KMITL Sci. Tech. J. Vol. 11 No. 2 Jul. - Dec. 2011

The Genetic Characterization of *Pseudomonas* strains Isolated from Aquatic Animals Based on the 16S-23S rRNA Intergenic SpacerRegions

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Abstract

Pseudomonas species are being used as a biological control, probiotics and bioremediation tools. Nevertheless, phenotypic properties of pseudomonads are the most heterogeneous among the species. Consequently, a thorough characterization of this genus is needed for accurate identification and may lead to a better understanding of the diversity of this genus. Here, 16S-23S rRNA internal spacer regions (ITS1) of *Pseudomonas* isolated from various aquatic animals were analyzed. PCR amplification of ITS1 region of the studied isolates generated one, two or three bands ranging from 384 bp to 705 bp. The analysis of the DNA products revealed that two genes namely $tRNA^{Ile}$ and $tRNA^{Ala}$ were detected in all six studied strains. Phylogenetic analysis using the unweighted pair group method of clustering (UPGMA) revealed 3 phylogenetic clusters including *Pseudomonas aeruginosa, P.putida* and *P. fluorescens*. This report for the first time suggests that there was intercistronic heterogeneity of the ITS1 among the pseudomonad strains in aquaculture environment in Thailand.

Keywords: *Pseudomonas*, 16S-23S rRNA, ITS1, aquatic animal

1. Introduction

The genus *Pseudomonas* has been used in various activities such as biological control, probiotics, and bioremediation. For example, *Pseudomonas fluorescens* was utilized as biological control of soil-borne phytopathogens [1-2]; *P. fluorescens* was employed to reduce the mortality in rainbow trout during the vibriosis outbreak [3]. *Pseudomonas* was also used to promote the growth performance and health of Nile tilapia, *Oreochromis niloticus* [4]; *P. fluorescens* was exploited to degrade the Direct Orange-102 effluent from textile industries [5], whereas *P. aeruginosa* and *P. putida* were used to degrade oil [6-7]. On the other hand, some *Pseudomonas* strains are known as plant and animal pathogens [8]. Phenotypic properties of pseudomonads are well known as the most heterogeneous across the genus. Much research has been elaborated on the genome characterization to understand this genus [8-13]. This will facilitate the better utilization of pseudomonad in each purpose.

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The rRNA genes (16S, 23S and 5S) are ideal gene candidates for bacterial identification and evolutionary studies as they are highly conserved within the species [14]. The ITS1 regions located between the 16S and 23S rDNAs have been proven to be under less evolutionary pressure [15]. Thus, they are widely used in differentiation and identification of closely related bacteria [16]. Intercistronic heterogeneity of the ITS1 has been observed among *Pseudomonas* strains isolated from various sources including plants [9-11], hospitalized patients [17], foods [18]. However, there has been no report on the heterogeneity of ITS1 of *Pseudomonas* isolated from aquaculture. Here, we describe the use of 16S-23S rRNA ITS1 to demonstrate the heterogeneity of six *Pseudomonas* isolates found in aquaculture environment in Thailand.

2. Materials and Methods

2.1 Bacterial strains and culture conditions

Six bacteria isolated from different aquatic animals (Table 1) were grown on tryptic soy agar (TSA) (Oxoid) at 30°C for 18 h. They were previously characterized by API 20NE (Biomerieux) to identify the species as shown in Table 2.

2.2 Bacterial DNA isolation and amplification of spacer region

Genomic DNAs were extracted as described by Boom *et al.* [19]. The DNAs of each isolates were then amplified in a DNA thermal cycler (OmniGene, Hybaid Ltd., UK) as described by Jaturapahu *et al*. [20]. Briefly, a typical reaction mixture (50 μL) consisted of reaction buffer (50 mM KCl, 10 mM Tris-HCl, pH 9.0, 0.1% Triton X-100, 3.0 mM $MgCl_2$), 200 μM (each) deoxynucleotide triphosphate, 2 U *Taq* DNA polymerase (Promega), 5 ng DNA sample, and 10 pmol of each primer (P16sf and P23sr, previously described by Sawada *et al.* [15]. The reaction mixture was cycled 35 times as follows: 1 min denaturation at 94°C, 1 min annealing at 52°C and 1 min 30 sec extension at 72°C. The vials were held at 4°C until the PCR product was detected by 1.5% agarose gel electrophoresis.

2.3 Cloning and sequencing

The PCR products were purified with phenol-chloroform. After precipitating with ethanol, the DNA pellet was dissolved in 50 μL TE buffer. The fragment was ligated into pGEM T-Easy vector (Promega), and then the recombinant plasmid was transformed into *Escherichia coli*. Plasmid was extracted from positive transformants by the alkaline lysis method [21]. Inserts were amplified with M13 primers using a Taq DyeDeoxy Terminator Cycle Sequencing Kit (Perkin-Elmer, Norwalk, CT). Finally, the products were sequenced by the ABI Prism 377 automatic sequencer (Applied Biosystems).

2.4 Sequence analysis

The ITS1 sequences with approximately equal lengths of the six *Pseudomonas* isolates were aligned with CLUSTAL W program [22]. A phylogenetic tree was constructed by the unweighted pair group method of clustering (UPGMA) using program MEGA version 4.0 [23] with a bootstrap of 1,000 samplings. Gaps were eliminated from the calculations. The ITS1 regions were analyzed by transfer RNAs (tRNAs) using tRNAscan-SE version 1.21 [24].

Table 1 Characterization of ITS1 in six *Pseudomonas* strains isolated from different aquatic animals.

Isolate	Strain	Host	No. of fragment	Fragment No.	PCR product size (bp)	ITS1 length (bp)	tRNA
AAHRI 01031	*P. fluorescens*	Guppy	1	1	579	476	$tRNA^{Ile}$, $tRNA^{Ala}$
AAHRI 01213	*P. putida*	Oscar	3	1	705	602	$tRNA^{Ile}$, $tRNA^{Ala}$
				2	614	511	$tRNA^{Ile}$, $tRNA^{Ala}$
				3	384	281	no
AAHRI 01342	*P. fluorescens*	Guppy	2	1	610	507	$tRNA^{Ile}$, $tRNA^{Ala}$
				2	420	317	no
AAHRI 01419	*P. fluorescens*	Flame gourami	1	1	611	508	$tRNA^{Ile}$, $tRNA^{Ala}$
AAHRI 02007	*P. aeruginosa*	Striped catfish	1	1	600	497	$tRNA^{Ile}$, $tRNA^{Ala}$
AAHRI 03418	*P. fluorescens*	Gold fish	2	1	608	505	$tRNA^{Ile}$, $tRNA^{Ala}$
				2	420	317	no

AAHRI: Aquatic Animal Health Research Institute

3. Results and Discussion

3.1 PCR amplification of ITS1

PCR amplification of genomic DNA derived ITS1 region of the six *Pseudomonas* strains showed different bands ranging from 384 bp to 705 bp (Table 1). The fragments consisted of the complete ITS1 sequences flanked upstream by 53 bp of 16S rRNA and downstream by 50 bp of 23S rRNA. The presence of multiple bands in PCRs suggested the presence of multiple rRNA operons (Figure 1).

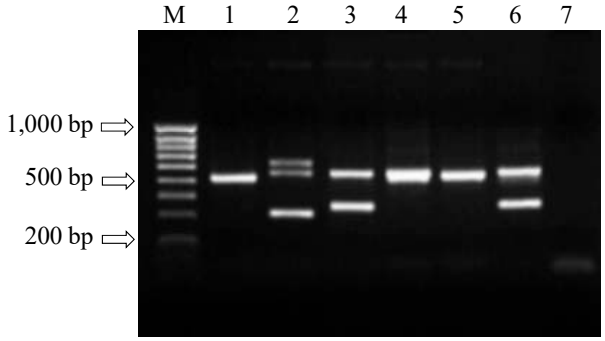

Figure 1 The PCR products of six *Pseudomonas* strains isolated from aquatic animals. Lanes: (1) AAHRI 01031, (2) AAHRI 01213, (3) AAHRI 01342, (4) AAHRI 01419, (5) AAHRI 02007, (6) AAHRI 03418. Lane M was DNA marker (100 bp ladder, BIOLINE). Lane 7 was negative control (dH_2O)

3.2 Comparison of ITS1 sequences and phylogeny

ITS regions from the six isolates were sequenced and then were generated from the PCR amplicons. The complete ITS1 nucleotide sequences were edited to eliminate the 16S and 23S portions for further analysis. The size of the 16S-23S ITS1 ranged from 281 to 602 bp. The analysis of the DNA sequences revealed that there were two genes, including $tRNA^{Ile}$ and $tRNA^{Ala}$ presenting in all six *Pseudomonas* strains, in the order 16S rRNA-$tRNA^{Ile}$-$tRNA^{Ala}$-23S rRNA.

The tRNA sequence for $tRNA^{Ile}$ and $tRNA^{Ala}$, which are highly conserved, and major areas among a sequence of tRNA genes are variable. The variable region followed the $tRNA^{Ala}$ gene and ended with antiterminator box B stem-loop structure equivalent to box B of *E. coli* [25]. The study area was conducted as a report of Milyutina *et al*. [11]. The conserved region following box B contained a block of nucleotides highly homologous to the sequence of the antiterminator box A of other bacteria [26] (Figure 2). In additional, isolates AAHRI 01213, AAHRI 01342 and AAHRI 03418 generated a smaller ITS1 281 bp, 317 bp and 317 bp respectively without tRNA features was detected confer the report Tamboung *et al*. [9]. These multiple non-identical rRNA operons may have an impact on studies in molecular systematic and population genetics in cyanobacteria [27]. ITS1 operon variability in the genomes of the *Pseudomonas* strains reported here confirms that may vary due to recombination events and/or horizontal transfers [9, 11].

The aligned ITS1 sequences were used to generate a phylogenetic relationship of the six *Pseudomonas* strains, three reference *Pseudomonas* sequences (including *P. aeroginosa* ATCC 27853, *P. putida* DMST 10603 and *P. fluorescens* TISTR 358 previous studied from Jaturapahu *et al*. [20]), and two sequences obtained from the GenBank database (*P. aeruginosa* accession number FM209186 and *P. fluorescens* accession number AM181176) were entries. The results showed three distinct clusters. By phylogenetic analysis, the use of UPGMA clustering revealed these three clusters were *P. aeruginosa, P. putida* and *P. fluorescens* (Figure 3). This result is consistent with that obtained from the biochemical test (Table 2).

In conclusion, the heterogeneity of the pseudomonad ITS1 conducted from aquatic environment was determined by sequence variants of variable region. However, the identical multiple rRNA operons are interesting for further analysis to understand the recombination and horizontal transfer.

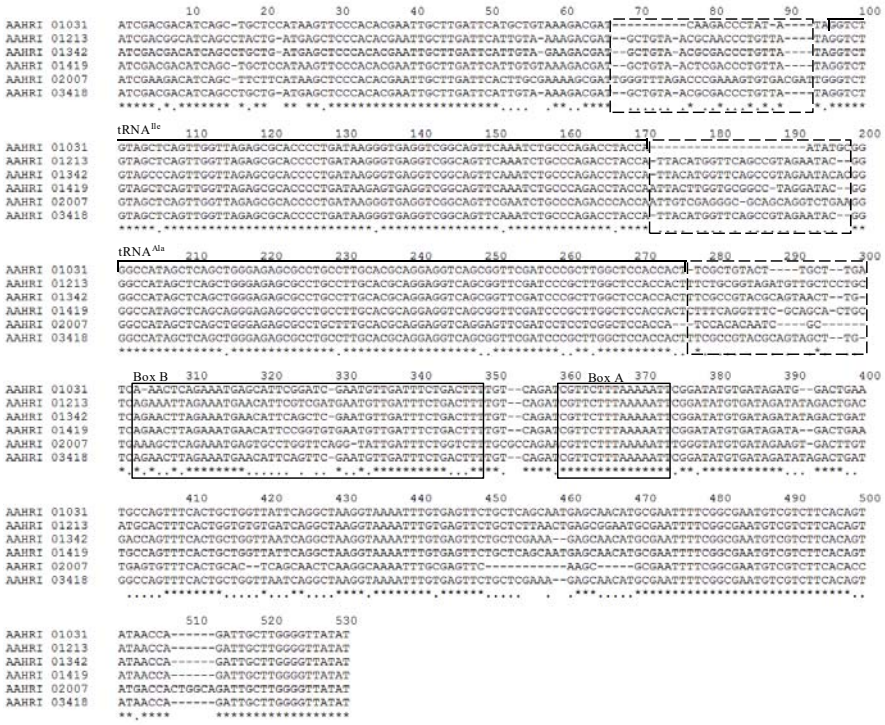

Figure 2 Total sequence alignments of the 16S-23S rRNA intergenic spacer regions of six *Pseudomonas* strains isolated from various aquatic animal. The highly variable region between positions 67-93, 171-198 and 277-300 are enclosed in dash boxes as well as box A and B and position of tRNA genes are indicated.

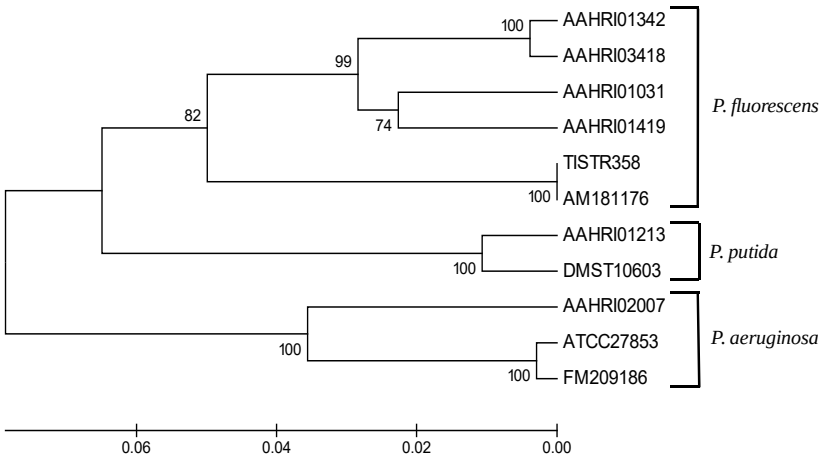

Figure 3 Phylogenetic tree based on the 16S-23S rRNA intergenic spacer regions of six *Pseudomonas* strains was inferred using the UPGMA clustering. (ATCC: American Type Culture Collection, DMST: Department of Medical Sciences Thailand, TISTR: Thailand Institute of Scientific and Technological Research, AAHRI: Aquatic Animal Health Research Institute).

Table 2 Phenotypes of six *Pseudomonas* strains (AAHRI 01031, AAHRI 01213, AAHRI 01342, AAHRI 01419, AAHRI 02007 and AAHRI 03418) isolated in this study and reference strains (ATCC 27853, DMST 10603 and TISTR 358) using the API 20NE biochemical test kit.

Characteristic	**AAHRI 01031**	**AAHRI 01213**	**AAHRI 01342**	**AAHRI 01419**	**AAHRI 02007**	**AAHRI 03418**	**ATCC 27853**	**DMST 10603**	**TISTR 358**
Gram strain	neagtive	neagtive	neagtive	neagtive	neagtive	neagtive	neagtive	neagtive	neagtive
Reduction of nitrates to nitrites	-	-	-	-	-	-	-	-	-
Reduction of nitrites to nitrogen	-	-	-	-	+	-	+	-	-
Indole production	-	-	-	-	-	-	-	-	-
Fermentation (Glucose)	-	-	-	-	-	-	-	-	-
Arginine dihydrolase	+	+	+	+	+	+	+	+	+
Urease	+	-	-	+	+	-	+	-	-
β-glucosidase hydrolysis	-	-	-	-	-	-	-	-	-
Protease hydrolysis	+	-	+	+	+	-	+	-	-
Para-nitrophenyl-βD-galactopyranosidase	-	-	-	-	-	-	-	-	-
Assimilation (glucose)	+	+	+	+	+	+	+	+	+
Assimilation (arabinose)	-	+	+	-	-	+	+	+	-
Assimilation (mannose)	+	+	-	+	-	+	-	+	+
Assimilation (mannitol)	+	-	+	+	+	+	+	-	+
Assimilation (N-acetyl-glucosamine)	-	-	+	+	+	+	+	-	+
Assimilation (maltose)	-	-	+	-	-	+	+	-	-
Assimilation (potassium gluconate)	+	+	+	+	+	+	+	+	+
Assimilation (capric acid)	+	+	+	+	+	+	+	+	+
Assimilation (adipic acid)	-	-	-	-	+	-	+	-	+
Assimilation (malate)	+	+	+	+	+	+	+	+	+
Assimilation (trisodium citrate)	-	+	+	+	+	+	+	+	+
Assimilation (phenylacetic acid)	-	-	+	+	-	+	-	-	-
Oxidase	+	+	+	+	+	+	+	+	+
Strain	*P. fluorescens*	*P. putida*	*P. fluorescens*	*P. fluorescens*	*P. aeruginosa*	*P. fluorescens*	*P. aeruginosa*	*P. putida*	*P. fluorescens*

Abbreviations: + = reaction occur, - = no reaction

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