In-silico Specificity Comparison between GMF-GMR and JMF-JMR Primers for Detecting *moaC* Genes of Food Spoilage Bacteria *Pseudomonas* spp

In-silico Specificity Comparison between GMF-GMR and JMF-JMR Primers for Detecting *moaC* Genes of Food Spoilage Bacteria *Pseudomonas* spp.

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Abstract. *Pseudomonas* spp. have been known as notorious food spoilage bacteria with ability to produce thermo-tolerant enzymes. They pose serious risk to public health as its most pathogenic member, *P. aeruginosa*, could cause nosocomial infections affecting peoplewith immunodeficiency. The use of GMF-GMR primers had been reported capable for detecting bacterial *moaC* of *Alcaligenes javaensis* JG3. The gene is suspected to be related with dormancy of pathogenic bacteria. This study aimed to investigate specificity of the GMR-GMF as well as a newly designed JMF-JMR pairs of primers (JMF: 5'-GGCGTACATCATCCACACTG-3' and JMR: 5'-GGCGTTGACCATCTATGACA-3') for detecting *moaC* genes of 57 members of *Pseudomonas* spp. retrieved from http://insilico.ehu.eus/ database using *in silico* PCR (Polymerase Chain Reaction). The results showed that GMF-GMR primers could selectively amplify 271-bp *in silico PCR* products from 14 out of 57 members of *Pseudomonas* spp. tested. However, BLASTn analysis on these 14 amplified DNA sequences showed that they were not part of *moaC*, yet *glpK* gene fragment sequences. Meanwhile, the newly designed primers from *moaC* sequence of strain JG3, JMF-JMR, could specifically amplify 214-bp *in silico PCR* products from 2 out of 57 members of *Pseudomonas* spp. matched to bacterial *moaC* gene fragment sequences. As conclusion, based on *in silico* study JMF-JMR primers are more specific than GMF-GMR ones for detecting *moaC* gene fragments of members of *Pseudomonas* spp. studied.

1. Introduction

Food spoilage by *Pseudomonas* spp. in particular food groups such as fish, meat, milk and dairy products, water, fruit, and vegetables. Despite the fact that *Pseudomonas* spp. do not cause serious risk to public health, most pathogenic *Pseudomonas aeruginosa* belongs to risk group II. It is categorized as opportunistic pathogen, which causes nosocomial infections in hospitals affecting immunocompromised persons [1].

Characterization of *moaC* of food-borne bacterium *Alcaligenes* sp. JG3, later known as *Alacligenes javaensis* JG3 using degenerate GMF-GMR primers had been reported. Previous result showed that strain JG3 has analogous moaC gene sequence, which is responsible for the synthesis of

MoaC (molybdenum cofactor biosynthesis protein C) of MoaC superfamily involved in molybdenum cofactor (MoCo) biosynthesis similar to that belongs to *Pseudomonas stutzeri* LMG 11199 [2,3]. It is therefore interesting to reveal possible relationship between *moaC* with *Pseudomonads*.

The term *in silico* was coined at the end of the 1980s, to refer to "virtual" experiments existing only "inside" computers. It complements the terms in vivo and in vitro, characterizing experiments that are accomplished, respectively, within a living [4]. In genomic studies, *in silico* PCR is aimed to calculate theoretical PCR results by using up-to-date sequenced bacterial genomes, a technique which allows amplification of specific DNA sequences [4,5]. *In silico* or virtual PCR could be used to predict or to calculate theoretical ability of pairs of primers to amplify targeted gene fragments by using up-to-date sequenced bacterial genomes stored in a database. It is a technique, which allows amplification of specific DNA sequences supporting successful DNA amplification using *in vivo* PCR [5-8]. Today, various web tools for *in silico* PCR including virtual oligonucleotide assembly and analysis have been well-developed. Currently, *in silico* approach is capable to reveal potential PCR biases in the use of DNA barcode. For example, it divulges such biases in the use of internal transcribed spacer (ITS) of nuclear DNA, which has been used as an environmental DNA barcode for fungi for 15 years. In silico analysis has been use to investigate virulence genes in an emerging dental pathogen *A. baumannii* and related species. In addition, successful determination of a real-time PCR (Q-PCR) based *in silico* amplification of signature genes for quantification of various bacteria causing sepsis has also been reported [9-11].

Biochemical and molecular methods aiming to detect and confirm the presence of *Pseudomonas* spp. had been studied. However, none of these studies targeting the *moaC* gene sequence of *Pseudomonas* group, while previous report had inferred the link between *moaC* with the food-spoilage agent [2]. This study compared the specificity detection between GMF-GMR and JMF-JMR primers in detecting *moaC* genes of food-spoilage bacterial group of *Pseudomonas* spp. by *in silico* PCR method.

2. Methods

2.1. GMF-GMR primer preparation

GMF-GMR primers were obtained from previous publication [2]. The primers were reported to be able to amplify *moaC* gene fragment of *Alcaligenes javaensis* JG3 using conventional PCR method.

2.2. JMF-JMR Primer design

A pair of gene specific primers were designed using Primer3Plus web-based tool, which was freely available from http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi [11]. A DNA template used to design the primers was a *moaC* gene fragment of *Alcaligenes javaensis* JG3 previously reported [2,3]. The sequence was accessed through Genbank accession code of AB894422.1 available from NCBI (National Center for Biotechnology Information) websitehttps://www.ncbi.nlm.nih.gov/nuccore/AB894422.1?report=fasta.

2.2.1. In silico PCR

An amplification was performed using in silico PCR amplification software freely available from http://insilico.ehu.es/PCR/ (ver. 2010, released by CONSOLIDER-INGENIO, Spain) using GMF-GMR and JMF-JMR primers [6,8]. As many as 57 genomic DNA sequences of members of *Pseudomonas* spp. retrieved from in silico database were used as virtual templates. The genomic sequences of members of *Pseudomonas* spp. encompass notable species from genus *Pseudomonas* such as *P. brassicacearum*, *P. denitrificans*, *P. entomophila*, *P. fluorescens*, *P. fulva*, *P. mendocina*, *P. monteilii*, *P. pose*, *P. protegens*, *P. resinovorans*, *P. aeruginosa*, and *P. syringae*. Separately, both pair of primers were used to amplify the set bacterial genomic DNA sequences. The parameters set included: Exclude plasmid (unchecked), option "allow 1-2 mismatches, but in nucleotides in 3' end",

and maximum length of band 3000 nucleotides. The amplified products from amplification using both pairs of primers were then analysed using BLASTn from NCBI website [12], and the results were compared based on specificity of products.

3. Results and Discussion

Sequence of moaC previously reported [2] as seen in Figure 1 was retrieved using GenBank accession number: AB894422.1

Article I. Alcaligenes sp. JG3 DNA, moaC gene fragment

GenBank: AB894422.1

>AB894422.1 Alcaligenes sp. JG3 DNA, moaC gene fragment
TGGGCAGTTCGGCAGCTACCTGATTGCGCGCGCACATCAGATTCTGCTCGCCGAGTACCTCCCAGACGCC
ACCGCGCGCCAGGAGGTACTGGCGTACATCATCCACACTGCCGAACGATTCATCCCATTGGATGCGCTCG
CCATCGCTGCCTAGGGTTTCCCGATAACGGGCAAAGAACTGAATCGAAATCATGCCTGCACCTGCCAATG
CCCACTCTTGCCGCCCAGCTTCTCCAGCAGGCGCACCCCTTCGATCACCATGCCGCGGTCAACGGCCTTG
CACATGTCATAGATGGTCAACGCCGCGACGCTGGCAGCGGTCAGCGCTTCCATTTCCACGCCCGTCTGCC
CCGCCAGCTTGCAGGCCGCGGGGATCAATACGCTGTCGTCACCATCGGCCTGGATCCTCGACCATGTACA
G

Figure1. DNA sequence of moaC gene fragment of *A. javaensis* JG3 under (Genbank acc. Code AB894422.1) [Ethica *et al*., 2018].

From the *moaC* sequence (**Figure 1**), a new pair of primers were designed, JMF: 5'-GGCGTACATCATCCACACTG-3' and JMR: 5'-GGCGTTGACCATCTATGACA-3' could be designed using Primer3Plus web tool (**Figure 2**).

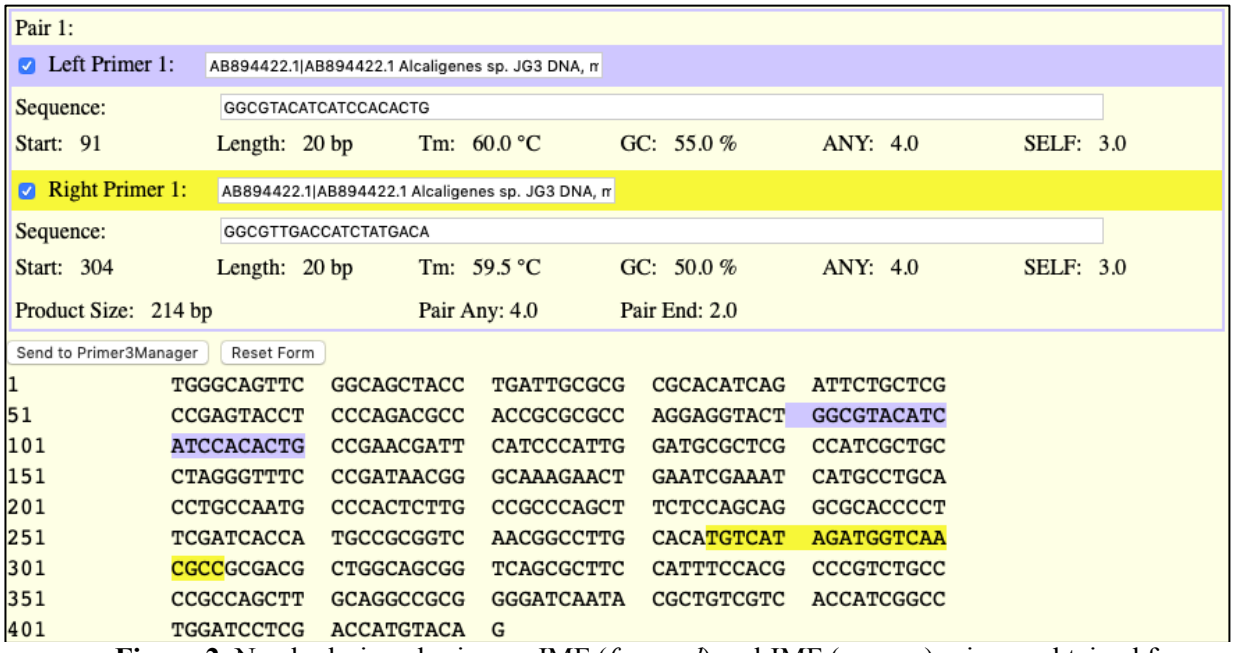

Figure 2. Newly designed primers, JMF (*forward*) and JMF (reverse) primers obtained from Primer3Plus primer design tool based on moaC sequence of *A. javaensis* JG3 (blue- and yellow- highlighted).

These newly designed pair of primers, along with the available GMF-GMR primers were used to amplify targeted sequence with 57 genome sequences of *Pseudomonas* spp. (**Table 1**) as templates.

Table 1. Genome sequences of 57 members of *Pseudomonas* spp. available from database used as templates of *in silico* PCR to evaluate specificity of GMF-GMR and newly designed JMF-JMR of primers for detection of *moaC*.

No.	NCBI Reference Sequence	Genome of Species	Author
1	NC_002516.2	*P. aeruginosa*	Winsor *et al*., 2018
2	CP004061.1)	*P. aeruginosa* B136-33	Lo *et al*., 2017
3	NC_018080.1	*P. aeruginosa* DK2	Rau *et al*., 2017
4	NC_002516.2	*P. aeruginosa* LES431	Jeukens *et al*., 2017
5	NC_011770.1	*P. aeruginosa* LESB58	Winstanley *et al*., 2017
6	NC_017548.1	*P. aeruginosa* M18	Wu *et al*., 2017
7	NC_023019.1	*P. aeruginosa* MTB-1	Ohtsubo *et al*., 2017
8	AP012280.1	*P. aeruginosa* NCGM2.S1	Miyoshi-Akiyama *et al*., 2017
9	NC_022808.2	*P. aeruginosa* PA1	Lu *et al*., 2017
10	NC_022806.1	*P. aeruginosa* PA1R	Le *et al*., 2017
11	NC_009656.1	*P. aeruginosa* PA7	Roy et al., 2018
12	NC_022594.1	*P. aeruginosa* PAO1-VE13	Yin *et al*., 2017
13	NC_022591.1	*P. aeruginosa* PAO1-VE2	Yin *et al*., 2017
14	NC_022361.1	*P. aeruginosa* PAO581	Yin *et al*., 2017
15	NC_021577.1	*P. aeruginosa* RP73	Jeukens *et al*., 2017
16	NC_023149.1	*P. aeruginosa* SCV20265	Eckweiler *et al*., 2017
17	NC_008463.1	*P. aeruginosa* UCBPP-PA14	Lee et al., 2017
18	NC_022360.1	*P. aeruginosa* c7447m	Yin *et al*., 2017
19	NC_015379.1	*P. brassicacearum* NFM421	Ortet *et al*., 2017
20	CP004143.1	*P. denitrificans* ATCC 13867	Ainala *et al*., 2014
21	NC_008027.1	*P. entomophila* L48	Vodovar *et al*., 2018
22	NC_017911.1	*P. fluorescens* A506	Loper *et al*., 2018
23	NC_016830.1	*P. fluorescens* F113	Redondo-Nieto *et al*., 2017
24	NC_004129.6	*P. fluorescens* Pf-5	Paulsen *et al*., 2017
25	NC_007492.2	*P. fluorescens* Pf0-1	Silby *et al.*, 2017
26	NC_012660.1	*P. fluorescens* SBW25	Silby *et al.*, 2017
27	CP002727.1	*P. fulva 12-X*	Lucas *et al.*, 2011
28	CP002620.1	*P. mendocina* NK-01	Guo *et al.*, 2014
29	NC_009439.1	*P. mendocina ymp*	Copeland *et al.*, 2017
30	NC_023075.1	*P. monteilii* SB3078	Dueholm *et al.*, 2017
31	NC_023076.1	*P. monteilii* SB3101	Dueholm *et al.*, 2017
32	NC_020209.1	*P. poae* RE*1-1-14	Mueller *et al.*, 2017
33	CP003190.1	*P. protegens* CHA0	Schuldes *et al.*, 2014
34	CP002290.1	*P. putida* BIRD-1	Matilla *et al.*, 2014
35	CP003734.1	*P. putida* DOT-T1E	Matilla *et al.*, 2014
36	CP000712.1	*P. putida* F1	Copeland *et al.*, 2017
37	CP000926.1	*P. putida* GB1	Copeland *et al.*, 2017
38	CP005976.1	*P. putida* H8234	Molina *et al.*, 2014
39	CP003738	*P. putida* H83267	Molina *et al.*, 2015
40	NC_002947.4	*P. putida* KT2440	Belda *et al.*, 2016
41	NC_021505.1	*P. putida* NBRC 14164	Ohji *et al.*, 2018
42	NC_017986.1	*P. putida* ND6	Li *et al.*, 2018
43	NC_015733.1	*P. putida* S16	Yu *et al.*, 2017
44	EU514690.1	*P. putida* UW4	Cheng *et al.*, 2010
45	NC_010501.1	*P. putida* W619	Copeland *et al.*, 2017
46	AP013068.1	*P. resinovorans* NBRC 106553	Shintani *et al.*, 2016
47	NC_023064.1	*Pseudomonas sp.* TKP	Ohtsubo *et al.*, 2017
48	NC_022738.1	*Pseudomonas sp.* VLB120	Volmer *et al.*, 2017
49	NC_009434.1	*P. stutzeri* A1501	Yan *et al.*, 2017
50	CP002881.1	*P. stutzeri* LMG 11199	Chen *et al.*, 2016
51	NC_018028.1	*P. stutzeri* CCUG 29243	Brunet-Galmes *et al.*, 2017
52	CP003725.1	*P. stutzeri* DSM 10701	Busquets *et al.*, 2015
53	CP002622.1	*P. stutzeri* DSM 4166	Yu *et al.*, 2015
54	CP003071.1	*P. stutzeri* RCH2	Lucas *et al.*, 2013
55	JPQU01000039.1	*P. syringae*	Baltrus *et al.*, 2014
56	CP000058.1	*P. syringae pv. phaseolicola* 1448A	Joardar *et al.*, 2017
57	NC_007005.1	*P. syringae pv. syringae* B728a	Feil *et al.*, 2016

All parameters set on *in silico* PCR using JMF-JMR primers could be seen in **Figure 3**.

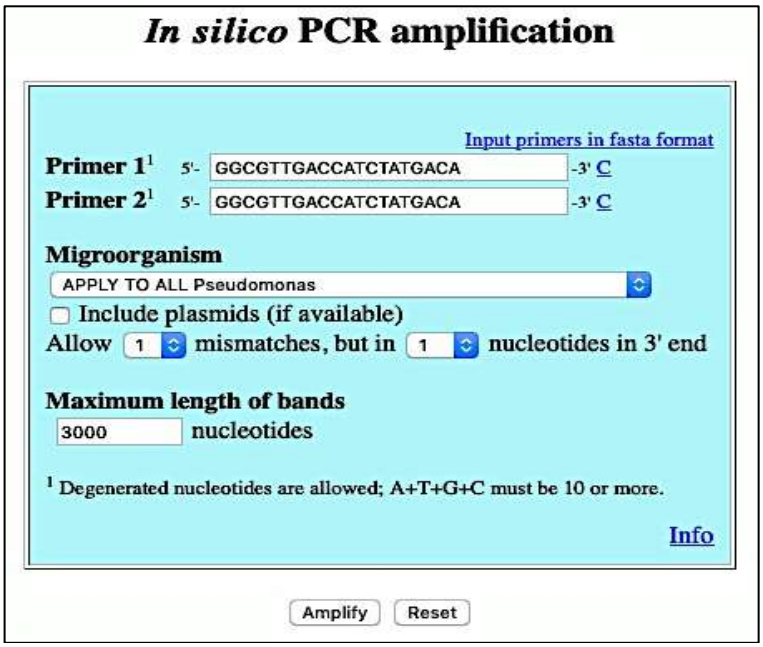

Figure 3. *In silico* PCR using newly designed primers, JMF (*forward*) and JMF (reverse) primers obtained from Primer3Plus primer design tool based on *moaC* sequence of *A. javaensis* JG3.

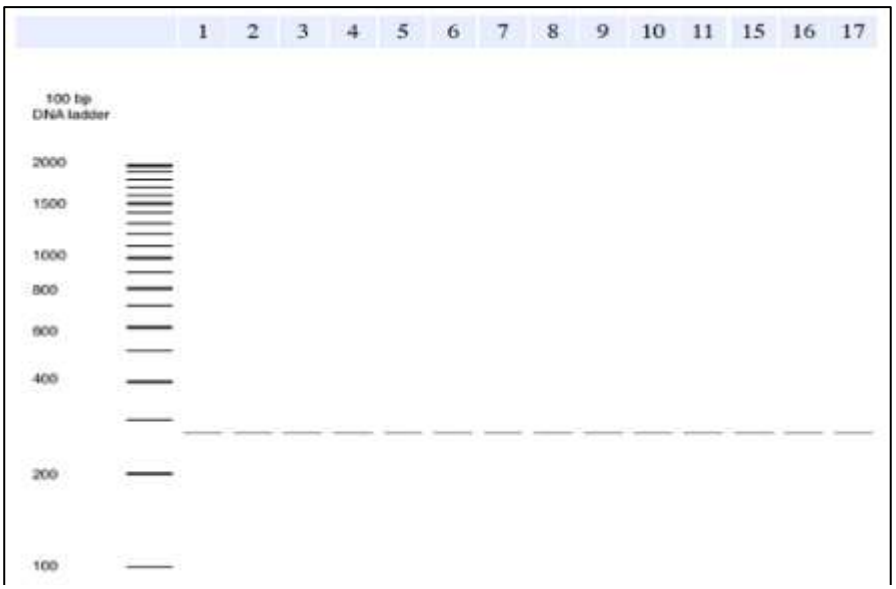

Figure 4. Products of *in silico* PCR amplification using a pair of GMF-GMR primers with genomic DNA sequences of 57 members *Pseudomonas* spp. as template, which were retrieved from *in silico* database (http://insilico.ehu.es/) (Bikandi, 2004). Single bands of 271-bp DNA matched to *glpK* gene fragments were obtained from 14 members of *Pseudomonas* spp., which consists of *Pseudomonas aeruginosa* strains represented by lanes: 1. *P. aeruginosa* sp., 2. *P. aeruginosa* B136-33, 3. *P. aeruginosa* DK2 4. *P. aeruginosa* LES431 5. *P. aeruginosa* LESB58 6. *P. aeruginosa* M18 7. *P. aeruginosa* MTB-1 8. *P. aeruginosa* NCGM2.S19. *P. aeruginosa* PA110. *P. aeruginosa* PA1R11. *P.*

aeruginosa PA7 15. *P. aeruginosa* RP73 16. *P. aeruginosa* SCV20265 17. *P. aeruginosa* UCBPP-PA14.

Meanwhile, results of *in silico* PCR using both GMR-GMF as well as JMF-JMR pairs of primers are shown in **Figure 4**and **Figure 5**, respectively. As seen in **Figure 4**, the amplified virtual PCR products are single bands sized ~271 bp. These results showed that GMF-GMR primers could selectively amplify ~271-bp *in silico PCR* products from 14 out of 57 genomes of members of *Pseudomonas* spp. tested. However, BLASTn analysis on these 14 amplified DNA sequences showed that all of them were not part of *moaC*, yet *glpK* gene fragment sequences. These results were not in line with conventional PCR using similar primers GMR-GMF with genomic DNA of *Alcaligenes javaensis* JG3 as template. As previously reported, DNA sequence of PCR product obtained from conventional PCR using GMF and GMR on DNA genome of strain JG3 resulted moaC gene fragment with assigned Genbank accession code of AB894422.1 [2]. Instead, the obtained *moaC* sequence, was then used to design new primers JMF-JMR reported in this study.

Meanwhile, the newly designed primers from *moaC* sequence of strain JG3, JMF-JMR, could specifically amplify 214-bp *in silico PCR* products from 2 out of 57 members of *Pseudomonas* spp. DNA sequences of these PCR products were matched to *moaC* gene fragment sequences belong to two strains of *P. stutzeri* species. Results of *in silico PCR* using JMF-JMR primers with 57 genome sequences of *Pseudomonas* spp. are shown in **Figure 5**.

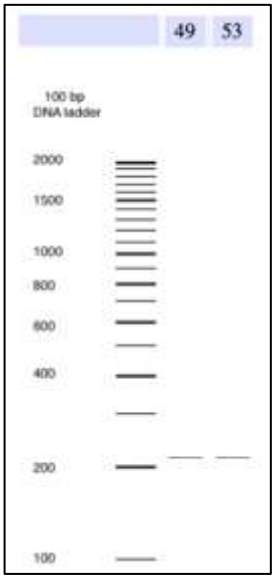

Figure 5. Products of *in silico* PCR amplification using a pair of GMF-GMR primers with genomic DNA sequences of 57 members *Pseudomonas* spp. as template, which were retrieved from *in silico* database (http://insilico.ehu.es/). Single bands of 214-bp DNA matched to *moaC* gene fragments were obtained from 2 members of *Pseudomonas* spp., which consists of *Pseudomonas stutzeri* strains represented by lanes: 49. *P. stutzeri* A1501 and 53. *P. stutzeri* DSM 4166.

As seen in **Figure 5**, lanes number 49 and 53 showed single bands sized ~214 bp. These lanes belong to *P. stutzeri* A1501 and *P. stutzeri* DSM 4166 genomic sequences, respectively. BLASTn analysis on these PCR products showed that both DNA sequences shared 100% similarity with *moaC* gene fragments of *P. stutzeri* A1501 **(Figure 6)** and *P. stutzeri* DSM 4166 (**Figure 7**), also with similar gene of *Alcaligenes javaensis* JG3 [3].

Conserved domains of both predicted protein sequences displayed in **Figure 6** and **Figure 7** have been reported in NCBI as cyclic pyranopterin monophosphate synthase (MoaC, Molybdenum Cofactor Biosynthesis Protein C). Based on information from protein data bank (PDB), MoaC is a

member of the MoaC superfamily cl00242, which hypothetical structure is displayed in **Figure 8**. Members of cI00242 superfamily are involved in molybdenum cofactor (Moco) biosynthesis, an essential cofactor of a diverse group of redox enzymes. MoaC, a small hexameric protein, converts, together with MoaA, a guanosine derivative to the precursor Z by inserting the carbon-8 of the purine between the 2' and 3' ribose carbon atoms initiating three phases of Moco biosynthesis [14-16].

Pseudomonas stutzeri A1501
>NC_009434, from 1284772 to 1284985 (214 bp); Pseudomonas stutzeri A1501
GGCGTTGACCATCTATGACATGTGCAAGGCCGTTGACCGCGGCATGGTGATCGAAGGGGTGCGCCTGC
TGGAGAAGCTGGGCGGCAAGAGTGGGCATTGGCAGGTGCAGGCATGATTTCGATTCAGTTCTTTGCCC
GTTATCGGGAAACCCTAGGCAGCGATGGCGAGCGCATCCAATGGGATGAATCGTTCGGCAGTGTGGAT
GATGTACGCC
Identical protein: Cyclic pyranopterin monophosphate synthase MoaC [Pseudomonas stutzeri] NCBI Reference Sequence: WP_011912355.1
>WP_011912355.1 cyclic pyranopterin monophosphate synthase MoaC [Pseudomonas stutzeri]
MLTHLDSLGRASMVDVTDKAVTAREAVAEARVRMLPQTLQLIQQGGHPKGDVFAVARIAGIQAAKKTH
ELIPLCHPLLLTSIKVELQADGEDSVLIRAVCKLAGQTGVEMEALTAASVAALTIYDMCKAVDRGMVI
EGVRLLEKLGGKSGHWQVQA

Figure 6. Predicted amplified DNA sequence on *Pseudomonas* spp. genome (lane 49) using JMF-JMR primers converted to protein sequence, which shared highest similarity with *moaC* of *Pseudomonas stutzeri* A1501.

Pseudomonas stutzeri DSM 4166
>NC_017532 from 1219396 to 1219609 (214 bp); *Pseudomonas stutzeri* DSM 4166
GGCGTTGACCATCTATGACATGTGCAAGGCCGTTGACCGCGGCATGGTGATCGAAGGGGTGCGCCTGC
TGGAGAAGCTGGGCGGCAAGAGTGGGCATTGGCAGGTGCAGGCATGATTTCGATTCAGTTCTTTGCCC
GTTATCGGGAAACCCTAGGCAGCGATGGCGAGCGCATCCAATGGGATGAATCGTTCGGCAGTGTGGAT
GATGTACGCC
Identical protein: Cyclic pyranopterin monophosphate synthase MoaC [Pseudomonas stutzeri] NCBI Reference Sequence: WP_014596023.1
>WP_014596023.1 cyclic pyranopterin monophosphate synthase MoaC [Pseudomonas stutzeri]
MLTHLDSQGRASMVDVTDKAVTAREAVAEARVRMLPQTLQLIQQGGHPKGDVFAVARIAGIQAAKKTH
ELIPLCHPLLLTSIKVELQADGEDSVLIRAVCKLAGQTGVEMEALTAASVAALTIYDMCKAVDRGMVI
EGVRLLEKLGGKSGHWQVQA

Figure 7. Predicted amplified DNA sequence on *Pseudomonas* spp. genome (lane 53) using JMF-JMR primers converted to protein sequence, which shared highest similarity with *moaC* of *Pseudomonas stutzeri* DSM 4166

Figure 8. Hypothetic structure of MoaC cI00242 superfamily which members include MoaC and MoaA proteins (Source: Protein Data Bank, PDB).

Boutros and Okey reported that the information in a primer pair is combined by an *in-silico* PCR to identify potential amplicons by both identity and size [17]. *In silico* PCR results allow the user to accept or reject potential primer pairs for experimental use. Based on this, the newly designed pair of primers JMF-JMR obtained in this study were theoretically accepted and were potential to proceed for *in vitro* PCR use to detect *moaC* of *P. stutzeri* from *Pseudomonas* spp. JMF-JMR showed theoretically potential uses to detect the occurrence of *P. stutzeri* contamination on foods based on its *moaC* genes amplified by these primers.

4. Conclusion

Based on *in silico* study, JMF-JMR primers are more specific than GMF-GMR ones for detecting *moaC* genes fragment of 57 members of *Pseudomonas* spp. studied, which include strains of species *P. brassicacearum*, *P. denitrificans, P. entomophila, P. fluorescens, P. fulva, P. mendocina, P. monteilii*, *P. pose*, *P. protegens*, *P. resinovorans*, and *P. syringae*. As conclusion, based on *in silico* study, JMF-JMR primers are more specific than GMF-GMR ones for detecting *moaC* genes fragment of members of *Pseudomonas* spp. studied.

5. Author Contribution Statement

Dr. SNE contributed in preparing and analyzing all research data reported. ARS contributed in preparing manuscript and summarizing research data, Dr. SD contributed in final editing the manuscript.

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