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RESEARCH PAPER

Genus-specific Primer Design for Molecular Identification of *Rhodobacter* sp. Origin of the *16S rRNA* Gene

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Abstract

Rhodobacter is one of the purple phototrophic bacteria widely used in aquaculture activity. It is particularly difficult in identifying and distinguishing *Rhodobacter* from other non-sulfur purple bacteria. This study aimed to evaluate primer pairs designed based on the *16S rRNA* gene from *Rhodobacter* as an effective and efficient molecular identification that can be performed in the laboratory using conventional PCR techniques. The *16S rRNA* sequences from *Rhodobacter* and other bacteria were aligned and analyzed for the high-variation region to capture the desired target amplicon. Forward and reverse primer pairs were designed based on the appropriate target amplicon. The primer pairs were also assessed for their feasibility based on ten assessment criteria for the quality of the PCR primers. The PCR optimization results obtained showed a specific band with a size of 548 bp according to the amplicon target size in the *Rhodobacter* samples tested and did not show any cross-reaction with other comparator bacteria, and no dimers or other non-specific bands were found. Based on the results obtained, it can be concluded that the primer pairs designed in terms of quality and specificity are good and can be used as alternative specific primers for the genus *Rhodobacter* sp.

Introduction

Rhodobacter is a Gram-negative bacterium, oval or rod-shaped, non-motile to motile, because it has flagella, produces capsules and mucus, and can form chain formations. *Rhodobacter capsulatus* is a kind of species with a characteristic zigzag chain formation. *Rhodobacter* sp. grows well in areas with high organic substrates and low oxygen concentrations, such as sewage ponds and eutrophic lakes. These bacteria is a mesophilic bacterium that has a habitat in freshwater with a neutral water pH and without salts, such as NaCl, and reproduce by binary fission and budding are taxonomically members of the phylum Proteobacteria, class Alphaproteobacteria, order Rhodobacterales, and family Rhodobacteraceae (Imhoff, 2015).

Rhodobacter is a phototrophic purple bacterium that uses bacteriochlorophylls α and carotenoid pigments in its photosynthesis process. Aerobic culture conditions will cause colonies of *Rhodobacter* in liquid media to be pink to red. Photoheterotrophic growth can occur when conditions contain high levels of organic matter, which provide carbon and electron sources in waters and under no oxygen conditions. Hydrogen, sulfide, or thiosulfate can be source of electron donors when the photoautotrophic mechanism occurs. Aerobic, low oxygen and dark conditions can cause chemotrophic processes such as denitrification, fermentation, and

oxidant-linked metabolism (Imhoff, 2015). Species of *Rhodobacter* sp. have many uses–*R. sphaeroides* can denitrify nitrite in the environment, so they can be used as a probiotic that can reduce nitrite levels in dark conditions and with little oxygen (Satoh et al., 1976; Shimizu et al., 2018). Brown and Herbert (1977) stated that *R. capsulatus* and *R. sphaeroides* use ammonia as a nitrogen source assimilated through the glutamine synthase or glutamate synthase reaction (NADPH-dependent).

The denitrification ability of the *Rhodobacter* is the main factor of using these bacteria in aquaculture activities as probiotics which function in bioremediation to break down inorganic nitrogen compounds in the aquaculture environment. Aside from being bioremediation of inorganic nitrogen compounds, *Rhodobacter* is reported to have a function in breaking down heavy metals. Li et al. (2016) stated that using *R. sphaeroides*, although with low activity, can reduce lead (Pb) levels in the environment. Bai et al. (2008) reported similar findings and that *R. sphaeroides* can help the cadmium (Cd) bioremediation process.

The process of identifying *Rhodobacter* to date has been guided by conventional identification, chemotaxonomy using lipopolysaccharide (LPS), and molecular using sequencing of the *16S rRNA* gene (Weckesser et al., 1995; Imhoff, 2015). However, this requires quite a long time, and not all probiotic development laboratories have the resources to conduct the sequencing process for identification. The objective of this study was aimed to design alternative specific primer pairs to detect the genus *Rhodobacter* because there is still limited specific polymerase chain reaction (PCR) primer pairs that can specifically detect the genus *Rhodobacter*.

Materials and Methods

Preparation of Forward and Reverse Primer Design of *Rhodobacter* sp.

The preparation of the primer design assembled from the *16S rRNA* gene sequence was carried out by analyzing the *16S rRNA* sequence of *Rhodobacter* sp. beforehand and comparing it with *16S rRNA* from several other bacterial species, such as *Streptococcus* sp., *Aeromonas* sp., *Vibrio* sp., *Bacillus* sp., and *Staphylococcus* sp. The preparation of unique sequences with high variation of *16S rRNA* from *Rhodobacter* sp. was performed using MEGA software version 11.0 (Tamura et al., 2021). We used *Rhodobacter capsulatus* strain ATCC 11166 *16S rRNA* gene (accession number NR_043407) (Hiraishi & Ueda, 1994) as reference data in comparing unique sequences with high variation from GeneBank's database (https://www.ncbi.nlm.nih.gov/nuccore/NR_043407.1?report=genbank) (NCBI, 1988).

After obtaining a high variation of the *16S rRNA* gene sequence from *Rhodobacter* sp, then a primer design search was performed using the primer design web (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Untergasser, 2007) and primer quality checking was carried out on the OligoAnalyzer™ Tool (Owczarzy et al., 2008)(https://sg.idtdna.com/pages/tools/oligoanalyzer?returnurl=%2Fcalc%2Fanalyzer), and primer specificity on the NCBI BLAST Primer (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) (NCBI, 1988).

Primer Design Assessment Criteria

The primer design assessment was carried out following the ten criteria that must be possessed by forward and reverse primer pairs and meets the requirements. Primers were ordered to Integrated DNA Technologies, Inc. (Coralville, Iowa, USA) as a third party. The ten criteria are primer length 18–22 bases, forward and reverse primer melting temperature (Tm) must be the same or nearly the same, primer annealing temperature (Ta) must be comparable to primer Tm, the difference in primer melting temperature (ΔTm) <5°C, GC content 40–60%, secondary structure like hairpin (ΔG = maximum −2 to −3 kcal/mol); self-dimer (ΔG = <−5 kcal/mol); and cross-dimer (ΔG = <−6 kcal/mol), self-complementary and pair-complementary <4 bases, repeats and runs no more than five base repeats or two base pairs more than two times, specificity high primer BLAST yield against target sequences, and amplicon product length <2000 bp (Lowe et al., 1990; Burpo, 2001; Lin et al., 2005; Sasmito et al., 2014).

Bacterial Sample DNA Extraction

The extraction method was performed according to the manufacturer's instructions in the IQ 2000 DNA Extraction kit (GeneReach Biotechnology Corp., Taiwan). Briefly, one mL of bacterial culture from both two *Rhodobacter* sp. archive isolates (PT. Marindolab Pratama, Serang, Banten, Indonesia) in Sistrom's minimal medium (SMM) was pelleted by centrifugation at 12,000 rpm or 11,269 ×g for three minutes. The pellet resuspended with Dodecyltrimethylammonium Bromide (DTAB) solution incubated at 75°C for five

Table 1. PCR amplification cycles used for primer design in the study

PCR Steps	Temperature (°C)	Duration	Annotation
Initial denaturation	95	3 minutes	
Denaturation	95	1 minute	
Annealing	50	1 minute	35 cycles
Elongation	72	1 minute	
End elongation	72	5 minutes	

minutes. After cooled to room temperature, the suspension was then homogenized by vortex and spun down for a few seconds to mix, and then 0.7 mL of chloroform was added and homogenized again by vortex for 20 seconds and centrifuged at 12,000 rpm or 13,800 ×g for five minutes. The top layer of the suspension was transferred 200 µL into a 1.5 mL microtube containing a mixture of 100 µL of Cetyltrimethylammonium Bromide (CTAB) solution and 900 µL of ddH_2O. The mixture was then homogenized with a rapid vortex and incubated at 75°C for five minutes. The suspension was cooled to room temperature and centrifuged at 12,000 rpm or 13,800 ×g for ten minutes. The supernatant discarded and, 150 µL of dissolve solution (patent material extraction kit) was added to the pellet, and homogenized. The suspension was incubated at 75°C for five minutes, cooled to room temperature, and then centrifuged at 12,000 rpm or 13,800 ×g for five minutes. A total of 130 µL of the supernatant was transferred into a new 1.5 mL microtube containing 300 µL of 96% ethanol, then homogenized by vortex quickly and centrifuged at 12,000 rpm or 13,800 ×g for five minutes, and the supernatant was removed. The pellet was washed with 200 µL 75% ethanol, centrifuged at 12,000 rpm or 13,800 ×g xg for five minutes, then the supernatant was discarded, and the pellet was dried and dissolved with Diethylpyrocarbonate (DEPC) H2O solution according to the size of the pellet.

Polymerase Chain Reaction (PCR) Method

The target bacteria was *Rhodobacter* sp., with the forward primer (Rh_c F) being 5'-ATCTGAAAGTCAGAGGTGAA-3' and the reverse primer (Rh_c R) being 5'-TGATGGCAACTGAAAGTGTG-3'. The total reaction volume was 25 µL with the premix composition used being 8.5 µL nuclease-free water (NFW), 1 µL each of forward and reverse primers (10 µM), 12.5 µL GoTaq® Green Master Mix (Promega, Wisconsin, USA), and 2 µL DNA template. The target amplicon product was 548 bp, with the target gene being *16S rRNA* at the 505th to 1056th base position. Pure isolates from *Streptococcus* sp., *Aeromonas* sp., *Vibrio* sp., *Bacillus* sp., and *Staphylococcus* sp. in tryptic soy broth (TSB) medium (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA) were used as a comparison control in the PCR process. The universal gene of *16S rRNA* was also used in PCR as quality control of genomic DNA (gDNA) samples which were examined with forward primer (27F) 5'-AGAGTTTGATCCTGGCTCAG-3', reverse primer (1492R) 5'-GGTTACCTTGTTACGACTT-3'., and the amplicon length of 1465 bp (Lane, 1991; Rizkiantino *et al.* 2020). The total reaction volume was 50 µL with the premix composition used being 13 µL nuclease-free water (NFW), 5 µL each of forward and reverse primers (10 µM), 25 µL GoTaq® Green Master Mix (Promega, Wisconsin, USA), and 2 µL DNA template.

The amplification cycles used for primer design and universal gene of *16S rRNA* were presented in Table 1. and Table 2. The PCR products were electrophoresed on agarose gel with a concentration of 1.5% (BIORON, Römerberg, Germany) in 1× Tris-Borate-Ethylenediaminetetraacetic acid (EDTA) (TBE) buffer solution at an electric voltage of 100 V with a duration of 35 minutes. Gel was visualized with the UVITEC Cambridge Gel Documentation System (Uvitec Ltd, Cambridge, UK). NEB N3200L 1 kb Plus DNA Ladder (New England Biolabs Ltd., UK) was used as the standard base size. The PCR product results were sent for sequencing (1st BASE, Singapore) for reconfirmation. The sequencing results were analyzed using MEGA software version 11.0 (Tamura et al., 2021), and BLAST was performed on GeneBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LI151 NK_LOC=blasthome).

Results

Preparation and Evaluation of Primer Designs from *Rhodobacter* sp.

Based on the unique gene sequences with hypervariation in the *16S rRNA gene* from *Rhodobacter capsulatus* strain ATCC 11166, target sequences at the 505th to 1056th base position was obtained (Figure 1. and Figure 2.). The sequence was then analyzed further to carry out the forward and reverse primer designs by searching the primer using the primer design web (https://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). The primer design results obtained for forward primers was 5'-ATCTGAAAAGTCAGAGGTGAA-3', and the reverse primer was 5'-TGATGGCAACTGAAAAGTGTG-3'. The primer pairs were

Table 2. PCR amplification cycles used for universal gene of *16S rRNA*.

PCR Steps	Temperature (°C)	Duration	Annotation
Initial denaturation	95	3 minutes	
Denaturation	95	30 seconds	
Annealing	50	30 seconds	35 cycles
Elongation	72	1 minute	
End elongation	72	5 minutes	

assessed and evaluated for primer quality on the OligoAnalyzer™ Tool and primer specificity on the NCBI BLAST Primer. The forward and reverse primer assessments obtained the following results, which are presented in Table 3.

Polymerase Chain Reaction **(PCR) Results**

Based on the results of PCR examination, it was found that only samples from *Rhodobacter* sp. showed a specific band at 548 bp (Figure. 3). Different isolates, such as *Vibrio* sp., *Aeromonas* sp., *Streptococcus* sp., *Staphylococcus* sp., and *Bacillus* sp. did not show a cross-reaction when amplified using a designed primer pair. Dimer results were also not seen in all wells, which showed well-optimized primers. Based on sequencing analysis found *Rhodobacter capsulatus* in well 1 with query coverage 100%, E-value 0, and percentage identity 100.00%; and *Rhodobacter sphaeroides* in well 2 with query coverage 100%, E-value 0, and percentage identity 99.67%.

```
ORIGIN
        1 aacgaacgct ggcggcaggc ctaacacatg caagtcgagc gagaccttcg ggtctagcgg
       61 cggacgggtg agtaacgcgt gggaacgtgc cctttgctac ggaatagccc cgggaaactg
      121 ggagtaatac cgtatgtgcc cttcggggga aagatttatc ggcaaaggat cggcccgcgt
      181 tggattaggt agttggtggg gtaatggcct accaagccga cgatccatag ctggtttgag
      241 aggatgatca gccacactgg gactgagaca cggcccagac tcctacggga ggcagcagtg
      301 gggaatctta gacaatgggg gaaaccctga tctagccatg ccgcgtgagc gatgaaggcc
      361 ttagggttgt aaagctcttt caggtgggaa gataatgacg gtaccaccag aagaagcccc
      421 ggctaactcc gtgccagcag ccgcggtaat acggaggggg ctagcgttgt tcggaattac
      481 tgggcgtaaa gcgcacgtag gcggatctga aagtcagagg tgaaatccca gggctcaacc
      541 ttggaactgc ctttgaaact cctggtcttg aggtcgagag aggtgagtgg aattccgagt
      601 gtagaggtga aattcgtaga tattcggagg aacaccagtg gcgaaggcgg ctcactggct
      661 cgatactgac gctgaggtgc gaaagcgtgg ggagcaaaca ggattagata ccctggtagt
      721 ccacgccgta aacgatgaat gccagtcgtc ggcaggcatg cctgtcggtg acacacctaa
      781 cggattaagc attccgcctg gggagaacgg tcgcaagatt aaaactcaaa ggaattgacg
      841 ggggcccgca caagcggtgg agcatgtggt ttaattcgaa gcaacgcgca gaaccttacc
      901 aacccttgac atcgggatcg cggttaccgg agacggtttc cttcagttcg gctggatccc
      961 agacaggtgc tgcatggctg tcgtcagctc gtgtcgtgag atgttgggtt aagtccggca
     1021 acgagcgcaa cccacacttt cagttgccat cattcagttg ggcactctgg aagaactgcc
     1081 gatgataagt cggaggaagg tgtggatgac gtcaagtcct catggcccct acgggttggg
     1141 ctacacacgt gctacaatgg tggtgacaat gggccaatcc caaaaagcca tctcagttcg
     1201 gattggggtc tgcaactcga ccccatgaag tcggaatcgc tagtaatcgc gtaacagcaa
     1261 gacgcggtga atacgttccc gggccttgta cacaccgccc gtcacaccat gggaattggg
     1321 tctaccctaa gatggtgcgc caacccgcaa gggaggcagc cagccacggt aggctcagtg
     1381 actggggtg
//
```

Figure 1. Analysis of unique sequences with high variation in the *16S rRNA* gene of the *Rhodobacter* sp. Unique sequences were at the 505th–1056th base position (red colour).

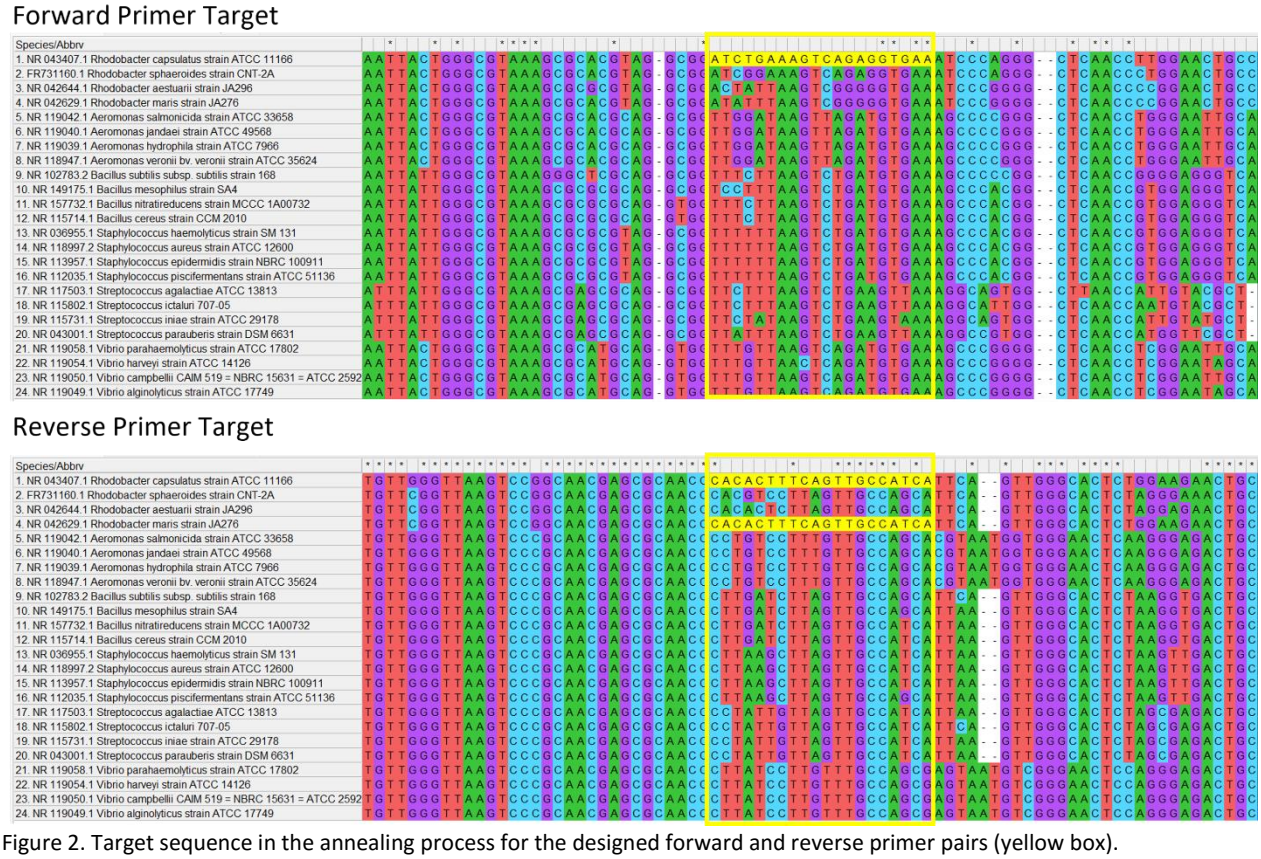

Figure 2. Target sequence in the annealing process for the designed forward and reverse primer pairs (yellow box).

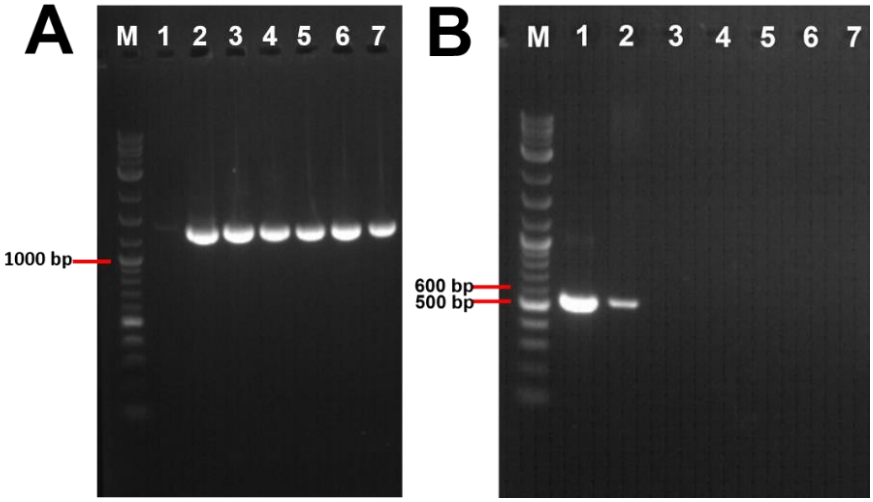

Figure 3. Results of PCR examination of the *16S rRNA* universal gene (A) and the primers that had been designed (B). The results showed a specific band only on isolates from the *Rhodobacter* sp. and did not show any cross-reaction with the *16S rRNA* gene from other bacteria as a comparison. M. Marker. 1. *Rhodobacter capsulatus*. 2. *Rhodobacter sphaeroides*. 3. *Vibrio* sp.. 4. *Aeromonas* sp.. 5. *Streptococcus* sp.. 6. *Staphylococcus* sp.. 7. *Bacillus* sp..

Table 3. The results of the assessment of the ten criteria for designed primer pairs

No.	Assessment criteria	Primer	
		Forward	Reverse
	Oligonucleotides	5′-ATCTGAAAGTCAGAGGTGAA-3′	5′-TGATGGCAACTGAAAGTGTG-3′
1.	Primer length Ideal: 18 – 22 bases	20 bases	20 bases
2.	Primer melting temperature (Tm) Ideal: Tm forward and reverse must be the same or nearly the same	53,59 °C	56,55 °C
3.	Primer annealing temperature (Ta) Ideal: Comparable to primer Tm	50,07 °C Gradient annealing optimization temperature suggestion: 49,00–53,00 °C	
4.	The difference in primer melting temperature (Tm) Ideal: <5 °C	(ΔTm) = 56,55 °C – 53,59 °C = 2,96 °C	
5.	GC content Ideal: 40–60%	40%	45%
6.	Secondary structure		
	- Hairpin (ΔG: maximum −2 to −3 kcal/mol)	−2.16 to −1.88 kcal/mol	−0,65 kcal/mol
	- Self-Dimer (ΔG : <−5 kcal/mol)	−1,47 to −6,70 kcal/mol	−1,34 to −3,14 kcal/mol
	- Cross-dimer (ΔG : <−6 kcal/mol)	−1,37 to −5,13 kcal/mol	−1,34 to −5,13 kcal/mol
7.	Self-complementary and pair-complementary Ideal: <4 bases	7,00 & 0,00	3,00 & 0,00
8.	Repeats and runs Ideal: No more than five base repeats or two base pairs more than two times	None	None
9.	Specificity Ideal: High primer BLAST yield against target sequences	Species of the genus *Rhodobacter* sp. dominate the BLAST results of the target sequence	
10.	Product length (amplicon) Ideal: <2000 bp	548 bp	

Discussion

The morphological characteristics possessed by *Rhodobacter* species and also possessed by *Rhodovulum* species are rod-shaped cells, an internal vesicular membrane, and carotenoids. The thing that can distinguish between the two genera is that *Rhodobacter* species can grow very optimally if there is no presence of NaCl salts due to their characteristics in a freshwater environment. The chemotaxonomy of the two genera (*Rhodobacter* and *Rhodovulum*) also have similarities, such as glucosamine content in lipopolysaccharides that are owned and can assimilate sulfate using the 3'-phosphoadenosine-5'-phosphosulfate pathway (Imhoff, 1982). Biochemical test such as NaCl requirements, sulfide oxidation end products, polar lipid composition, and tolerance to sulfides, take a long time to conduct because the colony of genus *Rhodobacter*, such as *R. sphaeroides*, will only grow on the day 4 of the incubation period (Paustian and Kurtz 1994; Suresh et al., 2019).

The most likely difference between the genus *Rhodobacter* and other purple phototrophic bacteria to be analyzed is based on their *16S rRNA* sequence. Unfortunately, time-consuming the *16S rRNA* gene sequencing methods and limitations require specific primers that can characterize the genus *Rhodobacter* without having to conduct the sequencing(Weckesser et al., 1995; Imhoff, 2015). The existing primers still have cluster targets in the genera *Rhodobacter* and *Pseudorhodobacter*, while the two of these genera are different in terms of having complete genes for photosynthesis, where genus *Rhodobacter* has bacteriochlorophyll a and carotenoids (Imhoff et al., 1984; Bräuer et al., 2011; Kim and Lee, 2019).

Therefore, it is necessary to have specific primers that can be used to detect genus *Rhodobacter* quickly, effectively and efficiently to accelerate the molecular identification process in the initial isolation purpose. The primer could be assembled from a unique sequence in the *16S rRNA* gene of a bacterium. Identical to each individual, can be used as an evolutionary chronometer, has conservative sequences, and has nine hypervariable regions (V1–V9) that make it easy to identify a type of bacteria are some of the advantages of using the *16S rRNA* gene in molecularly identifying bacterial species (Pangastuti, 2006). These hypervariable regions can indicate the presence of considerable sequence diversity among different bacterial species. These species-specific or genus-specific sequences in the hypervariable region are valuable targets for diagnostic tests and other scientific investigation purposes (Chakravorty et al., 2007).

The current study showed potential primer pairs that can be used to identify the genus of *Rhodobacter* molecularly. The results of testing the primer design based on the unique sequence of the *16S rRNA* gene of *Rhodobacter* showed the potential to identify this bacterium. Cross-reactions against Gram-positive bacteria (*Bacillus* sp., *Staphylococcus* sp., and *Streptococcus* sp.) and other Gram-negative bacteria (*Aeromonas* sp. and *Vibrio* sp.), as well as dimer bands, were not found during observation on the electrophoresis gel results. The results indicate that there is sufficient specificity and high potential to be able to detect the target amplicon of the designed primer pair. However, there is a difference in the band thickness found between the two samples of *Rhodobacter* used in this study. It is suspected that species differences cause less than optimal levels of primer attachment on the template DNA, thus affecting the resulting amplification products. Based on the results of the sequencing analysis performed on the PCR products from the two *Rhodobacter* samples, it was found that both are members of the *Rhodobacter* genus with the species of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*. *R. capsulatus* band was thicker and brighter, while the *R. sphaeroides* tend to be thinner when compared to *R. captulatus*. This is presumably because the primer pair was designed from the *R. capsulatus 16S rRNA* gene sequence, so it showed the greatest band on the *R. capsulatus* sample in well 1.The sequencing results were obtained to strengthen the primer design's ability to detect *Rhodobacter* specifically. However, primer pairs in the current study should be tested on other genera in the Rhodobacteraceae family that are more closely related, such as *Tabrizicola*, *Paenirhodobacter*, and *Thioclava*, should also be explored in future studies (Hördt et al., 2020).

Conclusion

Based on the results of the evaluation of the ten criteria for primer pairs, it can be concluded that the primer pairs designed in terms of quality and specificity are good and have the potential to be used as specific primers in detecting bacteria from the genus *Rhodobacter* sp.

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