A bacterial strain, designated PS9T, was isolated from soil in the Ryukyu Archipelago, Japan. The bacterium grew with racemic phenylsuccinate as the sole carbon and energy source. Phylogenetic analysis based on the 16S rRNA gene sequence showed that strain PS9T was closely related to *Citricoccus muralis*, *C. alkalitolerans* and *C. nitrophenolicus* with sequence similarities of 97.5, 97.8, and 98.3%, respectively, suggesting that the strain belonged to the genus *Citricoccus*. Strain PS9T was a Gram-positive, non-motile, circular-shaped and aerobic bacterium. The major respiratory quinone was MK-8 (H$_2$) and the predominant cellular fatty acid was C$_{15:0}$ anteiso, C$_{17:0}$ anteiso, and C$_{19:0}$ iso. The G+C content was 72.4 mol%. Based on the phylogenetic and phenotypic traits, it was concluded that the organism represents a new species in the genus *Citricoccus*, with the name *Citricoccus yambaruensis*. The type strain is PS9T (=NBRC102121T = DSM18783T).

**Key Words**——actinomycetes; *Citricoccus* sp.; phenylsuccinate

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**Introduction**

Carboxylate-substituted aromatic or heterocyclic (Nakano et al., 1999) compounds with high optical purity are one of the interesting targets either for biodegradation (Cao et al., 2008) or bioconversion (Yatake et al., 2008). In our attempt to find racemic phenylsuccinate (PS)-degrading bacteria, we succeeded in isolating two actinomycetes showing opposite stereospecific degradation from the soil in the Ryukyu Archipelago. They attacked PS from either side of the carboxylic acid moiety, followed by degradation via benzoate or phenylacetate (Matsui et al., 2009). While phylogenetic analysis based on the 16S rRNA gene sequence revealed that the one isolate, designated strain PS17, was *Microbacterium* sp., the other one, PS9T, was closely related to the genus *Citricoccus* originally proposed by Altenburger et al. (2002), with emendation for the quinone system by Nielsen et al. (2011). The members of the genus *Citricoccus* are all Gram-positive cocci and share the following chemotaxonomic characteristics: MK-9(H$_2$) and/or MK-8(H$_2$) are contained as the predominant menaquinone; diphosphatidylglycerol, phosphatidylglycerol, phos-
phatidylinositol and several unknown lipids are the major polar lipids; and anteiso-C15:0, anteiso-C17:0, iso-C16:0 and iso-C15:0 are the major cellular fatty acids (Altenburger et al., 2002; Li et al., 2005; Nielsen et al., 2011). The newly isolated strain possessed similar traits to these members, suggesting that it should be classified under the genus *Citricoccus*. However, some differences were obviously found between the isolate and related *Citricoccus* spp.

**Materials and Methods**

*Isolation and cultivation.* Approx. 200 mg soil taken from forest, river, seaside, and agricultural fields in the north part of Okinawa’s main island was inoculated into 4 ml of Minimal (MM) medium (Matsui and Furuhashi, 1995) with 1 g/L of *R,S*-phenylsuccinate (PS) as the sole carbon and energy source, and incubated aerobically by shaking at 180 rpm at 30°C for 2 weeks. 0.1 ml of the initial enrichment was transferred into the same fresh medium (4 ml). This transfer was repeated three times, and the enrichment culture was established. Strain PS9T was isolated from the enrichment using Nutrient Agar (Oxoid, Ltd., UK) (NA) plates.

*Physiological and chemotaxonomic characterization.* Physiological and biochemical tests were performed using the API systems (APICORYNE, bioMerieux, France), according to the manufacturer’s instructions. The test for organic substrate-oxidation activities was analyzed by the BioLOG system (GSI Creos, Japan). The respiratory quinones, cellular fatty acids and G+C content of the isolate were analyzed by the method described by Hanada et al. (2002) with cells grown in Nutrient Broth (NB, Oxoid, Ltd., UK) at 30°C. The cells grown in liquid NB medium were non-sporeforming oval to circular, 0.8–1 μm diameter (Fig. S1).

**16S rRNA gene sequence-based phylogenetic analyses.** Genomic DNA was extracted with ISOLPLANT (Nippon Gene, Tokyo, Japan) and used for polymerase chain reaction (PCR). The 16S rRNA gene locus was amplified by PCR using EX Taq DNA polymerase (TaKaRa-Bio, Kyoto, Japan) and the PC818 thermal cycler (Astec Co., Fukuoka, Japan). The PCR primers were EUB11f (5’T-GTT GTC GAT CCM TGG CTY AG-3’) and EUB1511r (5’T-GTA CAA CCT TGG TAC GAC TT-3’). The initial denaturation step was carried out at 94°C for 5 min; it was followed by 35 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1.5 min; and the last elongation step was performed at 72°C for 10 min. The PCR-amplified 16S rRNA gene fragments (approximately 1.5 kb long) were purified by agarose gel electrophoresis, ligated into the pT7blue vector (Novagen Merck Co., Germany), and used for the transformation of *Escherichia coli* JM109 (TaKaRa-Bio, Kyoto, Japan). The nucleotide sequences of both strands of the 16S rRNA genes were determined by using the M13-47 and RV-M primers (Novagen Merck Co., Germany) and the ABI model 3100 and BigDye terminator kit, version 1.1 (Applied Biosystems, Inc., CA), according to the manufacturer’s instructions. For phylogenetic analysis of the strain PS9T, CLUSTAL W program version 1.4 (Thompson et al., 1994) was used to align the 16S rRNA gene sequences. The tree was constructed on the basis of the evolutionary distances calculated by using the neighbor joining method (Saitou and Nei, 1987) with the Kimura two-parameter model (Kimura, 1980). The neighbor-joining analysis was performed with the CLUSTAL W program. All gaps in the alignment were excluded; 1,362 bases were compared. The sequences of the 16S rRNA fragments obtained from strains PS9T have been assigned the DNA Data Bank of Japan (DDBJ)/European Molecular Biology Laboratory (EMBL)/GenBank accession number AB473562.

**Results and Discussion**

*Morphology of the colonies grown on the NA plate* at 30°C for 48 h was a smooth surface of light yellow. The cells grown in liquid NB medium were non-spore-forming oval to circular, 0.8–1 μm diameter (Fig. S1).
The cells had no flagella and showed no motility. Neither finger-like nor amorphous flocculation was observed in liquid culture. On the NA plate, strain PS9<sup>T</sup> grew optimally at 25–30°C (growth was occurred between 10 and 37°C). The isolate had tolerance up to 8% NaCl, but could not grow in a 9% NaCl-containing NA medium. The isolate showed the following activities. Catalase, pyradinamidase, and β-glucuronidase were positive; neither nitrate reduction nor gelatin hydrolysis was observed; N-acetyl-β-glucosaminidase, alkaline phosphatase, β-galactosidase, α-glucosidase, β-glucosidase, pyrollonylarylamidase, were negative. The test for organic substrate-oxidation activities revealed that the cells aerobically oxidized various sugars and sugar alcohols, i.e. L-alanine, L-alanyl-glycine, acetic acid, 2'-deoxyadenosine, dextrin, L-glutamic acid, β-hydroxy butyric acid, p-hydroxy phenyl acetic acid, D-mannose, 3-methyl glucose, β-methyl-D-glucoside, pyruvic acid, D-raffinose, D-sorbitol, stachyose, succinamic acid, turanose, Tween 40, Tween 80, and uridine. This isolate, however, did not oxidize the following compounds: N-acetyl L-glutamic acid, N-acetyl-D-mannosamine, adenosine, adenosine 5'-monophosphate, D-alanine, amygdalin, arabinose, arabitol, arbutin, L-asparagine, cellobiose, α- and β-cyclodextrin, fructose, D-fructose 6-phosphate, fucose, gentibiose, glucose, α-D-glucose-1-phosphate, glycerol, D,L-α-glycerol-phosphate, glycogen, glycyl-L-glutamic acid, α- or γ-hydroxybutyric acid, inosine, inositol, inulin, α-ketoglutaric acid, α-ketovaleric acid, lactamide, lactic acid methyl ester, lactose, malic acid, maltose, maltotriose, mannitol, mannan, melezitose, α-methyl-D-galactoside, β-methyl-D-galactoside, α-methyl-D-glucoside, α-methyl-D-mannoside, methylypyruvate, methylsuccinate, paratinosine, propionic acid, psicose, putrescine, L-pyroglutamic acid, rhamnose, ribose, salicin, sedoheptulose, succinic acid, sucrose, tagatose, thymidine, thymidine 5'-monophosphate, trehalose, uridine 5'-monophosphate, xyitol, or xylose.

The phylogenetic tree (Fig. 1) based on the 16S
rRNA gene sequence was constructed by the neighbor joining method (Saito and Nei, 1987) with the Kimura two-parameter model as a distance corrector (Kimura, 1980). The tree showed that the isolate belonged to the order Actinomycetales of the class Actinobacteria with the genera Micrococcus, Citricoccus and Arthrobacter as the closest relatives. The sequence similarities between strain PS9\(^T\) and its relatives were as follows: Citricoccus nitrophonolicus PNP1\(^T\), 98.3%, C. alkalitolerans YIM70010\(^T\), 97.8%; C. muralis 4-0\(^T\), 97.5%, Micrococcus luteus DSM20030\(^T\), 96.8%. Phylogenetic analysis clearly suggested that the isolate represented at least a new species in the genus Citricoccus.

Strain PS9\(^T\) contained MK8 (H\(_2\)) and MK9 (H\(_2\)) as the respiratory quinone (86.0 and 10.7%, respectively). This result was consistent with the description of Altenburger et al. (2002) emended by Nielsen et al. (2011). An unknown menaquinone (MK-n) clearly different from MK7 (H\(_2\)) was also detected (3.3%). The fatty acid methyl ester analysis of the isolate along with two related species revealed that all three strains had a similar fatty acid composition. The main cellular fatty acid in strain PS9\(^T\) was C\(_{15:0}\) anteiso, which accounted for 55.3% of total cellular fatty acids (Table S1). C\(_{17:0}\) anteiso (24.2%), and C\(_{16:0}\) iso (9.1%) were also detected as the next major components. The G+C content of the DNA of strain PS9\(^T\) was 72.4 mol%.

Differential characteristics of strain PS9\(^T\) and its

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>PS9(^T)</th>
<th>1(^a)</th>
<th>2(^b)</th>
<th>3(^c)</th>
<th>4(^d)</th>
<th>5(^e)</th>
</tr>
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<tbody>
<tr>
<td>Growth at 9% NaCl</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Carbon utilization</td>
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<tr>
<td>galactose</td>
<td>-</td>
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<td>+</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td>PS(^f)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Quinones</td>
<td>Major</td>
<td>MK8(H(_2))</td>
<td>MK9(H(_2))</td>
<td>MK9(H(_2))</td>
<td>MK9(H(_2))</td>
<td>MK9(H(_2))</td>
</tr>
<tr>
<td>Others</td>
<td>MK9(H(_2))</td>
<td>MK9(H(_2))</td>
<td>n.i.</td>
<td>MK8(H(_2))</td>
<td>MK8(H(_2))</td>
<td>MK8(H(_2))</td>
</tr>
<tr>
<td>G+C content (mol%)</td>
<td>72.4</td>
<td>68</td>
<td>67.8</td>
<td>66</td>
<td>67.8</td>
<td>68.1</td>
</tr>
</tbody>
</table>

Strains are indicated as: 1, C. muralis; 2, C. alkalitolerans; 3, C. zhaacienensis; 4, C. parietis; 5, C. nitrophonolicus; n.i., no information.

\(^a\) Data from Altenburger et al. (2002), \(^b\) Data from Li et al. (2005), \(^c\) Data from Meng et al. (2010), \(^d\) Data from Schaefer et al. (2010), \(^e\) Data from Nielsen et al. (2011), \(^f\) Racemic phenyl succinate (results obtained in this study), \(^g\) Unknown menaquinone.
two unknown phospholipids and an unknown lipid, but phospha-tidylserin is not detected and phosphatidylserine is detected in the type strain of *C. yambaruensis*. The major fatty acids are ai-C_{15:0}, ai-C_{17:0}, iC_{16:0}.

**Description of *Citricoccus yambaruensis* sp. nov.** (yam. ba.ru.en’nsis. N.L. adj. *yambaruensis* referring to the Yambaru area of Okinawa Prefecture where the strain was isolated from).

Cells are Gram-positive, non-motile, non-spor-forming cocci, 0.8–1.0 μm in diameter. Aerobic, oxidase-negative and catalase positive, and no urease, α-glucosidase is produced. Nitrate is not reduced to nitrite. Growth occurs at 10–37°C, 0–8% NaCl and pH 4–12. D-Arabinose can support its growth. The menaquinone system contains MK-8 (H_{2}), MK-9 (H_{2}), and an unknown menaquinone. Polar lipids are diphospha-tidylglycerol, phosphatidylglycerol, and phosphatidyldserine. The peptidoglycan type is Ala/Gly/Glu/Lys, N-acetylβ-glucosaminidase, or α-glucosidase is produced. Nitrate is not reduced to nitrite. Growth occurs at 10–37°C, 0–8% NaCl and pH 4–12. D-Arabinose can support its growth. The menaquinone system contains MK-8 (H_{2}), MK-9 (H_{2}), and an unknown menaquinone. Polar lipids are diphasphatidylglycerol, phosphatidylglycerol, and phosphatidyldserine. The peptidoglycan type is Ala/Gly/Glu/Lys, although Orn and meso-diaminopimelic acid (corresponding to the case of A4β) and A4γ respectively were not detected. Therefore, the diamino acid (Lys) should be in position 3 (corresponding to variation A4) (Schleifer and Kandler, 1972). The genomic G+C content is 72.4 mol%. 16S rRNA gene sequence analysis indicates the genus *Citricoccus* is a member of the family *Micrococccaceae* of *Actinobacteria*. The cells oxidize various sugars and sugar alcohols, i.e. L-alanine, L-alanyl-glycine, acetic acid, 2’-deoxyadenosine, dextrin, L-glutamic acid, β-hydroxy butyric acid, p-hydroxy phenyl acetic acid, D-mannose, 3-methyl glucose, β-methyl-D-glucoside, pyruvic acid, D-raffinose, D-sorbitol, stachyose, succinamic acid, turanose, Tween 40, Tween 80, and uridine. This isolate, however, does not oxidize the following compounds: N-acetyl L-glutamic acid, N-acetyl-D-mannosamine, adenosine, adenosine 5’-monophosphate, D-alanine, amydalin, arabinose, arbutin, L-asparagine, cellobiose, α- or β-cyclodextrin, fructose, D-fructose 6-phosphate, fucose, gentibiose, glucose, α-D-glucose-1-phosphate, glycerol, D,L-α-glycerol-phosphate, glycerogen, glycyrl-L-glutamic acid, α- and γ-hydroxybutyric acid, inosine, inositol, inulin, α-ketoglutaric acid, α-ketovaleric acid, lactamide, lactic acid methyl ester, lactose, malic acid, maitose, maltotriose, mannotol, mannan, melezitose, α-methyl-D-galactoside, β-methyl-D-galactoside, α-methyl-D-glucoside, α-methyl-D-mannoside, methylpyruvate, methylsuccinate, paratinose, propionic acid, psicose, putrescine, L-pyroglutamic acid, rhamnose, ribose, salicin, sedoheptulose, succinic acid, sucrose, tagatose, thymidine, thymidine 5’-monophosphate, trehalose, uridine 5’-monophosphate, xylitol, or xylose. In addition, sucrose, glycerogen, acetate, starch and racemic phenyl succinate are assimilated. D-Arabinose, L-arabinose, ribose, D-xyllose, D-xylene, galactose, glucose, fructose, mannose, mannitol, sorbitol, N-acetyl glucosamine, amygdalin, salicin, cellobiose, maltose, lactose, glycone, and L-lysine are not assimilated. The major fatty acids are ai-C_{15:0}, ai-C_{17:0}, i-C_{17:0}, i-C_{18:0}, and i-C_{15:0}.

**Supplementary Materials**

Fig. S1. Phase-contrast photomicrograph of strain PS9T cells grown aerobically at 30°C in NBG medium.

Bar, 2 μm. Optical microscope type BX50F4 (Olympus, Tokyo, Japan) was used for the photomicrograph.

Fig. S2. Alignment of the 16S rRNA gene of strain PS9T with the family.


Table S1. Whole cell fatty acid composition of strain PS9T and related strains.

|---------|----------------|------------------------|--------------------|----------------|------------------------|
| Strains are indicated as: 1, *C. muralis*; 2, *C. alkalitolerans*; 3, *C. zhacaleinis*; 4, *C. peretis*; 5, *C. nitrophenolicus*.

Supplementary figures and table are available in our J-STAGE site (http://www.jstage.jst.go.jp/browse/jgam).

**References**


