**Pseudonocardia pini** sp. nov., an endophytic actinobacterium isolated from roots of the pine tree *Callitris preissii*

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**Abstract**
A Gram-positive, aerobic, actinobacterial strain with rod-shaped spores, **CAP47**\(^T\), which was isolated from the surface-sterilized root of a native pine tree (*Callitris preissii*), grown in South Australia is described. The major cellular fatty acid of this strain was **iso-H-C\(_{16:1}\)** and major menaquinone was **MK-8(H\(_4\)**). The diagnostic diamino acid in the cell-wall peptidoglycan was identified as **meso-diaminopimelic** acid. These chemotaxonomic data confirmed the affiliation of strain **CAP47**\(^T\) to the genus *Pseudonocardia*. Phylogenetic evaluation based on 16S rRNA gene sequence analysis placed this strain in the family *Pseudonocardiaceae*, being most closely related to *Pseudonocardia xishanensis* JCM 17906\(^T\) (98.8%), *Pseudonocardia oroxyli* DSM 44984\(^T\) (98.7%), *Pseudonocardia thailandensis* CMU-NKS-70 \(^T\) (98.7%), and *Pseudonocardia ailaonensis* DSM 44979\(^T\) (97.9%). The results of the polyphasic study which contain genome comparisons of AN\(_b\), AN\(_m\), and digital DNA-DNA hybridization revealed the differentiation of strain **CAP47**\(^T\) from the closest species with validated names. This strain represents a novel species and the name proposed for this microorganism is *Pseudonocardia pini* sp. nov., indicating the source of this actinobacterium from a pine tree. The type strain is **CAP47**\(^T\) (= **DSM 108967**\(^T\) = **NRRL B-65534**\(^T\)). Genome mining revealed that this strain contained a variety of genes encoding enzymes that can degrade hazardous chemicals.

**Keywords** *Pseudonocardia pini* sp. nov. · An endophytic actinobacterium

**Introduction**
The genus *Pseudonocardia* belonging to family *Pseudonocardiaceae* was first proposed by Henssen (1957). It is the only family in the Order *Pseudonocardiales* (Labeda and Goodfellow 2012; Franco and Labeda 2014). At the time of submission of this manuscript, the genus encompasses more than 60 valid species ([https://lpsn.dsmz.de/genus/Pseudonocardia](https://lpsn.dsmz.de/genus/Pseudonocardia)) (Parte et al. 2020). They were discovered from different environmental samples including soil, marine sediments, activated sludge, termite nests, and gold mine core and plant tissues. Most of them were isolated from the soil but there are at least 13 validly named species isolated as endophytes (Parte et al. 2020). *Pseudonocardia* strains were reported for their application in biodegradation including as bioactive compound producers (Warwick et al. 1994; Reichert et al. 1998). The endophytic actinobacterium, strain **CAP47**\(^T\) was discovered in the course of a study which focused on the biodiversity of endophytic actinobacteria from Australian native trees and screening for antimicrobial activity (Kaewkla and Franco 2013). In this report, the description of the phenotypic and genotypic properties including a genome comparative study of a *Pseudonocardia*-like strain, **CAP47**\(^T\), is presented. The data show that this strain represents a novel species, for which the name *Pseudonocardia pini* sp. nov. is proposed.

**Materials and methods**

**Isolation of the actinobacterium**
Root samples of a native pine tree (*Callitris preissii*) grown on the Bedford Park campus of Flinders University,
Adelaide, South Australia (35.024636 S 138.571604 E) were used as the plant source. Sample collection and surface sterilized methods were described by Kaewkla and Franco (2013). Crushed plant tissues were plated onto 10 different isolation media and incubated at 27 °C. The emergence time of each colony was recorded and whole colonies were removed from the isolation plates every week for 12 weeks. Actinobacteria were purified and maintained on half-strength potato dextrose agar (HPDA). 

Genomic DNA of strain CAP47RT was extracted by using GenElute™ (Sigma) and the library was prepared by using a short insert size library. Illumina HiSeq X-ten platform (Illumina) was used to sequence the whole genome of this strain (2 × 150 bp paired-end reads) at the Beijing Genome Institute (BGI), Hongkong. The reads were de novo assembled by using Unicycler (0.4.8) (Wick et al. 2017). The draft assemblies of strain CAP47RT genome have been submitted to GenBank. Genome of strain CAP47RT was annotated by using Prokka version 1.14.5 (Seemann 2014). As there was only one genome of the closest type strain; P. oroxyli DSM 44984T which was available in the public database, strain CAP47RT was studied for genome comparison with only P. oroxyli DSM 44984T. The phylogenetic tree of the genome of strain CAP47RT and related taxa was constructed by using the Type (strain) Genome Server (TYGS) (Meier-Kolthoff et al. 2013; Meier-Kolthoff and Göker 2019). The tree applied with FastME 2.1.6.1 (Lefort et al. 2015) from Genome BLAST Distance Phylogeny (GBDP) distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula $d^4$. The Digital DNA-DNA hybridization (dDDH) value was calculated by applying the Genome-to-Genome Distance calculator (GGDC 2.1; BLAST + method) which formula 2 (identities/HSP length) was applied for the incomplete draft genome (Meier-Kolthoff et al. 2013).

The average nucleotide identity (ANI) value between strain CAP47RT and P. oroxyli DSM 44984T was evaluated with pairwise genome alignment by using ANI-BLAST (ANiB) and ANI-MUMmer (ANIm) algorithms within the JSpeciesWS web service (Richter and Rosselló-Móra 2009; Richter et al. 2016).

### Chemotaxonomic study

For the chemotaxonomic analyses, diaminopimelic acid was detected by two-dimensional thin layer chromatography (TLC) using the method described previously (Bousfield et al. 1985) and whole-cell sugar was analysed by the TLC method of Hasegawa et al. (1983). The extraction and analysis of phospholipids were determined as described by Minnikin et al. (1984) and Komagata and Suzuki (1987) using 5% ethanolic molybdophosphoric acid, α-naphthol, Dragendorff reagent, ninhydrin, and molybdenum blue reagent and periodate-Schiff reagents.

Extraction and purification of isoprenoid quinones were performed using the method of Minnikin et al. (1984) with analysis of the samples by reverse phase LC–MS employing UV detection and electrospray mass spectrometry (ESI) as described by Kaewkla and Franco (2019). Strain CAP47RT and the type strains, P. xishanensis JCM 17906T and P. oroxyli DSM 44984T were studied together for the analysis of whole-cell fatty acids. Cells of all strains were grown for 10 days at 25 °C in Trypticase soy broth (Oxoid) at 150 rpm. Wet cells (100 mg) were saponified, methylated and the fatty acid methyl esters (FAMES) analysed using the MIDI (Microbial Identification) system (Sasser 2001).

### Sequencing, assembly and annotation of genome

Genomic DNA of strain CAP47RT was extracted by using GenElute™ (Sigma) and the library was prepared by using a short insert size library. Illumina HiSeq X-ten platform (Illumina) was used to sequence the whole genome of this strain (2 × 150 bp paired-end reads) at the Beijing Genome Institute (BGI), Hongkong. The reads were de novo assembled by using Unicycler (0.4.8) (Wick et al. 2017). The draft assemblies of strain CAP47RT genome have been submitted to GenBank. Genome of strain CAP47RT was annotated by using Prokka version 1.14.5 (Seemann 2014). As there was only one genome of the closest type strain; P. oroxyli DSM 44984T which was available in the public database, strain CAP47RT was studied for genome comparison with only P. oroxyli DSM 44984T. The phylogenetic tree of the genome of strain CAP47RT and related taxa was constructed by using the Type (strain) Genome Server (TYGS) (Meier-Kolthoff et al. 2013; Meier-Kolthoff and Göker 2019). The tree applied with FastME 2.1.6.1 (Lefort et al. 2015) from Genome BLAST Distance Phylogeny (GBDP) distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula $d^4$. The Digital DNA-DNA hybridization (dDDH) value was calculated by applying the Genome-to-Genome Distance calculator (GGDC 2.1; BLAST + method) which formula 2 (identities/HSP length) was applied for the incomplete draft genome (Meier-Kolthoff et al. 2013).

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Morphological study

Morphological characteristics of strain CAP47T and three type strains, *P. xishanensis* JCM 17906T, *P. oroxyli* DSM 44984T and *P. ailaonensis* DSM 44979T, were compared as described by Shirling and Gottlieb (1966) on 8 different media: ISP 2, ISP 3, ISP 4, ISP 5, ISP 7 (ISP; International Streptomycetes Project), Bennett’s agar, HPDA and Nutrient agar (Atlas and Parks 1993). Strain CAP47T was grown on HPDA for 10 days and sample preparation for the scanning electron micrograph was carried out according to Kaewkla and Franco (2019). Electron microscope (The Philips XL30 Field Emission Scanning Electron Microscope) at Adelaide Microscopy, Adelaide University, Adelaide, South Australia was used to visualize mycelial and spor morphology.

The physiological and biochemical study

The physiological characteristics of strain CAP47T and three closest type strains were studied together by using the following methods. Hydrolysis of skim milk, starch, utilization of four phenolic compounds as sole carbon source, catalase production, assimilation of seven organic acids were followed protocols of Kurup and Schmitt (1973). Acid catalase production, assimilation of four phenolic compounds as sole carbon source, decomposition of esculin, L-tyrosine, and urea were studied according to the methods of Gordon et al. (1974). Growth at different pH between 4 and 10 (in 1 pH unit intervals), temperatures (4, 15, 27, 37, 45 and 55 °C), and NaCl concentrations (1, 3, 5, 10, 15 and 20%, w/v) were evaluated after incubation for 10–14 days on ISP 2 medium (Kurup and Schmitt 1973).

Genome mining

Secondary metabolite Analysis Shell (anti-SMASH) version 5.1.0 (Blin et al. 2009) was applied to reveal biosynthetic gene clusters (BGCs) of strain CAP47T. The in silico approach was also applied to scan for genes relating to bioactive compound and enzyme production, bioremediation, and plant growth promotion. Blastp on the Uniprot database with matrix; blosum62 was used to uncover the gene products and the closest similarity of microorganisms (https://www.uniprot.org/blast; The UniProt Consortium 2019).

Results and discussion

Isolation of strain CAP47T

After surface sterilized root samples were placed on an isolation medium; Humic acid Vitamin B agar (HVA) (Hayakawa and Nonomura 1987), a white small colony of CAP47T emerged on the root tissue after incubation for 6 weeks.

16S rRNA gene analysis and phylogenetic trees construction

The results showed that strain CAP47T was closely embedded within the genus *Pseudonocardia*. Strain CAP47T showed the highest 16S rRNA gene sequence similarity with *P. xishanensis* JCM 17906T (98.8%), *P. oroxyli* DSM 44984T (98.7%), *P. thailandensis* CMU-NKS-70T (98.7%), and *P. ailaonensis* DSM 44979T (97.9%). The phylogenetic position of strain CAP47T was analysed and showed clearly that strain CAP47T was encompassed by other members of the genus *Pseudonocardia*. Strain CAP47T was positioned in a different clade with four closest relative type strains. The closest neighbour was *P. oroxyli* DSM 44984T on both trees with a low bootstrap values at 56 and 41%, respectively. *P. thailandensis* CMU-NKS-70T positioned in the farthest distance on both trees (Fig. 1 and Fig. S1).

Genome comparison

The draft genome sequence of strain CAP47T was 6.05 Mb with DNA G + C content determined by in silico genome sequence as 72.5 mol%. The genome analysis showed the ANIb, ANIm, and dDDH values of the draft genome between strain CAP47T and the type strain *P. oroxyli* DSM 44984T at 81.6, 86.6 and 27.5% (c.t. 25.2–30.0%), respectively. dDDH value was lower than the threshold of 70% used to define species level (Richter and Rosselló-Móra 2009; Chun et al. 2018). In addition, according to the report of Richter and Rosselló-Móra (2009), an ANI value lower than the 95–96% cut-off should delineate different species. The phylogenetic tree based on TYGS revealed the relationship between strain CAP47T and the related type strains. The result clearly showed that strain CAP47T was positioned in the same clade with the closest type strain, *P. oroxyli* DSM 44984T. However, the phylogenetic tree of the genome obviously showed that strain CAP47T was grouped in the different species cluster of this type strain (Fig. 2).

Chemotaxonomic study

The diagnostic diamino acid in the cell-wall peptidoglycan was identified as meso-diaminopimelic acid. Whole-cell sugars were arabinose and galactose. Lipids contained phosphatidylglycerol, phosphatidylmethylethanolamine, including three unknown glycolipids, an unknown phosphoglycolipid, an unknown phosphoglycolipid positive with the ninhydrin spray and two unknown lipids positive with the ninhydrin spray (Fig. S2). Strain CAP47T contained MK-8(H4) (89.7%) as the predominant menaquinone and small amounts of MK-8(H4) (9.7%) as the predominant menaquinone and small amounts of MK-8(H4) (10.3%). The whole-cell fatty acids of strain CAP47T were of the iso- and anteiso-branched fatty acids which were the same pattern with other type strains.
The major fatty acids were **iso-H-C**<sub>16:1</sub> (28.0%), **iso-C**<sub>16:0</sub> (22.3%) and **iso-C**<sub>14:0</sub> (13.8%) Table 1.

### Morphological study

This strain showed morphology belonging to the genus *Pseudonocardia*, with well-developed white aerial mycelium on most media tested. Substrate mycelium was yellowish white on all media used. Cultural characteristics on different media are shown in Supplementary Table S1 (Kornerup and Wanscher 1978). Strain CAP47<sup>T</sup> did not produce melanin pigment on ISP 7 and any diffusable pigment on the media tested. Mycelia fragmented to form zig zag spores, which were straight short rods with a smooth surface (approximately 1 × 5 µm, Supplementary Fig. S3 available in online version).

### The physiological and biochemical study

Other phenotypic data revealed that strain CAP47<sup>T</sup> was different from the closest type strains. CAP47<sup>T</sup> could produce acid from sorbitol but the type strain; *P. oroxyli* DSM 44984<sup>T</sup> could not. On the other hand, the type strains; *P. oroxyli* DSM 44984<sup>T</sup> and *P. xishanensis* JCM 17906<sup>T</sup> could produce acid from mannose, mannotol, raffinose and could hydrolyze starch and assimilate propionate but strain CAP47<sup>T</sup> could not. Also, these two type strains could grow at 37 °C and pH 4 but strain CAP47<sup>T</sup> could not (Table 2).
Genome mining for BGCs and other bioactive compounds

Based on the result of the gene cluster prediction derived from the ‘antiSMASH’ database, gene clusters which share gene similarity with known genes at 50% or higher may be likely to produce a variety of novel bioactive compounds. Strain CAP47RT contained only three BCGs gene clusters including siderophore; bacillibactin (60%), polyketides; alkylresorcinol which possess antioxidant activities (100%) and ectoine relating to drought tolerance (100%) (Supplementary Table S2).

In silico analysis of the genome revealed that strain CAP47RT contained genes encoding enzymes which are capable of degrading recalcitrant chemicals in the environment (Supplementary Table S2). For example, 4-hydroxyacetophenone monoxygenase degrades bisphenol and aromatic ring hydroxylase degrades polycyclic aromatic hydrocarbons (PAH). Alkansulfonate monooxygenase degrades organosulfonate and haloalkane dehalogenase degrades a variety of compounds which are alkanes containing one or more halogens. 2-halobenzoate 1,2-dioxygenase small subunit degrades xenobiotic compounds in the benzoate pathway. DMSO/TMAO reductase degrades dimethyl sulfone (DMSO2) (Reichert et al. 1998). Furthermore, carboxymethylenebutenolidase degrades hexachlorocyclohexane which was dimerized to produce Mirex, a banned organochlorine pesticide. Also, cyclohexanone monooxygenase and 2,4-dichlorophenol 6-monoxygenase degrade pesticides which are carcinogens. In addition, 2-nitropropane dioxygenase degrades 2-nitropropane which causes carcinomas and nitroreductase degrades polynitroaromatic explosive, 2,4,6-trinitrotoluene (TNT). The many genes relating to xenobiotic compounds degradation revealed that this strain can be applied to remove hazardous chemicals from the environment. Strain CAP47RT showed this ability which corresponds to many species of the genus Pseudonocardia (Huang and Goodfellow 2012).

Moreover, strain CAP47RT contained genes encoding universal stress protein and enzymatic free radical scavengers in the management of oxidative stress. Biodegradation enzymes including a variety of proteases and xylanases were also detected. Interestingly, strain CAP47RT contained genes encoding to polyhydroxyalkanoate synthase and poly-beta-hydroxybutyrate polymerase which relate to polyhydroxybutyrate (PHB) production; an alternative for production of biodegradable plastics (Getachew and Woldesenbet 2016).

**Conclusion**

In conclusion, strain CAP47RT can be distinguished from other members of the genus Pseudonocardia; P. xishanensis JCM 17906 T, and P. oroxyli DSM 44984 T. The many genes relating to xenobiotic compounds degradation revealed that this strain can be applied to remove hazardous chemicals from the environment. Also, strain CAP47RT contained genes relating to PHB production which can be applied to produce bioplastic.

**Description of Pseudonocardia pini sp. nov.**

Pseudonocardia pini (pi’ni. L. gen. n. pini of pine, isolated from root of pine tree) Aerobic, Gram-stain positive, non-acid/alcohol-fast. Catalase positive. Growth occurs between 25 and 32 °C but grows well at 27 °C. Growth occurs between pH 5.0 and 10.0 but grows well at pH 7.0. Growth occurs in the presence of NaCl 5% (w/v).
Colonies are small and tough and aerial mycelium develops well. Aerial mycelium fragments into long rod-shaped spores with a smooth surface (approximately 1 × 5 µm). Colony colour is yellowish white to pale white. Diffusible pigments are not produced on any medium used. Cells can produce acid from arabinose, fructose, fucose, galactose, glucose, maltose, myo-inositol, ribose, sucrose and trehalose but not from cellulose, meso-erythritol. Strain CAP47RT hydrolyzes esculin and urea. All strains could assimilate acetate, citrate, L-lactate, malate but not toluene, phenol, and benzene as sole carbon source. All strains could grow at 1 and 3% NaCl (w/v), pH between 5 and 10 and at 27 °C. They could not grow at 15 and 20% NaCl (w/v), at 4, 45 and 55 °C.

Strain: 1, Pseudonocardia pini CAP47RT; 2, Pseudonocardia orxyli DSM 44984T; 3, Pseudonocardia xishanensis ICM 17906T and 4, Pseudonocardia ailaonensis DSM 44979T. +, positive or present; w, weakly positive; −, negative or absent. Catalase was positive for all strains. All strains could produce acid from arabinose, fructose, fucose, galactose, glucose, maltose, myo-inositol, ribose, sorbitol, sucrose and trehalose but not from cellulose and meso-erythritol. They could hydrolyze esculin and urea. All strains could assimilate acetate, citrate, L-Lactate, malate but not benzoate and tartrate. They could not use toluene, phenol, and benzene as sole carbon source. All strains could grow at 1 and 3% NaCl (w/v), pH between 5 and 10 and at 27 °C. They could not grow at 15 and 20% NaCl (w/v), at 4, 45 and 55 °C.

### Table 2

| Characteristics | 1 | 2 | 3 | 4 |
|----------------|---|---|---|---|
| Acid production from Mannose | − | + | + | + |
| Mannitol | − | + | + | + |
| Methyl-D-glucopyranoside | − | − | + | − |
| Raffinose | − | + | + | + |
| Sorbitol | + | − | − | − |
| Xylose | − | − | − | + |
| Growth at NaCl 5% (w/v) | w | + | w | − |
| NaCl 10% (w/v) | − | w | − | − |
| 15 °C | − | w | w | w |
| 37 °C | − | + | − | − |
| pH 4 | − | + | w | − |
| Use of organic acid | − | − | − | + |
| Use of phenolic compounds | − | − | − | + |
| Acid production from Mannose | − | + | + | + |
| L-tyrosine | − | − | − | w |
| Skim milk | − | + | + | − |
| Starch | − | + | + | + |
| Propionate | − | + | + | + |
| Growth at Use of organic acid | − | − | − | + |

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00203-021-02309-3.

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**Author contributions** OK and CF planned the experiments. OK carried out the experimental work and prepared the draft manuscript. CF contributed to improving the manuscript and provided the facilities.

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**Declarations**

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** This article does not contain any studies with human participants and/or animals performed by any of the authors. Formal consent is not required in this study.

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