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Pseudomonas alcaligenes, Potential Antagonist Against *Fusarium oxysporum* f.sp.*lycopersicum* the Cause of *Fusarium* Wilt Disease on Tomato

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Abstract
Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici* is one of important diseases that potentially cause significant yield losses on tomato. There is no measure available to control the disease effectively. This study was done to isolate the antagonistic *Pseudomonas* sp. from rhizospeheres of plants of Leguminosae and Solanaceae and to determine their antagonistic activity against *F.oxysporum* f.sp.*lycopersicum*. Biochemical test and molecular analysis of the 16S rRNA gene were done to determine the species of *Pseudomonas* sp. There are three isolates of *Pseudomonas* sp. were obtained in this study namely isolates KtS1, TrN2, and TmA1. Results of 16S rRNA gene analysis showed that all three isolates had more than 99% similarity to *P. alcaligenes*. These isolates showed strong antagonistic activity against *F. oxysporum* f.sp. *lycopersici* with inhibitory activity more than 80%. Application of bacterial suspension of *P. alcaligenes* effectively suppressed the disease incidence of *Fusarium* wilt on tomato.
Keywords : antagonistic activity, *Pseudomonas alcaligenes*, *Fusarium* wilt disease.

1. Introduction

Fusarium wilt disease on tomato caused by *Fusarium oxysporum* f.sp. *lycopersici*, is an important disease on tomato plants and has been known to cause significant losses in various tomato-producing areas of the world (Wibowo, 2005). The outbreak of the disease in Indonesia occurred in 1970's, which had caused substantial losses. The incidence of *Fusarium* wilt disease on tomato in Lembang, West Java was 16.7% , while in Malang, East Java was 10.25% (Semangun, 2007). Survey on the disease incidence done in early February 2011 at three tomato's main growing areas in Bali, namely Kembang Merta, Perean, and Baturiti showed that *Fusarium* wilt disease occurred in all areas with disease incidence varied from 10 to 15%.

Various efforts have been done to control the *Fusarium* wilt disease, including the use of healthy seeds, crop rotation, intercropping and fungicides, but none gave satisfactory result. The use of bio-agents was reported quite effective to control *Fusarium* wilt disease on tomato (Freeman *et al*., 2002). Biocontrol organisms such as *F. oxysporum*, *Trichoderma hamatum*, *Gliocladium virens*, *Pseudomonas fluorescens*, and *Bacillus cepacia*, significantly reduced *Fusarium* wilt disease of tomato in greenhouse tests, with a reduction in disease incidence ranged from 35 to 100% (Larkin and Fravel, 1998). The results of a study done by Romero *et al*. (2004) showed that treatement with Bacillus pumilus strain SE34 and B. subtilis strain GBO3 suppressed the powdery mildew on pumpkins by 50% and 43% respectively when compared to control. Rhizobacteria such as *Pseudomonas* spp. could serve as biofertilizer, biological control agents to plant pathogens and may induce plant resistance against pathogens (McMilan, 2007). Rhizobakteria may be act directly as biofertilizer, and biostimulants through production of plant growth hormones such as indole acetic acid, gibberelin, cytokinin, ethylene, dissolved minerals, and also indirectly prevents the development of pathogenic microorganisms through siderofore, and antibiotics production (McMilan, 2007; Sarma *et al*., 2009).

Several researchers proved that treatments with *Pseudomonas* spp. could improve the plant growth of wheat and protect them from infection of *Phytium* spp. through seed treatment (Weller and Cook, 1986); could protect peanut plants from wilting and root pathogens *Sclerotium rolfsii* (Ganesan and Gnanamanickam, 1986) and could protect peas from the pathogen *F. oxysporum* f.sp. *fission* and *Phytium uLtimun* (Benhamou *et al*., 1996), could induce cucumber plant resistance to pathogens *Colletotrichum orbiculare* through seed treatment on root (Wei *et al*., 1991)

This study was conducted to isolate, characterize and determine the potential use of *P. alcaligenes* isolated from rhizozspheres of plants of Leguminoseae and Solanaceae to control *Fusarium* wilt disease on tomato.

2. Materials and Methods

2.1 Isolation of Pseudomonas spp.

Soil samples were taken from rhizospheres of plants of the families Solanaceae and Leguminoseae from several areas in Bali. Ten gram of soil sample was dissolved in 90 ml of sterile water and shaken in a mechanical shaker for 30 minutes. Each sample was serially diluted from 10^{-2} to 10^{-6}. One milliliter of the suspension was poured into 10 ml Kings'B medium, and incubated for 48 h at the room temperature. The emerged bacterial colonies were purified again in the same medium and the colonies that show the bright green or bluish-green color under ultra violet light (λ = 265 nm) were selected for further test.

2.2 Biochemical properties test

Biochemical tests such as TSIA (Triple Sugar Iron order) test, Voges-Proskauer test, Methyl Red test, Indole test were done. Several biochemical properties of *Pseudomonas* spp. were tested in this study such as Gram staining, oxidase, catalase, TSIA test, oksidase test, fermentation test, sulfide indol motility, metyl red, Voges Proskuer, growth on King,s A and King'S B.

2.3 DNA extraction

Bacterial isolates were grown in 100 ml of Luria-Bertani liquid medium in 250 ml Erlenmeyer then shaken on the orbital shaker at a speed of 2000 for 24 h at room temperature. Bacterial cultures were harvested by centrifugation, and the supernatants were discarded. Pellets were suspended in 480 l of 5% EDTA solution, then added with 120 l lysozyme. After incubation at 37^{o}C for 30-60 min, the cell suspension was centrifuged at a speed of 8,000 for 30 min at room temperature. Supernatant was discarded, and the residue was suspended in 350 l buffer solution with the aid of a vortex. The suspension was incubated in a water bath temperature of 65^{o}C for 10 min, and added with 150 l of buffer solution B and 500 ml of chloroform, then was mixed on a vortex. The mixture was centrifuged at 12,000 rpm for 10 min at room temperature.

Supernatant was transferred into 1.5 ml Eppendorf tube, then was added with 1 ml cooled ethanol, then incubated in an ice bath for 30 min. After centrifugation at 12,000 rpm for 10 min at room temperature, the supernatant was discarded. Tube were reversed and drained on tissue papers. The precipitates were added with 500 l cooled 70% ethanol, then incubated in an ice bath for 30 minutes.

The RNA molecules were removed by adding 4 l/ml RNAse and incubated at 37^{o}C for 30 min. After centrifugation at 12,000 rpm for 2 min at room temperature, the supernatant was discarded. Sediment in the tube was dried up in a vacuum oven at temperature 50^{o}C for 30 min. DNA pellets were suspended in 100 l TE buffer and ready for further use.

2.4 PCR amplification

The DNA was amplified by 16 S rRNA gene using two primers *i.e.* 63F (5'-GGT GGT GGA TTC ACA GTM TAY CAR GCW ACA GC-3 ') and 1387R (5'-TTC ATT GCR GGR TAG TAG TTW TT-3 ') which would amplify 1300 base pairs (bp) (Luton *et al*., 2002). The reactions was performed in PCR machine **GeneAmp® PCR System 2700** (Applied Biosystems, Foster City,California, USA) under the following conditions: pre-denaturation: 94^{0}C (2 min 2 sec) followed by 30 cycles of denaturation 94^{0}C (15 sec), annealing: 50 ^{0}C (30 sec.), extension: 72 ^{0}C (1 min 30 sec), and final extension 72^{0}C (10 min.). PCR reaction mixture: Pure taq using Ready To Go PCR Beads, Primary 16sV-F (50 pmol / l): 1 l, Primary 16sV-R (50 pmol / l): 1 l, Geno: 1 l, ddH2O: 22 l, final volume: 25 l. For 25 l vol. of reaction mixture contains: 2.5 units of pure taq DNA polymerase, 200 M dNTPs in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl and 1.5 mM MgCl2.

Agarose gel prepared with a concentration of 1%, and then inserted into the electrophoresis apparatus. Each 2 ml PCR results and DNA markers (1 kb) were added by 10 l loading buffer and electrophoresis was performed for 30 minutes. Electrophoresis results were observed in the mini transiluminator at a wavelength of 260 nm.

2.5 16S rRNA gene analysis and phylogeny analysis

Pure DNA was sequenced to determine the sequence of DNA bases. Then, the results of the data sequence was aligned with GenBank using BLAST-N program (Basic Local Alignment Search Tool-nucleotide) from the NCBI website (National Center for Biotechnology Information) to determine the similarity of the species of the tested isolates. Sequencing was performed using Genetic Analyzer machine brand Applied Biosystems ABI PRISM 310 (Applied Biosystems, Foster City, California – USA).

Phylogeny analysis was performed using the MEGA 4.0[11], Neighbor Joining method (NJ) with a bootstrap 1000x. The steps were as follows: (1) The connection sequence with the BLAST program at www.ncbi.nlm.nih.gov.site (2) Finding similarity between sequences. Sequences of data were stored on the notepad in FASTA format, analyzed using Blast-WU facility that was available online in the website www.ebi.ac.uk/ClustalW. Based on the analysis of the similarity, a new species might be found if the degree of base sequence similarity of the encoded genes 16S rRNA is less than 97% (Pangastuti, 2006). (3) Phylogeny tree was made with the MEGA program. Data processing results using ClustalW facility was then be used as a baseline to create a phylogeny tree using MEGA data facility.

2.6 Antagonistic activity test of P. alcaligenes on PDA

Inhibitory activity of three isolates of *P. alcaligenes* (isolates KtS1, TrN2, and TmA1) against *F.oxysporum* f.sp. l*ycopersici* was tested on PDA medium in Petri dishes. A mycelial plug of *F.oxysporum* f.sp. l*ycopersici* (5 mm diam*.)* taken from a 5-day old culture was put in the centre of Petri dish, and the isolates of *P. alcaligenes* were gown on the two sides of the mycelial plug. Ten Petri dishes were prepared for each isolate. The cultures were kept in the dark under room temperature (28 + 2^{o}C) for eight days. The inhibitory activity of *P. alcaligenes* against *F. oxysporum* f.sp. *lycopersici* was determined based on the following formula :

$$I = \frac{r_1 - r_2}{r_1} \times 100\ \%.$$

Note :
I = Inhibitory activity (%)
r_1 = Colony size of *F. oxysporum* f.sp. *lycopersici* on control
r_2 = Colony size of *F. oxysporum* f.sp. *lycopersici* grown with *P. alcaligenes*.

2.7 Application of Bacterial Suspension to Control Wilt Disease

This experiment was performed in a factorial pattern consisting of two factors *i.e*. factor 1was the isolates of *P. alcaligenes* and factor 2 was the application methods. Three isolates of *P. alcaligenes* were tested in three application methods namely dipping the root, soaking the seed, and dressing the bacterial suspension on seedbed. There were 12 combination treatments were tested in this experiment and each treatment was replicated 3 times. A randomized block design (RBD) was applied to allocate the treatments. Inoculation with fungal spore's suspension of *F.oxysporum* f.sp *lycopersici* was done by dressing 20 ml of spore suspension into the soil in polybags three days before planting of tomato seedlings. The number of wilted tomato plant was observed everyday after transplanting to determine the disease incidence. The disease incidence was determined using the following formula:

$$P = \frac{n}{n+N} \times 100\%$$

Note :
P = Disease incidence (%)
n = The number of infected plant
N = The total number of plants obser

2.8 Statistical analysis

Statistical analysis was done using software SPSS Windows version 17.0. The data were analyzed using analysis of variance (ANOVA) and followed by either least significance difference (LSD) at 5% level or Duncan's multiple range test at 5% level.

3. Results

3.1 Biochemical properties of the bacterial isolates

All three isolates obtained in this study (KrS1, TrN2 and TmA1) were gram negative bacteria. In general these isolates showed similar biochemical properties as presented in Table 1. Based on the biochemical properties, all three isolates showed similarity to *Pseudomonas*. *Pseudomonas fluorescens* is a common Gram-negative, oxidase test positif, Heat-stable lipases and proteases are produced, obligate aerobe, nonsaccharolytic bacteria (Palleroni, 1984). *Pseudomonas putida* is a gram-negative rod-shaped saprotrophic soil bacterium, have been used in organic synthesis. *Pseudomonas aeruginosa* is Gram-negative, aerobic, secretes a variety of pigments, including pyocyanin (blue-green), pyoverdine (yellow-green and fluorescent), and pyorubin (red-brown), King A positif, and King B positif (King *et al*., 1954). There are several similarities between *P.alcaligenes* and *P. fluorescens* and *P. putida*.

3.2 Genome analysis

Separation pattern of 16S rRNA-spesific fragments amplified with primers 16sV-F and 16sV-R is shown in Figure 1. Labeled band of approximately 1500 bp is corresponding to 16S rRNA gene. The Fragments of the 16S rRNA gene of *Pseudomonas* spp. isolates KtS1, TrN2, and TmA1 then sequenced to determine the species

of the bacteria on the percent of homology with other references of identified species. The 16S rRNA gene is a universal gene shared by all prokaryote microorganisms. Nucleotide sequence of each species or bacterial isolates are usually specific genes that can be used as a key in the identification of bacteria.

The results of 16S rRNA gene sequencing for three isolates of *Pseudomonas* spp. (KtS1, TrN2, and TmA) was further confirmed using Blast program at the Gene Bank to determine the similarity between the identified bacteria and the stored bacteria in the gene bank. Based on the 16S rRNA analisis showed that *Pseudomonas* spp. isolates KtS1, TrN1 and TmA1 showed similarity to *Pseudomonas alcaligenes* F78 by 99.44%, 99.79% and 99.72% respectively (Table 2). Genetic relationship of *Pseudomonas* spp. isolates KtS1, TrN2, and TmA1 with *Pseudomonas alcaligenes*, *Psudomonas fuorescens*, *Pseudomonas aeruginosa*, and *Pseudomonas putida* is presented in Figure 2.

3.3 Antagonistic activity of P. alcaligenes

Three isolates of *P. alcaligenes i.e.* KtS1, TrN2, and TmA1 exhibited antagonistic activity against *F. oxysporum* f sp *lycopersici* with the inhibitory activities by 90%, 88% and 86% respectively for isolates TmA1, TrN2, and KtS1 (Figure 3 and Table 3). Treatment of isolates of *P. alcaligenes* in combination with the method of application significantly ($P<0.05$) reduced the disease incidence of *Fusarium* wilt on tomato plant under green house test (Table 4). There was no significant interaction between the threatment of isolates of *P. alcaligenes* and the method of application. Treatment with *P. alcaligenes* TmA2 by soaking tomato seed or watering the nursery bed showed the lowest disease incidence when compared with other treatments. This result suggested that *P. alcaligenes* isolates KtS1, TrN2, and TmA1 are potential bio-control agent against *F. oxysporum* f.sp. *lycopersici*. Further study is needed to prove the effectiveness of the these agents under field condition.

4. Discussion

Based on 16S rRNA gene analysis, three isolates of *P. alcaligenes* isolated in this study belong to the group of *P. aeruginosa* (Anzai *et al*., 2000). *P. alcaligenes* is a gram-negative aerobic bacteria used as a soil inoculant for bioremediation purposes because it can degrade polycyclic aromatic hydrocarbons. It can also be a human pathogen yet the incidence rarely happens (Valenstein *et al*., 1983). Bacteria belonging to *P. aeruginosa* included human pathogens ranging from bacteria, bacterial plant pathogens, plant beneficial bacteria, to bacteria with the ability of soil bioremediation and other species that cause spoilage of milk and milk products (Vrije *et al*., 2008).

Several reports have been published on the use of bio-agents to control plant diseases (Freeman *et al*., 2002; Larkin and Fravel, 1998; Romero et al., 2004; McMilan, 2007; Sarma *et al*., 2009; Weller and Cook, 1986), however no report on the use of *P. alcaligenes* to control *Fusarium* wilt disease on tomato. *Trichoderma hamatum*, *Gliocladium virens*, *Psudomonas fluorescens*, and *Bacillus cepacia* significantly reduced *Fusarium* wilt disease on tomato under green house test (Larkin and Fravel, 1998). Treatment with *Bacillus pumilus* strain SE34 and *B. subtilis* strain GBO3 effectively suppressed the powdery mildew on pumpkins (Romero *et al*., 2004). The seed treatment using *Pseudomonas* spp. promoted the plant growth of wheat and protect them from infection caused by *Phytium* spp. (Weller and Cook, 1986), could protect peanut plants from wilting and root pathogen *Sclerotium rolfsii* (Ganesan and Gnanamanickam, 1986) and could protect peas from infection of *F. oxysporum* f.sp. *fission* and *Phytium ultimun* (Benhamou *et al*., 1996).

5. Conclusion

Three isolates of rhizobacteria isolated from the rhizospheres plants of the Families Solanaceae and Legumonosae namely KtS1, TrN2, and TmA1 were identified as *Pseudomonas alcaligenes*. All these isolates exhibited antagonistic activity against *Fusarium oxysporum* f.sp. *lycopersici,* the cause of tomato wilt disease. All three isolates of *P. alcaligenes* effectively reduced the incidence of wilt disease on tomato under green house experiment. This result suggested that the *P. alcaligenes* isolates KtS1, TrN2 and TmA1 may be further developed as bio-control agent to manage the wilt disease on tomato.

6. References

Anzai, K. H., Park, J.Y., Wakabayashi, H. and Oyaizu, H. (2000). "Phylogenetic affiliation of the Pseudomonads based on 16S rRNA sequence". Int. J. Syst. Evol. Microbiol **50** (4): 1563–89. http://en.wikipedia.org/wiki/ , 2012) Accessed on September 17 , 2012.

Benhamou, N., Belanger, R.R. and T.C. Paulitz, T.C. (1996). Induction of differential host responses by *Pseudomonas fluorescens* in Ri T-DNA-transformed pea roots after challenge with *Fusarium oxysporum* f.sp. *pisi* and *Phytium ultimum*. *Phytopathology* 86:1174-1185.

Freeman S., Zveibel, A., Vintal, H. and Maymon, M. (2002). Isolation of nonpatogenic mutants of *Fusarium oxysporum* f.sp. *melonis* for biological control of *Fusarium* wilts in cucurbits. *Phytopathology* 92:164-168.

Ganesan, P. and Gnanamanickam, S.S. (1986). Biological control of *Sclerotium rolfsii* Sacc. In peanut by

inoculation with *Pseudomonas fluorescens*. Centre of advanced study in Botany, University of Madras, India. Pp.35-37.
King, E.O., Ward, M.K. and Raney, D.E. (1954). "Two simple media for the demonstration of pyocyanin and fluorescein". *J. Lab. Clin. Med.* 44 (2): 301–307.
Kumar, S., Tamura, K., Jakobsen, L.B. and Nei, M. (2001). Molecular evolutionary genetics analysis version 3.1. Pennsylvania State Univ.: lnstitute of Molecular Evolutionary Genetics.
Larkin, R. P., and Fravel, D. R. 1998. Efficacy of various fungal and bacterial biocontrol organisms for control of *Fusarium* wilt of tomato. *Plant Dis*. 82:1022-1028.
McMilan, S. (2007). Promoting Growth with PGPR. The Canadian Organic Grower. www.cog.ca. Page 32-34
Palleroni, N.J. (1984). Pseudomonadaceae. Bergey's Manual of Systematic Bacteriology. Krieg, N. R. and Holt J. G. (editors) Baltimore: The Williams and Wilkins Co., pp. 141 - 199.
Pangastuti, A. (2006). Prokaryotic Species Definitions Bases Based Sequence encoder 16s rRNA gene and protein-coding genesDepartment of Biology FMIPA Sebelas Maret University (UNS) Surakarta 57126. BIODIVERSITAS ISSN: 1412-033X Volume 7, Number 3 July 2006. pp.: 292-296 (in Indonesian language)
Romero, D., Pérez-García, A., Rivera, M.E., Cazorla,F.M. and de Vicente, A. (2004). Isolation and evaluation of antagonistic bacteria towards the cucurbit powdery mildew fungus *Podosphaera fusca*. *Appl. Microbiol. Biotechnol*. 64: 263–269.
Sarma, M.V., Saharan, R.K., Prakash, K., Bisaria, A. and Sahai, V. (2009). Application of Fluorescent Pseudomonads Inoculant Formulations on *Vigna mungo* through Field Trial. *International Journal of Biological and Life Sciences* 1:41-47.
Semangun, H. (2007). Diseases of Horticultural Crops in Indonesia. Gadjah Mada University Press. Yogyakarta (in Indonesian language).
Valenstein, P., Bardy, G.H., Cox, C.C. and Zwadyk, P. (1983). *Pseudomonas alcaligenes* endocarditis. *American Journal of Clinical Pathology* 79(2):245-7
Vrije, P.C. (2008). Genomics and Molecular Biology,. Ed. *Universiteit Brussel, Belgium.* Caister Academic Press ISBN: 978-1-904455-19-6 , January 2008. http://www.horizonpress.com. Accessed on February 19, 2012
Weller, D.M. and Cook, R.J. (1986). Increased growth of wheat by seed treatments with fluorescens pseudomonads, and implications of Phytium control. *Can.J. Plant Pathol*. 8:328-334.
Wibowo, A. (2005). Colonization of tomato root by antagonistik bacterial strains to fusarium wilt of tomato. Department of entomology and phytopathology, Faculty of agriculture, Gajah Mada. *Journal of Plant Protection Indonesia* 11: 66-76

Table 1. Biochemical properties of *P. alcaligenes* isolates KtS1, TrN2, and TmA1

No	**Biochemical properties**		**Isolates**			**Remarks**
			KtS1	**TrN2**	**TmA1**	
1	Gram staining		-	-	-	
2	Oxidase		-	-	-	
3	Catalase		+	+	+	
4	TSIA	Butt	Yellow	Yellow	yellow	Triple Sugar Iron Agar
		Slant	Yellow	Yellow	yellow	
		Gas	-	-	-	
		H2S	-	-	-	
5	OF		Alkaline	-	alkaline	oxidase fermentation
6	SIM	Indol	-	-	-	
		Motil	+	-	-	Sulfide indol motility
		Gas	-	-	-	
		H2S	-	-	-	
7	MR		-	-	-	Metyl Red
8	VP		-	-	-	Voges Proskuer
9	KA		+	+	-	King,s A
10	KB		+	+	+	King'S B

Table 2. Comparisons of 16S rRNA gene homology levels of isolates *P. Alcaligenes* with multiple sequences in GenBank using the BLAST program

Isolates	Percent homology (%)		
	P. alcaligenes TrN2	*P. alcaligenes* TmA1	*P. alcaligenes* KtS1
Pseudomonas fluorescens strain YMN16	81.947	82.015	81.609
Pesudomonas alcaligenes faecalis	99.721	99.651	99.372
Pseudomonas alcaligenes sp. F78	99.791	99.721	99.442
Pseudomonas putida strain ECAn8-1	82.932	83.003	82.790
Pseudomonas putida	79.459	79.459	79.140
Pseudomonas aeruginosa strain HNYM11	83.228	83.298	82.947

Tabel 3. Inhibitory activities of *P. alcaligenes* against *F. oxysporum* f.sp. *lycopersici*

No	Isolates	Fungal colony size (mm^2)	Inhibitory activity (%)
1	Control	4,310 a	-
2	*P. alcaligenes* KtS1	610 b	86
3	*P. alcaligenes* TrN2	535 b	88
4	*P. alcaligenes* TmA1	440 b	90
LSD 5% = 2,052			

Note : Values in the same columns followed by the same letters are not significantly different (P>0.05) according to the Least Significance Difference (LSD) at 5% level.

Table 4. Effect of the isolates of *P. alcaligenes* in combination with application methods on the disease incidence of *Fusarium* wilt disease on tomato at 10th week after transplanting

Application method	Disease incidence (%)			
	Control	P. alcaligenes *KtS1*	P. alcaligenes *TrN2*	P. alcaligenes *TmA2*
Dipping the roots of seedlings (A)	100.00 a	67.00 b	55.56 b	55.56 b
Soaking tomato seed (B)	100.00 a	55.56 b	55.56 b	44.44 b
Watering the nursery beds (C)	100.00 a	55.56 b	55.56 b	44.44 b

Note : Values in the rows and columns followed by the same letters are not significantly different according to the Duncan's Multiple Ring Test (DMRT) 5%.

Figure 1. The Agarose Gel Electrophoresis of 16S rRNA gene of *P. Alcaligenes* isolates KtS1, TrN2, and TmA1. Lane 1, 2 and 3 is a fragment of 16S rRNA for each isolate KtS1, TrN2, and TmA1 (1500 bp). Line 2 (M2) is VC 100bp Plus DNA Ladder / Marker 0.1-3 kbp (*ready-to-use*) Vivantis

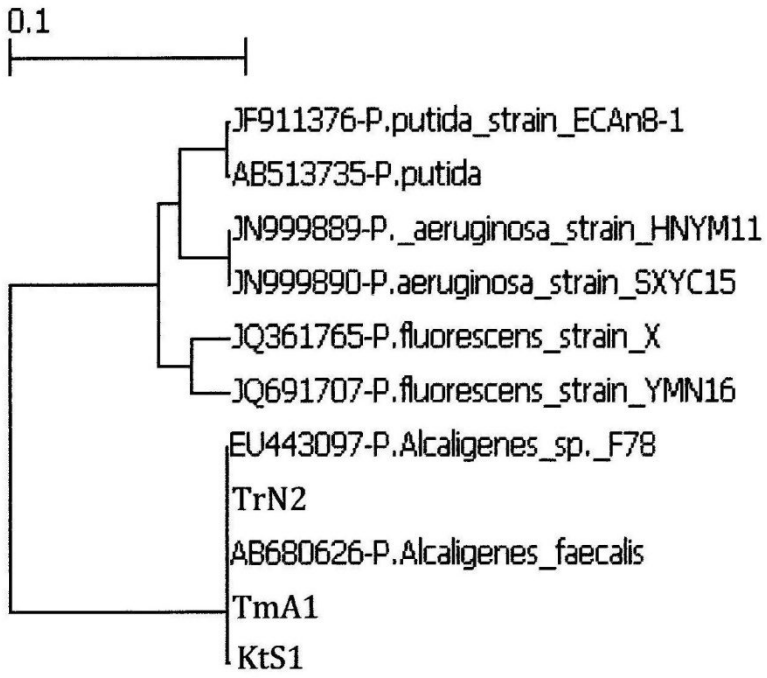

Figure 2. Phylogenetic relationship of the bacteria internal transcribed spacer sequence of the characterized clone library of *Pseudomonas*.