Pseudomonas azotifigens sp. nov., a novel nitrogen-fixing bacterium isolated from a compost pile

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A nitrogen-fixing bacterium, designated strain 6H33b^T, was isolated from a compost pile in Japan. The nitrogenase activity of this strain was detected based on its acetylene-reducing activity under low oxygen concentrations (2–4 %). An analysis of the genes responsible for nitrogen fixation in this strain, *nifH* and *nifD*, indicated a close relationship to those of *Pseudomonas stutzeri* A15 (A1501). Sequence similarity searches based on the 16S rRNA gene sequences showed that strain 6H33b^T belongs within the genus *Pseudomonas sensu stricto*; closest similarity was with *Pseudomonas indica* (97.3 %). A comparison of several taxonomic characteristics of 6H33b^T with those of *P. indica* and some type strains of the genus *Pseudomonas sensu stricto* indicated that 6H33b^T could be distinguished from *P. indica* based on the presence of nitrogen fixation ability, the absence of nitrate reduction and denitrification abilities and the utilization of some sugars and organic acids. Phylogenetic analyses and the results of DNA–DNA hybridization experiments also indicated that strain 6H33b^T represents a species distinct from *P. indica*. From these results, it is proposed that strain 6H33b^T (=ATCC BAA-1049^T=JCM 12708^T) is classified as the type strain of a novel species of the genus *Pseudomonas sensu stricto* under the name *Pseudomonas azotifigens* sp. nov.

The genus *Pseudomonas sensu stricto* includes species with various characteristics, such as the ability to degrade aromatic compounds and xenobiotics, and which are pathogenic to plants and animals. However, nitrogen fixation ability within the genus is poorly understood. It was believed that no strains in this genus were legitimate nitrogen-fixers (Young, 1992), but recent studies have demonstrated that several strains, classified as *Pseudomonas stutzeri*, do have the ability to fix nitrogen (Krotzky & Werner, 1987; Vermeiren *et al.*, 1999; Desnoues *et al.*, 2003). Among them, *P. stutzeri* A15 (A1501), isolated from rice paddies in China,

was studied in detail to determine the conditions under which nitrogen fixation occurred and the structure of the nitrogenase genes involved (Desnoues *et al.*, 2003).

A unique composting process has recently been developed, referred to as 'hyperthermal composting', which consists of rapid composting at high temperatures using a newly developed hyperthermal composting machine. When the final compost pile produced by this process was packed in polyethylene bags and sealed, several bags were found to be almost completely deflated after about 2 weeks at room temperature, similar to 'vacuum-packing'. Repacking of this compost produced the same result, i.e. a loss of atmospheric gas pressure after 2 weeks. It was considered that this result might have been achieved through biological nitrogen fixation, because nitrogen gas (80 % of the atmosphere) must have been lost. Screening of the nitrogen-fixing micro-organism(s) from the 'vacuum-packed' compost was therefore undertaken. We report the isolation of a novel nitrogen-fixing species of the genus *Pseudomonas sensu stricto* from this sample.

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA and *nifHD* gene sequences of strain 6H33b^T are respectively AB189452 and AB189453.

A phylogenetic tree based on the *nifH* gene sequence of 6H33b^T and other representative members of the β - and γ -Proteobacteria is available as a supplementary figure in IJSEM Online.

Five grams of a sample from the 'vacuum-packed' compost was inoculated in 100 ml N-free minimum medium (NFMM; 0.18 g K_2HPO_4 , 0.026 g KH_2PO_4 , 0.16 g NaCl, 0.24 g $MgSO_4 \cdot 7H_2O$, 2 mg $CaCl_2$, 2 mg $FeCl_3$, 0.2 mg $ZnSO_4 \cdot 7H_2O$, 0.06 mg $MnCl_2 \cdot 4H_2O$, 0.6 mg H_3BO_3 , 0.4 mg $CoCl_2 \cdot 6H_2O$, 0.02 mg $CuCl_2 \cdot 2H_2O$, 0.04 mg $NiCl_2 \cdot 6H_2O$, 10 mg $Na_2MoO_4 \cdot 2H_2O$, 5.0 ml acetic acid, 1000 ml distilled water, pH 7.0) and cultured for 2 weeks at 30 °C. A portion of this culture was transferred to fresh NFMM and cultivated again for 2 weeks. This treatment was repeated, and the final culture was then spread onto NFMM-agar plates to obtain single colonies. Many types of colonies were initially observed, but most did not grow when they were cultured again in liquid NFMM (data not shown). Only one colony, designated strain 6H33b^T, grew when re-cultured. This strain was then further investigated. Strain 6H33b^T grew in an aggregated form in NFMM and required several weeks to reach the stationary phase of growth. As 6H33b^T also grew on Luria-Bertani (LB) medium, this medium was used to maintain the strain for further work. Cells of strain 6H33b^T formed translucent, wrinkled, obscure-edged colonies on an LB agar plate. Microscopic observation revealed that cells were Gram-negative, straight rods, 2–5 µm long and 0.5 µm wide, and motile.

An acetylene reduction assay, a modification of the method of Desnoues *et al.* (2003), was used to detect the nitrogenase activity of 6H33b^T. Cells cultured in LB medium overnight were washed with NFMM three times and suspended in NFMM to give an OD₆₀₀ of 0.08. Five millilitres of this suspension was transferred to 20 ml tubes and sealed with rubber caps. After the tubes were degassed once, the headspace gas of the tubes was replaced by argon gas, and acetylene and oxygen gasses were added to final concentrations of 10 % (v/v) and a range from 0 to 6 % (v/v), respectively. The tubes were incubated at 30 °C with shaking, and 0.5 ml of the headspace gas was taken periodically for measurement of ethylene formation. Detection of ethylene was performed using GC with an HP 6890 series (Hewlett Packard) gas chromatograph equipped with a thermal conductivity detector. Injector and detector temperatures were set to 80 °C. The column used was a Porapak N (80/100 mesh, diameter 2.3 mm × 2.0 m; GL-Sciences), and the carrier gas was N₂ delivered at a flow rate of 30 ml min⁻¹. This assay was performed three times. Strain 6H33b^T was observed to reduce acetylene to ethylene at an oxygen concentration of 2–6 % (Fig. 1). The highest nitrogenase activity was detected at 4 % oxygen, with no activity at 0 %. This result indicated that the nitrogenase activity of 6H33b^T was controlled against oxygen tension, as reported for *P. stutzeri* A15 (A1501) (Desnoues *et al.*, 2003) and for some diazotrophs (Bergersen, 1991).

The *nifH* gene sequence of 6H33b^T, which encodes nitrogenase reductase, was analysed as follows. PCR amplification of a partial *nifH* fragment was performed using the method of Bürgmann *et al.* (2004) by using the total DNA of strain 6H33b^T, which was extracted using the method of

Fig. 1. Effect of oxygen concentration in the gas phase on the nitrogenase activity of strain 6H33b^T. Oxygen concentrations were 0 (□), 1 % (◇), 2 % (○), 4 % (△) and 6 % (▽) (v/v).

Perego *et al.* (1988). The product (331 bp) was cloned with the pGEM-T vector system (Promega). To obtain the full-length *nifH* gene sequence, a Southern blot analysis and subsequent colony hybridization were performed with the cloned PCR fragment as a probe using a DIG DNA labelling kit (Roche Diagnostics) and a DIG nucleic acid detection kit (Roche Diagnostics). In the Southern blot analysis, total DNA of strain 6H33b^T was digested with *EcoRI*, *HindIII* or *PstI* and blotted on a Hybond-N⁺ membrane (Amersham Biosciences). Hybridization signals were observed at about 9.0 kb, at more than 10 kb and at 3.2 kb, respectively, with these fragments (data not shown). The 3.2 kb *PstI* fragment was selected and cloned into pUC118 by colony hybridization, using a Hybond-C membrane (Amersham Biosciences). Sequencing of the cloned fragment was performed with a CEQ DTCS-quick start kit (Beckman Coulter) and a CEQ 2000 XL sequencing system (Beckman Coulter) using the M13 forward and reverse primers and primers generated from the internal sequence of the fragment. The sequence of the *PstI* fragment was 3196 bp in length (GenBank accession no. AB189453) and contained the complete *nifH* gene sequence (882 bp) and a partial *nifD* gene sequence (1428 bp). Similarity searches in BLASTN (Altschul *et al.*, 1997) and FASTA (Pearson, 2000) indicated that the *nifH* and *nifD* gene sequences of 6H33b^T showed the highest similarity to those of the *nifH* (GenBank accession no. X96609) and *nifD* (GenBank accession no. X95565) genes of *P. stutzeri* A15 (A1501) (96.6 and 93.2 % similarity, respectively). Moderate similarities were found to the *nifH* and *nifD* gene sequences of strains of other representative β - and γ -proteobacteria (see Supplementary Fig. S1 in IJSEM Online).

To determine the phylogenetic position of strain 6H33b^T, its 16S rRNA gene sequence was analysed. PCR amplification of a 16S rRNA gene fragment was conducted with primers Eubac27F and 1492R (DeLong, 1992) using Ex Taq (Takara

Shuzo) and a PCR System 9700 (PE Applied Biosystems). After agarose-gel electrophoresis, the fragment was purified using an EZNA gel extraction kit (Omega). Sequencing was performed with the same system described above for the *nifH* and *nifD* gene sequences using the primers reported by Hiraishi (1992) and Hiraishi *et al.* (1994). The 16S rRNA gene sequence of strain 6H33b^T was aligned at positions 32–1489 (*Escherichia coli* numbering system; Brosius *et al.*, 1978) with those of representative strains of the genus *Pseudomonas sensu stricto* using the CLUSTAL X software package (Thompson *et al.*, 1997). Distance values were calculated using the method described by Kimura (1980), which were then used in CLUSTAL X to construct a phylogenetic tree via the neighbour-joining method (Saitou & Nei, 1987) with bootstrap values (Felsenstein, 1985) based on 1000 replications. A maximum-likelihood analysis was performed in DNAML with bootstrap values based on 100 replications using the SEQBOOT and CONSENSE programs of the PHYLIP package, version 3.6a3 (Felsenstein, 2002). Visualization of these results was provided by TreeView software (Page, 1996). The 16S rRNA gene sequence of 6H33b^T (1458 bp) showed closest similarity to that of *Pseudomonas indica* IMT37^T (97.3 % similarity), and a relationship to strains of the genus *Pseudomonas sensu stricto* was tested. Phylogenetic analyses based on the 16S rRNA gene sequences, performed using the neighbour-joining (Fig. 2) and maximum-likelihood (data not shown) methods, indicated that strain 6H33b^T belongs to the genus *Pseudomonas sensu stricto*. Although differences in branching were found between the two trees, that between 6H33b^T and *P. indica* IMT37^T was supported with high bootstrap values (above 96 %) in both. These observations suggest that 6H33b^T and *P. indica* IMT37^T are representative of different species.

To characterize 6H33b^T in more detail, comparisons of several physiological characteristics were performed among 6H33b^T, *P. indica* DSM 14015^T (=IMT37^T) as its closest related strain, and *Pseudomonas aeruginosa* NCIMB 8295^T and *P. stutzeri* NCIMB 11358^T as typical strains of the genus

Pseudomonas sensu stricto. Production of pyocyanin and fluorescent pigments were tested via growth in medium A of King *et al.* (1954) and the medium described by Luisetti *et al.* (1972). Nitrate reduction and denitrification were tested according to the methods of Lelliott *et al.* (1966) and Stanier *et al.* (1966), respectively. Nitrogen fixation was tested using the method described by us above. Utilization of various carbon sources was determined in triplicate using a Biolog GN2 MicroPlate, as recommended by the manufacturer. The G+C content of the DNA of strain 6H33b^T and the reference strains was determined by the method of Tamaoka & Komagata (1984). Other physiological characteristics were tested as described by Palleroni (1984). Strain 6H33b^T grew over a pH range of 6.1–9.8 and at 28–41 °C in LB medium. It reacted positively in oxidase and catalase tests and in a test for hydrolysis of Tween 80, as did the three reference strains. Other physiological characteristics of strain 6H33b^T differed from those of the reference strains, such as the production of pigments, nitrate reduction, denitrification, resistance to NaCl and the utilization of some substrates (Table 1). Of the four strains tested, only 6H33b^T was able to fix nitrogen. Although nitrogen fixation was reported in *P. stutzeri* A15 (A1501), the type strain of *P. stutzeri* indicated a negative reaction. The G+C content of the DNA of 6H33b^T was 66.3 mol%, which was in good agreement with the values of the genus *Pseudomonas sensu stricto* (58–70 mol%; Palleroni, 1984).

Phylogenetic analysis based on the 16S rRNA gene sequence of strain 6H33b^T indicated that it is a member of the genus *Pseudomonas sensu stricto*. Differences in some of the physiological and biochemical characteristics and the phylogenetic position based on 16S rRNA gene sequence analysis between 6H33b^T and other members of the genus suggested that 6H33b^T represents a novel species. This was further suggested based on the low level of DNA–DNA hybridization, as determined using the method of Ezaki *et al.* (1989), between 6H33b^T and its closest relative, *P. indica* DSM 14015^T (1–5 % similarity; data not shown). Strain 6H33b^T therefore represents a novel species of the genus

Fig. 2. Phylogenetic tree based on the 16S rRNA gene sequences of strain 6H33b^T, type strains of the genus *Pseudomonas sensu stricto* and *P. stutzeri* A1501. The tree was drawn by the neighbour-joining method. Bootstrap percentages were calculated from 1000 repeats and those greater than 50 % are shown at branch points. Numbers in parentheses are the GenBank accession numbers. Bar, 0.01 substitutions per nucleotide position.

Table 1. Comparison of physiological and biochemical characteristics between strain 6H33b^T and three type strains of the genus *Pseudomonas sensu stricto*

Strains: 1, 6H33b^T; 2, *P. aeruginosa* NCIMB 8295^T; 3, *P. indica* DSM 14015^T; 4, *P. stutzeri* NCIMB 11358^T.

Characteristic	1	2	3	4
Production of:				
Pyocyanin	—	+	—	—
Fluorescent pigments	—	+	—	—
Nitrate reduction	—	+	+*	+
Denitrification	—	+	+	+
Nitrogen fixation	+	—	—	—
Growth in the presence of NaCl at:†				
5 %	+	+	+*	+
7 %	—	—	—	+
Hydrolysis of starch	+	—	+	+
Utilization of:‡				
<i>N</i> -Acetyl-D-glucosamine	—	+	—	—
L-Arabinose	—	—	+	—
2,3-Butanediol	—	+	+	—
DL-Carnitine	—	+	+	—
Dextrin	—	+	+	+
D-Galacturonic acid	—	—	+	—
D-Glucuronic acid	—	—	+	—
Glycerol	—	+	+	+
L-Histidine	—	+	+	—
Hydroxy-L-proline	+	+	—	—
Inosine	—	+	—	—
Malonic acid	—	+	+	+
Quinic acid	—	+	+	—
D-Saccharic acid	+	—	+	—
Urocanic acid	—	+	+	—
DNA G + C content (mol%)	66.3 ± 0.5	65.2 ± 0.5	63.5 ± 0.7	63.2 ± 0.3

*A negative reaction was reported by Pandey *et al.* (2002).

†All the strains grew in 2.5 % NaCl and did not grow in 10 % NaCl.

‡Only substrates for which a difference was observed among the four strains are listed.

Pseudomonas sensu stricto, for which the name *Pseudomonas azotifigens* is proposed.

From the phylogenetic tree constructed based on the *nifH* gene sequences, the *nifH* genes of *P. azotifigens* 6H33b^T and *P. stutzeri* A15 (A1501) appear to have evolved from a common ancestral gene (Supplementary Fig. S1). However, the nitrogenase genes were not found in other *Pseudomonas* species, and Southern blot analysis with the *nifH* fragment of strain 6H33b^T as a probe revealed the absence of *nifH*-like genes in the genomes of its most closely related type strains, *P. indica* DSM 14015^T and *P. stutzeri* NCIMB 11358^T (data not shown). This was in good agreement with the results of the nitrogenase assay. These results strongly indicated that nitrogen fixation ability in *Pseudomonas sensu stricto* is a strain-specific characteristic, and that there may have been a complex evolutionary history of the nitrogenase genes in the genus. One possible explanation is that the nitrogenase

genes may have been lost from the other *Pseudomonas* strains, especially the type strain of *P. stutzeri*, after the strains NCIMB 11358^T and A15 (A1501) diverged. Alternatively, the nitrogenase genes of *P. azotifigens* 6H33b^T and *P. stutzeri* A15 (A1501) may have been horizontally transferred from other diazotroph(s) after these strains were established. Isolation and characterization of novel nitrogen-fixers in the genus *Pseudomonas sensu stricto*, together with analysis of nitrogen fixation within recognized *Pseudomonas* species, may provide further data on the evolutionary history of the nitrogenase genes in this genus.

Description of *Pseudomonas azotifigens* sp. nov.

Pseudomonas azotifigens [a.zo.ti.fi'gens. French n. *azote* (from Gr. pref. *a-* and Gr. n. *zoê*) nitrogen; N.L. n. *azotum* -i

nitrogen; L. part. *figens* (from L. v. *figo*) fixing; N.L. part. adj. *azotifigens* nitrogen fixing].

Cells are Gram-negative straight rods, 2–5 µm long and 0.5 µm wide, and motile. Colonies are translucent, wrinkled and with obscure edges when grown on LB agar. Growth in LB medium is observed at pH 6.1–9.8, 28–41 °C and in the presence of 2.5–5 % NaCl. Nitrogen fixation in NFMM is observed in the presence of 2–6 % oxygen in the gas phase. Catalase and oxidase are produced. No pigments are produced on King medium A or Luisetti medium. Nitrate reduction and denitrification are negative. Starch and Tween 80 are hydrolysed. The following substrates are oxidized: acetic acid, *cis*-aconitic acid, L-alaninamide, D-alanine, L-alanine, D-arabitol, L-asparagine, bromo-succinic acid, citric acid, formic acid, D-gluconic acid, α-D-glucose, L-glutamic acid, glycogen, hydroxy-L-proline, α-hydroxybutyric acid, β-hydroxybutyric acid, itaconic acid, α-ketobutyric acid, α-ketoglutaric acid, α-ketovaleric acid, DL-lactic acid, L-leucine, D-mannitol, methyl pyruvate, monomethyl succinate, L-proline, propionic acid, D-psicose, L-pyroglutamic acid, D-saccharic acid, sebacic acid, succinamic acid, succinic acid and Tweens 40 and 80. The following substrates are oxidized weakly: L-alanylglycine, L-aspartic acid, D-cellobiose, D-fructose, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, D-serine, L-serine and L-threonine. The G+C content of the type strain is 66.3 mol%.

The type strain, 6H33b^T (=ATCC BAA-1049^T=JCM 12708^T), was isolated from a compost pile in Japan.

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