteria [39].

The rare actinomycete-like bacteria in this research require incubation of 3 weeks since the isolation time as described by Zheng et al. [15]. A similar time is also required to obtain rare-actinomycete bacteria from the environment [40]. This phenomenon is likely due to the rare bacteria species being adapted to specific niches that cause low abundance or some species experience dormancy as a strategy to overcome unfavorable conditions [41]. Dormancy can result in the failure of rare species growing in the short-term incubation approach. Re-activation of the dormancy causes some bacteria that are “rare” to require a longer incubation time until the morphology of the bacteria can be seen macroscopically [42].

Reasoner’s 2A (R2A) medium is a medium containing yeast extract, difco protease peptone, casamino acids, glucose, starch, K2HPO4, gram MgSO4, and sodium pyruvate [43]. Most of the rare actinomycete-like bacteria isolates were successfully obtained with 10% R2A isolation media [4,14,15]. Nearly all the species that were successfully obtained were members of the *Dictyobacteraeae* family. Whereas in phylum *Actinobacteria*, most of the isolation media used are specific media in the form of the International Streptomyces Project (ISP) [44] or Actinomycetes isolation agar [45]. Thus, This research confirmed that rare-actinomycete-like bacteria successfully obtain by the R2A medium.

Another possibility according to Stepanovic et al. [46], nutrient composition in R2A the medium affects the quantity of colony formation in different ways and the nature or character of bacterial colonies. The growth medium also contains several types of amino acids that may be able to reactivate bacteria, in this case, rare actinomycete-like bacteria, from the dormancy process. In terms of media composition, R2A contains amino acids in the form of casamino acids [47] while the medium of ISP or Actinomycyes isolation contains asparagine [44,45]. In addition, according to Berg and Sandine [48], the low pH content in the isolation medium (5.5) can also result in a reduction in S-S chain bonds resulting in the opening of a key system in the enzyme system or changing the structure that controls dormancy. Therefore, the pH used in this study perhaps gives effect to there dormancy of rare actinomycete-like bacteria from the soil.

Further analysis using phylogenetic using the neighbor-joining method gave a significant indication that all rare actinomycete-like bacteria formed a monophyletic group with the type strain *Dictyobacteraurantiacus* S-27T as the most closely related species with strong bootstrap support (88%). Besides, a comparison using maximum-likelihood and maximum-parsimony method showed more than 70% bootstrap support (78 % and 88 % respectively). The length of the sequence (>1300bp) with good quality of chromatogram in this research was ideal enough to conduct the phylogentic position [49,50]. Thus, the result in this study was comprehensive enough to give the taxonomic position of rare actinomycete-like bacteria based on 16S rRNA genes.
The data in the phylogenetic tree also showed that all strains in this research belong to the Ordo Ktedonobacterales and the family Dictyobacteraceae which are supported with strong bootstrap value (>90%) for all tree-making method. Yabe et al. [14] reported that, aurantiacus as the type strain in the genus Dictyobacter has heterotrophic mesophilic character. Additional data from Wang et al. [4] could be suggested that all members of family Dictyobacteraceae were gram-positive and showed positive catalase activity. The optimum growth for temperature and pH for this family was range 20-30 °C and 6.0-7.0 respectively. This evidence suggested that rare actinomycete-like bacteria in this research has a similar growth temperature and pH with all of the members of Dictyobacteraceae.

Another interesting point based on figure 2 separated all Cisolok’s strains from the cluster of several Uno strains bacteria originating from soil-like microbe mass in Japan [4] which supported by bootstrap value more than of 60%. The similarity of the 3.2.1 strains group with the Uno strain bacterial group was about 96% while the similarity with Tenguinobactertsumagoensis in the separate genus was 91.88%. Therefore, it could be concluded that the cluster strain at location 3.2.1 is a member of rare actinomycete-like bacteria in the genus Dictyobacter and with D. aurantiacus forming cluster rare actinomycete-like bacteria which originally from Indonesia.

**Figure 2.** Neighbour-joining tree based on 1214 aligned positions of the 16S rRNA gene sequence. Numbers at nodes are bootstrap percentages from the neighbour-joining method based on 1000 replicated datasets; only values greater than 60% are shown. The outgroup consisted of sequences from Caldlinaea aerophila and Streptomyces griseus. Closed circles indicate nodes that were supported by bootstrap values greater than 60% with the neighbour-joining [25], Maximum-likelihood [26] and Maximum-parsimony [27] tree-making algorithms.
4. Conclusion
Analysis of the 16S rRNA gene sequence showed that all strains of rare actinomycete-like bacteria originating from the Cisolok geothermal area have the closest relationship with Dictyobacter aurantiacus S-27T in the Ktedonobacteria class. It also indicated that species of the rare actinomycete-like bacteria within genus Dictyobacter was present in forest soil in the geothermal area besides paddy soil and soil-like microbe mass. Further research is polyphasic characterization to determine the more accurate taxonomic position of the four strains.

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References
[1] Trujillo, M E 2008 Actinobacteria. In: Encyclopedia of Life Sciences (ELS) Chicester John Wiley & Sons, Ltd
[2] Sharma M, Pinki D, and Meenakshi C 2014 Int. J. Curr. Microbiol. App. Sci. 3(2) 801-832
[3] Rosenberg E, DeLong E F, Lory S, Stackebrandt E, Thompson F 2014 Berlin: Springer-Verlag Berlin Heidelberg Chapter 40 pp 515-529
[4] Wang, C, Zheng Y, Sakai Y, Toyoda A, Minakuchi Y, Abe K, Yokota A and Yabe S 2019 Int. J. Syst. Evol. Microbiol. D 10.1099
[5] Yabe S, Sakai Y, Abe K, and Yokota A 2017 Microb. Env. 32 (1) 61-70
[6] Cavaletti L, Monciardini P, Bamonte R, Schumann P, Rohde M, Sozio M, and Donadio S 2006 J. Appl. Environ. Microbiol. 43 60–69
[7] Adams T H and Yu J H 1998. Curr. Opin. Microbiol. 1 (6) : 674-677
[8] Calvo AM, Wilson R A, Bok J W, and Keller N P 2002 Microbiol. Mol. Bio.Rev. 6 447-459
[9] Chang Y J, Land M, Hauser L, Chertkov O, Del Rio T G, Nolan M, et al. 2011 Stand. Genomic Sci. 5 97–111
[10] Zheng Y, Saitou A, Wang C, Toyoda A, Minakuchi Y, Sekiguchi Y, Ueda K, Takano H, Sakai Y, Abe K, Yokota A, and Yabe S 2019 Front. Microbiol. 10 1-21
[11] Igarashi Y, Kazuki Y, Chiaki U, Nodoka Y, Katsuya S, Kazuki T, Masaru E, Shigefumi K, Tsutomu O, Etsu T, Masaya I, Ye X, Tao Z, Enduro H and Naoya Oku 2019 J. Antibiotics 72 653–660
[12] Stal L J and Mariana S C 2016 The Marine Microbiome (Switzerland: Springer Nature) pp 350
[13] Frohlich J and Konig H 2000 FEMS. Microbiol. Rev. 24 567-572
[14] Yabe S, Sakai Y, Abe K and Yokota A 2017 J. Syst. Evol. Microbiol. 67 2615–2621.
[15] Zheng Y, Wang C, Sakai Y, Abe K, Yokota Aand Yabe S 2019. Int. J. Syst. Evol. Microbiol. 10.1099 (Preprint ijsen0.0003388)
[16] Taroutkian S R, Palmer R M and Wade W G 2010 FEMS Microbiol. Lett.309 1–7
[17] Stewart J E 2012 J. Bacteriol. 194(16) 4151-60
[18] Pham V H T and Kim J 2012 Trends Biotechnol. 30 (9) 475-486
[19] Yanzi, Richter M, Peples J, Euzeyb J, Aman T, Schleifer K, Ludwig W, Glockner F O,
[20] Rosello-Mora R 2008 Syst. Appl. Microbiol. 31 241-250
[21] Madigan M T, Bender K S, Buckley D H, Sattley W M and Stahl D A 2019 Brock Biology of Microorganisms 15th Ed. Harlow Pearson Education Limited
[22] Yang B, Wang Y and Qian P 2016 BMC Informatics 12 (135) 1-8
[23] Ali A, Hameed S, Oresnik IJ, Shahid M, Tariq M, and Ahmad N 2015 Pak. J. Agri. Sci. 52 (2) 285-291
[24] Stott M B, Crowe M A, Mountain B W, Smirnova A V, Hou, Alam S M, and Dunfield P F 2008 Environ. Microbiol.10 (8) : 2030-41
[25] King C E and King M G 2014 Int. J. Syst. Evol. Microbiol.64 1244–1251.
[26] Mawarid A T, Sjamsuridzal W, Yabe S, Santoso I and Ningsih F 2016 AIP Conference Proceedings 1792
[27] Yokota A, Ningsih F, Nurlaili D G, Sakai Y, Oetari, A, Santoso I and Sjamsuridjal W 2016 Int. J. Syst. Evol. Microbiol. 66(8) 3088-94
[28] Syafitri W A, Ningsih F, Setyaningsih P P, Rachmania M K, Sari D C F, YabeS, YokotaA, Oetari A and Sjamsuridzal W 2019 Biodiv. 20(7) 1929-38
