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Characterization of *moaC* and a nontarget gene fragments of food-borne pathogen *Alcaligenes* sp. JG3 using degenerate colony and arbitrary PCRs

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Abstract

Alcaligenes faecalis, is an opportunistic, pathogenic, Gram-negative, food-borne bacterium appearing to be of public health importance due to its resistance to common antibiotics and frequent involvement in nosocomial infections and water contamination. Yet, the Betaproteobacterium showed potential benefit in fat degradation of food waste. It is suggested that pterin-based Molybdenum cofactor (Moco) biosynthesis involving *moa* gene is related with the pathogenesis of several Gram-negative bacteria, while in addition to lipase genes, glycerol coding genes including *glpD* are involved in effective degradation of fat waste. In this study, a degenerate colony and an arbitrary PCR-based methods were involved in gene isolation steps. We designed a pair of degenerate primers to detect *moaC* (GMF: 5′-ATCGGCATCACCAACCAGC-3′ and GMR: 5′-TGTCGATGGTGCCGAAGC-3′) and two gene specific primers to amplify 5′ (5′-TGATGAATGTTCCGTTGCGCTGCC-3′) and 3′ (5′-GACCTGCAGGCATGCAAGCTCGGC-3′) ends of *glpD* of strain JG3 using degenerate colony and arbitrary PCR methods, respectively. The developed degenerate colony PCR resulted amplification of targeted 421-bp DNA, while the set arbitrary PCR resulted in non-targeted 436-bp DNA. Deduced amino acid analysis showed that both sequences shared 94% amino acid similarity with molybdenum cofactor biosynthesis (MoaC) of *Pseudomonas stutzeri* and benzoate membrane transporter (BenE) of *A. faecalis*, respectively.

Practical applications

It is suggested that pterin-based Molybdenum cofactor (Moco) biosynthesis involving *moa* gene is related with the pathogenesis of several Gram-negative bacteria, while in addition to lipase genes, glycerol coding genes including *glpD* are involved in effective degradation of fat waste. However, information about these genes in pathogenic, food-borne, Gram-negative *Alcaligenes* sp. JG3 bacterium originated from root of *Zea mays* is barely found. A pair of degenerate primers (GMF: 5′-ATCGGCATCACCAACCAGC-3′ and GMR: 5′-TGTCGATGGTGCCGAAGC-3′) designed in this study could detect 421-bp *moaC* gene fragment using degenerate colony PCR, while another pair of gene specific primers 5′ (5′-TGATGAATGTTCCGTTGCGCTGCC-3′) and 3′ (5′-GACCTGCAGG CATGCAAGCTCGGC-3′) aiming to amplify ends of *glpD* of *Alcaligenes* sp. JG3 detected a nontargeted, 436-bp *benE* gene of the strain using an arbitrary PCR method.

1 | INTRODUCTION

Species *Alcaligenes faecalis* has been known as one of food-borne pathogens and spoilage bacteria (Hoque, Bari, Inatsu, Juneja, & Kawamoto, 2007). It was first isolated from stale beer in 1896 by Petruschky and later also found in marine food waste composed of shrimp shells and crab shells (Annamalai, Rajeswari, Vijayalakshmi, & Balasubramanian, 2011; O'Hara, Brenner, & Miller, 2000). *Alcaligenes* sp. JG3 has been known as an opportunistic, Gram-negative, fat and glycerol degrading rhizobacterium isolated from root of *Zea mays* in

Central Java. Based on identification using SEM, BD phoenix Automated Microbiology System and 16S rRNA analysis, the strain was detected as *A. faecalis* showing the closest relationship with the subspecies of *faecalis* and *parafaecalis* of *A. faecalis* and species *Alcaligenes aquatilis* LMG 22996 (Ethica & Raharjo, 2014; Lestari, Handayani, & Oedjijono, 2009).

Alcaligenes faecalis appears to be of public health importance due to the fact that that this ubiquitously opportunistic organism is usually resistant to common antibiotics and closely related with nosocomial infections, and commonly responsible for water contamination. However, its virulence and resistance mechanism is not well understood. Activities involving molybdenum cofactor biosynthesis (MoCo) has been implicated in pathogenesis of a number of Gram-negative bacterial infections. For example, recent advances in elucidating the MoCo biosynthetic pathway in *Mycobacterium tuberculosis* (Mtb) highlighted the evidence implicating the biosynthesis of this cofactor, as well as the enzymes depending on it for activity, in Mtb pathogenesis (Andreae, Titball, & Butler, 2013; Williams et al., 2014). The cofactor is actually ubiquitous in nature, and the pathway for MoCo biosynthesis is conserved in all three domains of life (Nichols, Xiang, Schindelin, & Rajagopalan, 2007). However, very little is known about the roles of MoCo-dependent enzymes encoded by *moaA* and *moaC* to the relatedness of MoCo biosynthesis with pathogenesis of *A. faecalis*. This study aimed to obtain DNA primers useful to isolate partial gene coding for Moco biosynthesis enzymes in *A. faecalis* using colony PCR method in order to study the structural feature of the genes of *A. faecalis*, to understand its role in glycerol metabolism of this organism.

Meanwhile, in addition to its lipolysis ability, strain *Alcaligenes* sp. JG3 is capable of glycerol degradation giving it potential as effective degradator of fat and oil wastes. Genes responsible for glycerol utilization by strain JG3, *glpD* and *glpK* have been recently detected (Ethica et al., 2014). In previous study, genetic engineering involving these genes from a Gram-negative, anaerobic bacterium, *Shewanella oneidensis*, was able to give another bacterium, *Escherichia coli*, the ability to synthesize succinic acid from glycerol (Flynn et al., 2010). Nevertheless, the full length of *glpD* and *glpK* from strain JG3 has not been reported. This study aimed to obtain DNA primers useful to isolate partial gene coding for Moco biosynthesis enzymes in *A. faecalis* using colony PCR method in order to study the structural feature of the *glpD* gene of *A. faecalis*, to understand the pathogenic role and virulence of this organism.

Today, various rapid PCR techniques including colony PCR are being offered to isolate various parts of target gene. Colony PCR utilizes microbial cells directly as the template, with no DNA extraction and purification prior to PCR. It utilizes a small amount of a single colony picked with a sterile micropipette tip and added to the tubes as the DNA template (Mirhendi et al., 2007). Luo and Mitchell (2002) showed that colony PCR would be the most convenient and a rapid protocol for amplification of target DNA and could be routine for the amplification of DNA for many purposes in laboratory work. It is not time intensive and additional steps such as mechanical high speed cell disruption, sonication, or toxic chemical are not needed. Degenerate colony PCR is a colony PCR method employing a pair of degenerate primers of conserved internal regions of targeted sequence.

Arbitrarily primed-PCR can be used to generate characteristic DNA fingerprint patterns. However, small changes in reaction conditions can cause band irreproducibility (Cusick & O'Sullivan, 2000). In previous study, Knobloch et al. (2003) successfully utilized a site-specific arbitrary PCR for the rapid and easy identification of Tn*917* insertion sites in *Staphylococcus epidermidis*. As result, fragments containing Tn*917* flanking chromosomal DNA could be amplified by the arbitrary PCR for all of the six investigated mutants.

In this study, the use of the degenerate colony and the arbitrary PCRs was demonstrated for the amplification of partial *moaC* and the rest of *glpD* of *Alcaligenes* sp. JG3, and their applicability was evaluated. It was expected that using both methods, the presence of *moaC* of strain JG3 could be detected, while the full length of its *glpD* gene could be obtained.

2 | MATERIALS AND METHOD

2.1 | Bacterial specimen

2.1.1 | Bacterial cultivation and identification

Azospirillum sp. JG3 strains originated from root of *Zea Mays* was received as pure culture in NA medium from Microbiology Laboratory, Faculty of Biology, University of Jenderal Soedirman (Purwokerto, Central Java, Indonesia). The culture was immediately stored at 8°C after received to be used as starter. Bacterial strains were subcultured and plate-purified in 2% nutrient agar medium (Merck, Germany) for 24 hr at 37°C. Other plates containing instant nutrient agar Merck, Germany, TSA (TSA II™, Beckton and Dickinson Company, USA) and Mac Conkey (Thermo Scientific, UK) media were also used for further colony identification. Cellular morphology of strain JG3 was further examined using scanning electron microscopy (SEM, JEOL, 5310-LV; 20 kV). Identification of strain JG3 was continued by biochemical tests. Identification was further carried out by obtaining partial sequence of 16S rRNA gene of the strain. Next, taxonomy status of the strain was determined by and complying all of the gathered information with Bergey's Manual of Determinative Bacteriology (Ethica & Raharjo, 2014; Garrity et al., 2005).

2.1.2 | Degenerate primer design

Sequences of *moaC* genes belong to *Proteobacteria* required to design degenerate primers were obtained from GenBank. Global alignments were performed using ClustalW (Thompson, Gibson, & Higgins, 2002) and the output was used directly as input for Primaclade (Gadberry, Malcomber, Doust, & Kellogg, 2005). A pair of primers that have the least possibility of hairpin formation, self-complementarity, and dimerization was selected.

2.1.3 | Isolation of *moaC* using degenerate colony PCR

Bacterial colony was prepared from pure and fresh 24-hr culture by using a sterile tip of an automatic pipettor and the cells adhering to the tip were directly dispersed in 6.5 μl dH_2O in a 1.5 ml microtube under

aseptic condition. Bacterial cell wall destruction was carried out by boiling bacterial colony for several minutes in the microtube as previously described (Ethica et al., 2013a, 2013b).

Amplification by colony PCR method aiming to amplify *moaC* partial region was performed in a 25 μl reaction volume with final concentrations as follows: 12.5 μl Ready-Mix Polymerase Kit (Kapa), 3 μl of 0.3 μM primers and a loopful of single bacterial colony. The reaction had an initial denaturation step of 1 min at 95°C, then 35 cycles as follows: 95°C 30 s, 50°C 1 min and 72°C 1 min. A final elongation step of 7 min was performed at 72°C.

2.1.4 | Isolation of 5′ and 3′ ends of *glpD* gene using arbitrary PCR

Several visible bacterial colonies on agar media and 9.5 ml of the bacterial cell suspension with $OD_{600} = 1$ were used to prepare DNA templates for arbitrary PCR. The calculated concentration of genomic DNA obtained from both conventional methods was determined based on absorbance at 260 nm measured by UV-Vis Spectrophotometer.

Arbitrary PCR consisting two rounds of amplification was performed to obtain the rest of the *glpD* gene using previously described procedure (Espinosa-Urgel, Salido, & Ramos, 2000). For amplification of 3′ end, the first round of PCR was performed by using the genomic DNA of *Azospirillum* sp. JG3 as a template, with an arbitrary primer (ARB1; 5′-GGCACGCGTCGACTAGTACNNNNNNNNNGATAT-3′) and an internal primer of *glpD* internal fragment, unique for the right end (GSP3′). The themocycling condition was: 3 min at 95°C; 6 cycles of 30 min at 95°C, 30 min at 30°C, and 1 min at 72°C; 30 cycles of 30 min at 95°C, 30 min at 50°C, and 1 min 72°C; and an extension period of 7 min at 72°C. The second round of PCR was done with 5 ml of the first-round reaction as the template as follows: 3 min at 95°C; 30 cycles of 30 min at 95°C, 30 min at 57°C, and 1 min at 72°C; and 7 min at 72°C. Primers used for the second round were those corresponding to the conserved region of ARB1 (ARB2; 5′-GGCACGCGTCGACTAG-TAC-3′) and similar GSP3′ primer. Similar condition was applied for amplification of 5′ end using similar arbitrary primers ARB1and ARB2, but different gene specific primer, GSP5′.

2.2 | Gel electrophoresis

Ten microliter of each DNA isolate from four different methods and 10 μl of each associated PCR products were loaded on 1.5% agarose gel with 0.5× TAE running buffer and 6x loading buffer stained with 0.25 μg/ml EtBr at 50 V for 40 min.

2.2.1 | DNA purification and sequencing

Purification of DNA amplicons from both colony PCR and arbitrary PCR was performed using Quick Gel Extraction Kit according to instructions suggested by the manufacturer (Promega, 2010). The sequencing of all PCR products was proceeded using BigDye® Terminator v3.1 sequencer system (Applied Biosystem, USA). The obtained sequences were ready for analysis.

2.2.2 | BLAST and sequence analysis

The sequence of internal part of *moaC*, and a sequence of arbitrary PCR products were deposited in the GenBank database. Homology analyses of these sequences at the level of nucleotide and amino acid were performed using BLASTn and BLASTx (Altschul et al., 1997), while the alignments based on amino acids were conducted using ClustalW (Thompson et al., 2002). The phylogenetic relationship and estimated divergence numbers of these sequences with other sequencess referred by BLAST from other organisms already deposited in GenBank was determined using MEGA 6.0 (Tamura, Nei, & Kumar, 2004; Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

3 | RESULTS AND DISCUSSION

3.1 | Bacterial strain cultivation

Uniform shapes of cells and consistent red color of Gram-stained cells were eventually obtained after third colony purification step on analogue plates of NA medium referring signs of purity. Based on the result, single colonies from the solid medium was then proceeded to colony cultivation in TSA with 5% sheep blood. In TSA medium strain JG3 showed punctiform, dry, irregular shapes with grayish color. The ability of the strain to grow in various media without the need of strict temperature condition or specific treatment showed that the organism is easy to grow. Yet, the strain did not show growth in MacConkey medium commonly used to cultivate Gram-negative, enteric, pathogenic bacteria. Inability to grow in MacConkey agar medium is typically a sign that bacteria could not ferment lactose.

A list of 44 biochemical characteristics of strain JG3 resulted from panel for Gram-negative bacteria of BD Phoenix 100 Automated Microbiology System was compared to the BD Phoenix system database of organisms showing that strain JG3 was detected as species *A. faecalis* rhizobacterium with 99% confidence. Genotypic identification of strain JG3 based on 16S rRNA gene using universal or species-strain specific primers resulting in 1511-bp nucleotide sequence deposited in GenBank under accession number of AB914514 showing the closest relationship (96%) with strain *A. faecalis* (Ethica & Raharjo, 2014).

3.2 | Primer design

As the first step to isolate *moaC* fragment, degenerate primers were designed using Primaclade software (Gadberry et al., 2005) based on the conserved regions of *moaC*-like sequences of other *Proteobacteria* retrieved from GenBank. The selected sequences as the basis of degenerate primer design in this study is listed in Table 1. To ensure that there were conserved regions containing *glpD*-specific signature nucleotides essential to design the genus-specific primers, a MULTALIN multialignment was conducted on the sequences where theoretical positions of these primers could be displayed as seen in Figure 1. A pair of degenerate primers were designed from conserved regions of targeted genes showed by MULTALIN multialignment. After being checked for any theoretical hairpin and other self-complementarity formations, the primers were then synthesized with characteristics listed

TABLE 1 List of sequences used in degenerate and gene specific primer design

Organism name	ENA GenBank accession no.	Associated gene	Length (bp)	Primer type (D = Degenerate, G = Gene specific)
Azospirillum brasilense Sp245	CCC97915.1	*moaC*	495	D
Azospirillum sp. B510	BAI72242.1	*moaC*	510	D
Azospirillum lipoferum 4B	CBS86419.1	*moaC*	501	D
Rhodospirillum rubrum ATCC 11170	ABC23610.1	*moaC*	498	D
Pseudomonas stutzeri DSM 4166	AEA83042.1	*moaC*	492	D
Rhodospirillum rubrum ATCC 11170	ABC23610.1	*moaC*	498	D
Alcaligenes sp. JG3	AB862285.1	*glpD*	427	G

in Table 2. As validation step, the specificity of the amplicons targeted by the designed primers using the chosen PCR method was verified by using the BLAST.

3.3 | Characteristics of isolated gene fragments

Degenerate colony PCR using a pair of designed primers (GMF/GMR) performed in a total volume of 25 μl containing a loopful of bacterial single colony, which was immediately boiled before used as template resulted a single band on gel corresponding to DNA size of 490 bp. Amplification of an internal control was carried out in every assay to leave out false negative results due to interference of inhibitors or poor DNA extraction procedure. After sequencing, the nucleotide sequence of the isolated DNA from gel was obtained as 421-bp fragments deposited in GenBank database under accession numbers of AB894422.

Conversely, the first and second rounds of the arbitrary PCR for the isolation the rest of *glpD* following a protocol described by Espinosa-Urgel et al. (2000) resulted nonspecific bands on gel electrophoresis. Two dominant bands from each second-round arbitrary PCR were isolated, purified, and sequenced. From the sequencing process, only one of four obtained bands corresponding to DNA size of 500 bp resulted in 436-bp nucleotide sequence with particular function. The sequence was deposited in GenBank under accession number of AB914513.

3.4 | Nucleotide sequence characteristics of isolated gene fragments

Sequence analysis and phylogenetic construction of isolated gene fragments obtained in this study was performed to identify the function of proteins coded by the DNA sequences and to identify relationship of these sequences with those from other species already deposited in GenBank.

3.5 | Characteristics of 421-bp sequence

Results from NCBI BLASTn search indicated that the 421-bp nucleotide sequences obtained from colony PCR shared high similarity with *moaC* of *Pseudomonas stutzeri* A1501 by 97%.

Analysis on deduced amino acid sequences of the isolated gene fragments from strain JG3 was carried out to find out the diversity of these sequences among those of other species that have been previously deposited in GenBank. The analysis was also done to identify possible protein domain coded by these sequences, to understand their particular functions. NCBI BLASTx analysis and alignment of 421-bp sequence converted to amino acid revealed its closest relationship with Molybdenum cofactor biosynthesis protein C (MoaC) of *P. stutzeri* LMG11199 by 94%, similarity on amino acid sequence. The conserved regions from the alignment are observed as displayed in Figure 2. The evolutionary divergence numbers (amino acid substitutions per site) between referred species based on 421-bp sequence of strain JG3

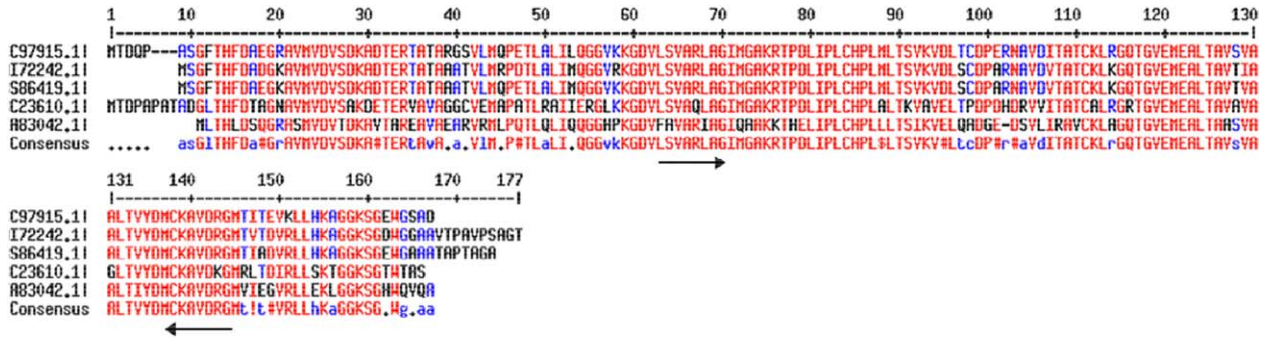

FIGURE 1 MULTALIN multialignment outputs showing conserved regions of targeted genes from members of *Proteobacteria*. C. Multialignment of *moaCsNote*. Arrows show positions of primers, red fonts are the conserved amino acids

TABLE 2 Degenerated primers designed from available *moaC*s retrieved in GenBank and gene specific primers designed from internal *glpD* of *Alcaligenes* sp. JG3

Primer name	Sequence	Nt (bases)	Tm (°C)	GC (%)	Degeneracy
GMF	5′-ATCGGCATCACCAACCAGC-3′	19	58.4	55.6	0
GMR	5′-TGTCGATGGTGCCGAAGC-3′	18	58.3	55.0	0
GSP3′	5′-TGATGAATGTTCCGTTGCGCTGCC-3′				n.a.
GSP5′	5′-GACCTGCAGGCATGCAAGCTCGGC-3′				n.a.

determined using MEGA 6 (Tamura et al., 2004, 2013) are shown in Table 3.

3.6 | Characteristics of 436-bp sequence

The first and second rounds of the arbitrarily-primed PCR for the isolation the rest of *glpD* following a protocol described by Espinosa-Urgel et al. (2000) resulted nonspecific bands on gel electrophoresis. Two dominant bands from each second-round arbitrarily-primed PCR were isolated, purified and sequenced resulting in 436-bp nucleotide sequence with particular function. Thus, it was deposited in GenBank under accession number of AB914513.

Further, NCBI BLASTx analysis on 436-bp revealed the highest identity with membrane protein of *A. faecalis* subsp. *faecalis* NCIB8687 by 95%. Amino acid alignment between 436-bp sequence of strain JG3 with that of the closest organism showing limited conserved regions of the sequence as displayed in Figure 3. The alignment of 436-bp sequence converted to amino acid result showed that strain JG3 posseses analogous sequence responsible for the synthesis of Benzoate membrane transport protein (BenE) as possesed by *A. faecalis* subsp. *faecalis* NCIB8687. According to BLASTx both sequences shared 95% identity on 89% query of amino acid sequences with five amino acid gaps that do not show type of amino acid between two sequences compared.

4 | DISCUSSION

This study was done as part of efforts to elucidate genes involved in MoCo biosynthesis and glycerol metabolism; which later could be used to further study the relatedness of MoCo biosynthesis with pathogenesis of *A. faecalis* as well as to the study know genes involved in glycerol metabolism of the organism becomes necessary. In this study, a degenerate colony and an arbitrary PCR-based methods were carried out using new primers in order to isolate both targeted gene fragments. We designed a pair of degenerate primers to detect *moaC* (GMF: 5′-ATCGGCATCACCAACCAGC-3′ and GMR: 5′-TGTCGATGGTGCC GAAGC-3′) and two gene specific primers to amplify 5′ (5′-TGATGAAT GTTCCGTTGCGCTGCC-3′) and 3′ (5′-GACCTGCAGGCATGCAAG CTCGGC-3′) ends of *glpD* of strain JG3 using degenerate colony and arbitrary PCR methods, respectively. The developed degenerate colony PCR resulted amplification of targeted 421-bp DNA, while the set arbitrary PCR resulted in nontargeted 436-bp DNA.

Sequencing the 421-bp degenerate PCR amplification product was performed to the purified PCR product without cloning prior to sequencing while it was performed to the 436-bp DNA. The approach was chosen because clear single fragment was clearly observed on the gel electrophoresis for the 421-bp PCR product in addition it was also aimed to check the purity of the PCR product. If the PCR product is mixture of DNA fragments with similar size then the sequencing process could not result in a good sequence. The direct sequencing of the obtained PCR fragments successfully proved that each of the PCR product was associated with a single DNA fragment (Raharjo, Haryono, Nataningtyas, Alfiraza, & Pranowo, in press).

Analysis of the 421-bp DNA sequence was based on Table 3 showing that the lowest evolutionary divergence number based on 421-bp sequence and Molybdenum cofactor biosynthesis protein C (MoaC) of *P. stutzeri* LMG11199 and other *P. stutzeri* species was the lowest (2.62), while the highest was between the strain with *Pseudomonas mandeli* by 2.98. This means that amino acid substitutions per site between sequences of were the least between strain JG3 and *P. stutzeri* LMG11199 based on 421-bp and MoaC sequence. These results showed that strain JG3 possesses analogous sequence responsible for the synthesis of MoaC (Molybdenum cofactor biosynthesis protein C) of MoaC superfamily involved in Molybdenum cofactor (MoCo) biosynthesis similar to that possessed by *P. stutzeri* LMG 11199.

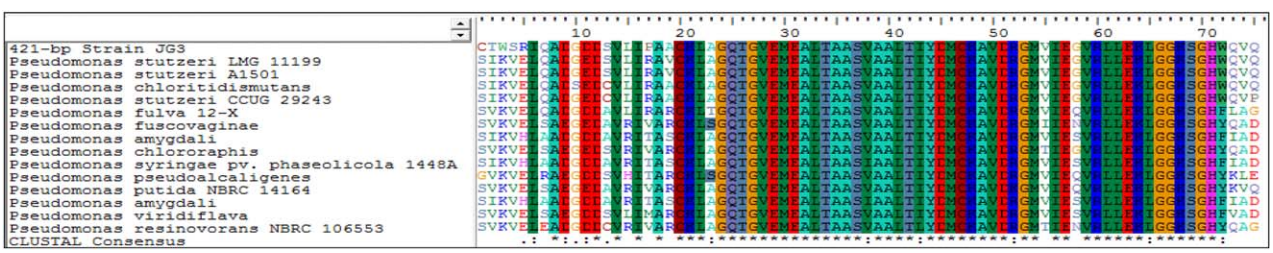

FIGURE 2 Alignment of amino acid based on 421-bp sequence with associated sequence of referred strain, *Pseudomonas stutzeri* LMG 11199*Note*. Clustal consensus below the list show conserved amino acid position between referred strains

TABLE 3 Estimates of evolutionary divergence numbers between strain JG3 with 14 other referred species based on 421-bp sequence (Tamura et al., 2004; Zuckerkandl & Pauling, 1965)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. AB894422.1| Alcaligenes sp. JG3															
2. YP 004713445.1|Pseudomonas stutzeri LMG 11109	0.13														
3. YP 001171710.1|Pseudomonas stutzeri A1501	0.13	0.00													
4. WP 023446804.1|Pseudomonas chloritidismutans	0.14	0.04	0.04												
5. YP 006458828.1|Pseudomonas stutzeri CCUG 20243	0.14	0.04	0.04	0.03											
6. YP 004473106.1|Pseudomonas fulva 12-X	0.22	0.14	0.14	0.16	0.14										
7. WP 019360174.1|Pseudomonas fuscovaginae	0.29	0.19	0.19	0.21	0.19	0.16									
8. WP 005746014.1|Pseudomonas amygdali	0.24	0.17	0.17	0.19	0.17	0.14	0.17								
9. WP 009047007.1|Pseudomonas chlororaphis	0.24	0.14	0.14	0.17	0.16	0.17	0.05	0.17							
10. YP 273390.1|Pseudomonas syringae pv. phaseolicola 1448A	0.26	0.19	0.19	0.21	0.19	0.16	0.19	0.01	0.19						
11. WP 003459465.1|Pseudomonas pseudoalcaligenes	0.26	0.21	0.21	0.24	0.22	0.16	0.16	0.21	0.16	0.22					
12. YP 008111841.1 |pseudomonas putida NBRC 14164	0.26	0.16	0.16	0.17	0.17	0.16	0.10	0.19	0.10	0.21	0.14				
13. WP 002552260.1|Pseudomonas amygdali	0.26	0.19	0.19	0.21	0.19	0.16	0.19	0.01	0.19	0.00	0.22	0.21			
14. WP 004886220.1|pseudomonas viridiflava	0.24	0.17	0.17	0.21	0.19	0.13	0.14	0.14	0.11	0.16	0.16	0.16	0.16		
15. YP 008101528.1|Pseudomonas resinovorans NBRC 106553	0.26	0.19	0.19	0.19	0.17	0.14	0.11	0.17	0.10	0.19	0.19	0.16	0.19	0.17	

Molybdenum (Mo) are redox enzymes involved in the global carbon, sulfur and nitrogen cycles, while Moco is the essential component of Mo allowing the redox properties of molybdenum to be harnessed by enzymes in order to catalyze redox reactions in the carbon, sulfur and nitrogen metabolism (Shi & Xie, 2011; Williams et al., 2012). MoCo is known as an essential cofactor of a diverse group of redox enzymes (NCBI, server 2014). In mycobacteria, several important physiological functions, such as dormancy regulation, the metabolism of energy sources, and nitrogen source were exerted by Mo enzymes. Pterin-based Mo cofactor (Moco) is the common cofactor of the Mo enzymes in mycobacteria but the cofactor biosynthesis is nearly an untapped area (Shi & Xie, 2011). Result of this study gave initial information about the pterin-based Mo cofactor biosynthesis in *A. faecalis*, which hopefully could lead to better understanding about probable dormancy regulation of this pathogenic species.

Sequencing the 436-bp arbitrary PCR amplification product was performed to the purified PCR product with cloning prior to sequencing because more than one DNA bands observed on the gel electrophoresis. BLASTn on a 436-bp nucleotide sequence obtained from arbitrary PCR indicated that the sequence did not correspond to *glpD* gene. Instead, the sequence showed high identity with genome part of *Pusillimonas* sp. T7-7 by 73%, which means the rest of the *glpD* gene could not be obtained using the set arbitrary PCR. However, NCBI BLASTx analysis on 436-bp revealed the highest identity with membrane protein responsible for the synthesis of benzoate membrane transport protein (BenE) of *A. faecalis* subsp. *faecalis* NCIB 8687 by 95%.

As seen from Table 4, the lowest evolutionary divergence number based on 436-bp sequence was 2.18, which was between strain JG3 and *Halomonas elongata* DSM 2581, while the highest was between strain JG3 and *Pusillimonas* sp. T7-7, *Azoarcus* sp. KH32C and *Azoarcus toluclasticus*, respectively by 2.47. This means that amino acid substitutions per site between sequences of were the least between strain JG3 and *Halomonas elongata* DSM 258, revealing high similarity between two sequences compared.

Based on the result, analysis using NCBI database showed that strain JG3 could produce benzoate transporter functioning as a

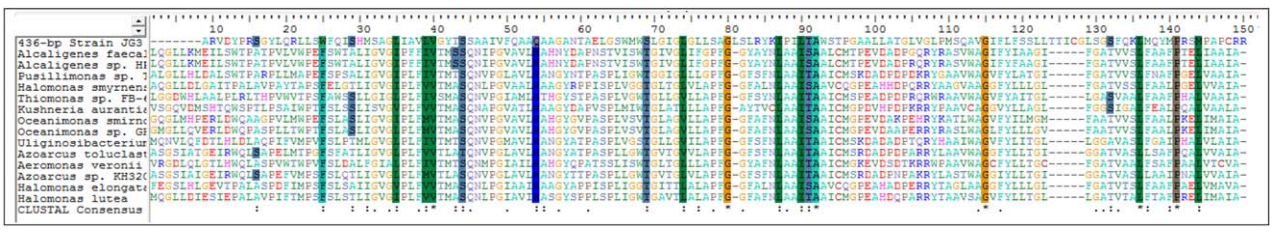

FIGURE 3 Alignment of amino acid based on 436-bp sequence with associated sequence of referred strain, *Alcaligenes faecalis* subsp. *faecalis* NCIB8687*Note*. Clustal consensus below the list show conserved amino acid positions between referred strains

TABLE 4 Estimates of evolutionary divergence numbers between strain JG3 with 14 other species based on 436-bp sequence (Tamura et al., 2004; Zuckerkandl & Pauling, 1965)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1. AB914513.1 4 Alcaligenes sp. JG3															
2. WP 003799370.1| Alcaligenes faecalis subsp. faecalis NCIB8687	2.32														
3. WP 009458870.1| Alcaligenes sp. HPC1271	2.32	0.01													
4. YP 004416547.1| Pusillimonas sp. T7-7	2.47	0.43	0.43												
5. WP 016854293.1| Halomonas smymensis	2.32	0.61	0.61	0.50											
6. WP 018914618.1| Thiomonas sp. FB-6	2.25	0.63	0.61	0.44	0.59										
7. WP 019949959.1| Kushneria aurantia	2.25	0.69	0.69	0.61	0.57	0.63									
8. WP 019933474.1| Oceanimonas smirnovii	2.39	0.43	0.44	0.46	0.49	0.55	0.63								
9. YP 005093266.1| Oceanimonas sp. GK1	2.25	0.44	0.44	0.47	0.46	0.56	0.57	0.14							
10. WP 018605100.1| Uliginosibacterium gangwonense	2.32	0.68	0.68	0.40	0.59	0.40	0.71	0.55	0.53						
11. WP 018992757.1| Azoarcus toluclasticus	2.47	0.68	0.68	0.37	0.50	0.49	0.61	0.53	0.50	0.44					
12. WP 021231649.1| Aeromonas veronii	2.39	0.60	0.60	0.50	0.57	0.51	0.60	0.56	0.49	0.54	0.55				
13. YP 007598386.1| Azoarcus sp. KH32C	2.47	0.69	0.69	0.40	0.50	0.53	0.65	0.55	0.51	0.42	0.15	0.60			
14. YP 003897379.1| Halomonas elongata DSM 2581	2.18	0.64	0.64	0.55	0.26	0.64	0.60	0.54	0.49	0.61	0.57	0.55	0.56		
15. WP 019018715.1| Halomonas lutea	2.47	0.60	0.60	0.48	0.36	0.54	0.57	0.50	0.49	0.50	0.50	0.54	0.49	0.33	

benzoate/proton symporter (transport and binding proteins, carbohydrates, organic alcohols, and acids) (NCBI, server 2014). Nishikawa et al. (2008) reported *Pseudomonas putida benF*, *benK*, *benE1*, and *benE2* genes encode proteins belonging to benzoate transporter super family, but those functions have not yet been clarified. In the study *P. putida* gene products expressed in *Saccharomyces cerevisiae* cells were localized to the yeast plasma membrane and were involved in taking up benzoate into the cells. Based on the sensitivity of yeast cell-growth to benzoate, it is proposed that *benK*, *benE1*, and *benE2* gene products act as transporters taking up benzoate into the cells. This supported chemoorganotrophic characteristic of *Alcaligenes* sp. JG3 as member of genus *Alcaligenes*.

5 | CONCLUSION

A pair of primers designed using Primaclade software (GMF: 5′-ATCGGCATCACCAACCAGC-3′ and GMR: 5′-TGTCGATGGTGCCGAAGC-3′) could detect 421-bp *moaC* gene fragment of *Alcaligenes* sp. JG3 using degenerate colony PCR method with thermal treatment, while another pair of primers designed from internal part of *glpD* (5′-TGATGAATGTTCCGTTGCGCTGCC-3′) and 3′ (5′-GACCTGCAGGCATGCAAGCTCGGC-3′) detected a nontargeted, 436-bp *benE* gene of the strain using an arbitrary PCR method.

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