NOTE

***Paenibacillus azoreducens* sp. nov., a synthetic azo dye decolorizing bacterium from industrial wastewater**Colette Meehan,¹ Anthony J. Bjourson² and Geoffrey McMullan¹¹ School of Environmental Studies, University of Ulster, Coleraine, County Londonderry BT52 1SA, Northern Ireland, UK² Department of Agriculture for Northern Ireland, Applied Plant Sciences Division, Newforge Lane, Belfast BT9 5PX, County Antrim, Northern Ireland, UK

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An azo-dye-reducing, endospore-forming bacterium isolated from textile industry wastewater has been taxonomically studied. Particularly interesting was the ability of this organism to decolorize the azo dye Remazol Black B by 98% within 24 h. Levels of 16S rRNA similarity between the isolate and *Paenibacillus* species ranged from 92.1 to 95.0%. The DNA G+C content was 46.8 mol% and anteiso-branched C_{15:0} was the major fatty acid. Based upon the phenotypic properties and the phylogenetic inference, it is proposed that the bacterium should be designated *Paenibacillus azoreducens* sp. nov. The type strain of *Paenibacillus azoreducens* is CM1^T (= DSM 13822^T = NCIMB 13761^T).

Keywords: *Paenibacillus*, azo dye, wastewater

Synthetic azo dyes frequently found in effluents from the textile industry pose an environmental threat because potential breakdown products resulting from anaerobic microbial actions are toxic, carcinogenic and mutagenic amines (Conneely *et al.*, 1999). As a result, considerable interest has been generated in studying microbial azo dye degradation (Banat *et al.*, 1997). In addition to the comprehensively studied white-rot fungi, several *Bacillus* species have been reported as azo dye decolorizers (Wuhrmann *et al.*, 1980; Knapp & Newby, 1994; Ogawa & Yatome, 1990; Yatome *et al.*, 1991; Zissi *et al.*, 1997).

Traditionally, lack of incisive differentiating criteria caused all aerobic, endospore-forming bacteria to be classified as *Bacillus*. In addition, the absence of discriminating assays has limited recognition of new species, and promoted grouping of organisms that failed to show clear-cut differences (Yoon *et al.*, 1998). Despite the comprehensive studies of Smith *et al.* (1952) and Gordon *et al.* (1973), identification of *Bacillus* species remains difficult for most bacteriologists because of taxonomic and characterization problems. To alleviate the latter, Logan & Berkeley (1984) developed an identification scheme using the API system of biochemical and enzymic tests. However, new species have been discovered and the

taxonomic problems continue (Heyndrickx *et al.*, 1996). Molecular biological methods used to study microbial diversity have revealed that the genus *Bacillus* is a phylogenetically heterogeneous taxon with species varying greatly in DNA base composition (32–69 mol% G+C) (Claus & Berkeley, 1986; Slepecky & Hemphill, 1991; Stackebrandt & Liesack, 1993). Subsequent phylogenetic studies using 16S rRNA gene sequencing revealed that the genus *Bacillus* could be separated into several distinct genera such as *Alicyclobacillus* (Wisotzkey *et al.*, 1992), *Aneurinibacillus* (Shida *et al.*, 1996), *Brevibacillus* (Shida *et al.*, 1996), *Halobacillus* (Spring *et al.*, 1996) and *Paenibacillus* (Ash *et al.*, 1991).

To date no *Paenibacillus* species have been implicated in azo dye decolorization. During investigation of a previously uncharacterized consortium described by Banat *et al.* (1997), we isolated a bacterium capable of azo dye decolorization, designated CM1^T. Based on phylogenetic and phenotypic evidence, we propose that the isolate be classified as *Paenibacillus azoreducens* sp. nov. CM1^T has been deposited in NCIMB and DSMZ with the accession numbers NCIMB 13761^T and DSM 13822^T, respectively.

DSM 13822^T was maintained at 4 °C on nutrient agar slopes containing 5 mg MnSO₄·H₂O l⁻¹ to enhance sporulation. The isolate was routinely grown at 37 °C and 150 r.p.m. for 24 h in liquid broth containing (l⁻¹): 5 g yeast extract; 0.5 g (NH₄)₂SO₄; 2.66 g KH₂PO₄;

The GenBank accession number for the 16S rRNA sequence of strain DSM 13822^T is AJ272249.

Table 1. 16S rRNA sequence similarities to DSM 13822^T

The 16S rRNA sequence was compared with each of the other sequences by using a FASTA search.

Species name	Accession no.	Similarity (%)
<i>Paenibacillus chibensis</i> NRRL B-142 ^T	D85395	95.0
<i>Paenibacillus macquariensis</i> NCTC 10419	X60625	94.6
<i>Paenibacillus illinoisensis</i> NRRL NRS-1356 ^T	D85397	94.6
<i>Paenibacillus jamilae</i> CECT 5266	AJ271157	93.3
' <i>Paenibacillus burgondia</i> ' B2	AJ011687	93.2
<i>Paenibacillus amylolyticus</i> NRRL NRS-290 ^T	X60606	93.1
<i>Paenibacillus alginolyticus</i> DSM 5050 ^T	D88517	93.1
<i>Paenibacillus polymyxa</i> NCDO 1774	X60632	93.1
<i>Paenibacillus lautus</i> NRRL B-377	D85609	92.6
<i>Paenibacillus pabuli</i> NCIMB 12781	X60630	92.4
<i>Paenibacillus apiarius</i> NRRL NRS-1438	U49247	92.3
<i>Paenibacillus larvae</i> subsp. <i>pulvificiens</i> NCDO 1141	X60636	92.3
<i>Paenibacillus azotofixans</i> NRRL B-14372	D78318	92.1

and 4.32 g Na₂HPO₄·H₂O. Standard phenotypic characterization of DSM 13822^T using the methods described by Gordon *et al.* (1973) and by Logan & Berkeley (1984) was carried out. Cell morphology and motility were examined using electron and phase-contrast microscopy. The G+C content was determined by the method of Tamaoka & Komagata (1984). Cellular fatty acid content was analysed by the method described by Pukall *et al.* (1999) with the exception that DSM 13822^T was grown in the liquid broth described above. Decolorization experiments were carried out in 250 ml Erlenmeyer flasks containing 100 ml mineral media containing (g l⁻¹): yeast extract, 5.0; (NH₄)₂SO₄, 0.5; and Remazol Black B, 0.1. A 10% (v/v) inoculum was added from a liquid culture of DSM 13822^T grown for 24 h in nutrient broth. Flasks were sealed, flushed with sterile oxygen-free nitrogen gas and incubated at 37 °C and 50 r.p.m. for 24 h. Culture filtrate was removed aseptically at regular intervals and analysed spectrophotometrically (600 nm) for dye decolorization. 16S rRNA gene sequencing of DSM 13822^T was carried out at NCIMB using protocols and universal primers as detailed for the MicroSeq 16S rDNA Bacterial Identification System (Applied Biosystems) and then compared with the 16S rRNA gene sequences obtained from the EMBL and MicroSeq databases. The GenBank accession number for the 16S rRNA sequence of strain DSM 13822^T is AJ272249.

The need for novel technology developments for effective textile wastewater treatment has been widely reported (Banat *et al.*, 1996). One potentially attractive strategy is bioremediation, a process aimed at providing a low cost, environmentally acceptable treatment solution to this waste problem. Several research groups, including our own, have investigated the ability of a wide range of micro-organisms to decolorize and metabolize textile dyes (Conneely *et al.*,

1999; Kirby *et al.*, 2000). Whilst many studies have focused upon the capabilities of white-rot fungi such as *Phanerochaete chrysosporium*, the ability of axenic bacterial cultures to decolorize textile dyes has been relatively poorly studied.

Previously, Banat *et al.* (1997) reported the isolation of a microbial consortium capable of decolorizing a range of commercially important textile dyes. Of interest to us was an isolate distinguishable from other members of the consortium by its very rapid dye-decolorizing capabilities. In order to identify this bacterium, an almost complete 16S rRNA gene sequence (1500 bp) was determined, which had highest similarity with *Paenibacillus chibensis* NRRL B-142^T (95%), *Paenibacillus illinoisensis* NRRL NRS-1356^T (94.6%) and *Paenibacillus macquariensis* NCTC 10419^T (94.6%) (Table 1). Somewhat lower matching sequence identities (93.3–92.1%) also occurred with other *Paenibacillus* isolates (Table 1).

Cells of this isolate, DSM 13822^T, are motile (by peritrichous flagella), Gram-variable rods that form single ellipsoidal endospores in the subterminal/terminal region of the cell. The colonies on nutrient agar containing glucose (5%, w/v) are flat, smooth, circular, entire and cream/yellowish in colour (not shown). Table 2 shows the distinct biochemical profiles of a range of *Paenibacillus* species which should allow the ready separation of DSM 13822^T using routine tests. When the characteristics of DSM 13822^T were compared with those reported for *P. chibensis* NRRL B-142^T, the species showing the highest sequence identity to DSM 13822^T, the two species exhibited 74% similarity (28 of 38 tests; full list not shown). Strain DSM 13822^T was unable to ferment either L-arabinose or inositol or reduce nitrate to nitrite. In contrast, *P. chibensis* NRRL B-142^T exhibited positive reactions for these traits (Shida *et al.*, 1997b). Strain

Table 2. Distinctive phenotypic characteristics of type species and subspecies in the genus *Paenibacillus*

1, *Paenibacillus polymyxa* ATCC 842^{T*}; 2, *Paenibacillus azotofixans* ATCC 35681^{T*}; 3, *Paenibacillus peoriae* LMG 14832^{T*}; 4, *Paenibacillus macerans* ATCC 8244^{T*}; 5, *Paenibacillus durus* ATCC 27763^{T*}; 6, *Paenibacillus lautus* NRRL NRS-666^{T*}; 7, *Paenibacillus amylolyticus* NRRL NRS-290^{T*}; 8, *Paenibacillus macquariensis* ATCC 23464^{T*}; 9, *Paenibacillus pabuli* NRRL NRS-924^{T*}; 10, *Paenibacillus alvei* ATCC 6344^{T*}; 11, *Paenibacillus apiarius* NRRL NRS-1438^{T*}; 12, *Paenibacillus validus* ATCC 43897^{T*}; 13, *Paenibacillus larvae* subsp. *larvae* ATCC 9545^{T*}; 14, *Paenibacillus larvae* subsp. *pulvifaciens* ATCC 13537^{T*}; 15, *Paenibacillus campinasensis* KCTC 0364BP^{†‡}; 16, *Paenibacillus curdianolyticus* IFO 15724[‡]; 17, *Paenibacillus kobensis* IFO 15729[‡]; 18, *Paenibacillus lentimorbus* ATCC 14707[‡]; 19, *Paenibacillus popilliae* ATCC 14706[‡]; 20, *Paenibacillus thiaminolyticus* JCM 8360[§]; 21, *Paenibacillus dendritiformis* T168[§]; 22, *Paenibacillus illinoisensis* NRRL NRS-1356^{T ||}; 23, *Paenibacillus chibensis* NRRL B-142^{T ||}; 24, *Paenibacillus glucanolyticus* DSM 5162^{T ||}; 25, *Paenibacillus koreensis* KCTC 2393^{T¶}; 26, DSM 13822^{T#}. +, Positive reaction; –, negative reaction; v, variable reaction; NR, not reported.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
Anaerobic growth	+	+	+	+	+	+	+	+	+	+	+	–	+	+	+	–	–	+	+	+	+	+	–	+	+	+
Catalase	+	+	+	+	NR	+	+	+	+	+	+	+	–	–	+	–	+	–	–	+	+	+	+	NR	+	+
Oxidase	–	–	–	NR	NR	–	–	NR	–	NR	–	–	NR	NR	–	–	–	–	–	+	+	–	–	+	+	–
Nitrate reduction	+	–	+	+	–	+	+	–	–	–	–	V	V	V	NR	+	+	–	–	+	–	–	+	V	+	–
Production of:																										
Acetylmethylcarbinol	+	+	+	–	+	–	–	–	–	+	–	V	V	V	NR	–	–	–	–	–	–	–	–	NR	–	–
Indole	–	–	–	–	–	–	–	–	–	+	–	–	+	+	NR	–	–	–	–	+	+	NR	–	NR	–	–
Dihydroxyacetone	+	–	–	–	NR	NR	NR	–	NR	+	–	NR	+	+	NR	NR	NR	–	NR	–	–	NR	–	NR	+	–
pH in VP broth	4.5–6.8	4.5–5.1	5.5–6.5	4.5–5.0	NR	< 5.5	< 5.5	< 6.0	< 5.5	4.6–5.2	4.4–5.4	NR	NR	NR	NR	NR	NR	5.9–6.5	NR	4.9–5.5	< 6.0	5.0–5.2	4.6–4.7	NR	NR	4.6–4.7
Decomposition of:																										
Tyrosine	–	–	–	–	NR	–	–	–	–	V	+	NR	–	–	NR	NR	NR	–	–	+	–	–	–	NR	NR	–
Hydrolysis of:																										
Casein	+	–	+	–	NR	–	–	–	V	+	+	NR	+	+	+	–	–	–	–	+	+	+	–	V	+	–
Starch	+	–	+	+	–	+	+	+	+	+	+	NR	–	–	+	+	+	–	–	+	+	+	+	NR	+	+
Utilization of:																										
Citrate	–	–	+	V	NR	–	–	–	–	–	+	–	–	–	NR	–	–	–	–	+	–	–	–	V	–	–
Litmus milk	NR	NR	NR	+	NR	+	+	NR	+	+	NR	NR	NR	NR	NR	NR	NR	NR	NR	–	+	+	–	NR	NR	+
Optimum growth temp (°C)	30	30–37	30	30	30	28–30	28–30	20	28–30	28	28	28–35	35–37	28–30	40	NR	NR	NR	NR	NR	28	37	37	37	NR	38–40
Growth at:																										
pH 5.6	+	+	+	+	+	–	+	–	+	–	+	NR	–	–	–	NR	NR	–	–	–	+	+	+	+	+	+
50 °C	–	–	–	+	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	–	+	+	NR	+	+
Growth in presence of:																										
0.001 % lysozyme	+	–	V	NR	NR	+	–	–	–	+	+	NR	+	+	NR	+	+	+	+	–	NR	V	+	NR	+	+
5 % NaCl	–	–	–	–	NR	+	–	–	V	V	+	NR	–	–	+	–	–	–	–	V	+	–	–	+	–	–
Fermentation of:																										
L-Arabinose	+	–	+	+	–	+	+	–	+	–	–	+	–	–	+	+	+	–	–	V	–	+	+	V	+	–
Mannitol	+	+	+	+	–	+	+	+	+	–	–	+	–	+	–	–	–	–	–	–	–	+	+	NR	+	+
D-Xylose	+	–	+	+	–	+	+	+	+	–	–	+	–	–	+	+	+	–	–	–	–	+	+	NR	–	+
Glycerol	+	–	–	NR	NR	NR	NR	NR	NR	NR	NR	+	+	+	–	NR	NR	NR	NR	NR	NR	+	–	V	NR	+
G + C content (mol%)	43–46	48–53	45–47	52–53	50	50–52	53	39	48–50	45–47	52–54	50–52	42–43	42–43	60	NR	NR	NR	NR	NR	52–54	55	48	53	49–50	54

* Shida *et al.* (1997a).† Yoon *et al.* (1998).‡ Pettersson *et al.* (1999).§ Tcherpakov *et al.* (1999).|| Shida *et al.* (1997b).¶ Chung *et al.* (2000).

Data obtained in this study.

Table 3. Cellular fatty acid composition of DSM 13822^T and several species of the genus *Paenibacillus*

The percentage of the total cellular fatty acid content is shown. ND, Not detected. Data for fatty acid composition of DSM 13822^T were obtained as described in Methods.

Species and strain	Saturated acids									Unsaturated acids	
	Straight-chain			Iso-branched				Anteiso-branched		C _{16:1} ω11	Iso-C _{17:1} ω10
	C _{14:0}	C _{15:0}	C _{16:0}	C _{14:0}	C _{15:0}	C _{16:0}	C _{17:0}	C _{15:0}	C _{17:0}		
Isolate DSM 13822 ^T	3.5	0.1	22.1	0.8	5.9	8.6	5.6	33.9	19.8	ND	ND
<i>Paenibacillus chibensis</i> NRRL B-142 ^T *	0.3	0.6	5.3	0.8	4.0	12.2	3.2	57.8	14.3	ND	ND
<i>Paenibacillus illinoisensis</i> NRRL NRS-1356 ^T *	2.0	1.0	24.7	1.8	1.5	6.3	1.4	56.6	4.9	ND	ND
<i>Paenibacillus macquariensis</i> CIP 103269 ^T †	0.7	1.2	2.9	0.8	5.2	2.6	0.4	81.0	0.7	0.6	0.2
<i>Paenibacillus pabuli</i> NRRL NRS-924 ^T †	0.7	0.1	10.1	0.7	2.2	4.8	1.3	73.7	4.1	ND	ND
<i>Paenibacillus amylolyticus</i> NRRL NRS-290 ^T *	0.6	0.5	20.1	0.2	2.9	4.1	5.8	36.9	11.5	0.5	1.0

* Data from Shida *et al.* (1997b).

† Data from Shida *et al.* (1997a).

DSM 13822^T was facultatively anaerobic while *P. chibensis* NRRL B-142^T was strictly aerobic. Other differences found between the two *Paenibacillus* species were the production of hydrogen sulfide, fermentation of glycerol, mannose and lactose, reaction of litmus milk and liquefaction of gelatin (Table 2). Strain DSM 13822^T showed clear positive reactions for these features, while *P. chibensis* NRRL B-142^T was reported to be negative. The G+C content of the isolate DSM 13822^T was 46.8 (mol %), a level included within the range found in the members of the genus *Paenibacillus* (Shida *et al.*, 1997a, b). The major fatty acids found in isolate DSM 13822^T are shown in Table 3 and are compared with values available for closely related *Paenibacillus* strains. Like other species in the *Paenibacillus* cluster, the major fatty acid found was the anteiso-branched C_{15:0} comprising 33.9% of the total. This value is generally lower than that reported for other *Paenibacillus* spp., only *Paenibacillus lautus* NRRL B-379 (34.8%) and *Paenibacillus macerans* IAM 1243 (34.5%) having comparable levels of anteiso-branched C_{15:0} (Shida *et al.*, 1997a).

One notable feature of isolate DSM 13822^T is its ability to rapidly decolorize synthetic textile dyes such as Remazol Black B by 98% within 24 h. The decolorization rate of this dye varied with concentration (25–400 mg l⁻¹); optimum rate was achieved at a dye concentration of 100 mg l⁻¹ at 37 °C (results not shown). As the decolorization rate for Remazol Black B compares very favourably with previous reports (Banat *et al.*, 1996), DSM 13822^T may be of future use in textile wastewater biotreatment.

In conclusion, the phenotypic and phylogenetic data clearly demonstrate that DSM 13822^T is a member of the genus *Paenibacillus*. Furthermore, the notable phenotypic ability to rapidly degrade the synthetic azo dye Remazol Black B and the genetic distinctiveness inferred from the 16S rRNA analysis warranted the

proposal of this isolate as the new species *Paenibacillus azoreducens*.

Description of *Paenibacillus azoreducens* sp. nov.

Paenibacillus azoreducens (azo.reduc'ens. Gr. n. *azo* a combining form meaning nitrogen; M.L. pres. part. *reducens* reducing; N.L. adj. *azoreducens* nitrogen-reducing, referring to the ability to decolorize azo-based textile dyes by reductive cleavage of the azo linkage).

Rod-shaped 0.5–0.8 by 3.0–6.0 µm. Gram-variable. Motile by means of peritrichous flagella. Ellipsoidal spores formed in swelling sporangia. On nutrient agar containing 5% glucose, colonies are flat, smooth, circular, entire and creamish-yellow, 1.0–2.0 mm diameter. Facultatively anaerobic. The pH in Voges–Proskauer broth is 4.6. Catalase-positive, starch is hydrolysed and gelatin is decomposed. Litmus is reduced in litmus milk, and acid is produced from the fermentation of glycerol, ribose, D-xylose, galactose, glucose, fructose, mannose, mannitol, salicin, cellibiose, maltose, lactose, melibiose, sucrose, trehalose, inulin, melezitose and starch. Grows in the presence of 0.001% lysozyme and at pH 5.6. The organism is negative for oxidase, and production of indole and dihydroxyacetone but positive for hydrogen sulfide production. Nitrate is not reduced to nitrite, casein and urea are not hydrolysed. There is no degradation of tyrosine and citrate is not utilized. L-Arabinose, adonitol, sorbose, rhamnose, inositol and sorbitol are not fermented. Growth occurs at temperatures between 10 and 50 °C and optimal growth temperature is 37 °C at pH 7. Growth is inhibited by 5% NaCl. The G+C content is 47 mol%. The predominant fatty acid is anteiso-C_{15:0} (34%). The species also has the ability to completely decolorize the azo dye Remazol Black B (25–400 mg l⁻¹) within 24 h. The type strain is CM1^T (= DSM 13822^T = NCIMB 13761^T).

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