Journal of Agricultural Science and Technology B 5 (2015) 292-296
doi: 10.17265/2161-6264/2015.04.008

Phenotypic and Genotypic Comparison of *Pseudomonas stutzeri* in Freshwater Fish in Indonesia

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Abstract: *Pseudomonas stutzeri* caused an outbreak of freshwater fish in Luwuk Banggai (tilapia and catfish), Bali (tilapia), Jambi (tilapia and catfish) and Tanjung Pinang (catfish). The study was purposed to comprehensively identify special phenotypic and genotypic characteristics of *P. stutzeri* isolated from several areas in Indonesia, including its morphometric and biochemical characteristics and molecular variation. Bacteria were isolated from internal organs (kidney, ulcer and eye) of fish. They were then identified using morphology and biochemical test. DNA isolates were entirely extracted, amplified and reversed on 16S rRNA region, and further then were sequenced. Phylogenetic trees of bacteria were constructed using neighbor-joining and maximum-parsimony methods. The colony were similar, such as rod shape (Jambi, Tanjung Pinang, Bali), bacil shape (Luwuk Banggai), transparant in tryptic soy agar (TSA) (Luwuk Banggai), creamy beige in glutamate starch phenol red (GSP) (Bali), gram negative, motile, no reaction in the oxidative-fermentative test, positive result in catalase and oxidase test, negative in lysine decarboxylase and ornithine decarboxylase test and positive result in indole test; gelatin was degraded (only Bali), urea was not degraded, no color change in Methyl-red and Voges-proskaeur (MR-VP) test; acid not produce from glucose, inositol or sucrose. Citrate was utilized by some isolates: positive (Jambi, Tanjung Pinang) and negative (Bali, Luwuk Banggai). Results showed us that isolates of Jambi, Bali and Tanjung Pinang were monophyletic species with *P. stutzeri* S8 and ZH-1 comparing to gen bank. However, merely phenotypic analysis among *Pseudomonas* sp. was confused compared to each other.

Key words: *P. stutzeri*, genotype, phenotype.

1. Introduction

Aquaculture industry in Indonesia was developing rapidly to export and fulfill internal consumption. *Pseudomonas stutzeri* was a non-fluorescent denitrifying bacterium, widely distributed in the environment and also been isolated as an opportunistic pathogen from humans. The species was received much attention because of its particular metabolic properties: (1) it was proposed as a model organism for denitrification studies; (2) many strains have natural transformation properties, which made it relevant for study of the transfer of genes in the environment; (3) several strains were able to fix dinitrogen; (4) others were participated in the degradation of pollutants or interaction with toxic metals [1].

Since 1956, the pathogenicity of *P. stutzeri* was reported to occur in human. However, there was no clear association of this species with an infectious process. In fact, 15 of 17 strains studied in 1966 [2] were of clinical origin. In 1973, the first well documented case of *P. stutzeri* infection appeared in the literature. It involved a non union fracture of a tibia [3]. Another bone infection involved fracture infection, joint infection, osteomyelitis, arthritis [3], and pneumonia and/or empyema [4].

In fish and/or aquatic life, the infection of *P. stutzeri* was rarely reported. However, *P. stutzeri* was isolated from marine turtle (*Caretta caretta*) in South Carolina, USA, during a study of patterns of antibiotic resistance in bacteria isolated from marine turtles by Pasquino [5]. In 2009, *P. stutzeri* was isolated in wastewater from catfish ponds in Delta Mekong River,

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Vietnam [6]. Meanwhile, clinical sign of *P. stutzeri* infection in fish was reported in the pearl spot (*Etroplus suratensis*), which commonly known as "Karimeen" in Kerala as an indigenous fish extensively found along the East and Southwest coasts of Peninsular India. The infection of *P. stutzeri* in *E. suratensis* was called as a fin rot disease associated with loss of natural colour, fraying of tail/fin tissue, swimming near water surface and ecchymosis [7]. *P. stutzeri* was first reported from freshwater fish in Indonesia, so it will be supported by molecular analysis.

The study was aimed to comprehensively identify special phenotypic and genotypic characteristics of *P. stutzeri* isolated from several areas in Indonesia, including its morphometric and biochemical characteristics and molecular variation.

2. Materials and Methods

2.1 Isolation and Identification

P. stutzeri was isolated from naturally infected fish, from different localities in Indonesia (tilapia from Bali; tilapia and catfish from Jambi and Luwuk Banggai; catfish from Tanjung Pinang). The clinical signs were darkness and petechiae of the skin, petechial hemorrhage on the skin and detached scales. Some fish showed slight abdominal distension and exophthalmia. Bacteria were isolated from internal organs (kidney, ulcer and eye) of fish. Identification was based on morphology and biochemical tests according to Austin, B. and Austin, D. A. [8].

2.2 Molecular Analysis

The extraction of DNA used Qiagen DNA isolation kit, then was amplified with universal primers PAR: 5'-ATGCAGCACCTGTGTCTGAG-3' and PAF: 5'-GGACGGGTCAGTAATGCCTA-3'. Purification and sequencing of DNA were conducted by genetic science corp. A similarity search with 16S rRNA sequence was performed with 16S rRNA sequences available in the Gen Bank/EMBL/DDBJ databases, using the basic local alignment search tool (BLAST) algorithm [9]. The sequence results were aligned with CLUSTAL W multiple sequence alignment program version 1.8 [10]. The genetic distances matrix was obtained using Kimura's two parameter model [11] and an evolutionary tree was created using the neighbor-joining and maximum-parsimony method with MEGA 4.1 [12].

3. Result and Discussion

3.1 Bacterial Identification

In pure culture, the isolates exhibited slow growth on tryptic soy agar (TSA), glutamate starch phenol red (GSP) and blood agar plates (BAP) producing tiny translucent colonies. The colony were similar, such as rod shape (Jambi, Tanjung Pinang, Bali), bacil shape (Luwuk Banggai), convex shape, colored white in BAP (Jambi) and TSA (Tanjung Pinang), transparant in TSA (Luwuk Banggai), creamy (beige) in GSP (Bali). Bacteria were gram-negative, motile, no reaction in the oxidative-fermentative test; catalase and oxidase were produced; indole test was positive but negative was lysine decarboxylase and ornithine decarboxylase, respectively; gelatin are degraded (only Bali); urea was not degraded; Methyl-red and Voges-proskauer (MR-VP) was negative; acid was not produce from glucose, inositol or sucrose; citrate was utilized by some isolates: positive (Jambi, Tanjung Pinang) and negative (Bali, Luwuk Banggai). The result of phenotypic analysis was described in Table 1.

3.2 Molecular Results

The polymerase chain reaction (PCR) results of isolates from Jambi, Tanjung Pinang and Bali in 16S rRNA showed 1,200 bp of bands (Fig. 1).

The DNA sequences of isolates from Jambi, Bali and Tanjung Pinang were confirmed within the world gen data base (BLAST), they were more closely related to *P. Stutzeri* than *P. anguilliseptica* (Table 2).

Table 1 Morphometric and biochemical characters of *P. stutzeri* isolated from Jambi, Luwuk Banggai, Tanjung Pinang and Bali.

Characters test	Character based on Austin, B. and Austin, D. A. [8]	Jambi	Luwuk Banggai	Tanjung Pinang	Bali
Morphology					
Shape of colony	Bacil	Rod	Bacil	Rod	Rod
Edge shape		Convex	Convex	Convex	Convex
Colour		White	Transparant	White	Cream (beige)
Media		BAP	TSA	TSA	GSP
Morphology cell		Haemolysis			
Gram	Gram negative	Gram negative	Gram negative	Gram negative	Gram negative
Biochemistry					
Oxidative/fermentative	Negative	-	-	-	-
Motility	Positive	+	+	+	+
Catalase	Positive	+	+	+	+
Oxydase		+	+	+	+
Lysine decarboxylase		-	-	-	-
Ornithin decarboxylase		-	-	-	-
TSIA		K/K	A/K	K/K	A/K
Indole	Negative	-	-	-	-
Metyl-red		-	-	-	-
Voges-proskaeur		-	-	-	-
Simon citrate	Positive/negative	+	-	+	-
Gelatin	Positive	-	-	-	+
Urea	Negative	-	-	-	-
Myo-inositol		-			
Sorbitol		-		-	-
Glucose	Negative	-	-	-	-
Sukrose	Negative	-	-	-	-
Lactose	Negative	-	-	-	-

+: positive test result; -: negative test result; TSIA: triple sugar iron agar; K/K: non glucose-sucrose-lactose fermentation; A/K: glucose fermentation and non lactose-sucrose fermentation.

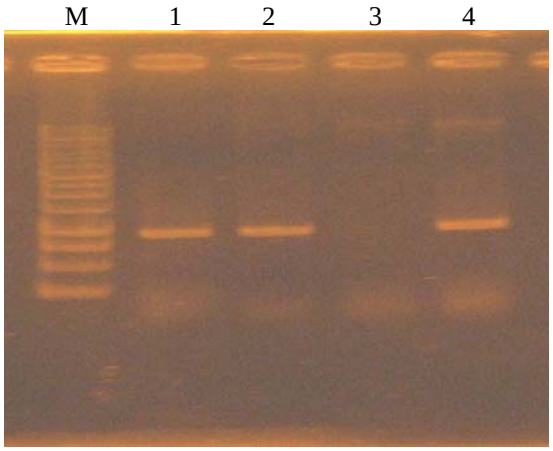

Fig. 1 PCR product of control (M) and isolates from Jambi (1), Tanjung Pinang (2), Luwuk Banggai (3) and Bali (4), showing molecular weight of 1,200 bp on 2% agarose gel.

M = marker 1 kb; lanes 1-4 = *P. stutzeri* isolates obtained from several areas in Indonesia.

Phylogenetic tree by neighbour-joining showed that the isolates from Jambi, Bali and Tanjung Pinang were 99% closely related to *P. stutzeri* S8 and ZH-1, and different from *P. anguilliseptica* groups with sea bream, sea bream 2, Europe, China, Baltic sea and strain M-S-TSA 17 based on neighbor-joining method (Fig. 2). This results suggest that isolates from Jambi, Bali and Tanjung Pinang are monophyletic species with *P. stutzeri* S8 and ZH-1.

Those results were supported by phylogenetic tree that was analyzed using maximum-parsimony method (Fig. 3). Phylogenetic tree showed that each component of the species in every clade is nearly similar. The result however specifically can not to be exactly similar. The neighbour-joining method is characterized by several advantages: analyzing the

Table 2 Molecular characteristics of isolates and their homology to gen bank references.

No.	Isolates	Strain	Homology	Strain	Homology
1	PJ_Jambi	*P. anguilliseptica*	97%	*P. stutzeri*	99%
2	PB_Bali	*P. anguilliseptica*	97%	*P. stutzeri*	99%
3	PT_Tj. Pinang	*P. anguilliseptica*	97%	*P. stutzeri*	99%

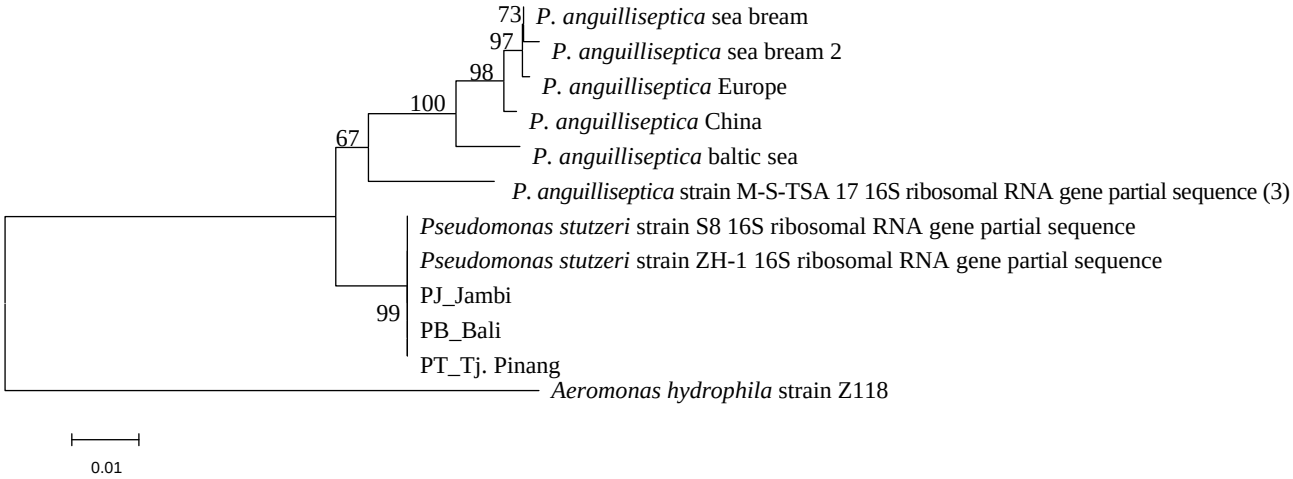

Fig. 2 Phylogenetic tree of *P. stutzeri* (PJ_Jambi, PB_Bali and PT_Tj. Pinang) using neighbor-joining method.

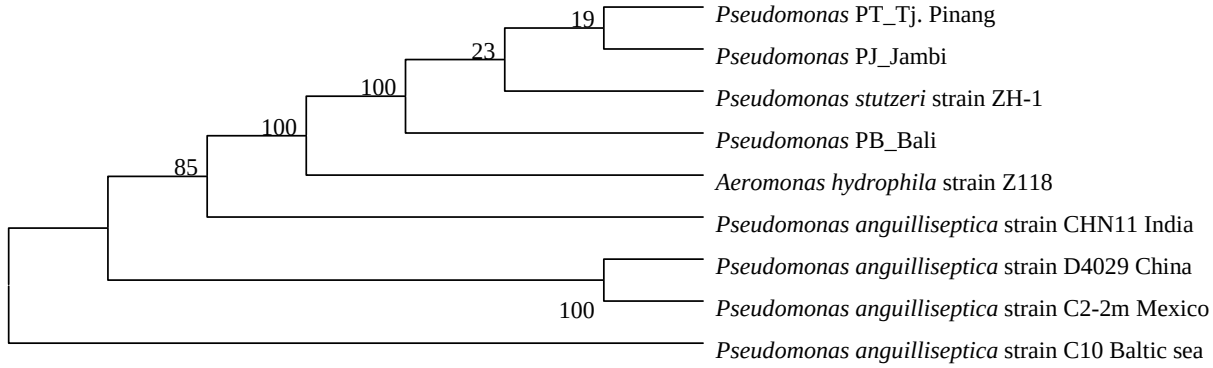

Fig. 3 Phylogenetic tree of *P. stutzeri* (PJ_Jambi, PB_Bali and PT_Tj. Pinang) using maximum-parsimony method.

phylogeny is extremely rapid, cluster analysis is very simple, it often is the fastest approach to tree construction and feasible to handle very large data sets/numbers of sequences. In the other hand, maximum-parsimony methods trees are created to minimize the number of changes needed to explain the data [13]. In many cases, both methods returned to the same or different results, while neighbor-joining was more quick and detail. On the other hand, maximum-parsimony may be considered as the most accurate method when dealing with low divergence level [14]. Instead of maximum-parsimony method, the 16S rRNA gene segments were accurately grouped to all the five taxa using neighbor-joining. It is suggested the possible superiority of the former method for phylogenetic analysis using similar data structure. The maximum-parsimony method appears to be a more reliable method for phylogenetic inference among the five taxa using 16S rRNA gene sequences [15]. Jin and Nei [16] studied the effect of various types of substitution rate variation among different nucleotide sites on *Pc* values (the probability of obtaining the correct tree) for the neighbor-joining and maximum-parsimony methods, the results showed that this effect was important only when *U* (half the expected distance/number of nucleotide substitutions

between two most distant DNA sequences) is large. Jin and Nei [16] also showed that nucleotide substitution patterns different from those of one-parameter and two-parameter models do not seriously affect the *Pc* values unless *U* is large.

4. Conclusions

The phenotypic analysis of *P. stutzeri* was confused and took a longer time. The identification of *P. stutzeri* using molecular analysis provided more accurate results when compared with the phenotypic analysis. Genotypic analysis of bacteria was relatively stable.

Isolates of Jambi, Bali and Tanjung Pinang were a monophyletic species with *P. stutzeri.*

Acknowledgments

The authors would like to thank to Project of Penelitian Unggulan Perguruan Tinggi-Lembaga Penelitian dan Pengabdian Kepada Masyarakat (PUPT-LPPM) (College Priority Research-Institute of Research and Society Empowerement), Faculty of Veterinary Medicine, Gadjah Mada University that has supported the completion of this research.

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