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ORIGINAL ARTICLE

Acidophilic actinomycetes from rhizosphere soil: diversity and properties beneficial to plants

Nalin Poomthongdee[1], Kannika Duangmal[1,2] and Wasu Pathom-aree[3]

Three hundred and fifty-one isolates of actinomycetes were recovered from 21 rhizospheric soil samples using acidified media of pH 5.5. They were evaluated for their antifungal, siderophore production and phosphate solubilization activities. The total count of actinomycetes growing on acidified starch casein agar and Gause no. 1 agar were below 2.48×10^4 CFU g^{-1} soil. Two hundred and twelve isolates were assigned to acidophiles and the remaining 139 isolates were neutrophiles. Of these actinomycetes, 57.8, 32.5 and 50.4%, showed antagonistic activity against three rice pathogenic fungi; *Fusarium moniliforme, Helminthosporium oryzae* and *Rhizoctonia solani*, respectively. More than half of the isolates (68.1%) inhibited at least one tested pathogenic fungus, whereas 25.9% exhibited antifungal activities against all tested fungi. Three hundred and thirty-eight isolates (96.3%) produced siderophore and 266 isolates (75.8%) solubilized phosphate. A greater proportion of the acidophilic actinomycetes exhibited antifungal, siderophore production and phosphate solubilization activity compared with the neutrophiles. Three hundred and twenty-five isolates (92.6%) were classified as streptomycetes based on their morphological characteristics and the presence of the *LL*-isomeric form of diaminopimelic acid in whole-cell hydrolysates. The 16S ribosomal RNA (rRNA) gene analysis of representative non-streptomycete strains showed that the isolates belonged to seven genera, that is, *Allokutzneria*, *Amycolatopsis*, *Mycobacterium*, *Nocardia*, *Nonomuraea*, *Saccharopolyspora* and *Verrucosispora*. The potential antifungal acidophilic isolates, R9-4, R14-1, R14-5 and R20-5, showed close similarity to *Streptomyces misionensis* NBRC 13063^T (AB184285) in terms of morphological characteristics and 16S rRNA gene sequences.
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INTRODUCTION

Actinomycetes are a diverse group of Gram-positive bacteria with high GC content, which are common in soil and widely distributed in various environments. They represent the most economically and biologically valuable bacteria among prokaryote, producing various biologically active substances such as antibiotics, antitumors and enzymes.[1–3] Although actinomycetes are commonly known as neutrophiles, which grow well in neutral or slightly alkaline conditions, a few actinomycetes such as *Streptomyces acidiphilus*[4] and members of the genus *Streptacidiphilus*[5] have been reported to require acidic conditions (pH 2.6–5.5) for growth. In addition, the members of the genera *Actinospica*[6] and *Catenulispora*[7] are also known as acidophilic actinomycetes.

Acidophilic actinomycetes can be divided into two main groups, which are neutrotolerant acidophiles and strict acidophiles. Typical neutrotolerant acidophiles grow in media at pH 4.5–7.5 with optimum growth between pH 5.0 and 5.5. Members of strictly acidophilic group typically grow in media between pH 3.5 and 6.5, with an optimum growth at pH 4.5.[8,9] In acidic habitats, the actinomycetes commonly found belong to the genus *Streptomyces*.[10]

Rice is a staple crop that supports about half of the world's population. However, fungal rice diseases are major problems in rice cultivation.[11] The use of chemical synthetic compounds is regarded as an effective method for prevention and therapy, but has deleterious effects on health and the environment.[12,13] It is necessary to find an environmental-friendly alternative for sustainable protection management. Actinomycetes have been considered as potential biocontrol agents against various phytopathogenic fungi because of their production of bioactive metabolites[14–16] or of enzymes that hydrolyzed fungal cell walls.[17–20] Most of the previous works were focused on the ability of actinomycetes isolated from neutral pH isolation media. However, acidophilic actinomycetes were reported to inhibit fungi better than neutrophilic actinomycetes under acidic condition.[21] Crawford *et al.*[22] also reported that acidophilic actinomycetes exhibited strong antagonism toward multiple fungal root pathogens. In addition, Basilio *et al.*[14] demonstrated that actinomycetes isolated under alternative selective pH conditions possess a significant capacity to produce compounds with antimicrobial activity. In rice field soil, the pH is usually slightly acidic, which is favorable for the growth of pathogenic fungi. The use of acidophilic actinomycetes in the rice field would be more effective than neutrophilic strains. However, little attention has been given to determining the diversity and antifungal activity of acidophilic actinomycetes. Therefore, in this study, actinomycetes were isolated

[1]Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand; [2]Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Bangkok, Thailand and [3]Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand
Correspondence: Dr K Duangmal, Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok 10900, Thailand.
E-mail: fscikkd@ku.ac.th or kannikaduangmal@hotmail.com
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from rhizospheric soils under acidic selective conditions and their ability to inhibit the rice pathogenic fungi *Fusarium moniliforme*, *H. oryzae* and *R. solani* was assessed. The diversity of the actinomycetes obtained was also investigated.

MATERIALS AND METHODS

Sample collection and selective isolation of actinomycetes

Rhizospheric soil samples (17 samples from rice plants and 4 samples from rubber trees) were collected from several provinces of Thailand (Chiang Mai, Nakhon Sawan, Pathum Thani, Phatthalung, Phetchaburi, Phra Nakhon Si Ayuttaya, Ratchaburi and Suphan Buri). The samples were air dried, and sieved to remove unwanted particles. The pH of soil was determined according to Davet.[23] Air dried soil sample was mixed with distilled water at a ratio of 1:2.5, thoroughly mixed and left to stand for 30 min before pH measurement with pH meter. The isolation of actinomycetes was conducted using a dilution plate technique. One gram soil sample was diluted in 4 ml of 0.85% (w/v) NaCl solution, mixed well and then heat treated at 55 °C for 10 min in a water bath to eliminate fast growing bacteria. Serial 10–fold dilutions were prepared for each pretreated sample and aliquots (0.1 ml) of each dilution were spread onto acidified starch casein agar (SCA)[24] and acidified Gause no. 1,[25] pH 5.5, supplemented with nalidixic acid, nystatin and ketoconazole at final concentration of 25 μg ml^{-1}, 50 μg ml^{-1} and 100 μg ml^{-1}, respectively. Media of pH 5.5 was prepared by mixing sterile double strength media with an equal volume of sterile citrate/phosphate buffer of pH 5.5 (0.1 M citrate/0.2 M Na_2HPO_4). The method for pH adjustment using buffer was applied to all media used in this experiment, otherwise indicated. The plates were incubated at 28 °C for 28 days, and then the number of actinomycete colonies, recognized by their morphological characteristics, was counted. The colonies were then selected and restreaked on acidified International *Streptomyces* Project (ISP) medium 2[26] (pH 5.5) for purity checking and maintained on the same medium at room temperature. Spores and mycelial suspensions were stored in 20% (v/v) glycerol solution at −20 °C as stock culture.

Actinomycete characterization

Putative actinomycete isolates were characterized by their morphological, chemical and physiological characteristics. Their morphological characteristics were observed on acidified ISP medium 3[26] (pH 5.5), after incubation at 28 °C for 14 days. The color of spore mass and diffusible pigment production were determined and recorded by comparison with a color chart.[27] The isomeric form of 2,6-diaminopimelic acid (DAP) of whole organism hydrolysates was determined using paper chromatography according to the method as described by Hasegawa *et al.*[28] and Becker *et al.*[29]

The assignment of actinomycetes as acidophilic or neutrophilic strains was based on their ability to grow on ISP medium 2 at pH 4.5 and 7.5. The growth of each isolate was observed after being incubated at 28 °C for 3, 7 and 14 days. The isolates that grew better or grew only on acidified media were regarded as acidophiles and the isolates that grew better or grew only on media of pH 7.5 were regarded as neutrophiles.[30]

In vitro antifungal activity

Antagonistic activity of the isolates against three rice pathogenic fungi *F. moniliforme* (bakanae), *H. oryzae* (brown spot) and *R. solani* (sheath blight), were evaluated on potato dextrose agar (PDA, pH 5.5) using a dual culture technique.[31] These fungi were maintained on PDA at room temperature. Two 7-day-old actinomycete discs (5 mm) grown on acidified ISP medium 2 (pH 5.5) were placed on opposite sides, 3 cm away from the center of the PDA plate. After incubation at 28 °C for 7 days, a fungal mycelia disc (5 mm in diameter) was placed in the center of the plate. PDA plates with fungal mycelia discs in the center of the plate (without actinomycete) served as control. The plates were incubated at 28 °C until the fungal mycelium of control reached the edge of the plate. The percentage of inhibition was calculated as $[(r1 - r2)/r1] \times 100$. Where, $r1$ is the radial mycelia growth in control and $r2$ is the radial mycelia growth that occurs toward the actinomycetes.[32] The experiment was conducted in duplicate.

Screening for siderophore production

Discs (5 mm) containing good lawns of actinomycete isolates grown on ISP medium 2 (pH 5.5) at 28 °C for 7 days were placed on chrome azurol S agar.[33] Sterile ISP medium 2 (pH 5.5) discs were also placed on chrome azurol S agar as controls. The inoculation plates were incubated at 28 °C for 5 days. An orange halo around the agar discs was considered as siderophore-producing activity and the diameter of the halo was recorded. The experiment was conducted in duplicate.

Screening for phosphate-solubilizing actinomycetes

All actinomycete isolates were cultured on ISP medium 2, at pH 5.5 and incubated at 28 °C for 7 days. Discs (5 mm) containing good lawn of the isolates were placed on Pikovskaya agar.[34] The inoculation plates were incubated in the dark at 28 °C for 14 days. The presence of a clear zone around the agar discs was considered as phosphate-solubilizing activity and the diameter of clear zone was measured. The experiment was conducted in duplicate.

DNA extraction, PCR amplification and sequencing of 16S ribosomal RNA (rRNA) gene

Genomic DNA was extracted as described by Kieser *et al.*[35] PCR amplification of 16S rRNA gene was carried out using the primers: STR1F (5′-TC ACGGAGAGTTTGATCCTG-3′) and STR1530R (5′-AAGGAGATCCAGCC GCA-3′).[36] The PCR consisted of an initial denaturation step at 94 °C for 4 min, followed by 30 cycles of 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1 min and a final extension step for 10 min at 72 °C. Sequencing of 16S rDNA was performed using the service of Macrogen (Seoul, Korea) and 1st BASE Laboratory (Selangor, Malaysia), with the same primers as those used for PCR amplification. The resultant sequences were compared with other sequences of related type strains available in the EzTaxon-e database (http://eztaxon-e.ezbiocloud.net/). The 16S rDNA sequences were aligned with related

Table 1 Soil pH values, total actinomycete count (CFU per g^{-1} dry soil) and total number of actinomycete isolated from each soil sample

Samples	Soil pH	Total actinomycetes count (CFU g^{-1}) on SCA medium	Total isolates
R1	5.3	1.36×10^4	11
R2	5.1	2.48×10^4	25
R3	7.1	<30[a]	9
R4	6.0	<30[a]	8
R5	5.5	5×10^3	25
R6	7.3	4.78×10^3	14
R7	7.6	<30[a]	16
R8	7.2	<30[a]	27
R9	5.4	<30[a]	20
R10	3.1	<30[a]	3
R11	7.0	<30[a]	28
R12	6.7	<30[a]	1
R13	6.6	7.02×10^3	21
R14	7.0	7.18×10^3	15
R15	5.6	6.32×10^3	21
R16	5.1	<30[a]	23
R17	4.7	<30[a]	9
R18	4.7	<30[a]	24
R19	4.7	<30[a]	10
R20	4.8	<30[a]	16
R21	6.5	<30[a]	25
			351

Abbreviation: SCA, starch casein agar.
[a]actinomycetes number lower than 30 CFU/plate after spread the plate with soil suspension at a dilution of 1:4 and incubated for 4 weeks.

species using CLUSTALW program.[37] A neighbor-joining[38] phylogenetic tree was generated using MEGA version 5.0 software,[39] evaluated by bootstrap analysis of 1000 replications; a distance matrix was generated using Kimura's 2-parameter model.[40]

RESULTS AND DISCUSSION

Selective isolation of actinomycetes

Both rice plants and rubber trees can grow in acid soil and the characteristic acidic pH of paddy soils and of rubber tree plantation soils have been reported.[41,42] In this study, the pH of rice rhizosphere soils (R1 to R17) ranged from strongly acidic to slightly alkaline (pH 3.1–7.6) and the rubber tree rhizosphere soils (R18 to R21) were highly acidic to slightly acidic (pH 4.7–6.5) (Table 1).

In a preliminary test, five soil samples were used to isolate acidophilic actinomycetes at pH 4.5. One gram of each soil sample was suspended in 4 ml of 0.85% NaCl, heated at 55 °C for 10 min before being spread on acidified SCA medium with pH adjusted to 4.5, supplemented with nalidixic acid (25 μg ml^{-1}) and nystatin (50 μg ml^{-1}). However, after a few days of incubation, most isolation plates were covered with fungal mycelium, which overgrew the small colonies of actinomycetes. As Muramatsu *et al.*[43] observed, the isolation of acidophilic actinomycetes is difficult due to fungal contamination, which grow well under acidic conditions. In addition, most antifungal agents such as nystatin are unstable at low pH. Therefore, the isolation media used were evaluated with those soil samples to selectively isolate acidophilic actinomycetes by adjusting the pH of the media within the range of 4.0–6.0 and supplemented with fungal inhibitors such as salt (sodium chloride and sodium propionate), dyes (rose Bengal and Congo red) and antibiotics (nalidixic acid, nystatin and ketoconazole). The results showed that acidified SCA and Gause no. 1 agar plates of pH 5.5, supplemented with 25 μg ml^{-1} antibiotic nalidixic acid , 50 μg ml^{-1} nystatin and 100 μg ml^{-1} ketoconazole had fewer fungal contamination and gave higher number of actinomycete colonies (data not shown). Therefore, these two media were then applied for all soil samples. After incubating the plates at 28 °C for 28 days, colonies of actinomycetes were counted. Most soil samples showed actinomycete colonies <30 CFU per plate on Gause no. 1 medium, which had been spread with soil suspension at a dilution of 1:4. The highest number of actinomycete colonies (2.48×10^4 CFU g^{-1} soil) was found in rice plant rhizospheric soil sample (R2) on SCA medium (Table 1). In total, 351 actinomycete isolates were obtained from 21 soil samples (276 isolates from rice rhizospheres and 75 isolates from rubber tree rhizospheres). The highest number of actinomycete isolates ($n = 28$) were obtained from sample R11, and the lowest number of only one isolate from sample R12.

Characterization of the isolates

The detection result of 2,6-DAP from whole-cell hydrolysates showed that 325 isolates (93.2%) contained *LL*-DAP. From their morphological characteristics and the DAP type, this majority of isolates were then assigned to the streptomycete group. This number included members of the genus *Streptacidiphilus*, which has similar colony morphology and DAP type as the genus *Streptomyces*. These 325 isolates were classified into five color groups based on their spore color on ISP medium 3. The isolates which produced gray spore color were dominant (53.5%), followed by brown (18.5%), white (4.9%), yellow (4.6%) and green (1.5%). Fifty-five isolates (16.9%) in the streptomycete group did not produce spores on acidified ISP medium 3 (pH 5.5). The remaining 26 isolates (6.8%), containing *meso*-DAP, were assigned to the non-streptomycete or rare actinomycete group. Representatives of these non-streptomycetes (23 isolates) and streptomycetes (35 isolates) were identified to the genus level by 16S rRNA gene sequences analysis.

All 351 isolates were evaluated for their pH requirement for growth. The results showed that 212 isolates could grow better on pH 4.5 medium when compared with pH 7.5 and were assigned to acidophilic actinomycetes. However, they were not strictly acidophilic actinomycetes because these isolates could grow on medium pH 7.5. Ninety isolates grew better on pH 7.5 medium when compared with pH 4.5 and the remaining 49 isolates which grew only on pH 7.5 medium were assigned to neutrophilic actinomycetes. A previous study reported that acidophilic actinomycetes were present in soils in which the pH did not exceed 6.8 and were not found in slightly alkaline soil.[44] However, in this study acidophilic actinomycetes were found in all soil samples including those with slightly alkaline pH. One possible explanation is that soils are heterogeneous systems with multiple microzones, where pH may considerably differ from the average value. In acidic soil, neutrophilic actinomycetes grew and generated spores in microsites of ammonia adsorption on organic fragments and the spores survived.[8] On the other hand, acidophilic actinomycetes could grow in alkaline soils around plant roots that release H^+ for nutrient exchange, causing acid microsite, and they were recovered on an acidified isolation media.

Among 325 streptomycete isolates, 205 isolates were classified to acidophilic strains and 120 isolates to neutrophilic strains. Nineteen isolates of non-streptomycetes were classified to neutrophilic strains,

Table 2 Percentage of acidophilic and neutrophilic actinomycete antagonist against *F. moniliforme*, *H. oryzae* and *R. solani* based on inhibition level of fungal growth

	Percentage of actinomycetes antagonist against pathogenic fungi					
	F. moniliforme		*H. oryzae*		*R. solani*	
Inhibition level	*Acidophiles*	*Neutrophiles*	*Acidophiles*	*Neutrophiles*	*Acidophiles*	*Neutrophiles*
−	33.96	54.68	61.79	76.26	31.60	76.98
+	7.55	15.11	15.57	9.35	4.72	0.00
++	35.85	22.30	11.79	9.35	20.75	10.79
+++	18.40	6.47	8.02	3.60	28.30	9.35
++++	4.25	1.44	2.83	1.44	14.62	2.88
Total	100.00	100.00	100.00	100.00	100.00	100.00

Ratings: −, No inhibition; +, % inhibition ⩾30; ++, % inhibition ⩾50; +++, % inhibition ⩾70; ++++, % inhibition >90.

whereas the other seven isolates were acidophilic strains. According to previous studies, streptomycetes were the most notable acidophilic actinomycetes, which is probably related to the fact that they are predominant in most soils.[10,43,45]

Antifungal activity

The *in vitro* antifungal activity assay showed that, 203 isolates (57.8%), 114 isolates (32.5%) and 177 isolates (50.4%) suppressed *F. moniliforme, H. oryzae* and *R. solani*, respectively. Two hundred and thirty-nine isolates (68.1%) inhibited at least one tested pathogenic fungus and 91 isolates (25.9%) exhibited antifungal activity against all tested fungi. All antagonistic isolates belong to the streptomycete group except R8-39 and R13-3, which were non-streptomycete strains. Isolate R8-39 inhibited the growth of all tested fungi and R13-3 inhibited only *F. moniliforme*. The percentage of inhibition activity of isolate R8-39 to inhibit *F. moniliforme*, *H. oryzae* and *R. solani* were 74%, 84% and 71%, respectively.

Nine streptomycete isolates exhibited >80 percent of inhibition against all pathogenic fungi. Among these, eight strains (R9-4, R14-1, R14-5, R14-6, R14-10, R18-16, R20-5 and R21-2) were acidophiles and only one strain (R19-3) was neutrophile. Strains R9-4, R14-1, R14-5 and R20-5, which gave the highest antagonistic activity, were selected for 16S rRNA gene analysis.

Previous studies using actinomycetes isolated from neutral pH isolation media, reported lower percentages of antagonistic behavior toward *F. moniliforme* than that found in this study. Baniya and Vaidya[46] isolated 28 actinomycetes from vemicompost, of which only 4 isolates (10.5%) were active against *F. moniliforme*. Hatamy *et al.*[47] studied the inhibition activity of 100 actinomycetes isolated from greenhouse soil in Iran, reported that 18 strains showed high activity against *F. moniliforme*, and other neutrophilic actinomycetes showed antagonistic activity against *H. oryzae* and *R. solani*. Kathiresan *et al.*[48] isolated 160 actinomycetes from sediment samples in India and found that about 51% and 31% of isolates were effective against *H. oryzae* and *R. solani*, respectively.

Acidophilic actinomycetes are usually scattered in acid terrestrial systems,[45,49] in which they were important saprophytes and antagonistic microorganisms.[21,30] It is important to explore actinomycetes having antifungal activity in acidic condition for direct use as biocontrol in acidic environment. Therefore, both acidophilic and neutrophilic actinomycetes were evaluated for their antifungal activity on acidic medium. Comparison of antifungal activity between acidophilic and neutrophilic actinomycetes (Table 2) showed that acidophilic isolates gave higher percentage of inhibition with all tested pathogenic fungi. In this experiment, all actinomycetes were evaluated for their antifungal activity on acidified agar, pH 5.5, and a greater proportion of acidophilic actinomycete isolates showed antifungal activity compared with neutrophilic strains under acidic condition. This may be due to the acidic condition that supported the growth of acidophilic more than neutrophilic isolates.

Siderophore production

Streptomycete isolated from roots of a Thai jasmine rice plant with siderophore production was reported to promote plant growth.[50] In this experiment, the ability of all 351 isolates to produce siderophore was tested. Three hundred and thirty-eight isolates exhibited yellow to orange halos on chrome azurol S agar plate, and were thus considered to be siderophore producers. Two hundred and forty-five isolates (69.8%) exhibited positive zone diameters in the range of 1.1–2.0 cm, and 13 isolates exhibited zone diameters >3.0 cm. There were reports suggesting that the siderophore-producing bacteria could inhibit

Table 3 Identification of non-streptomycete isolates based on partial sequence of the 16S rRNA gene with their closest type strain from the EzTaxon-e database

Genus	*Strains*	*pH group*	*Accession no.*	*Closest type strains (Accession no.)*	*Similarity (%)*
Allokutzneria	R8-39	Neutrophile	AB841026	*A. albata* DSM 44149^T (AJ512462)	98.8
Amycolatopsis	R8-21	Acidophile	AB841020	*A. dongchuanensis* YIM 75904^T (JN656710)	98.7
	R13-25	Acidophile	AB841035	*A. dongchuanensis* YIM 75904^T (JN656710)	98.7
	R13-26	Acidophile	AB841036	*A. dongchuanensis* YIM 75904^T (JN656710)	98.7
	R12-7	Acidophile	AB841033	*A. tolypomycina* DSM 44544^T (AJ508241)	99.2
	R15-35	Acidophile	AB841044	*A. dongchuanensis* YIM 75904^T (JN656710)	98.3
Mycobacterium	R13-27	Neutrophile	AB841037	*M. poriferae* ATCC 35087^T (AF480589)	99.0
	R13-28	Neutrophile	AB841038	*M. poriferae* ATCC 35087^T (AF480589)	98.8
	R13-29	Neutrophile	AB841039	*M. poriferae* ATCC 35087^T (AF480589)	98.5
	R13-30	Neutrophile	AB841040	*M. poriferae* ATCC 35087^T (AF480589)	98.9
Nocardia	R5-25	Neutrophile	AB841007	*N. elegans* IMMIB N-402^T (AJ854057)	99.9
	R5-26	Neutrophile	AB841008	*N. elegans* IMMIB N-402^T (AJ854057)	99.9
Nonomuraea	R2-45	Neutrophile	AB841002	*N. wenchangensis* 210417 ^{T} (FJ261959)	99.4
	R8-35	Neutrophile	AB841023	*N. wenchangensis* 210417^T (FJ261959)	99.7
	R8-36	Neutrophile	AB841024	*N. wenchangensis* 210417^T (FJ261959)	99.7
	R5-34	Neutrophile	AB841009	*N. jabiensis* A4036^T (HQ157186)	99.3
	R6-19	Neutrophile	AB841014	*N. endophytica* YIM 65601^T (GU367158)	99.9
	R6-20	Neutrophile	AB841015	*N. candida* HMC10^T (DQ285422)	98.8
	R5-35	Neutrophile	AB841010	*N. pusilla* IFO 14684^T (U48978)	99.8
	R5-37	Neutrophile	AB841012	*N. pusilla* IFO 14684^T (U48978)	99.7
	R5-36	Neutrophile	AB841011	*N. candida* HMC10^T (DQ285421)	99.2
Saccharopolyspora	R7-2	Neutrophile	AB841016	*S. shandongensis* 88^T (EF104116)	99.2
Verrucosispora	R8-37	Neutrophile	AB841025	*V. maris* AB-18-032^T (AY528866)	99.2

Abbreviation: rRNA, ribosomal RNA.

fungi by Fe ion uptake competition. The produced siderophore could inhibit the growth of fungi or induce the resistance of the plant to phytopathogenic fungi.[51–53] Rhizospheric soil actinomycetes were also reported to inhibit the growth of phytopathogens and produce siderophore.[54] In this study, 70.4% of siderophore-producing strains exhibited antifungal activities. On the other hand, 12 of 13 strains shown no siderophore production and could not inhibit any pathogenic fungi (*F. moniliforme, H. oryzae* and *R. solani*).

The comparison of acidophilic and neutrophilic actinomycetes for siderophore production showed that the percentage of siderophore-producing acidophilic strains was slightly higher than neutrophilies. The proportion of siderophore production by

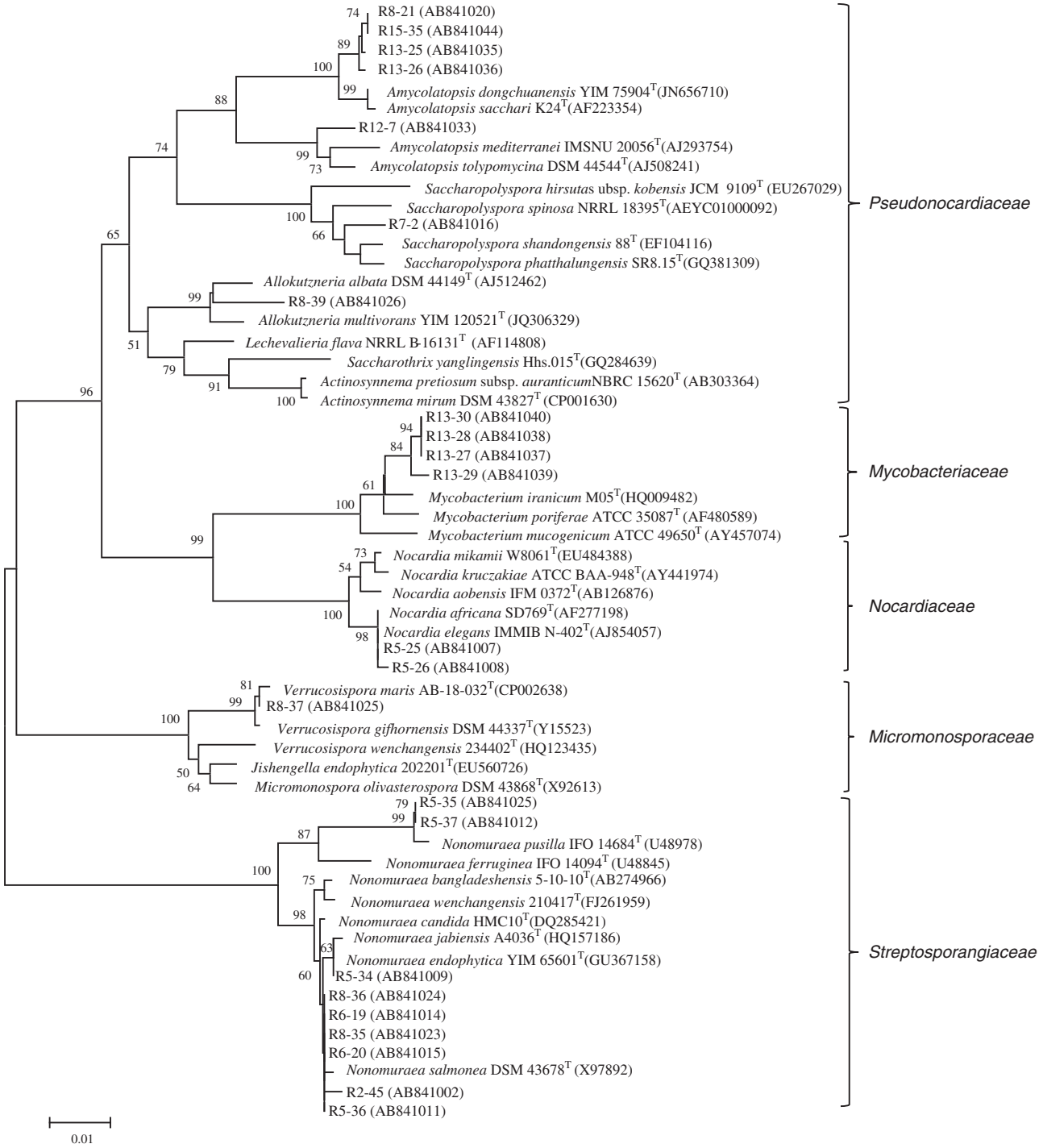

Figure 1 Neighbor-joining tree based on 851 aligned positions within the 16S rRNA gene, omitting regions of ambiguous alignment, showing relationships between the non-streptomycete isolates and related type strains. Numbers at the nodes indicate bootstrap values based on 1000 replicates; only values >50% are given. The scale bar indicates 0.01 substitutions per nucleotide position.

Table 4 Identification of representative streptomycete isolates based on the sequence of the 16S rRNA gene with their closest type strain from the EzTaxon-e database

Isolates	*pH group*	*Accession no.*	*Closest type strains (accession no.)*	*Similarity (%)*
R1-17	Acidophile	AB840998	*S. misionensis* NBRC 13063^T (AB184285)	99.6
R2-26	Acidophile	AB840999	*S. shenzhenensis* 172115^T (HQ660226)	99.9
R2-36	Neutrophile	AB841000	*S. niveiscabiei* S78^T (AF361786)	98.0
R2-38	Neutrophile	AB841001	*S. filipinensis* NBRC 12860^T (AB184198)	98.8
R3-2	Acidophile	AB841003	*S. misionensis* NBRC 13063^T (AB184285)	99.6
R4-2	Acidophile	AB841004	*S. misionensis* NBRC 13063^T (AB184285)	99.6
R5-13	Acidophile	AB841005	*S. misionensis* NBRC 13063^T (AB184285)	99.6
R5-21	Acidophile	AB841006	*S. griseorubens* NBRC 12780^T (AB184139)	100
R6-2	Neutrophile	AB841013	*S. griseorubens* NBRC 12780^T (AB184139)	100
R7-6	Neutrophile	AB841017	*S. albogriseolus* NRRL B-1305^T (AJ494865)	100
R7-28	Acidophile	AB841018	*S. corchorusii* NBRC 13032^T (AB184267)	100
R8-11	Neutrophile	AB841019	*S. coeruleorubidus* NBRC 12844^T (AB184849)	99.6
R8-29	Neutrophile	AB841021	*S. parvulus* NBRC 13193^T (AB184326)	100
R8-31	Neutrophile	AB841022	*S. geysiriensis* NBRC 15413^T (AB184661)	99.5
R9-4	Acidophile	AB841027	*S. misionensis* NBRC 13063^T (AB184285)	99.8
R9-5	Acidophile	AB841028	*S. corchorusii* NBRC 13032^T (AB184267)	100
R9-20	Acidophile	AB841029	*S. corchorusii* NBRC 13032^T (AB184267)	100
R11-2	Neutrophile	AB841030	*S. coeruleorubidus* NBRC 12844^T (AB184849)	99.4
R11-21	Acidophile	AB841031	*S. viridobrunneus* LMG 20317^T (AJ781372)	99.6
R11-28	Neutrophile	AB841032	*S. carpinensis* NBRC 14214^T (AB184574)	99.4
R13-24	Neutrophile	AB841034	*S. albogriseolus* NRRL B-1305^T (AJ494865)	99.8
R14-1	Acidophile	AB841041	*S. misionensis* NBRC 13063^T (AB184285	99.8
R14-5	Acidophile	AB841042	*S. misionensis* NBRC 13063^T (AB184285)	99.7
R14-7	Neutrophile	AB841043	*S. graminearus* LMG 19904^T (AJ781333)	98.6
R16-4	Acidophile	AB841045	*S. shenzhenensis* 172115^T (HQ660226)	99.3
R16-29	Neutrophile	AB841046	*S. rubrogriseus* LMG 20318^T (AJ781373)	99.8
R16-30	Acidophile	AB841047	*S. misionensis* NBRC 13063^T (AB184285)	99.5
R16-35	Acidophile	AB841048	*S. chromofuscus* NBRC 12851^T (AB184194)	99.3
R16-37	Acidophile	AB841049	*S. misionensis* NBRC 13063^T (AB184285)	99.6
R18-16	Acidophile	AB841050	*S. olivaceoviridis* NBRC 13066^T (AB184288)	100
R18-18	Acidophile	AB841051	*S. spiralis* NBRC 14215^T (AB 184575)	99.9
R18-25	Acidophile	AB841052	*S. capoamus* JCM 4734^T (AB045877)	100
R18-31	Acidophile	AB841053	*S. thermoviolaceus* DSM 41392^T (Z68095)	99.2
R19-5	Acidophile	AB841054	*S. misionensis* NBRC 13063^T (AB184285)	99.9
R19-6	Acidophile	AB841055	*S. misionensis* NBRC 13063^T (AB184285)	99.9
R20-1	Acidophile	AB841056	*S. misionensis* NBRC 13063^T (AB184285)	100
R20-5	Acidophile	AB841057	*S. misionensis* NBRC 13063^T (AB184285)	99.7
R21-27	Neutrophile	AB841058	*S. chiangmaiensis* TA4-1^T (AB562507)	99.2
R21-45	Acidophile	AB841059	*S. althioticus* NRRL B-3981^T (AY999791)	99.7

acidophilic strains was 98.6% compared with 92.8% in neutrophiles.

Phosphate solubilization

Among 351 actinomycetes, 266 isolates (75.8%) showed an ability to solubilize phosphate on Pikovskaya agar with the diameters of clear zones ranging from 0.7–1.6 cm. Only six isolates produced clear zones with diameters >1.5 cm. Five isolates were acidophiles and one isolate was neutrophile.

Phosphorus is an important element for plant growth. However, most of the phosphorus in soil are in insoluble forms, which are unavailable for plants. Deficiency of phosphate has negative effect on agricultural yield. Actinomycetes were reported to possess the ability to solubilize phosphate and thus significantly promote the growth of plants.[55,56] In acidic soils, phosphorus limitation is a serious problem because phosphorus forms complexes with metal ions.[57] In this experiment, it was found that 93.9% of the acidophilic group and 48.2% of the neutrophilic group were able to solubilize phosphate. This indicated that phosphate-solubilizing acidophilic actinomycetes might have an important role in the phosphorus cycle in acidic soils.

16S rRNA gene analysis

The 16S rRNA gene sequencing analysis was carried out on 23 non-streptomycete isolates, which were preliminary identified on the basis of morphological characteristics and chemotaxonomic analysis. The resultant sequences were determined and compared with the sequences available in the EzTaxon-e database. The percentages of 16S rRNA gene sequence identity of these isolates to the closest type strain are presented in Table 3. The results revealed that they were members of the following genera: *Allokutzneria* (one isolate, family *Pseudonocardiaceae*), *Amycolatopsis* (five isolates, family *Pseudonocardiaceae*), *Nocardia* (two isolates, family *Nocardiaceae*), *Nonomuraea* (nine isolates, family *Streptosporangiaceae*), *Mycobacterium* (four isolates, family *Mycobacteriaceae*), *Saccharopolyspora* (one isolate,

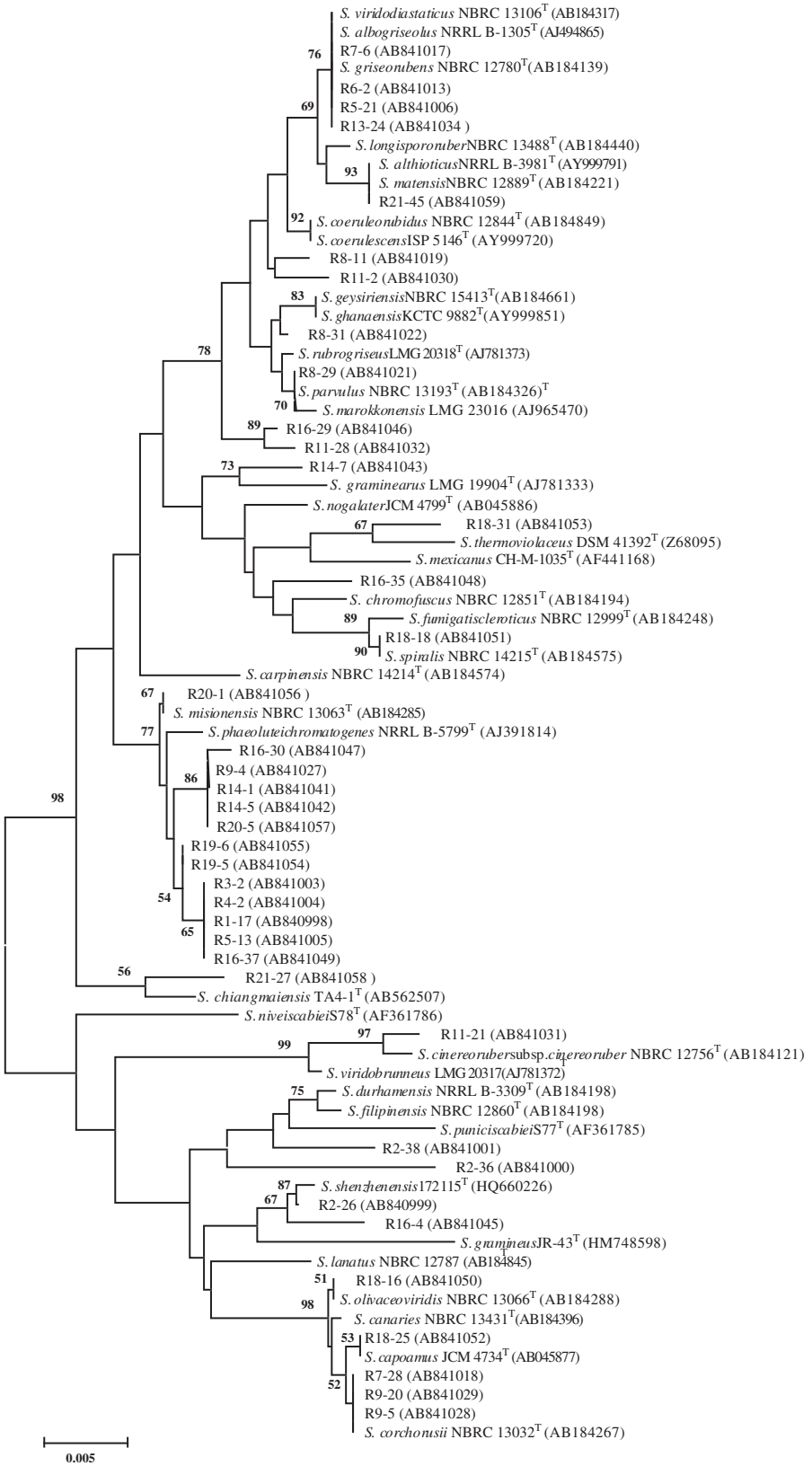

Figure 2 Neighbor-joining tree based on 1422 aligned positions within the 16S rRNA gene, omitting regions of ambiguous alignment, showing relationships between the *Streptomyces* isolates and related *Streptomyces* type strains. Numbers at the nodes indicate bootstrap values based on 1000 replicates; only values >50% are given. The scale bar indicates 0.005 substitutions per nucleotide position.

family *Pseudonocardiaceae*) and *Verrucosispora* (one isolate, family *Micromonosporaceae*). The genus *Nonomuraea* was the most frequently found. Most of non-streptomycete isolates were neutrophiles; the five exceptions were members of the genus *Amycolatopsis* (isolates R8-21, R12-7, R13-25, R13-26 and R15-35), which were acidophiles. Phylogenetic tree constructed based on the 16S rRNA gene sequences, which was supported by high bootstrap values, confirmed their affiliation to these families is shown in Figure 1. Based on colonial characteristics and morphological characteristics under light microscope, another non-streptomycete isolate R13-3, which showed ability to inhibit *F. moniliforme* was presumed a member of isolates R8-21, R13-25, R13-26 and R15-35, which identified to the genus *Amycolatopsis* by 16S rRNA gene sequence analysis.

In this study, one strain of the genus *Allokutzneria* (strain R8-39) was isolated from a rice rhizosphere soil. The genus *Allokutzneria* encompasses only two species, *Allokutzneria albata* and *Allokutzneria multivorans*, which were isolated from soils in the Philippines and China, respectively.[58–60] Only *A. albata* has been reported to produce a highly active antiviral antibiotic (cycloviracin).[58] The strain R8-39 shared the highest 16S rRNA gene similarity of 98.8% to *A. albata* and clustered with *A. albata* and *A. multivorans* in the 16S rRNA gene tree, which formed a well separated clade supported by a 99% bootstrap value in the family *Pseudonocardiaceae* (Figure 1). This observation suggests that the strain R8-39 may represent a new species in the genus *Allokutzneria*. However, detailed polyphasic taxonomic characterization of the strain is required to clarify its status.

Analysis of 16S rRNA gene sequences of 35 representative *LL*-DAP-containing isolates and 4 isolates ,which showed the highest antifungal activity, could be confirmed and assigned to the genus *Streptomyces*. The isolates with high similarity to *S. misionensis* were frequently found in different soil samples (Table 4). Phylogenetic tree of the resulting 16S rRNA gene sequences assigned them into clusters in the *Streptomyces* genus (Figure 2). The biggest cluster consisted of 13 acidophilic strains and the closest neighbor was *S. misionensis*, which had 16S rRNA sequence similarities of 99.5–100%. The 16S rRNA gene sequences of four effective antifungal isolates (R9-4, R14-1, R14-5 and R20-5) also fell into this cluster although these strains had been isolated from different soil samples. These four isolates produced whitish aerial mycelium, grayish spore and yellowish to grayish brown reversed color on acidified ISP medium 3 (pH 5.5). The isolates R9-4, R14-5 and R20-5 produced yellow diffusible pigment which was not detected in R14-1. Melanoid pigment was not produced on ISP medium 7. Microscopic observation revealed the formation of compact spiral chain of spores.

Acidic environments are major selective pressures of members of the genus *Streptacidiphilus*.[49] A previous work using soil samples collected from rice field soil from Thailand reported the isolation of *Streptacidiphilus oryzae* JCM 13271^{T}.[61] However, no member of the genus *Streptacidiphilus* was recovered in this study. This may be due to the fact that the pH of the isolation media not being acidified to the optimum pH for members of the *Streptacidiphilus*.

S. misionensis strains have been previously reported to exhibit antimicrobial activity such as *S. misionensis* strain NRRL 3609, which produced substance that could inhibit growth of various fungi including *Candida albicans* and *Cryptococcus neoformans*.[62] *S. misionensis* strain PMS101 isolated from the rhizosphere of healthy lily bulbs was reported to have antagonistic activity, reducing the incidence of seedling blight caused by *Fusarium proliferatum* and effectively control basal rot caused by *Fusarium oxysporum* f. sp. *lilii*.[63] There is no report on pathogenicity of *S. misionensis* to human, animals or plants. This suggests that *S. misionensis* strains could be used as safe biocontrol agents. In rice field soil, the pH is usually slightly acidic. *S. misionensis* strains R9-4, R14-1, R14-5 and R20-5 were neurotolerant acidophiles that exhibited efficient antagonistic activity against rice pathogenic fungi (*F. moniliforme, H. oryzae* and *R. solani*). In addition, all except R9-4 produced siderophore, and all of them solubilized phosphate. As a result, *S. misionensis* strains R9-4, R14-1, R14-5 and R20-5 are potential candidates for use in a sustainable development of agriculture especially in acidic paddy soil. The evaluation of active metabolites, produced by actinomycetes in acidic conditions, indicates the importance to isolate certain groups of species that have useful biological activities under such condition and may not be usually recovered by standard isolation procedures.

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