Subtercola endophyticus sp. nov., a cold-adapted bacterium isolated from Abies koreana

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Abstract

A novel Gram-stain-positive, aerobic bacterial strain, designated AK-R2A1-2^T, was isolated from surface-sterilized needle leaves of an *Abies koreana* tree. Strain AK-R2A1-2^T was closely related to *Subtercola boreus* K300^T and *Subtercola lobariae* 9583b^T with 97.3% and 96.7% 16S rRNA gene sequence similarities, respectively, and it formed a distinct phyletic lineage from these two strains. Growth of strain AK-R2A1-2^T was observed at 4–25°C with pH 5.0–8.0. Strain AK-R2A1-2^T contained menaquinone 9 (MK-9) and menaquinone 10 (MK-10) as the predominant respiratory quinones. The major cellular fatty acids were anteiso-$C_{15:0}$ and summed feature 8 ($C_{18:1}\omega7c$ or/and $C_{18:1}\omega6c$), and the polar lipids comprised diphosphatidylglycerol (DPG), and three unknown aminolipids (AKL2, AKL3, and AKL4). The complete genome of strain AK-R2A1-2^T was sequenced to understand the genetic basis of the survival strategy of the strain in low temperatures. Multiple copies of cold-associated genes involved in cold-active chaperon, stress response, and DNA repair supported survivability of the strain at low temperatures. Finally, this strain could significantly improve rice seeding growth under low temperatures. Strain AK-R2A1-2^T is concluded to represent a novel species of the genus *Subtercola*, and the proposed name is *Subtercola endophyticus* sp. nov. The type strain is AK-R2A1-2^T (= KCTC 49721^T = GDMCC 1.2921^T).

Introduction

Abies koreana, is a species of native fir that grows on the high mountains (1000–1900 m) of South Korea[1]. The tree is tolerant to alkaline soils and prefers to grow at cool locations with heavy winter snowfall[2]. *Abies koreana* was classified as endangered by the International Union for Conservation of Nature due to many unverified reasons leading to a massive decline in numbers and sudden dried death of these trees. Simultaneously, the tree has potential applications in traditional medicine to treat colds, stomachache, indigestion, and rheumatic disease[3]. Other important secondary metabolites of *Abies koreana*, such as essential oils, have exhibited antioxidant, antimicrobial, and anti-inflammatory activities[1, 4].

The genus *Subtercola*, a member of the family *Microbacteriaceae* within the class *Microbacteriales*, was first proposed by Männistö et al.[5] with the type species *Subtercola boreus* DSM 13056^T. In January 2022, this genus contained four child taxa with validly published names (https://lpsn.dsmz.de/genus/subtercola). Species of the genus *Subtercola* have been isolated from extremely high-altitude cold environmental niches, including groundwater[5], snowy mountains[6], and lakes[7]. Members of the genus grow optimally at low temperatures[5–7] and are aerobic, Gram-stain-positive and non-endospore-producing, with pale to yellow, circular, convex, and smooth colonies. The polar lipids of this genus are phosphatidylglycerol, diphosphatidylglycerol, one unknown phospholipid, and two glycolipids[5], the major fatty acid is anteiso-$C_{15:0}$, and MK-9 and MK-10 are the major respiratory quinones[5–7]. The genus *Subtercola* has a high G + C content, ranging from 64.4 to 69.0% (https://ncbi.nlm.nih.gov/genome/?term=subtercola).

The swift rise of omics-approaches has facilitated the investigation of microbial diversity and plant host-endophyte interactions across diverse ecological communities. Many endophytes possess the activity of biosynthesis of novel compounds to maintain a mutualistic relationship. During an investigation of the bacterial community of *Abies koreana* trees, with the aim of finding plant-microbe interactions and exploring some important secondary metabolites, 81 endophytic strains were isolated from surface-sterilized needle leaves of *Abies koreana*. One of the novel strains, designated AK-R2A1-2^T, was selected for further exploration in the present study. The taxonomic status of strain AK-R2A1-2^T was evaluated based on phenotypic, phylogenetic, genotypic, and chemotaxonomic data. Moreover, genome annotation indicated that this strain has multiple copies of cold-associated genes and the potential to produce secondary metabolites with antioxidant activities. Finally, the plant growth promotion effects of strain AK-R2A1-2^T were assessed in rice as a cold-sensitive crop. Our study indicated that novel species in *Subtercola* isolated from *Abies koreana* growing in Mount Halla have potential application values in the cultivation of crops.

Results And Discussion

Phylogenetic analysis

The assembled 16S rRNA gene amplicon of strain AK-R2A1-2^T was 1457 base pairs (bp). Comparative sequence analysis showed that strain AK-R2A1-2^T appeared to belong to the genus *Subtercola*, sharing the highest 16S rRNA gene sequence similarity with *S. boreus* DSM 13056^T (97.3%), followed by 96.7% sequence similarity with *Agreia bicolorata* VKM Ac-1804^T, *Agreia pratensis* VKM Ac-2510^T, and *S. lobariae* KCTC 33586^T. Sequence similarities with the other two members of the genus *Subtercola* were 96.2% and 96.1%. Strain AK-R2A1-2^T was regarded as a novel species of the genus *Subtercola* based on the novel species recognition threshold value of 98.6%[8]. To identify the phylogenetic location of strain AK-R2A1-2^T, phylogenetic trees based on 16S rRNA gene sequences were reconstructed using the MEGA program. The NJ phylogenetic tree showed that strain AK-R2A1-2^T formed a cluster with *S. boreus* DSM 13056^T, and all members of the genus *Subtercola* comprised a single group. The ML and MP phylogenetic trees supported this result (Fig. 1). Thus, strain AK-R2A1-2^T was confirmed as a member of the genus *Subtercola* rather than a member of the genus *Agreia*. Based on the 16S rRNA gene sequence similarity and phylogenetic analysis, strains *S. boreus* DSM 13056^T, *S. vilae* DSM 105013^T, *S. frigoramans* KCTC 49696^T, and *S. lobariae* KCTC 33586^T were selected for further comparative study under the same conditions.

Phenotypic characteristics

Phenotypic characteristics including optimal growth medium, temperature, pH, and salt tolerance were investigated. Strain AK-R2A1-2^T grew well on R2A, PD, MEA, YEP, ISP2, and GYM (optimum growth on PD and R2A), but did not grow on MA, LB, NA, or TSA. Cells of strain AK-R2A1-2^T were short rod-shaped without flagella (0.2–0.3 μm in width and 0.3–1.6 μm in length, Fig. S1), and were Gram-stain-positive,

non-motile, catalase-positive, and oxidase-negative. Growth of strain AK-R2A1-2^T was observed on R2A broth medium at 4–25°C (optimal 20°C), at pH 5–8 (optimal 5), and with 0–1% NaCl (optimal 0%). Differential physiological characteristics of strain AK-R2A1-2^T that distinguished it from closely related strains are shown in Table 1.

Chemotaxonomy features

The cellular fatty acid profiles (>1%) of strain AK-R2A1-2^T and closely related strains are shown in Table 2. The major fatty acids (>10%) of strain AK-R2A1-2^T were anteiso-$C_{15:0}$ (12.3%) and summed feature 8 (76.7%). This was most similar to *S. lobariae* KCTC 33586^T, which also contained anteiso-$C_{15:0}$ (25.8%) and summed feature 8 (52.1%) as the major fatty acids. The major fatty acids of *S. boreus* DSM 13056^T were anteiso-$C_{15:0}$ (46.0%), iso-$C_{15:0}$ (20.7%), 2-OH $C_{14:0}$ (10.4%), and summed feature 8 (11.8%), while *S. vilae* DSM 105013^T contained anteiso-$C_{15:0}$ (64.1%), iso-$C_{16:0}$ (12.6%), and 2-OH $C_{14:0}$ (15.6%) as major fatty acids, and *S. frigoramans* KCTC 49696^T contained summed feature 8 (40.4%) and summed feature 3 (30.2%) as major fatty acids. The differences in major and minor fatty acids can differentiate strain AK-R2A1-2^T from other closely related members of the genus *Subtercola* (Table 2). The respiratory quinones of strain AK-R2A1-2^T were menaquinone 9 (MK-9) and menaquinone 10 (MK-10), which is congruent with other closely related members of the genus *Subtercola*. The major polar lipids in strain AK-R2A1-2^T were diphosphatidylglycerol (DPG) and three unknown aminolipids (AKL2, AKL3, AKL4). The polar lipids profile of strain AK-R2A1-2^T was simple compared with those of type strains of other species of the genus *Subtercola* (Fig. S2).

Genome analyses

General and functional features of the genome of strain AK-R2A1-2^T

After being assembled by Canu (version 1.7) de novo assembler, the whole genome of strain AK-R2A1-2^T comprised a single circular chromosome of 4,318,731 bp, and the N50 value was 12,176 bases, with coverage of 95×. The G+C content calculated based on the respective whole-genome sequence was 65.8%. Comparison of two copies of 16S rRNA gene fragments with the whole-genome sequence indicated that DNA sequence contamination did not occur during genome assembly of strain AK-R2A1-2^T. The whole-genome sequence of strain AK-R2A1-2^T was deposited to NCBI under accession number CP087997.1. The genome sequence was annotated with RAST[9, 10], and protein-coding sequences were determined using PGAP[11]. The NCBI PGAP annotation revealed that the genome of strain AK-R2A1-2^T contains 3,874 protein-coding genes and 56 RNA genes, including two 5S rRNA genes, two 16S rRNA genes, two 23S rRNA genes, three non-coding RNA (ncRNA) genes, and 47 tRNA genes (Table S1). A total of 3,674 protein-coding sequences (CDs) were annotated by cluster orthologous group (Fig. 2). The largest group of CDs was classified as unknown (1,106 of total CDS, 30.1%), while the groups with the lowest numbers of CDs were classified as cytoskeleton and RNA processing and modification (1 of total

CDS, 0.02%). Transcription (8.9%) and carbohydrate transport and metabolism (7.8%) were the groups with the next highest numbers of CDs annotated from the whole genome (Fig. 2 and Fig. S3).

A whole-genome-based phylogenetic tree was also reconstructed using the UBCG program (version 3.0). The whole genome-based phylogenetic tree indicated that strain AK-R2A1-2^T forms a subgroup with *S. lobariae* 9583b^T, and that all members of the genus *Subtercola* comprise one group (Fig. 3). Concurrently, members of the genus *Agreia* form a single cluster, consistent with the phylogenetic tree based on 16S rRNA gene sequences. These findings support that strain AK-R2A1-2^T represented a novel species of the genus *Subtercola*.

Genomic features associated with cold-adaption of strain AK-R2A1-2^T

The RAST server, PGAP, and BlastKOALA pipeline were used to perform genome annotation and determine the metabolic pathways in strain AK-R2A1-2^T. Information in the following sections is predicted from the genome sequence analyses.

Stress response: Members of the genus *Subtercola* grow well in low temperatures, and although strain AK-R2A1-2^T was isolated at 25°C, it was proven to grow at 4°C; the type strains of other members of this genus can even grow at –2°C[5]. The potential mechanism(s) by which bacteria invoke various stress responses to adapt to environmental changes have yet to be fully elucidated. Bacterial adaptations to low temperature to facilitate survival include cell membrane fluidity adaptation (increased unsaturated fatty acids), protein response (refolding cold-damaged proteins), and so on[12]. Cold-shock proteins (Csp) are produced by bacteria in response to a rapid decrease in temperature, with the Csps acting as RNA chaperones to help prevent mRNA misfolding during cold stress[13]. The PGAP analysis in the current study showed that the genome of strain AK-R2A1-2^T contained two genes encoding a cold-shock protein (UFS59601.1; UFS60376.1) and a cold shock domain-containing protein (UFS59609.1). When whole-genome mining of strain AK-R2A1-2^T was performed via RAST SEEDS and BlastKOALA, many well-studied, cold-inducible genes[14-20] were annotated by the BlastKOALA pipeline, including *aceE* (encoding pyruvate dehydrogenase E1 component [EC:1.2.4.1]), *aceF* (pyruvate dehydrogenase E2 component, dihydrolipoamide acetyltransferase [EC:2.3.1.12]), *cspA* (cold shock protein), *deaD* (ATP-dependent RNA helicase DeaD [EC:3.6.4.13]), *dnaA* (chromosomal replication initiator protein), *gyrA* (DNA gyrase subunit A [EC:5.6.2.2]), *hupB* (DNA-binding protein HU-beta), *infA* (translation initiation factor IF-1), *infB* (translation initiation factor IF-2), *infC* (translation initiation factor IF-3), *nusA* (transcription termination/antitermination protein NusA), *otsA* (trehalose 6-phosphate synthase [EC:2.4.1.15 2.4.1.347]), *otsB* (trehalose 6-phosphate phosphatase [EC:3.1.3.12]), *pnp* (polyribonucleotide nucleotidyltransferase [EC:2.7.7.8]), *rbfA* (ribosome-binding factor A), *recA* (recombination protein RecA), and *tig* (trigger factor) (Table 3). Compatible solutes, a group of compounds that are important for osmotic stress and cold shock, can reduce membrane stress via various mechanisms. Glycine betaine, choline, and proline, which are recognized cold-protective solutes[21, 22], were annotated from the whole genome of strain AK-R2A1-2^T. In addition, genes encoding for the synthesis of the osmoprotectant proline from glutamate (*proA*,

glutamate-5-semialdehyde dehydrogenase [EC:1.2.1.41]; *proB*, glutamate 5-kinase [EC:2.7.2.11]; *proC* (pyrroline-5-carboxylate reductase; [EC:1.5.1.2]), two choline dehydrogenases (*betA*, [EC:1.1.99.1]), and the glycine betaine transport system (*opuC*, *opuBD*, *opuA*) were identified in the genome of AK-R2A1-2^{T}[23-27] (Table 3). From the RAST SEED annotation of strain AK-R2A1-2^{T} (Fig. S4), 22 genes were identified as related to the stress response. Among these genes, three of them were involved in osmotic stress (osmoregulation), 11 were related to oxidative stress [oxidative stress (6), glutathione: redox cycle (3), glutaredoxins (1), glutathionylspermidine and trypanothione (1)], and eight were related to detoxification. Some freezing-protective solutes, which can contribute to cold stress remission[28, 29], such as amino sugar and nucleotide sugars (26), amino acids (96), starch, and sucrose (27) were also detected in the genome information of strain AK-R2A1-2^{T}.

Motility: Two genes related to motility including one for flagellar assembly (*rpoD*) and one for bacterial chemotaxis (*rbsB*) were annotated from the whole genome of strain AK-R2A1-2^{T}. The incomplete pathway of motility explains why strain AK-R2A1-2^{T} was non-motile.

Fatty acid metabolism: The beta-oxidation-related biosynthesis pathway, including 3-hydroxyacyl-CoA dehydrogenase [EC:1.1.1.35], acyl-CoA oxidase [EC:1.3.3.6], acyl-CoA dehydrogenase [EC:1.3.8.7], acetyl-CoA acyltransferase [EC:2.3.1.16], and enoyl-CoA hydratase [EC:4.2.1.17], was detected in the whole genome of strain AK-R2A1-2^{T}. Concurrently, long-chain acyl-CoA synthesis (EC:6.2.1.3), related to acyl-CoA synthesis, was reconstructed from the whole genome of strain AK-R2A1-2^{T} by BlastKOALA pipeline.

Genome comparison and biosynthetic potential of the novel strain

The proposed threshold values of dDDH and ANI for delineating novel bacterial species were recommended as lower than 70% and lower than 95–96%, respectively[30]. In the current study, the ANI values between strain AK-R2A1-2^{T} and type strains of members of the genus *Subtercola* were 76.8–80%, followed by 73.6–74% with members of the genus *Agreia*, and less than 74.7% with other genera in the family *Microbacteriaceae*. Consistently, strain AK-R2A1-2^{T} exhibited a dDDH value of 22.5–24.5% with members of the genus *Subtercola*, 20.1–20.5% with members of the genus *Agreia*, and less than 20.7% with other genera in the family *Microbacteriaceae* (Table S2). Similar results were obtained for orthoANI values, which were less than 80.3% with most closely related strains in the family *Microbacteriaceae* (Table S2).

Bacteria can produce a large number of secondary metabolites that act as a reservoir of bioactive metabolites, with some exhibiting unique functions such as salt resistance[31]. In the genus *Subtercola*, there are limited studies on the production of specialized metabolites and the potential source of natural products in this genus remains untapped. In recent years, many computational methods have been developed to identify the biosynthetic gene clusters (BGCs) in genomic data. AntiSMASH is a platform that is widely used for genome mining of secondary metabolites. The secondary metabolite regions of the genome of strain AK-R2A1-2^{T} were searched using antiSMASH (V. 6.0.1). Seven regions were

identified from the whole genome, and these regions contained non-alpha poly-amino acids like e-Polylysine (NAPAA), beta-lactone (microansamycin), T3PKSS (alkylresorcinol), RRE-containing (kosinostatin), beta-lactone, terpene (carotenoid), and redox-cofactor (lipopolysaccharide) (Table S3 and Figs. S5–S11). Some of these compounds play vital roles in antioxidant, antimicrobial, and anti-inflammatory activities. For example, NAPAA is a cationic peptide proven to prevent microbe proliferation and is approved as a food-grade cationic antimicrobial metabolite[32], which has natural antioxidant and antimicrobial activities[33]. Carotenoid can be used to protect against oxidative stress in model systems[34-37], and regulates membrane fluidity at low temperature by cell membrane adaptation[38, 39]. Furthermore, increasing carotenoid levels might increase resistance against cold shock[40]. Alkylresorcinol can protect epithelial cells against oxidative damage, showed antioxidant activity[41-43], and has antigenotoxic activity[43] and potential antiglioma activity[44]. Kosinostatin has antitumor, antimicrobial, and antiproliferative functions[45, 46], while beta-lactone is a natural product with significant clinical applications through its antimicrobial, anticancer, and anti-obesity properties[47, 48]. Microansamycin is a member of the macrolactams family, which are important bioactive compounds that have anti-tuberculous and antitumor activity[49]. Those secondary metabolites are highly matched with its host plant.

Effect of strain AK-R2A1-2^T on rice seedling growth under low temperature

Rice is a cold-sensitive crop, and its exposure to low-temperature stress (<20 ^oC), during germination and early seedling growth, can negatively affect the initial stand establishment[50, 51]. To evaluate the strain AK-R2A1-2^T on rice seedling growth under low temperature, rice seeds were co-cultivated with strain AK-R2A1-2^T for 10 days at 20°C, and then total fresh weight, shoot weight, shoot length, root number, root length, and chlorophyll contents were examined in rice seedling (Fig. 4). Shoot weight and shoot length were increased 1.67-fold and1.36-fold, respectively compared to control (CK), while the root length and root number were increased 1.42-fold and 1.40-fold, respectively in rice seedling with strain AK-R2A1-2^T (Fig.4). Strain AK-R2A1-2^T significantly improved the rice seedling biomass and root morphological parameters under low temperatures.

Conclusion

Based on phylogenetic, phenotypic, and chemotaxonomic data, strain AK-R2A1-2^T belongs to the genus *Subtercola*. The differentiating phenotypic properties and chemotaxonomic data presented in Table 1 preliminarily distinguish the strain as representing a novel species of the genus *Subtercola*. The genome analyses of strain AK-R2A1-2^T using RAST SEEDS, BlastKOALA, and antiSMASH identified genes for a cold-active chaperone, general stress response, osmotic stress, oxidative stress, and DNA repair mechanisms, as well as the gene clusters responsible for host plant secondary metabolites. These data support the physiological properties of the psychrophilic genus *Subtercola*, with the demonstration of

bacterial and plant viability at low temperatures, and the secondary metabolites isolated from the host plant.

Description of Subtercola endophyticus **sp. nov**

Subtercola endophyticus (en.do.phy'ti.cus. Gr. pref. *endo-*, within; Gr. neut. n. *phyton*, plant; L. masc. adj. suff. *-icus*, adjectival suffix used with the sense of belonging to; N.L. masc. adj. *endophyticus*, endophytic, within plants, pertaining to the original isolation from plant tissues).

Colonies grown on R2A medium are white, circular (1–4 mm in diameter), smooth, and convex after culture for 4 days at 25°C. Cells (0.2–0.3 μm in width and 0.3–1.6 μm in length) are short rod-shaped, strictly aerobic, Gram-stain-positive, non-motile, without flagella, catalase-positive, and oxidase-negative. Grows occurs at 4–25°C (optimum, 20°C), at pH 5–8 (optimal 5), and with 0–1% NaCl (optimal 0%). Grows on R2A, PD, MEA, YEP, ISP2, GYM (optimum, PD and R2A), but not on MA, LB, NA, or TSA. Strain AK-R2A1-2^T was positive for the following enzyme activities in API ZYM strips: esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, valine arylamidase, cysteine arylamidase, trypsin, acid phosphate, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase, β-glucosidase, α-mannosidase. In the API 20NE strips, strain AK-R2A1-2^T was positive for aesculin hydrolysis and β-galactosidase activities, but negative for all other activities. In the API strips, all tests were negative except aesculin ferric citrate. The major fatty acids detected in strain AK-R2A1-2^T were summed featured 8 and anteiso-$C_{15:0}$. MK-9 and MK-10 were the respiratory quinones in strain AK-R2A1-2^T, and the major polar lipids were DPG, AKL2, AKL3, and AKL4.

The GenBank accession numbers of the 16S rRNA gene and the whole-genome sequences of strain AK-R2A1-2^T are OL314543 and CP087997.1, respectively. The strain is available from the Korea Collection for Type Culture (KCTC 49721^T) and the Guangdong Microbial Culture Collection Center (GDMCC 1.2921^T).

Materials And Methods

Sample isolation and culture conditions

Plant samples were collected from the top parts of Mount Halla, Jeju Island, Republic of Korea (33°21'32.4" N, 126°31'68" E) during July 2020. Two grams of the needle leaves of *Abies koreana* tree samples were surface-sterilized with 1% sodium hypochlorite, rinsed five times in sterile distilled water, and then ground with 20 mL of 1× phosphate-buffered saline (PBS) in a grinder. Ground samples were serially diluted from 10^{-1} to 10^{-4} with 1× PBS, and 100 μL aliquots were spread onto 10-fold diluted Reasoner's 2A agar (R2A) and incubated at 25°C for 7 days. Morphologically distinct colonies were selected and subsequently streaked on fresh R2A medium. All strains were preserved in sterile skimmed milk (10%, w/v) at -80°C. Among all the isolates, one white, circular, convex, and smooth colony, designated AK-R2A1-2^T, was selected for further study. *Subtercola boreus* DSM 13056^T, *Subtercola vilae*

DSM 105013^T, *Subtercola frigoramans* KCTC 49696^T, and *Subtercola lobariae* KCTC 33586^T were obtained from the corresponding culture collections as closely related strains to analyze the taxonomic characteristics under comparable culture conditions. Unless otherwise stated, bacterial strains were grown on R2A or ISP2 for 5 days for subsequent tests.

Strain identification and phylogenetic analysis

The 16S rRNA gene of strain AK-R2A1-2^T was amplified by PCR using universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') with bacterial DNA as the template. The purified PCR product was sequenced by BioFact company (Daejeon, Republic of Korea) using primers 27F, 1492R, 518F (5'-CCAGCAGCCGCGGTAATACG-3'), and 800R (5'-TACCAGGGTATCTAATCC-3'). Sequencing reads were assembled via Vector NTI software (1.6.1), and an almost full-length 16S rRNA gene sequence was obtained and subjected to similarity-based BLAST analyses against the GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and EzBioCloud (http://www.ezbiocloud.net)[30] databases. Phylogenetic trees based on 16S rRNA gene sequences were reconstructed via the Molecular Evolutionary Genetics Analysis (MEGA V.7.0) program[52], using the neighbor-joining (NJ), maximum-parsimony (MP), and maximum-likelihood (ML) algorithms with 1,000 bootstrap iterations. *Litorihabitans aurantiacus* HIsM16-52^T (GenBank accession number LC203065) was included as an outgroup.

Phenotypic characteristics

Cell morphology was observed by scanning electron microscopy at the Chuncheon Center, Korea Basic Science Institute (KBSI) after growing the strain on R2A agar plates for 4 days. Gram staining was conducted with a Gram-staining kit (Difco) according to the manufacturer's instructions. Cell motility was tested by growing the strain on a semi-solid R2A medium (0.4%). Catalase activity was determined by the production of bubbles after adding 3% (v/v) hydrogen peroxide solution to fresh cells[53], while oxidase activity was gauged with the aid of oxidase test strips (bioMérieux) that changed color to purple. Different media including nutrient agar (NA, beef extract 3 g/L, peptone 5 g/L, and agar 15 g/L), trypticase soy agar (TSA), Luria-Bertani agar (LB), marine agar 2216 (MA), R2A, potato dextrose agar (PD), malt extract agar (MEA), yeast extract peptone Agar (YEP), ISP Medium No.2 (ISP2), GYM (glucose 4 g/L, yeast extract 4.0 g/L, malt extract 10 g/L, $CaCO_3$ 2 g/L, and agar 15 g/L) were screened for optimal growth of the tested strains. Various temperatures (4, 10, 15, 20, 25, 30, 37, 40, 45, and 60°C) were assayed to identify the growth range of the novel strain. The pH range (3.0 to 12.0, adjusted with 1 N HCl and NaOH) for growth and NaCl tolerance (0 to 15%, w/v; 1% concentration increments) were measured in R2A liquid medium, monitoring the optical density at 600 nm (OD_{600}) using a microplate spectrophotometer (Multiskan skyhigh, Thermo Fisher Scientific). Other biochemical features, such as the utilization of substrates, acid production from carbohydrates, and enzyme activities, were tested using API 20NE (bioMérieux) or API ZYM with NaCl 0.85% medium (bioMérieux) and API 50CH according to the manufacturer's protocols. All closely related type strains were tested in the same conditions.

Chemotaxonomy features

Biomass for analysis of cellular fatty acids of strain AK-R2A1-2^T and closely related type strains were extracted from cells grown in R2A medium. According to the standard instructions of the Sherlock Microbial Identification System version 6.0 (MIDI), whole-cell fatty acid methyl esters were extracted, then analyzed by gas chromatography (model 6890N; Agilent) based on the Microbial Identification software package[54]. Quinones were extracted from 100 mg freeze-dried cells by shaking in chloroform:methanol mixture (2:1, v/v) and were purified by thin-layer chromatography as described by Collins et al.[55]. Analysis of the purified quinones was performed using reverse-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) absorbance detection at 270 nm. For extraction and analysis of polar lipids, 100 mg freeze-dried cells were boiled in methanol, mixed and shaken with chloroform, evaporated, and then identified by two-dimensional thin-layer chromatography (TLC) on Kieselgel 60 F254 plates (silica gel, 10 × 10 cm; Merck). Lipids spots were visualized by spraying with 0.2% ninhydrin (Sigma-Aldrich), α-naphthol, molybdenum blue (Sigma-Aldrich), 4% phosphomolybdic acid reagent, and Dragendorff's solution to detect amino group-containing lipids, sugar-containing lipids, phosphorus-containing lipids, total lipids, and quaternary nitrogen-containing lipids, respectively.

Genome sequencing and assembly

Whole-genome sequencing was performed by the Macrogen facility (Macrogen, Republic of Korea) using the PacBio Sequel System (Pacific Biosciences, Inc.) and the Illumina sequencing platform. Raw sequencing data were assembled by Canu (version 1.7) de novo assembler. Error corrections were performed by Pilon (version 1.21). Potential contamination of the assembled genome was checked using the ContEst16S algorithm[56] to compare 16S rRNA within the whole genome. To support that strain AK-R2A1-2^T belonged to the genus *Subtercola*, a whole-genome-based phylogenetic tree was reconstructed using the up-to-date bacterial core gene set and pipeline (UBCG) as described by Na et al.[57].

Genome annotation and comparative analysis

The National Center for Biotechnology Information Prokaryotic Genome Annotation Pipeline (PGAP) and Rapid Annotation of microbial genomes using Subsystem Technology (RAST) SEEDS[9–11] were used for genome annotation. Metabolic pathways were reconstructed using BlastKOALA, which is based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database[58]. Genome mining for the presence of secondary metabolite gene clusters was performed via the antiSMASH program (https://antismash.secondarymetabolites.org/) with relaxed detection strictness[59]. Comparative genome analysis including digital DNA-DNA hybridization (dDDH), average nucleotide identity (ANI) values, and orthoANI values between strain AK-R2A1-2^T and the most closely related members of the family *Microbacteriaceae* have performed with the Genome-to-Genome Distance Calculation (GGDC) webserver (http://ggdc.dsmz.de/)[60], ANI calculator (https://www.ezbiocloud.net/), and standalone Orthologous Average Nucleotide Identity (OAT) software[61], respectively. A graphical circular map of the genome of strain AK-R2A1-2^T was constructed via CGView (https://cgview.ca/).

Rice growth and low-temperature conditions

Seeds of the *Japonica* rice cultivar Haepung were surface-sterilized with 1% (v/v) sodium hypochlorite for 10 min followed by repetitive washes with distilled water and soaked in AK-R2A1-2^T suspension (1x10^8 CFU/mL) or R2A broth as control as described as Jiang et al.[31]. The control and AK-R2A1-2^T bacteria coating seeds were incubated at 14°C of dark for 4 days in three replicated square plates (245 mm × 245 mm) which contained 0.15% water agar. All the plates were incubated at 20°C with a 16-h light/8-h dark photoperiod for 10 days. To estimate shoot weight, shoot length, root weight, root number, root length, fifteen seedling plants from each experiment were considered. The chlorophyll contents were calculated as described by Fu et al.[62]. All experiments were performed in triplicate yielding similar results.

Declarations

Acknowledgments

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Author contributions

Conceptualization by JL and HCP; Funding acquisition by JL and CYK; LJ designed the research and carried out the experiment; YP, JS, DJ, M-GJ, JHL, JCJ, and CYK edited the manuscript; LJ wrote the manuscript; JL and LJ modified the manuscript. All authors read and approved the manuscript.

Competing interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The strain is available from the Korea Collection for Type Cultures (KCTC 49721^T) and the Guangdong Microbial Culture Collection Center (GDMCC 1.2921^T). The GenBank accession number of the strain AK-R2A1-2^T for the 16S rRNA gene is OL314543. Whole-genome sequences of strain AK-R2A1-2^T is CP087997.1. The associated BioSample and BioProject accession numbers are SAMN23075623 and PRJNA678113, respectively. The taxonomy ID is 2895559.

Additional information

The following are supplementary data to this article can be found online at:

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Tables

Tables 1 to 3 are available in the Supplementary Files section

Figures

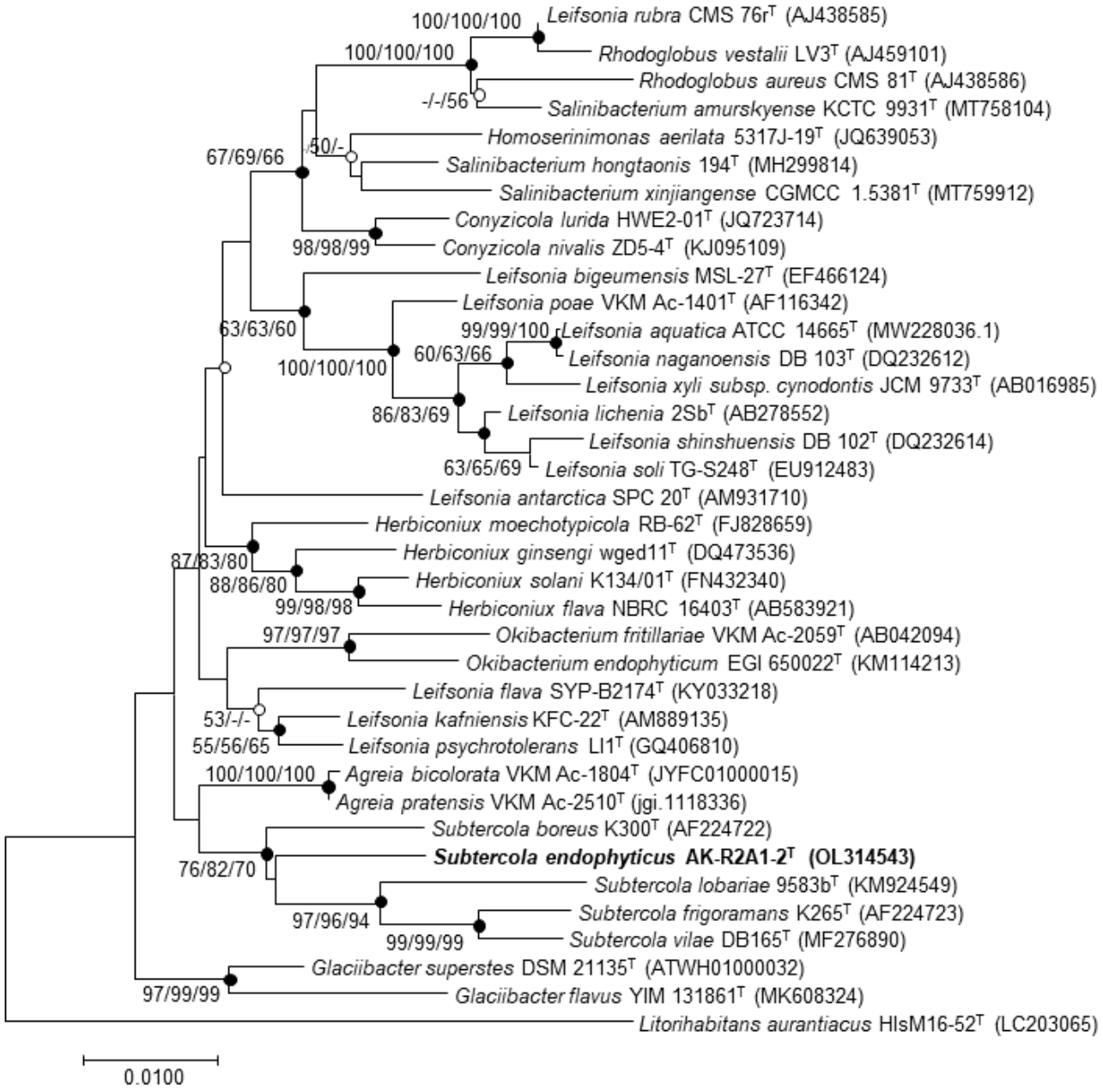

Figure 1

Neighbor-joining (NJ) phylogenetic tree based on 16S rRNA gene sequences showing the position of strain AK-R2A1-2^T. Bootstrap values (>70%) calculated using the NJ, maximum-likelihood (ML), and minimum evolution (ME) algorithms are shown. Filled circles on the nodes indicate that the relationships were also recovered by ML and ME algorithms, whereas open circles indicate nodes recovered by either ML or ME algorithms. Scale bar: 0.0100 substitutions per nucleotide position.

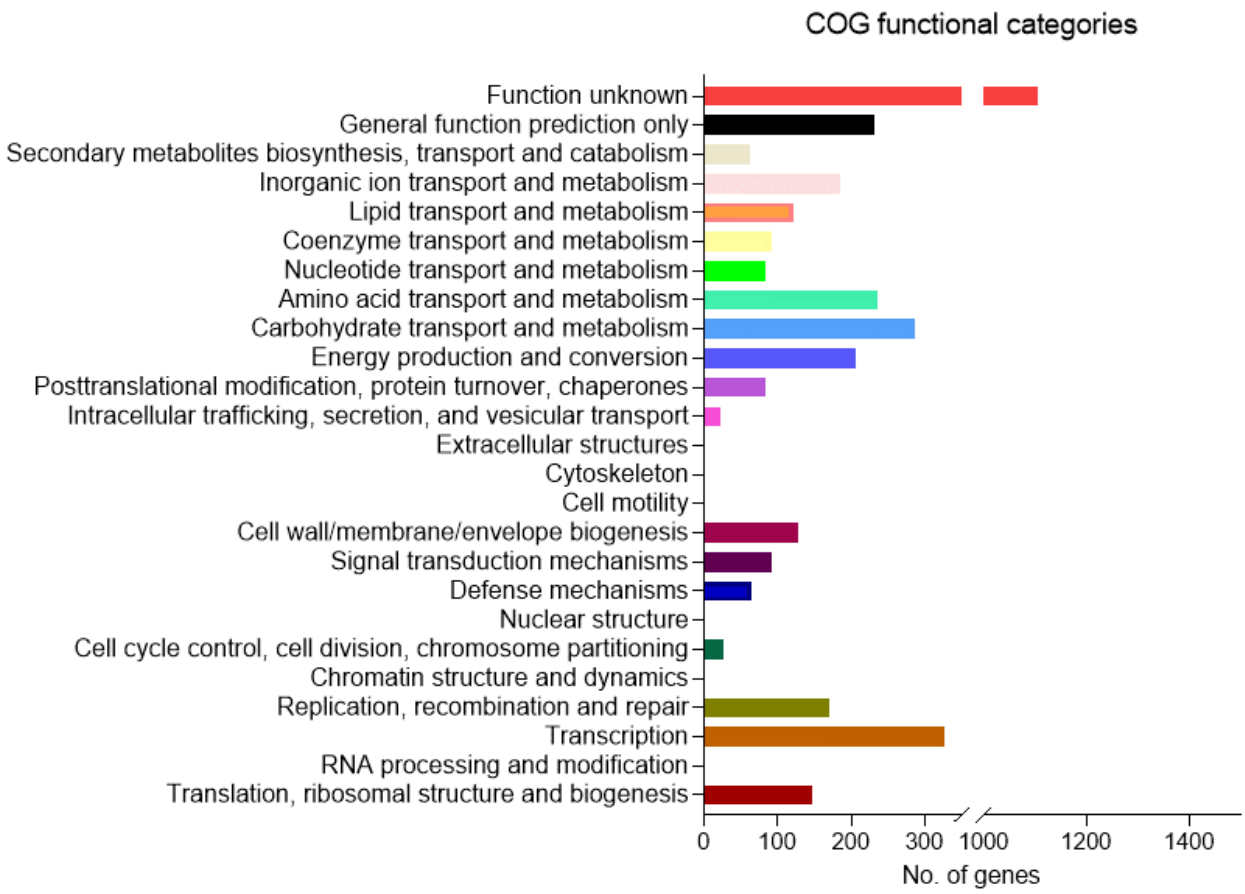

Figure 2

The cluster of orthologous groups (COG) classification of putative proteins in the whole-genome sequence of strain AK-R2A1-2^T.

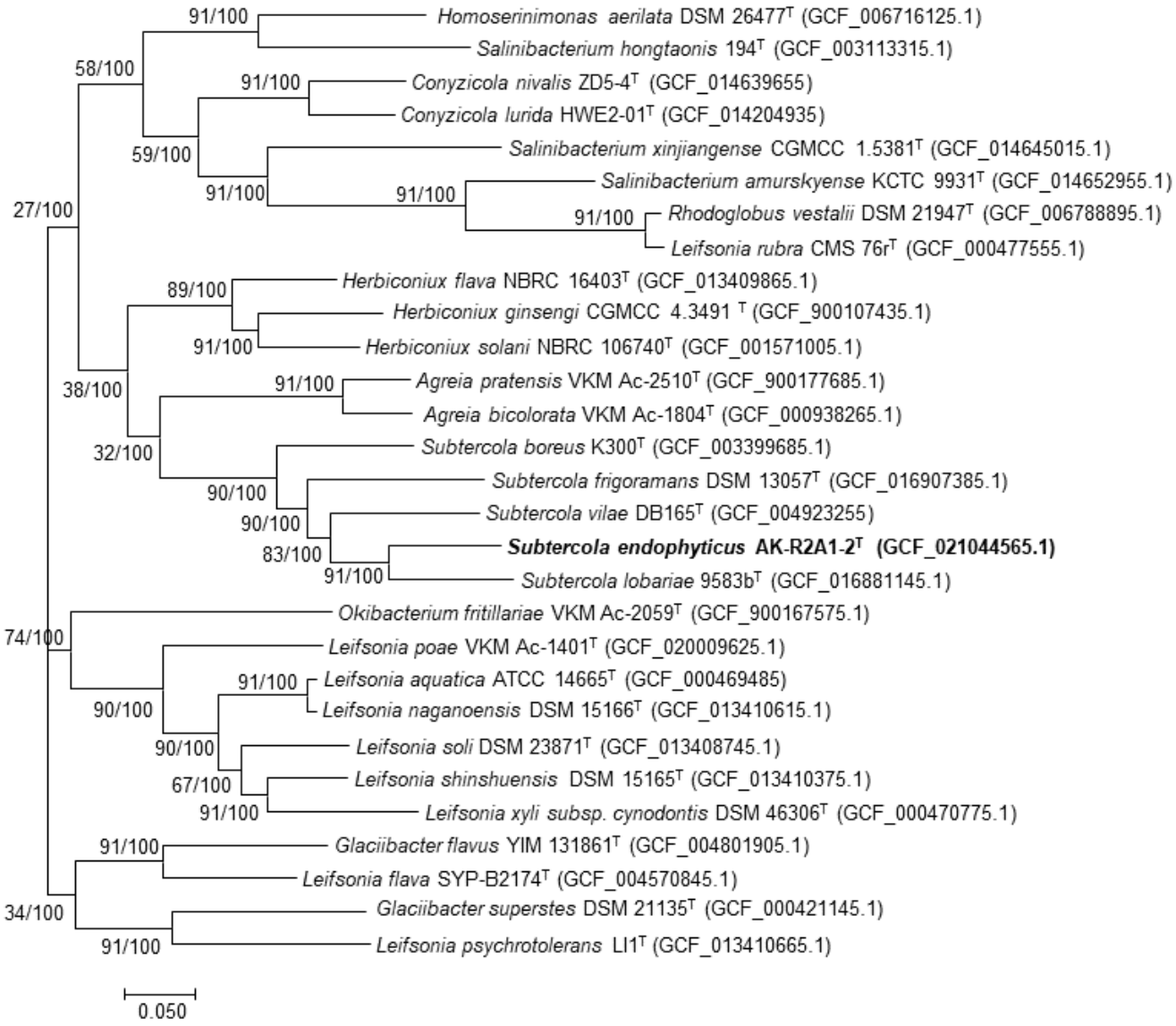

Figure 3

ML phylogenomic tree based on UBCGs (concatenated alignment of 92 core genes). Gene support index (GSI, left) and bootstrap values (right) are indicated at the nodes. Scale bar, 0.050 substitutions per position.

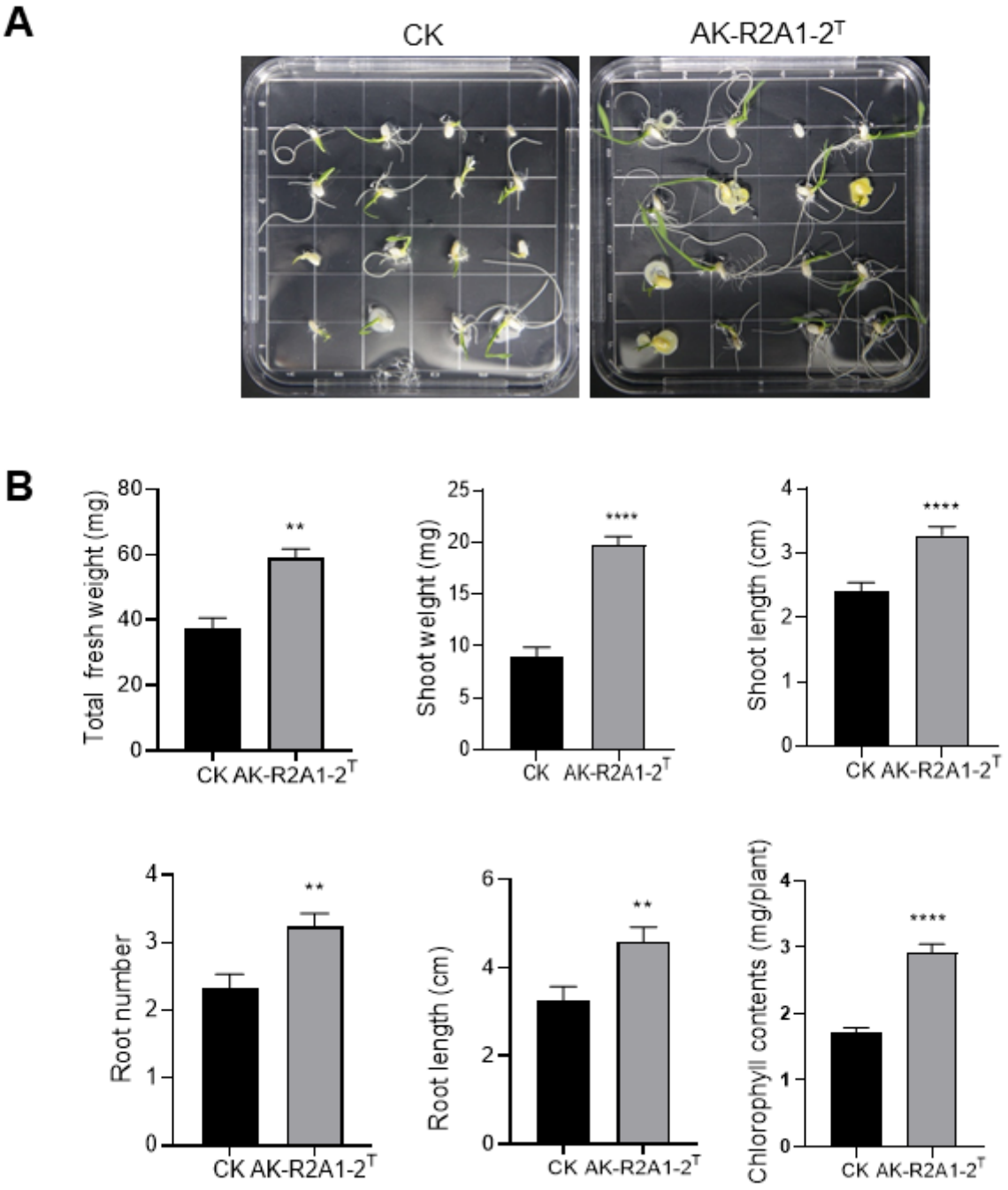

Figure 4

Effects of coating strain AK-R2A1-2^T on rice growth under low temperature. (A) Comparison of rice seedling growth by control (CK, R2A broth) and AK-R2A1-2^T bacterial suspension (1x10^8 cfu/mL) on water agar plates at 20 ^oC for 10 days. (B) Quantification of total fresh weight, shoot weight, shoot length, root number, root length, and chlorophyll contents. Error bars indicate the standard deviation of the mean (*n*=15). Asterisks indicate a statistically significant difference between control and coating bacteria suspension (two-way ANOVA, **p<0.01, and ****p<0.0001). Experiments were repeated twice with similar results.

Supplementary Files

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