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# *Pseudomonas canavaninivorans* sp. nov., isolated from bean rhizosphere

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**Abstract**

A novel canavanine-degrading bacterium, strain HB002$^T$, was isolated from rhizosphere soil of a catch crop field collected from the island of Reichenau in Konstanz, Germany, and characterized by using polyphasic taxonomy. The facultative aerobe, rod-shaped, Gram-stain-negative bacterium was oxidase- and catalase-positive. The isolate was able to grow on canavanine as a sole carbon and nitrogen source. Results of phylogenetic analysis based on 16S rRNA gene sequences revealed highest similarities to *Pseudomonas bijieensis* (L22-9$^T$, 99.93%), *Pseudomonas brassicacearum* subsp. *neoaurantiaca* (ATCC 49054$^T$, 99.76%), *Pseudomonas brassicacearum* subsp. *brassicacearum* (DBK 11$^T$, 99.63%), *Pseudomonas thivervalensis* (DSM 13194$^T$, 99.51%), *Pseudomonas kilonensis* (DSM 13647$^T$, 99.39%) and *Pseudomonas corrugata* (ATCC29736$^T$, 99.39%). Marker gene analysis placed the strain in the intrageneric group of *Pseudomonas fluorescens*, subgroup *P. corrugata*. *In silico* DNA–DNA hybridization and average nucleotide identity values were both under the recommended thresholds for species delineation. The predominant fatty acids of strain HB002$^T$ were $C_{16:0}$, $C_{17:0}$ cyclo $\omega 7c$ and $C_{18:1}$ $\omega 7c$. The major respiratory quinone was Q9, followed by Q8 and minor components of Q7 and Q10. Results from the phenotypic characterization showd the strain's inability to hydrolyse gelatin and to assimilate *N*-acetyl glucosamide and a positive enzymatic activity of acid phosphatase and naphthol-AS-BI phosphohydrolase that distinguish this strain from closely related type strains. Taken together, these results show that strain HB002$^T$ represents a novel species in the genus *Pseudomonas*, for which the name *Pseudomonas canavaninivorans* sp. nov. is proposed. The type strain is HB002$^T$ (=DSM 112525$^T$=LMG 32336$^T$).

## INTRODUCTION

Since its first description in 1894 [1], the genus *Pseudomonas* has been found in a variety of habitats ranging from soil, rhizosphere, waste water, open ocean and insects, to sites in and on the human body [2, 3]. According to the List of Prokaryotic Names with Standing in Nomenclature [4], more than 350 species of the genus *Pseudomonas* have been validly published to date. Representative members have been identified as plant growth-promoting microbes, bioremediation agents, and hosts for industrial bio-manufacturing [5]. Due to their plethora of ecological niches and metabolic versatility, they offer a wide range of potential industrial applications. In addition, so far uncharacterized metabolic pathways can be identified [6, 7] which makes the identification of novel *Pseudomonas* species highly interesting. In a quest for metabolic reactions that result in the accumulation of guanidine in nature, we isolated a novel *Pseudomonas* species that was able to grow on canavanine as the sole C- and N-source via an up to date unknown degradation pathway (manuscript in preparation). Here, we describe the taxonomic position of the novel species using a polyphasic approach, including phylogenetic analysis based on 16S rRNA gene and whole genome sequencing, phenotypic characterization, and the identification of chemotactic features.

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Keywords: bean rhizosphere; canavanine; polyphasic taxonomy; *Pseudomonas canavaninivorans*.
Abbreviations: ANI, average nucleotide identity; dDDH, digital DNA–DNA hybridization; LB, lysogeny broth; R2A, Reasoner's 2A.
The Whole Genome Shotgun (WGS) project of strain HB002$^T$ (=DSM 112525$^T$=LMG 32336$^T$) was deposited at DDBJ/ENA/GenBank under the accession number JAEKIK000000000. The 16S rRNA gene sequence of strain HB002$^T$ was deposited at DDBJ/ENA/GenBank under the accession number MZ644983.

## ISOLATION

Serial dilutions of bean rhizosphere soil samples were plated out on M9 (without addition of ammonium) minimal salt medium agar with 10 mM canavanine as the sole carbon and nitrogen source and incubated at 30 °C until unique colonies could be identified. Several rounds of enrichment were done on single colonies to ensure the purity of the isolate. Uniformly growing, single colonies were cultivated in liquid media for further analysis and identification. The strains were maintained at −80 °C in a suspension of lysogeny broth (LB) supplemented with 50% (w/v) glycerol.

## PHYLOGENY AND GENOME FEATURES

Isolates were randomly picked and identified by 16S rRNA gene analysis after colony Phusion PCR using primers 16S_27 (5′-AGAGTTTGATCCTGGCTCA-3′) [8] and 16S_1492 (5′- CGGCTACCTTGTTACGAC-3′) [8]. The amplicon was sequenced by Sanger sequencing (Eurofins Genomics) using primer 16S_907 (5′-CCGTCAATTCMTTTGAGTTT-3′) [8]. Running the sequence against the EzBioCloud 16S database [9] revealed that the bacterium belongs to the genus *Pseudomonas* with its closest relatives being *P. piscium* (P50$^T$, 100% similarity, 84.4% completeness), *P. bijieensis* (L22-9$^T$, 99.93% similarity, 100% completeness), *P. brassicacearum* subsp. *neoaurantiaca* (ATCC 49054$^T$, 99.76% similarity, 99.7% completeness), *P. brassicacearum* subsp. *brassicacearum* (DBK 11$^T$, 99.63% similarity, 99.9% completeness), *P. thivervalensis* (DSM 13194$^T$, 99.51% similarity, 100% completeness), *P. kilonensis* (DSM 13647$^T$, 99.39% similarity, 100% completeness) and *P. corrugata* ATCC 29736$^T$, 99.39% similarity, 98.6% completeness). All identified strains belong to the intrageneric group of *Pseudomonas fluorescens* [10]. A summary of the top valid hits and the gene sequences used for the analysis can be found in the supplementary material (Table S1 and File S1, available in the online version of this article).

For whole genome sequencing gDNA was isolated using DNeasy Blood and Tissue Kit (Qiagen), following the manufactures protocol for bacterial gDNA isolation. Sequencing was carried out by Novogene (Cambridge, UK). In short, sequencing libraries were generated using the NEBNext Ultra DNA Library Prep Kit for Illumina following manufacturer's recommendations. Sequencing was done using an Illumina NovaSeq PE150 system and *de novo* assembly was performed using SOAPdenovo software (version 2.04). Genome coverage was 159.0× with a G+C content of 61.04 mol%, which is close to the genus average of 61.2 mol% [11].

Further identification of close type strain relatives and digital DNA–DNA hybridization (dDDH) was done using the Type (Strain) Genome Server (TYGS) [12–17]. A detailed description of the determination of closely related type strains and the calculation procedure for pairwise comparison, phylogenetic inference and species clustering can be found in the supplementary material (table S2). Based on the MASH algorithm [18] in combination with 16S rRNA gene sequence analysis

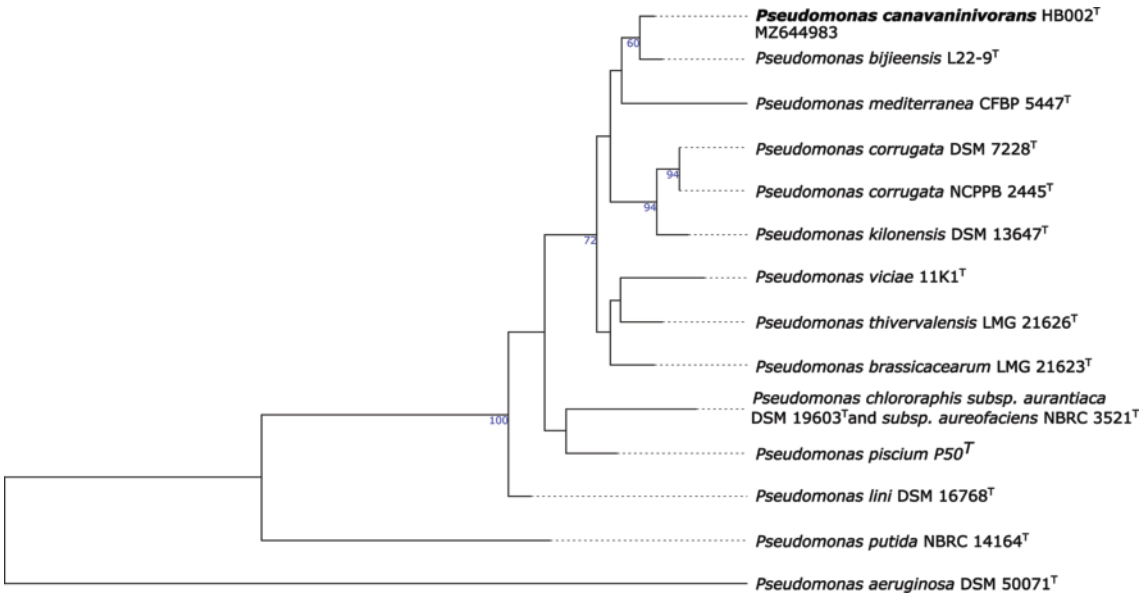


**Fig. 1.** Tree inferred with FastME 2.1.6.1 [15] from GBDP distances calculated from 16S rRNA gene sequences. The branch lengths are scaled in terms of GBDP distance formula d5 (for a more detailed description see File S2). The numbers above branches are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 74.1%. The tree was rooted at the midpoint. *P. putida* and *P. aeruginosa* were chosen as outgroups. Strain HB002$^T$ is highlighted in bold.

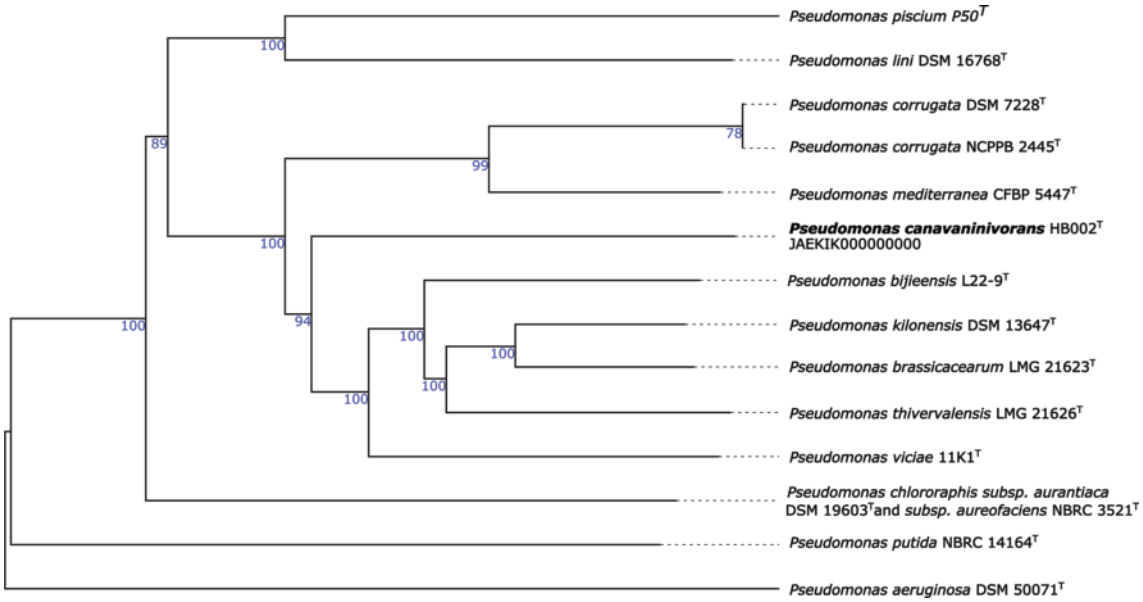


**Fig. 2.** Tree inferred with FastME 2.1.6.1 [15] from GBDP distances calculated from genome sequences. The branch lengths are scaled in terms of GBDP distance formula d5 (for a more detailed description see File S2). The numbers above branches are GBDP pseudo-bootstrap support values >60% from 100 replications, with an average branch support of 74.1%. The tree was rooted at the midpoint. *P. putida* and *P. aeruginosa* were chosen as outgroups. Strain HB002$^T$ is highlighted in bold.

[13, 19, 20], a phylogenetic tree was built (Fig. 1) which confirmed the preliminary 16S rRNA gene amplification result that strain HB002$^T$ clustered with known members of the *P. corrugata* subgroup of *P. fluorescens*. dDDH values for closely related type strains were all below the 70% cut-off value for species delineation [21]. Fig. 2 shows the phylogenetic tree inferred from the genome BLAST distance phylogeny (GBDP) calculated on the genome sequences. Table 1 shows the calculated dDDH values and the difference in G+C content between the query sequence and closely related type strains of the genus *Pseudomonas*.

**Table 1.** Digital DNA–DNA hybridization (dDDH) calculations for HB002$^T$ and closely related type strains

The dDDH cut-off value for the identification of a novel species is <70%. The three GBDP formulas d0, d4 and d6 are explained in more detail in the supplementary material (see TYGS summary).

| Hit taxon | dDDH (d0; %) | dDDH (d4; %) | dDDH (d6; %) | G+C content difference (mol%) |
|---|---|---|---|---|
| *Pseudomonas thivervalensis* LMG 21626$^T$ | 64.6 | 35.4 | 57.4 | 0.14 |
| *Pseudomonas bijieensis* L22-9$^T$ | 60.2 | 34.2 | 53.6 | 0.20 |
| *Pseudomonas kilonensis* DSM 13647$^T$ | 61.0 | 35.2 | 54.6 | 0.19 |
| *Pseudomonas brassicacearum* LMG 21623$^T$ | 60.7 | 35.1 | 54.3 | 0.25 |
| *Pseudomonas bijieensis* L22-9$^T$ | 59.5 | 33.4 | 52.7 | 0.19 |
| *Pseudomonas viciae* 11K1$^T$ | 55.1 | 33.6 | 49.4 | 0.71 |
| *Pseudomonas mediterranea* CFBP 5447$^T$ | 50.6 | 32.0 | 45.4 | 0.18 |
| *Pseudomonas corrugata* NCPPB 2445$^T$ | 50.0 | 31.1 | 44.7 | 0.43 |
| *Pseudomonas corrugata* DSM 7228$^T$ | 49.9 | 31.1 | 44.6 | 0.42 |
| *Pseudomonas chloroaphis* subsp. *aureofaciens* NBRC 3521$^T$ | 29.5 | 27.2 | 27.9 | 1.7 |
| *Pseudomonas piscium* P50$^T$ | 26.7 | 26.1 | 25.4 | 2.55 |
| *Pseudomonas lini* DSM 16768$^T$ | 33.0 | 26.8 | 30.5 | 2.23 |

**Table 2.** TrueBac ID results showing the average nucleotide identity and sequence similarity of the respective genes of HB002$^T$ with those of closely related species

The ANI cut-off value for the identification of a novel species is <95%.

| Hit taxon | ANI (%) | ANI coverage (%) | 16S (%) | *recA* (%) |
|---|---|---|---|---|
| *Pseudomonas brassicacearum* subsp. *neoaurantiaca* ATCC 49054$^T$ | 89.08 | 73.8 | 99.79 | 94.87 |
| *Pseudomonas thivervalensis* DSM 13194$^T$ | 89.32 | 74.8 | 99.59 | NA |
| *Pseudomonas bijieensis* L22-9$^T$ | 87.61 | 48.2 | 99.93 | 93.9 |
| *Pseudomonas corrugata* LMG 2172$^T$ | 87.67 | 64.2 | 99.58 | 95.06 |
| *Pseudomonas kilonensis* DSM 13647$^T$ | 89.18 | 72.9 | 99.52 | 94.87 |
| *Pseudomonas brassicacearum* subsp. *brassicacearum* LMG 21623$^T$ | 89.08 | 73.0 | 99.52 | 94.78 |
| *Pseudomonas moorei* DSM 12647$^T$ | 86.15 | 41.1 | 97.85 | NA |
| *Pseudomonas umsongensis* LMG 21317$^T$ | 86.05 | 39.4 | 97.67 | N/A |
| *Pseudomonas reinekei* DSM 18361$^T$ | 85.99 | 41.5 | 97.60 | NA |

Average nucleotide identity (ANI) was calculated using the TrueBac ID server [22–24] and confirmed the previous dDDH analysis based classification of the isolated strain as belonging to a new species of the genus *Pseudomonas*. The ANI scores of closest relatives were all below the threshold of 95% for species delineation [25]. Also, the housekeeper gene *recA* showed high differences in similarity (see Table 2). *In silico* PCR on nine marker genes was performed as described by Garrido-Sanz *et al*. [26] and, as expected from the previous classification results, strain HB002$^T$ obtained the specific markers of the *P. corrugata* phylogroup.

Taken together, the genomic evidence strongly indicates the discovery of a novel species belonging to the group of *P. corrugata* within the lineage of *Pseudomonas fluorescens*.

## PHENOTYPIC AND CHEMOTAXONOMIC CHARACTERIZATION

Strain HB002$^T$ was routinely incubated at 30 °C with 200 r.p.m. shaking. Growth was positive on LB agar, Simmons citrate agar, R2A agar and M8 minimal salt agar supplemented with canavanine as a sole carbon and nitrogen source. The tests for DNA and gelatin hydrolysis were conducted by using DNA [27] and gelatin hydrolysis test agars. The ability to produce pigments was tested on King A and King B agars [28]. Catalase activity was determined by bubble production using 3% (v/v) hydrogen peroxide and oxidase activity using Kovács' reagent [29]. Anaerobic growth behaviour was tested in liquid M9 (without addition of ammonium) minimal salt medium with 10 mM canavanine as sole carbon source and 10 mM $KNO_3$ as electron acceptor. All media and cultivation bottles were flushed with $N_2$ prior to inoculation. Growth behaviour under varying NaCl concentrations, pH conditions and different temperatures was investigated. To assess the phenotypic traits of strain HB002$^T$, API20NE and API ZYM commercial kits (bioMérieux) were used by following the manufacturer's protocol and compared to closely related strains. Motility, size and shape were checked by polarization microscopy (Zeiss Axiolmagar) after growing cells in LB medium at 30 °C for 24 h. The cellular fatty acid and respiratory quinone analyses were carried out by the identification service of Leibiz-Institute DSMZ – Deutsche Sammlung von Mikroorganismen und Zellkultur GmbH (Braunschweig, Germany).

The key physiological traits allowing differentiation between strain HB002$^T$ from its closely related species are listed in Table 3. The results of the whole phenotypic characterization are given in the species description.

## DESCRIPTION OF *PSEUDOMONAS CANAVANINIVORANS* SP. NOV.

*Pseudomonas canavaninivorans* (ca.na.va.ni.ni.vo'rans. N.L. neut. n. *canavaninum*, canavanine; L. pres. part. *vorans*, eating; N.L. fem. part. adj. *canavaninivorans*, canavanine-eating).

A rod-shaped, Gram-stain-negative bacterium. Forms translucent, round, yellow-beige coloured colonies on LB agar. Cells are motile, 1–1.2 µm wide and 2.5–3.3 µm long. It is a facultative anaerobe in the presence of $KNO_3$ and growth occurs in 0–4.6% (w/v) NaCl, at pH varying from pH 5.5 to 8.0, and within the temperature range of 4–37 °C. Fluorescent colonies are detected on King B but not on King A agar. DNA hydrolysis is weak positive, whereas gelatin hydrolysis is negative. Catalase and oxidase tests are both positive. In the API 20NE test the bacterium is positive for the hydrolysis of aesculin and the assimilation of D-glucose, L-arabinose, D-mannose, D-mannitol, gluconate, capric acid, malic acid and citric acid. Negative for indole production, glucose fermentation, arginine dihydrolase, urease, 4-nitroso-β-D-methyl galactose, *N*-acetyl glucosamine, maltose, adipic acid

**Table 3.** Differential characteristic phenotype of Pseudomonas *canavaninivorans* and closely related species

Strains: 1, HB002$^T$; 2, *P. bijieensis* L22-9$^T$ [30]; 3: *P. corrugata* NCPPB 2445$^T$ (BacDive ID: 13044); 4, *P. viciae* 11K1$^T$ [31]; 5, *P. kilonensis* DSM 13647$^T$ [32]; 6, *P. brassicacearum* DBK11$^T$ [33]; 7, *P. thivervalensis* DSM13194$^T$ [33]. Data for strain HB002$^T$ from this study and data for the related strains obtained from indicated respective references/database. +, Positive; –, negative; w, weak positive; v, variable; na, data not available.

| Characteristics | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| API 20NE assay: | | | | | | | |
| Gelatin hydrolysis | – | + | + | – | + | + | + |
| Aesculin hydrolysis | w | – | – | – | – | – | – |
| Assimilation of arabinose | w | + | + | + | + | + | + |
| Assimilation of mannitol | + | + | + | + | + | – | + |
| Assimilation of *N*-acetyl glucosamine | – | + | + | – | – | + | + |
| API ZYM assay: | | | | | | | |
| Alkaline phosphatase | + | + | v | + | na | na | na |
| Esterase (C4) | + | + | + | – | na | na | na |
| Esterase lipase (C8) | + | + | + | w | na | na | na |
| Lipase (C14) | – | – | – | + | na | na | na |
| Leucine aryl amidase | + | + | + | – | na | na | na |
| Valine aryl amidase | + | + | v | – | na | na | na |
| Cystine aryl amidase | – | – | – | w | na | na | na |
| Trypsin | – | – | + | + | na | na | na |
| α-Chymotrypsin | – | + | v | + | na | na | na |
| Acid phosphatase | + | – | v | – | na | na | na |
| Naphthol AS-BI-phosphohydrolase | + | + | – | – | na | na | na |

and phenylacetic acid. In the API ZYM system positive for alkaline phosphomonoesterase, esterase (C4), esterase lipase (C18), leucine arylamidase, acid phosphatase and naphthol AS-BI-phosphohydrolase; weak positive for valine arylamidase; and negative for lipase (C14), cysteine arylamidase, α-chemotrypsine, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. The predominant fatty acids of strain HB002$^T$ are $C_{16:0}$, $C_{17:0}$ cyclo ω7*c* and $C_{18:1}$ ω7*c*. The major respiratory quinone is Q9, followed by Q8 and only minor amounts of Q7 and Q10. A more detailed overview of the fatty acid and respiratory quinone profile can be found in Table S2.

The type strain, HB002$^T$ (=DSM 112525$^T$=LMG 32336$^T$), was isolated from the rhizosphere soil of a runner bean (*Phaseolus coccineus*) field that was collected from the island of Reichenau, Lake Constance, Germany in mid-August 2020. The DNA G+C content of strain HB002$^T$ is 60.02 mol%.

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

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