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Variovorax palleresanus sp. nov., a novel plant growth-promoting rhizobacterium isolated from sulphur-rich soil in Spain

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Abstract

During a screening of sulphur-oxidizing bacteria from different Spanish soils, a strain designated AFSI2.2^T was isolated from the rhizosphere soil of a mulberry plant thriving in a sulphur-rich environment. Cells of this strain were Gram-stain-negative motile rods with flagella. Its taxonomic classification was determined through a polyphasic approach. Phylogenetic analysis of the 16S rRNA gene revealed that strain AFSI2.2^T shares high similarity (99.64%) with certain *Variovorax* species, suggesting that the isolate belongs to this genus. Whole-genome phylogeny showed that the novel strain forms a distinct lineage within the genus *Variovorax*. Whole-genome analysis of AFSI2.2^T exhibited average nucleotide identity values of 78.37–89.63% and digital DNA–DNA hybridization values of 21.90–38.90% with closely related *Variovorax* species, both below the established thresholds for species delineation. The predominant cellular fatty acids of strain AFSI2.2^T were $C_{16:0}$, Summed Feature 3 ($C_{16:1}\omega6c$ and/or $C_{16:1}\omega7c$) and Summed Feature 8 ($C_{18:1}\omega7c$ and/or $C_{18:1}\omega6c$). Apart from the ability of oxidizing inorganic sulphur compounds, AFSI2.2^T presented further *in vitro* plant growth-promoting (PGP) activities such as production of indole-3-acetic acid and siderophores and 1-aminocyclopropane-1-carboxylate deaminase activity. Genes associated with these PGP traits, as well as with cytokinin and gamma-aminobutyric acid biosynthesis, were identified in the AFSI2.2^T genome, supporting its potential as a PGP bacterium. In conclusion, phylogenetic, genomic, phenotypic and chemotaxonomic analyses support the classification of the isolate as a novel species within the genus *Variovorax*, for which the name *Variovorax palleresanus* sp. nov. is proposed. The type strain is AFSI2.2^T (=CECT 31217^T=CIP 112526^T).

INTRODUCTION

The genus *Variovorax* is a group of Gram-stain-negative and rod-shaped betaproteobacteria, which belongs to the *Comamonadaceae* family. This genus was established with the reclassification of *Alcaligenes paradoxus* as *Variovorax paradoxus*, considered the type species [1]. As of May 2025, there are 13 validly published *Variovorax* species on the List of Prokaryotic Names with Standing in Nomenclature (LPSN) and 2 invalidly published species (https://lpsn.dsmz.de/genus/variovorax) [2]. Most species have been isolated from the rhizosphere or soil environments [3]. However, a few *Variovorax* species have also been recovered from other sources, including plants [4], sewage [5] and even humans [6].

As suggested by the name *Variovorax*, this group of bacteria can 'devour a variety of substrates'. They exhibit highly diverse metabolisms, with some strains capable of degrading a wide range of xenobiotics, including certain plastics [7], trichloroethylene [8] and 2,4-dinitrotoluene [9], as well as natural compounds like chitin [10] and cellulose [11]. Additionally, several *Variovorax*

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Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; ANI, average nucleotide identity; API, analytical profile index; BGCs, biosynthetic gene clusters; BLAST, Basic Local Alignment Search Tool; CECT, Colección Española de Cultivos Tipo; dDDH, digital DNA-DNA hybridization; GABA, gamma-aminobutyric acid; GBDP, Genome BLAST Distance Phylogeny; IAA, indole-3-acetic acid; IAN, indole-3-acetonitrile; LPSN, List of Prokaryotic Names with Standing in Nomenclature; ML, maximum-likelihood; NJ, neighbour-joining; PGP, plant growth-promoting; RAST, Rapid Annotation using Subsystem Technology; SOB, sulphur-oxidizing bacteria; TSA, trypticase soy agar; TYGS, type strain genome server.
The AFSI2.2^T sequences of the 16S rRNA partial gene and genome have been respectively deposited under accessions PQ725731 and JBJNVP000000000 in GenBank.
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strains are recognized as plant growth-promoting (PGP) rhizobacteria due to their potential to enhance plant growth. For instance, they are reported to produce the phytohormone indole-3-acetic acid (IAA) [12], the enzyme 1-aminocyclopropane-1-carboxyl ate (ACC) deaminase [13] and siderophores [14]. These multifaceted capabilities make the genus *Variovorax* of special interest for various agrobiotechnological applications, including the development of biostimulants and biofertilizers [15].

Sulphur-oxidizing bacteria (SOB) are a diverse group of micro-organisms that can oxidize inorganic sulphur compounds such as hydrogen sulphide (H_2S), elemental sulphur (S^0) and thiosulphate ($S_2O_3^{2-}$) as energy sources [16]. SOB play a crucial role in global sulphur cycling, contributing to biogeochemical processes and influencing soil fertility and nutrient availability [17]. Prominent examples of SOB include members of the genera *Thiobacillus*, *Acidithiobacillus*, *Beggiatoa* and *Thiomargarita*. These bacteria have significant biotechnological applications, including bioremediation, bioleaching, wastewater treatment and the production of sulphuric acid [16]. While various *Variovorax* strains are known to metabolize organic sulphur compounds, to the best of our knowledge, no member of the genus has yet been shown to oxidize inorganic sulphur compounds such as thiosulphate [18, 19]. This gap limits our understanding of the metabolic versatility of the genus and its potential contribution to sulphur cycling.

In this study, we isolated a rhizobacterium displaying thiosulphate-oxidizing activity, designated as strain AFSI2.2^T. Our objective was to determine its taxonomic status through a polyphasic approach. Analyses based on 16S rRNA gene phylogeny, whole-genome sequencing and phenotypic and chemotaxonomic data evidence that strain AFSI2.2^T represents a novel species within the genus *Variovorax*. We propose the name *Variovorax palleresanus* sp. nov. for this species.

ISOLATION AND ECOLOGY

The novel strain AFSI2.2^T was isolated from the rhizosphere soil of a mulberry plant at the facilities of AFEPASA S.A.U., a sulphur-specialized company in Constantí, Tarragona, Spain (41° 9′ 45.20″ N 1° 9′ 20.93″ E). This sulphur-rich environment, due to the company's commercial activities, provided optimal conditions for the isolation of SOB. The strain was obtained following an enrichment protocol, where soil samples were shaken for 2 h, serially diluted and inoculated into thiosulphate broth, a minimal medium with thiosulphate as the sole energy source [20]. Cultures were incubated at 28 °C with shaking (160 r.p.m.) for 5 days, followed by subculturing into fresh media and a further 25-day incubation under the same conditions. Aliquots from these secondary cultures were plated onto thiosulphate agar supplemented with bromocresol purple as a pH indicator. SOB were identified by colonies that changed the medium colour from purple to yellow, indicating acid production from thiosulphate oxidation [20]. Individual colonies were subsequently re-streaked on thiosulphate agar to obtain pure isolates. Following this protocol, the strain designated AFSI2.2^T was selected for its remarkable activity on thiosulphate agar. The isolate was also capable of growing on trypticase soy agar (TSA) under aerobic conditions at 28 °C within 48–72 h.

16S rRNA GENE PHYLOGENY

The 16S rRNA gene sequence was analysed as part of the isolate's preliminary characterization. DNA of strain AFSI2.2^T was extracted from an isolated colony grown on a culture plate using the QIAcube robot and the QIAamp DNA Blood Mini kit (Qiagen) according to the manufacturer's instructions. PCR amplification of the 16S rRNA gene was performed using the universal primers 16SF and 1492R [21], followed by Sanger sequencing with a 3730xl DNA Analyzer (Thermo Scientific). The resulting sequence was of 1,387 bp and was deposited in GenBank under accession number PQ725731. This partial sequence matches identically the corresponding region of the complete 16S rRNA gene (locus tag ACJ7Z0_13330, with 1,535 bp) retrieved from the whole-genome assembly. The full-length 16S rRNA gene was compared against the EzBioCloud database (https://www.ezbiocloud.net/) [22] to identify its closest known relatives, which were *V. paradoxus* NBRC 15149^T (99.66% similarity) and *Variovorax guangxiensis* DSM 27352^T (99.45% similarity). The rest of the most similar sequences were obtained from the EzBioCloud server and aligned with strain AFSI2.2^T using Clustal W [23]. Phylogenetic analysis was then performed using the MEGA program (v.11) [24], with the maximum-likelihood (ML) and neighbour-joining (NJ) methods [25, 26], applying the Kimura two-parameter model to calculate genetic distances and determining bootstrap values based on 1,000 replications [27, 28]. The phylogenetic tree based on the ML method shows that strain AFSI2.2^T groups within the genus *Variovorax* and represents a distinct lineage separated from other species in the genus (Fig. 1). Similarly, the phylogenetic tree constructed using the NJ method displayed comparable clustering of strain AFSI2.2^T with its closest relatives (Fig. S1, available in the online Supplementary Material).

GENOME FEATURES AND PHYLOGENOMICS

For whole-genome sequencing, DNA of strain AFSI2.2^T was extracted using the MagAttract HMW DNA Kit (Qiagen) according to the manufacturer's instructions and sequenced with the Nanopore duplex technology on the MinION platform (ONT). Library preparation and barcoding were performed using the SQK-LSK114 kit and the native barcoding Kit 96 V14 (SQK-NBD114.96) (ONT), respectively, following the manufacturer's guidelines. The quality analysis was done with Nanoplot (v.1.36.2) [29], the assembly with Flye (v.2.9.1) [30], Nanopolish for polishing the assembly [31] and

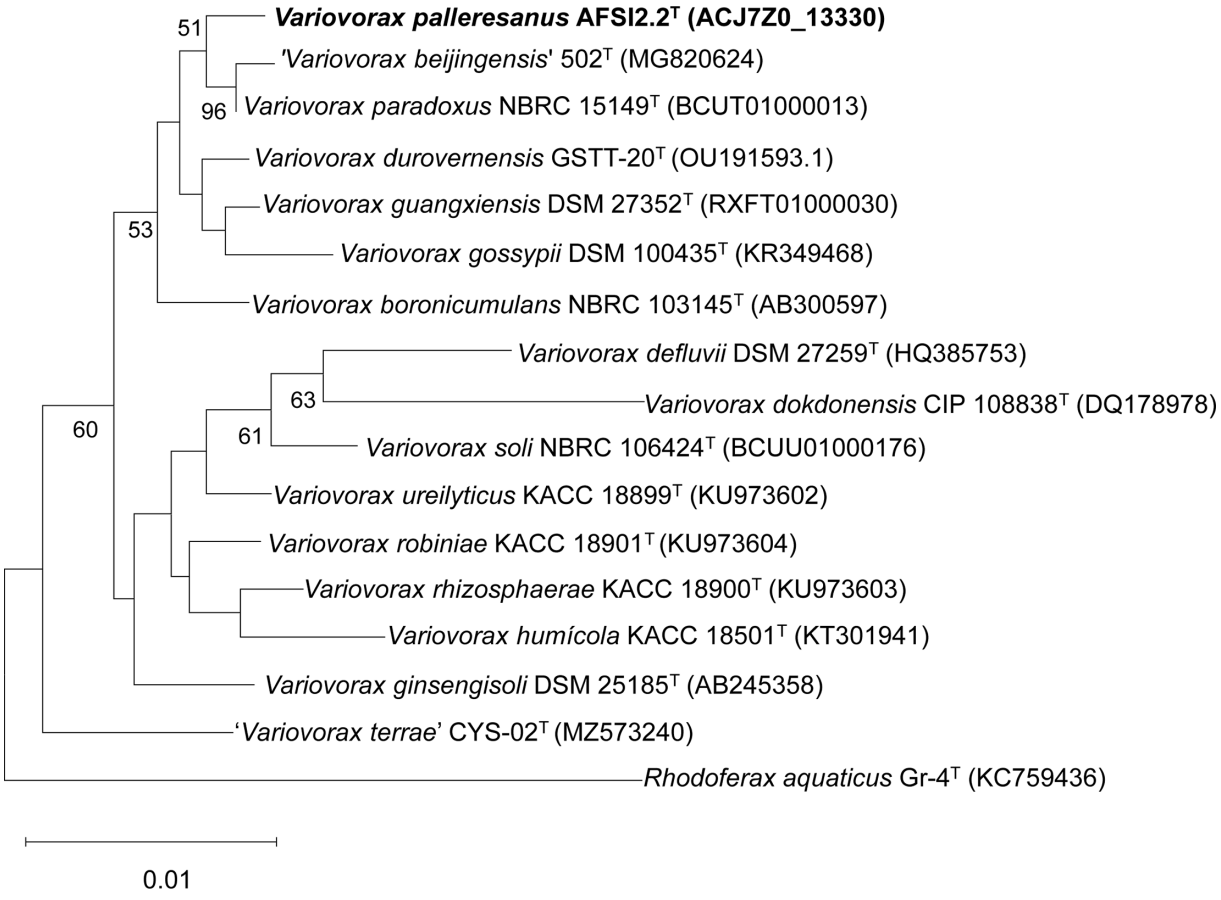


Fig. 1. Unrooted ML tree based on 16S rRNA gene sequences of strain AFSI2.2^T and its closely related type species. Bootstrap values, expressed as percentages from 1,000 replicates, are displayed at branch points where they exceed 50%. Accession numbers from the database are included in parentheses. Species names written in quotation marks are not validly published (https://lpsn.dsmz.de). *Rhodoferax aquaticus* Gr-4^T was used as an outgroup. Bar, 0.01 substitutions per nucleotide position.

Unicycler for improving the assembly with short reads [32]. The genome was annotated utilizing the Rapid Annotation using Subsystem Technology (RAST) server [33], and rRNA and tRNA were predicted using Barrnap [34] and tRNAscan [35]. The final assembly had 80× sequencing coverage with an estimated completeness of 100% and contamination of 0.93%, based on the CheckM program (v1.0.18) [36]. The resulting assembled genome was composed of a chromosome of 5,305,463 bp and 1 megaplasmid of 791,413 bp. In total, the genome of AFSI2.2^T was 6,096,876 bp; contained 5,712 CDSs, 3 rRNA genes and 59 tRNA genes; and showed a DNA G+C content of 66.2 mol% (Table 1). All these features, as well as the genome organization of AFSI2.2^T, are consistent with the data reported for other closely related *Variovorax* species [3, 37, 38]. The complete genome sequence of strain AFSI2.2^T has been deposited in the GenBank database under accession number JBJNVP000000000.

Whole genome-based approaches were used to confirm the taxonomic status of AFSI2.2. Average nucleotide identity (ANI) calculations were performed using the online ANI calculator available on EzBioCloud, which utilizes the OrthoANIu algorithm (https://www.ezbiocloud.net/tools/ani) [39]. The estimated digital DNA–DNA hybridization (dDDH) values were calculated using Formula 2 by Genome-to-Genome Distance Calculator 3.0 (https://ggdc.dsmz.de/) [40]. A whole-genome phylogenetic tree was generated using the Genome BLAST Distance Phylogeny (GBDP) approach from the Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de/) [41]. The analysis employed the 'coverage' algorithm and the d5 distance formula [40]. The ANI and dDDH values derived from the genome comparison between strain AFSI2.2^T and its closest related type species ranged from 78.37 to 89.63% for ANI and 21.90 to 38.90% for dDDH (Table 2). These results are well below the established thresholds for defining prokaryotic species, which are 95–96% for ANI and 70% for dDDH [42]. Moreover, the whole-genome phylogenetic analysis places strain AFSI2.2^T in a distinct

Table 1. General genome features for strain AFSI2.2^T and closely related species. Species names written in quotation marks are not validly published (https://lpsn.dsmz.de)

		Size (bp)	CDS	rRNA	tRNA	G+C content (mol%)
Variovorax palleresanus AFSI2.2^T	Chromosome	5,305,463	4,982	3	49	65.9
	Plasmid	791,413	730	0	10	66.3
	Total	6,096,876	5,712	3	59	66.2
Variovorax paradoxus SPNA7	Chromosome	5,887,536	5,459	2	46	67.5
	Plasmid	1,192,185	1,112	0	10	67.5
	Total	7,079,721	6,571	2	56	67.5
'*Variovorax beijingensis*' 502^T	Assembly total	6,765,052	6,307	6	58	67.4

Data for *V. paradoxus* SPNA7 and '*Variovorax beijingensis*' 502^T retrieved from [37] and [3], respectively.

clade within the genus *Variovorax* (Fig. 2). Collectively, these findings indicate that strain AFSI2.2^T represents a novel genomic species within the genus *Variovorax*.

GENES INVOLVED IN PLANT GROWTH PROMOTION AND SECONDARY METABOLITES

The genome of AFSI2.2^T was analysed via the SEED server using the RAST to conduct a preliminary evaluation of its PGP capabilities [43, 44]. Genes associated with plant growth promotion activities were identified using NCBI BLASTp with PGP-related proteins from closely related species (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The antiSMASH software (v8) was applied to determine secondary metabolite-associated biosynthetic gene clusters (BGCs) [45].

Analysis of the AFSI2.2^T genome revealed the presence of genes associated with common PGP features reported in different *Variovorax* strains, such as the biosynthesis of auxins, cytokinins, gamma-aminobutyric acid (GABA), siderophores and ACC deaminase activity [12–14]. Regarding IAA production, genome mining suggests that strain AFSI2.2^T may synthesize IAA from the intermediate indole-3-acetonitrile (IAN) via two pathways: a nitrilase-dependent route (*nitA*) and a nitrile hydratase (*nthA*,

Table 2. ANI and dDDH values between strain AFSI2.2^T and most closely related type species. Species names written in quotation marks are not validly published (https://lpsn.dsmz.de)

Strains	OrthoANIu (%)	dDDH (%)	Accession no.
Variovorax paradoxus NBRC 15149^T	89.63	38.70	GCA_001591365
'*Variovorax beijingensis*' 502^T	89.58	38.90	GCA_003951285
Variovorax boronicumulans NBRC 103145^T	85.63	30.10	GCA_001591345
Variovorax durovernensis GSTT-20^T	85.49	30.00	SAMEA8682661
Variovorax gossypii DSM 100435^T	85.32	29.70	GCA_003965815
Variovorax. guangxiensis DSM 27352^T	84.87	29.20	GCA_003952165
Variovorax ginsengisoli DSM 25185^T	81.31	24.60	GCA_030486595
Variovorax defluvii DSM 27259^T	80.61	23.50	GCA_039541515
Variovorax ureilyticus KACC 18899^T	80.22	23.40	GCA_037889935
Variovorax robiniae KACC 18901^T	80.00	23.10	GCA_037889955
Variovorax soli NBRC 106424^T	79.93	23.00	GCA_001591385
Variovorax rhizosphaerae KACC 18900^T	79.85	23.30	GCA_037889975
Variovorax humicola KACC 18501^T	79.79	23.00	GCA_037889915
'*Variovorax terrae*' CYS-02^T	78.37	21.90	GCA_022809125
Variovorax dokdonensis CIP 108838^T	77.77	21.20	GCA_030316895

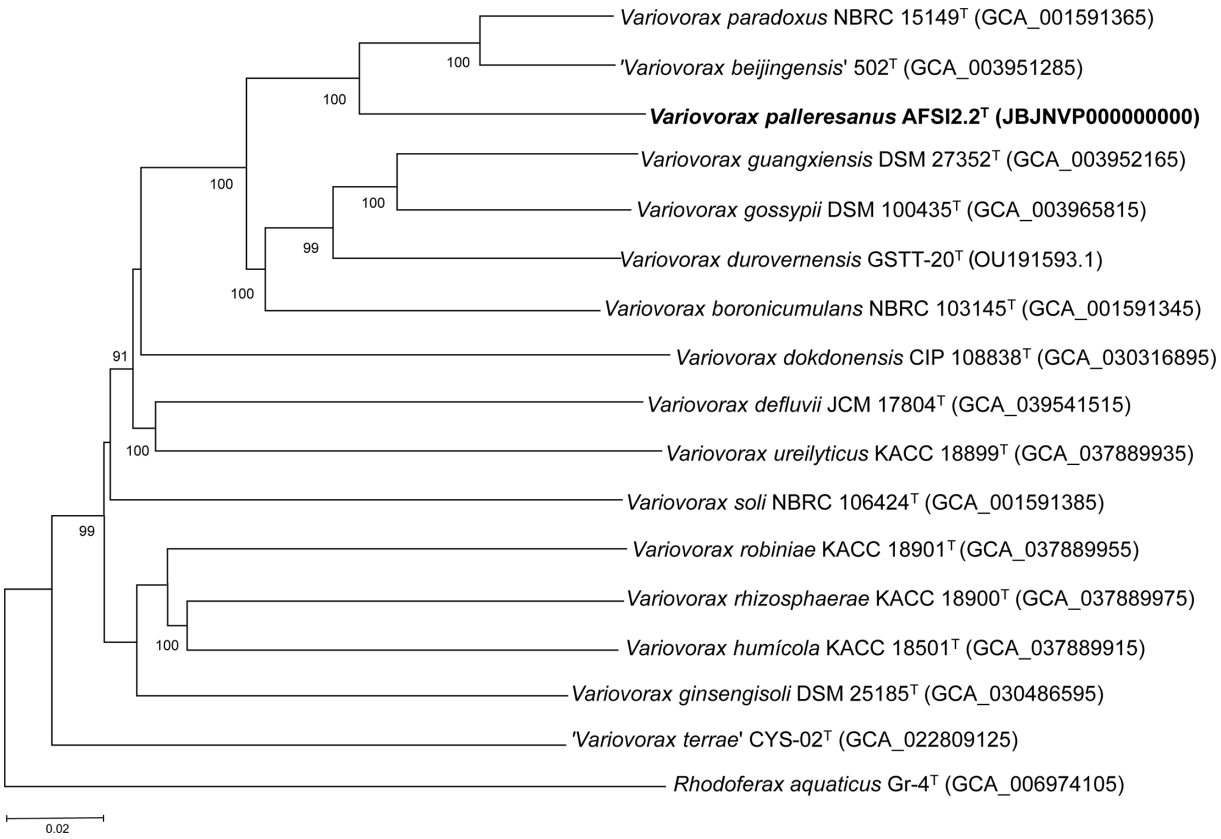


Fig. 2. Whole-genome tree inferred with FastME 2.1.6.1 from GBDP distances calculated from AFSI2.2T genome sequence and its closely related type species. The branch lengths are scaled in terms of GBDP distance formula d5. The numbers above the branches are GBDP pseudo-bootstrap support values when >70% from 100 replications. The tree was rooted at the midpoint. Genome accession numbers are given in parentheses. Species names written in quotation marks are not validly published (https://lpsn.dsmz.de). *R. aquaticus* Gr-4^T was used as an outgroup. Bar, 0.02 substitutions per site.

nthB)/amidase (*iaaH*)-dependent route. The IAN pathway has been identified as the primary route for IAA biosynthesis in its closely related strain *V. boronicumulans* CGMCC 4969 [12]. The AFSI2.2^T genome harbours the tryptophan operon (*trpSEGDCABF*) and the genes encoding indolepyruvate ferredoxin oxidoreductases (*ior*), enzymes recently proposed to be involved either in IAA catabolism or in an alternative IAA biosynthetic pathway where indole-3-pyruvic acid is directly converted into IAA [46]. Interestingly, the AFSI2.2^T genome also harbours an auxin degradation (*iad*) operon, widely conserved among *Variovorax* genomes, which is proposed to mediate the reversion of root growth inhibition, thereby promoting root development and rhizosphere colonization [47].

Genes encoding other phytohormones and additional plant-stimulating molecules were also identified through functional genome analysis. The ACC deaminase gene (*acdS*), well known for enhancing plant growth by reducing levels of the stress-associated phytohormone ethylene [13], was detected in the AFSI2.2^T genome. The potential for cytokinin production in strain AFSI2.2^T is supported by the presence of *miaA* and *miaB* genes, which encode tRNA dimethylallyltransferase and tRNA-2-methylthio-N6-dimethylallyladenosine synthase, respectively, as well as *xdhA* and *xdhB*, encoding xanthine dehydrogenase subunits [48]. Additionally, the detection of the *puuD* gene (gamma-glutamyl-gamma-aminobutyrate hydrolase), along with genes involved in GABA degradation (*gabT* and *gabD*), suggests that this strain also has the potential for GABA biosynthesis.

SOB enhance soil fertility by converting elemental sulphur or thiosulphate into sulphate, a form that plants can readily absorb and utilize [16]. AFSI2.2^T was found to encode two thiosulphate sulphurtransferase (rhodanese), which may be involved in energy generation from thiosulphate and in the oxidation of reduced sulphur compounds. The annotation also showed a thiosulphate/3-mercaptopyruvate sulphurtransferase, which has been associated with the biosynthesis of hydrogen sulphide [49]. AFSI2.2^T genome presented additional genes related to inorganic sulphur assimilation and transport, including *cysP*, *cysT*, *cysW* and *cysA* (involved in inorganic sulphate uptake); *cysN* and *cysD* (involved in the activation of the sulphate through adenylation); and *cysE* and *cysK*, which participate in cysteine biosynthesis. Moreover, the AFSI2.2^T genome also harboured genes encoding enzymes for the metabolism of alkanesulfonates, a primary form of organosulphur compounds in farming soils. This included the *ssuD*

and *ssuE* genes, which encode a monooxygenase system required for desulphonation, as well as *ssuA*, *ssuB* and *ssuC*, which form an ABC transporter system responsible for alkanesulfonate uptake [49].

Siderophore production is a key trait involved in iron homeostasis, commonly reported in plant-beneficial bacteria. AntiSMASH analysis identified in the AFSI2.2^T genome a siderophore gene cluster (*varABCDEFGHIJKLMNOP*) (Table S1), similar to other *Variovorax* strains, which are reported to be involved in the biosynthesis of lipopeptide-like siderophores, including variochelin, vacidobactin, variobactin and imaqobactin [50–52]. Genes related to the biosynthesis of additional siderophores were identified in the AFSI2.2^T genome, including *pvdF* (formyltransferase family protein), *aroC* (chorismate synthase) and *dhbB* (isochorismatase family protein) [49]. The genome also harboured genes related to siderophore transport and iron sequestration, such as those encoding TonB-dependent receptors, bacterioferritin (*bfr*), ferrochelatase (*hemH*) and iron ABC transporter permeases. Moreover, an NRPS-T1PKS BGC with similarity to the *var* cluster was identified through AntiSMASH analysis, suggesting a potential role in the production of additional siderophores (Table S1).

Apart from the siderophore-related BGCs, AntiSMASH identified ten additional clusters, highlighting the diverse secondary metabolism of strain AFSI2.2^T (Table S1). These include one terpene and two arylpolyene BGCs potentially involved in pigment synthesis, consistent with the yellow coloration of colonies on nutrient-rich media (Fig. S2). Such pigments are known to protect bacterial cells by neutralizing reactive oxygen species and shielding against light, which may enhance survival in the rhizosphere and indirectly support plant growth under stress [53, 54]. The presence of a redox cofactor BGC suggests that AFSI2.2^T may synthesize molecules such as riboflavin derivatives, which can function as signalling compounds in the rhizosphere, enhancing root colonization and stress mitigation [55]. Lastly, the two hydrogen cyanide BGCs may support plant growth by enhancing nutrient availability in certain environments, as previously suggested [56].

PHYSIOLOGY AND CHEMOTAXONOMY

Cell morphology, size and the presence of flagella were examined using phase-contrast microscopy (BH2, Olympus) and transmission electron microscopy (FEI Tecnai Spirit 120kV). Gram staining was performed following a standard protocol [57]. To determine the optimal growth conditions, we followed the growth of AFSI2.2^T in TSA for up to 5 days, at different temperatures (4–40 °C, at 4 °C intervals), pH levels (4–10, at 0.5 pH unit intervals) and salt concentrations [0–10%, at 1% intervals of NaCl (w/v)]. Traditional and standard biochemical tests were used to further characterize the bacterium. Oxidase activity was assessed by the oxidation of 1% (w/v) tetramethyl-p-phenylenediamine, whereas catalase activity was evaluated by the formation of oxygen bubbles in a 3% (v/v) aqueous hydrogen peroxide solution. We also conducted API 20 NE, API 50 CH and API ZYM test kits according to the manufacturer's instructions (bioMérieux, France). To further characterize strain AFSI2.2^T according to its PGP characteristics, we tested different culture media: chrome azurol S agar for siderophore production [58]; IAA production was determined with Salkowski reagent following the protocol in [59]; and modified Dworkin and Foster minimal salt medium, with ACC as a sole nitrogen source, for ACC deaminase activity [60]. The cellular fatty acid composition was determined at Colección Española de Cultivos Tipo (CECT) (Valencia, Spain) following the protocol recommended by MIDI Microbial Identification System [61]. Cells were grown in TSA at 28 °C for 48 h, and the cellular fatty acid content was obtained using an Agilent 6850 gas chromatograph, with the MIDI Microbial Identification System employing the CLIN6 method [62]. *In silico* chemotaxonomic analysis was carried out mainly using the SEED server with RAST annotations [43, 44] and further examined with BlastKOALA KEGG Mapper to assign KEGG Orthology terms and to obtain KEGG mapping [63]. When specific genes were not annotated in these resources, additional searches were performed using the NCBI Basic Local Alignment Search Tool (BLAST) with query sequences from related species (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Strain AFSI2.2^T grown for 72 h on TSA exhibited yellowish-pigmented, moist, convex and circular colonies with a diameter of 0.8–2.0 mm (Fig. S2). Microscopic examination revealed Gram-stain-negative, short rod-shaped cells with flagella, measuring ~0.50–0.61×1.04–1.30 µm in size (Fig. S2). Strain AFSI2.2^T showed growth within a temperature range of 5–40 °C (optimum, 28–30 °C), a pH range of 5–9 (optimum, 6.5) and in the presence of 0–3% (w/v) NaCl (optimum, 1%). These characteristics align with the general description of the genus *Variovorax* [3, 6]. Similarly to its closest phylogenetic relatives, AFSI2.2^T tested positive for catalase, oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities, and it was able to produce acid from D-fructose. However, it can be distinguished from these related species by its ability to assimilate D-mannitol, its inability to assimilate D-sorbitol and capric acid and its very weak assimilation of D-glucose (Table 3). Additionally, it differed from at least one of its phylogenetic neighbours in several biochemical traits, including urease activity, aesculin hydrolysis, nitrate reduction, valine arylamidase and cystine arylamidase. It also showed distinct assimilation profiles for a wide range of carbon sources, including malic acid, glycerol, D-xylose, D-adonitol, D-galactose, L-rhamnose, dulcitol, methyl-α-D-glucopyranoside, D-melibiose, sucrose, D-trehalose, D-fucose, D-arabitol, L-arabitol, D-mannose, adipic acid and phenylacetic acid. A detailed comparison of differential phenotypic traits between AFSI2.2^T and its closest relatives is outlined in Table 3, while results from biochemical commercial tests can be found in Table S2.

In addition to oxidizing inorganic sulphur, strain AFSI2.2^T exhibited other *in vitro* traits typical of biostimulant rhizobacteria, including ACC deaminase activity, siderophore production and synthesis of the phytohormone IAA, which reached a maximum

Table 3. Differential phenotypic traits of AFSI2.2^T and its closest phylogenomic relatives. Species names written in quotation marks are not validly published (https://lpsn.dsmz.de)

	V. palleresanus AFSI2.2^T	*V. paradoxus* NBRC 15149^T	'*Variovorax beijingensis*' 502^T	*V. boronicumulans* NBRC 103145^T	*V. gossypii* DSM 100435^T
Morphology and growth conditions:					
Colony colour	Yellowish	Yellow	Yellow	Yellow	Beige
Cell shape	Rod	Rod	Rod	Rod	Rod
Cell size (µm)	0.5–0.6×1.0–1.3	0.5–0.6×1.2–3.0	0.5–0.6×0.6–1.1	0.5–0.7×1.0–2.0	0.5–0.8×1.5–2.0
Motility	+	+	NP	+	+
Temperature range (°C)	5–40	4–40	20–40	4–37	10–37
pH range	5–9	6–9	5–9	5–9	4.5–9
Salinity tolerance up to (NaCl %)	3	2	2	1	3
MacConkey agar	–	–	ND	–	+
Activity:					
Urease	–	–	+	+	+
Aesculin hydrolysis	–	+	W	–	+
Nitrate reduction	–	+	+	–	NP
Valine arylamidase	–	+	W	W	NP
Cystine arylamidase	–	+	W	W	NP
Assimilation of:					
D-Mannitol	+	–	–	–	–
D-Sorbitol	–	+	+	+	+
Capric acid	–	+	+	+	NP
D-Glucose	W	+	+	+	+
Malic acid	+	+	–	–	NP
Glycerol	–	W	+	+	–
D- Xylose	–	+	–	+	–
D-Adonitol	–	+	–	+	W
D-Galactose	W	+	W	+	–
L-Rhamnose	–	–	–	+	–
Dulcitol	–	–	–	+	–

Continued

Table 3. Continued

	V. palleresanus AFSI2.2^T	*V. paradoxus* NBRC 15149^T	'*Variovorax beijingensis*' 502^T	*V. boronicumulans* NBRC 103145^T	*V. gossypii* DSM 100435^T
Methyl-α-D-glucopyranoside	–	–	+	–	–
D-Melibiose	–	–	–	+	–
Sucrose	–	+	+	+	–
D-Trehalose	–	–	+	+	–
D-Fucose	w	–	w	+	NP
D-Arabitol	w	–	w	+	–
L-Arabitol	–	–	–	+	NP
D-Mannose	–	+	–	–	–
Adipic acid	–	+	+	–	NP
Phenylacetic acid	–	+	w	–	–

Data for *V. paradoxus* NBRC 15149^T, '*Variovorax beijingensis*' 502^T, *V. boronicumulans* NBRC 103145^T and *V. gossypii* DSM 100435^T, retrieved from [1, 3, 4, 64], respectively. All strains grew well in nutrient and trypticase soya agar; were positive for catalase, oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase activity; and produced acids from D-fructose. +, positive; w, weak positive; –, negative; NP, not provided.

Table 4. Relative cellular fatty acid content (%) of AFSI2.2^T and its closest phylogenetic species. Species names written in quotation marks are not validly published (https://lpsn.dsmz.de)

Cellular fatty acid	*V. palleresanus* AFSI2.2^T	*V. paradoxus* NBRC 15149^T	'*Variovorax beijingensis*' 502^T	*V. boronicumulans* NBRC 103145^T	*V. gossypii* DSM 100435^T
$C_{8:0}$ 3-OH	0.6	4.7	–	–	–
$C_{9:0}$	1.1	–	–	–	–
$C_{9:0}$ 3-OH	–	2.1	–	–	–
$C_{10:0}$	0.8	–	2.8	–	–
$C_{10:0}$ 3-OH	3.1	1.8	26.2	2.3	8.0
$C_{12:0}$	10	3.5	8.1	3.5	4.1
$C_{12:0}$ 2-OH	1.1	–	–	0.7	–
$C_{14:0}$	1.8	–	1.6	–	–
$C_{14:0}$ 2-OH	2.4	2.4	6.2	2.6	4.1
$C_{15:0}$ *ω6c*	–	2.2	–	–	–
$C_{16:0}$	27.4	35.8	12.9	29.0	26.9
$C_{16:1}$ *ω5c*	–	1.6	–	–	–
$C_{17:0}$	–	2.4	–	–	–
$C_{17:0}$ cyclo	4.4	22.9	14.5	24.2	9.4
$C_{18:0}$	5.5	–	–	–	–
$C_{18:3}$ *ω6c* (6, 9, 12)	1.8	–	–	–	–
$C_{19:0}$ cyclo *ω8c*	–	1.5	–	2.0	–
Summed Feature 3	25.6	17.7	21.4	14.8	31.6
Summed Feature 8	13.6	11.5	5.5	17.4	15.2

All data were retrieved from [6], except data for *V. beijingensis* 502^T [3] and for AFSI2.2^T (this study). Summed Features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total. Summed Feature 3 consists of $C_{16:1}$ *ω7c* and/or $C_{16:1}$ *ω6c*, while Summed Feature 8 includes $C_{18:1}$ *ω7c* and/or $C_{18:1}$ *ω6c*.

of 123±8 ng ml^{-1} in TSA medium supplemented with 500 µg ml^{-1} L-tryptophan. These phenotypic results are consistent with the detection of genes associated with these PGP traits in the AFSI2.2^T genome, which have also been reported in closely related *Variovorax* species such as *V. beijingensis* [3], *V. paradoxus* [15] and *V. boronicumulans* [12].

Chemotaxonomic analysis of strain AFSI2.2^T revealed a fatty acid profile consistent with other *Variovorax* species, displaying as major components $C_{16:0}$ (27.4%), Summed Feature 3 containing $C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c* (25.6%) and Summed Feature 8 containing $C_{18:1}$ *ω7c* and/or $C_{18:1}$ *ω6c* (13.6%) (Table 4). Minor quantitative differences were observed in several low-abundance fatty acids, including $C_{17:0}$ cyclo, $C_{18:0}$, $C_{18:3}$ ω6*c* and $C_{9:0}$, when compared with previously reported profiles for the genus [6]. *In silico* chemotaxonomic profiling of strain AFSI2.2^T revealed the presence of *ubiABCEFGIJ*, indicating the genomic capacity for ubiquinone biosynthesis, consistent with the ubiquinone-8 profiles reported for closely related species [3, 4]. Genes encoding the synthesis of phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol were also detected (Table S3), suggesting that these polar lipids are likely major constituents of the strain, as observed in phylogenetically close taxa [3, 4].

Genomic, phylogenetic, biochemical and chemotaxonomic evidence supports that strain AFSI2.2^T constitutes a new species within the genus *Variovorax*. The proposed name for this species is *V. palleresanus* sp. nov.

DESCRIPTION OF *VARIOVORAX PALLERESANUS* SP. NOV.

Variovorax palleresanus (pal.le.res.a'nus. N.L. masc. adj. *palleresanus*, derived from the name Pallarés, in honour of Joan Pallarés, who founded AFEPASA in 1893, the company responsible for the isolation of this bacterium). Cells are Gram-stain-negative, motile rods (~0.50–0.61×1.04–1.30 µm) with flagella. Colonies on TSA agar are yellowish, circular, moist, convex with 0.8–2.0 mm

in diameter after 72 h at 30 °C. Growth occurs at 5–40 °C, at a pH range of 5–9 and in the presence of 0–3% (w/v) NaCl. Catalase, oxidase, alkaline phosphatase, esterase (C4), esterase lipase (C8), leucine arylamidase and naphthol-AS-BI-phosphohydrolase activities are positive; valine arylamidase, cystine arylamidase, trypsin, *α*-chymotrypsin, acid phosphatase, *α*-galactosidase, *β*-galactosidase, *β*-glucuronidase, *α*-glucosidase, *β*-glucosidase, n-acetyl-*β*-glucosaminidase, *α*-mannosidase and *α*-fucosidase activities are negative. Assimilate L-arabinose, D-mannitol, potassium gluconate, malic acid; weakly assimilate glucose; does not assimilate D-manose, *N*-acetyl-glucosamine, D-maltose, capric acid, adipic acid, trisodium citrate and phenylacetic acid. Acids are produced from D-fructose and D-mannitol; weakly produced from D-galactose, D-fucose and D-arabitol; and not produced from glycerol, erythritol, D-arabinose, L-arabinose, D-ribose, D-xylose, L-xylose, D-adonitol, methyl-β-D-xylopyranoside, D-glucose, D-mannose, L-sorbose, L-rhamnose, dulcitol, inositol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, n-acetyl-glucosamine, amygdalin, arbutin, aesculin ferric citrate, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, sucrose, D-trehalose, inulin, D-melezitose, D-raffinose, starch, glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, L-fucose, L-arabitol, gluconate K, 2-ketogluconate K and 5-ketogluconate K. The predominant fatty acids are $C_{16:0}$, Summed Feature 3 containing $C_{16:1}$ *ω6c* and/or $C_{16:1}$ *ω7c* and Summed Feature 8 containing $C_{18:1}$ *ω7c* and/or $C_{18:1}$ *ω6c*.

The type strain, AFSI2.2^T (=CECT 31217^T=CIP 112526^T), was isolated from the rhizosphere soil of a mulberry plant in Constantí, Tarragona, Spain. The genome size of the type strain is 6,096 Mb, comprising a chromosome and 1 megaplasmid. The DNA G+C content is 66.2 mol% (calculated from the whole-genome sequence). The GenBank accession numbers for the 16S rRNA partial gene and genome sequences of strain AFSI2.2^T are PQ725731 and JBJNVP000000000, respectively.

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Author contributions
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Conflicts of interest
The authors declare that there are no conflicts of interest.

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