Collimonas arenae sp. nov. and *Collimonas pratensis* sp. nov., isolated from (semi-)natural grassland soils

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A polyphasic taxonomic study was performed to compare 26 novel bacterial isolates obtained from (semi-)natural grassland soils and a heathland soil in the Netherlands with 16 strains that had previously been assigned to the genus *Collimonas*. Genomic fingerprinting (BOX-PCR), whole-cell protein electrophoresis, matrix-assisted laser desorption ionization time-of-flight mass spectrometry of intact cells and physiological characterization (Biolog) of the isolates confirmed the existence of different strain clusters (A–D) within the genus *Collimonas*. Until now, only cluster C strains have been formally classified, as *Collimonas fungivorans*. In this study, DNA–DNA hybridizations were performed with a selection of strains representing the four clusters. The results showed that cluster B strains also belong to *C. fungivorans* and that strains of clusters A and D represent two novel species within the genus *Collimonas*. The latter novel species could be differentiated by means of phenotypic and genotypic characteristics and are classified as *Collimonas arenae* sp. nov. (cluster A; type strain Ter10^T =LMG 23964^T =CCUG 54727^T) and *Collimonas pratensis* sp. nov. (cluster D; type strain Ter91^T =LMG 23965^T =CCUG 54728^T).

The genus *Collimonas* was described on the basis of 22 isolates that were obtained from slightly acidic dune soils from the Wadden island Terschelling in the Netherlands (De Boer *et al.*, 2001, 2004). *Collimonas* strains have the interesting ability to grow at the expense of living fungal hyphae (mycophagy), albeit under laboratory conditions (De Boer *et al.*, 2001, 2004). The taxonomy of these bacteria was examined using genomic fingerprinting (BOX-PCR), sequencing of 16S rRNA genes and physiological characterization, which revealed four clusters of strains (De Boer *et al.*, 2004). So far, only cluster C strains have been formally classified, as the novel species *Collimonas fungivorans*. The present investigation was designed to establish the taxonomic position of the three other clusters and of 26 new *Collimonas* isolates from different types of soils in the Netherlands (Table 1).

The new *Collimonas* isolates were taken from chitin agar enumeration plates of soil samples of eight (semi-)natural grasslands and a heathland in the Netherlands, as described previously (Höppener-Ogawa *et al.*, 2007) (Table 1). Based on colony morphology in combination with *Collimonas*-specific restriction fragment length polymorphism analysis of 16S rRNA genes, these 26 isolates were identified as *Collimonas* isolates (Höppener-Ogawa *et al.*, 2007). All isolates were stored at −80 °C and maintained on 10-fold-diluted tryptone soy broth (TSB) agar for routine culturing. The 10-fold-diluted TSB agar contained (l^{-1}) 1 g KH_2PO_4, 5 g NaCl, 3 g TSB (Oxoid) and 20 g agar. Media were adjusted to pH 6.5 with 1 M NaOH before autoclaving.

Repetitive sequence-based PCR profiles of the isolates were determined using the BOX-A1R primer, as described by Rademaker *et al.* (1997). Colony PCR was performed using fresh colonies that were taken from 10-fold-diluted TSB agar after 24 h of incubation (Rademaker *et al.*, 1997). Visual comparison of the banding profiles and UPGMA clustering of strains using Pearson's product–moment correlation coefficients in the Bionumerics version 3.5

Abbreviation: MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains LMG 23964^T and LMG 23965^T are AY281146 and AY281137, respectively. Those for other strains included in this study are listed in Table 1.

Detailed DNA–DNA hybridization results are available as supplementary material with the online version of this paper.

Table 1. Strains used in this study and their origins

Strain	Original strain name*	16S rRNA gene GenBank accession no.	Phylogenetic position*	Origin†	Ref.‡
LMG 23964^T	Ter10^T	AY281146	Cluster A	Dune grassland	1
LMG 23966	Ter282	AY281142	Cluster A	Dune grassland	1
LMG 23967			Cluster A	Flooded dune grassland, 27	2
R-35550	Ter252	AY281149	Cluster A	Dune grassland	1
R-35551	Ter146	AY281147	Cluster A	Dune grassland	1
LMG 23971	Ter266	AY281141	Cluster B	Dune grassland	1
LMG 23972	Ter228	AY281148	Cluster B	Dune grassland	1
LMG 23973			Cluster B	Ex-agricultural land, 22	2
R-35505			Cluster B	Unfertilized grassland, 5	2
R-35506			Cluster B	Unfertilized grassland, 5	2
R-35507			Cluster B	Unfertilized grassland, 5	2
R-35508			Cluster B	Unfertilized grassland, 5	2
R-35509			Cluster B	Unfertilized grassland, 4	2
R-35522			Cluster B	Ex-agricultural land, 18	2
R-35523			Cluster B	Ex-agricultural land, 18	2
LMG 21973^T	Ter6^T	AJ310394	Cluster C	Dune grassland	1
R-35554	Ter300	AY281145	Cluster C	Dune grassland	1
R-35555	Ter330	AY281150	Cluster C	Dune grassland	1
R-35556	Ter166	AY281140	Cluster C	Dune grassland	1
LMG 23965^T	Ter91^T	AY281137	Cluster D	Dune grassland	1
LMG 23968			Cluster D	Dune grassland, 26	2
LMG 23969	Ter90	AY281136	Cluster D	Dune grassland	1
LMG 23970	Ter291	AY281143	Cluster D	Dune grassland	1
R-22726	Ter227	AJ496445	Cluster D	Dune grassland	1
R-35510			Cluster D	Dune grassland, 26	2
R-35511			Cluster D	Dune grassland, 26	2
R-35512			Cluster D	Unfertilized grassland, 4	2
R-35516			Cluster D	Dune grassland, 26	2
R-35518			Cluster D	Dune grassland, 26	2
R-35521			Cluster D	Dune grassland, 26	2
R-35524			Cluster D	Ex-agricultural land, 18	2
R-35529			Cluster D	Unfertilized grassland, 6	2
R-35530			Cluster D	Unfertilized grassland, 6	2
R-35552	Ter113	AY281151	Cluster D	Dune grassland	1
R-35553	Ter118	AJ496444	Cluster D	Dune grassland	1
R-35513			Outside the four established clusters	Heathland, 15	2
R-35514			As above	Heathland, 15	2
R-35515			As above	Heathland, 15	2
R-35517			As above	Dune grassland, 26	2
R-35520			As above	Dune grassland, 26	2
R-35525			As above	Unfertilized grassland, 19	2
R-35526			As above	Unfertilized grassland, 19	2

*From De Boer *et al.* (2004).

†Sites from which Ter strains were isolated are described by De Boer *et al.* (1998); numbers refer to sites described by Höppener-Ogawa *et al.* (2007).

‡References: 1, De Boer *et al.* (2004); 2, Höppener-Ogawa *et al.* (2007).

software package revealed that the majority (n=18) of the new isolates fell within clusters B and D described previously (De Boer *et al.*, 2004). Only one of the new isolates fell in cluster A. The remaining seven isolates occupied distinct positions in the dendrogram (Fig. 1).

Isolates grown for 48 h at 28 °C on phosphate-buffered nutrient agar (pH 6.8), which contained (l^{-1}) 0.45 g KH_2PO_4, 2.39 g $Na_2HPO_4 . 12H_2O$, 2.39 g nutrient agar (Oxoid) and 20 g agar, were subjected to SDS-PAGE analysis of whole-cell proteins. One-dimensional analytical

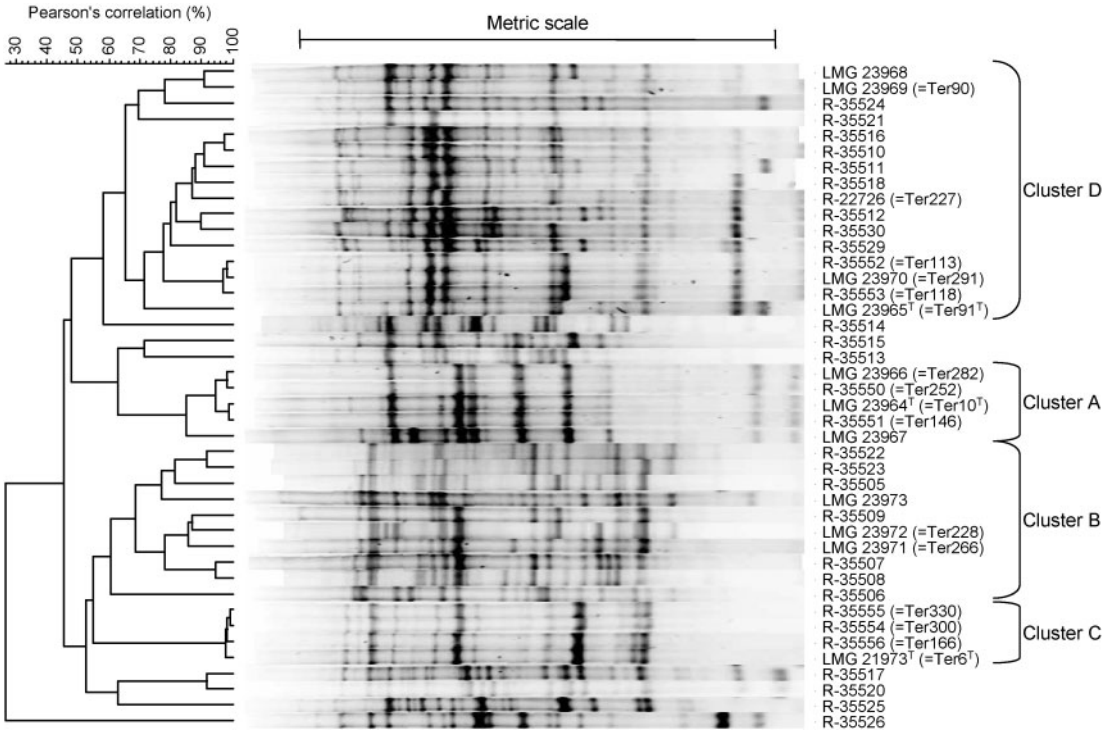

Fig. 1. Grouping of normalized digitized BOX-PCR fingerprint patterns of the 42 *Collimonas* isolates in a dendrogram based on UPGMA clustering of Pearson's correlation similarity coefficients.

SDS-PAGE was performed with a 12 % separating gel and a 5 % stacking gel using a previously described protocol (Pot *et al.*, 1994). Analysis of the whole-cell protein patterns supported the clustering result of the BOX-PCR fingerprint patterns (results not shown).

Twenty isolates (Fig. 2) were analysed by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) of intact cells. Bacterial cultures were grown and maintained on Columbia blood agar, containing 5 % (v/v) sheep blood. Incubation was standardized to 24 h and strains were grown aerobically at 28 °C. All strains were subcultured three times prior to MALDI-TOF analysis. Sample and target plate preparation, data acquisition using a M@LDI Linear TOF Mass Spectrometer [Waters Corporation (Micromass)] and data processing (with the aid of the MassLynx/MicrobeLynx^{TM} software; Micromass) were performed as described previously (Keys *et al.*, 2004). After cluster analysis of the spectral profiles (Fig. 2), 17 isolates formed four clusters, confirming results obtained by BOX-PCR fingerprinting and whole-cell protein electrophoresis (Fig. 1 and data not shown). The mass range m/z 2500–7500 Da contained the most discriminatory peaks, whereas the low (m/z 500–2500 Da) and high (m/z 7500–10 000 Da) mass ranges were very similar (data not shown). Strain R-35526, which occupied a distinct position in the BOX-PCR analysis (Fig. 1), and strains LMG 23973 (cluster B) and R-35529 (cluster D) represented a fifth cluster in the numerical analysis of the MALDI-TOF MS profiles.

To analyse whether the different clusters represent distinct species, DNA–DNA hybridization experiments were performed using a modification of the microplate method (Ezaki *et al.*, 1989) as described by Willems *et al.* (2001). Genomic DNA was prepared as described by Marmur (1961). A hybridization temperature of 45 °C was used. Isolates with different DNA fingerprints (Fig. 1) were selected as representatives of each cluster and were subsequently hybridized with each other (Supplementary Table S1, available in IJSEM Online). The cluster B strains (LMG 23971 and LMG 23973) showed DNA–DNA hybridization values of 75 and 70 %, respectively, towards *C. fungivorans* LMG 21973^{T} (cluster C). Strains LMG 23968 and R-35524 exhibited DNA–DNA hybridization values towards strain LMG 23965^{T} of 75 and 87 %, respectively, indicating that these three cluster D strains represent a single genospecies. All DNA–DNA hybridizations between strains representing distinct clusters yielded low to intermediate values in the range of 31 to 64 % (Supplementary Table S1). These data indicate that the cluster B isolates belong to *C. fungivorans*, whereas the cluster A and D isolates represent two novel genospecies. The DNA G+C contents of *C. fungivorans* LMG 21973^{T} and strains LMG 23964^{T}, LMG 23965^{T} and LMG 23971, as determined by HPLC (Mesbah *et al.*, 1989), were 59, 57, 59 and 59 mol%, respectively.

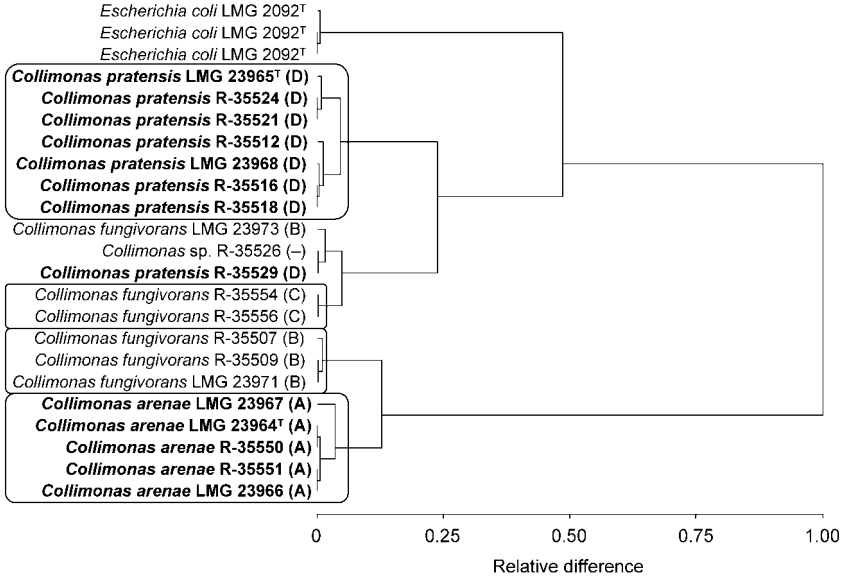

Fig. 2. Numerical analysis of MALDI-TOF MS spectral patterns generated using the MicrobeLynx^{TM} software package. Cluster A–D strains are shown in boxes. Three repeat measurements of *Escherichia coli* LMG 2092^T (included as a positive control) are shown to illustrate the reproducibility of the profiles.

Biochemical tests were performed for isolates representing clusters A (LMG 23964^T, LMG 23966, LMG 23967, R-35550 and R-35551), B (LMG 23971, LMG 23972, LMG 23973, R-35508 and R-35509), C (LMG 21973^T, R-35554, R-35555 and R-35556) and D (LMG 23965^T, LMG 23968, R-35510, R-35511, R-35512, R-35516, R-35518, R-35524, R-35529 and R-35530). Strains were examined for catalase and oxidase activities (King *et al.*, 1954). The ability to oxidize various carbon sources was tested using Biolog GN plates following the manufacturer's instructions (Table 2). Detection of enzyme activities was done using the API 20NE and API ZYM microtest systems (bioMérieux) according to the manufacturer's instructions (Table 2). The presence of the *nifH* gene was examined as described by Rosch & Bothe (2005). Test results and differential biochemical characteristics are listed in Table 2 and in the species descriptions.

Cellular fatty acid contents reported by De Boer *et al.* (2004) could not be used to distinguish clusters within the genus *Collimonas* (results not shown).

In summary, data from the present study demonstrate that cluster B isolates belong to *C. fungivorans* and that cluster A and D isolates represent two novel *Collimonas* species, which can be differentiated from each other and from *C. fungivorans* by means of genotypic (Fig. 1; De Boer *et al.*, 2004) and phenotypic (Table 2) characteristics. We propose to classify the cluster A and D strains formally into the novel species *Collimonas arenae* sp. nov. and *Collimonas pratensis* sp. nov., respectively. The taxonomic status of seven isolates (Table 1) identified as members of the genus *Collimonas* by their ability to clear colloidal chitin and by their *Collimonas*-specific 16S rRNA restriction patterns (Höppener-Ogawa *et al.*, 2007) needs further study. These strains may represent additional species within the genus *Collimonas*. The data obtained by MALDI-TOF MS indicate that this novel technology is useful for the rapid identification of *Collimonas* strains at the genus and species levels, although two of 20 isolates examined clustered aberrantly.

Description of *Collimonas arenae* sp. nov.

Collimonas arenae (a.re′nae. L. gen. n. *arenae* of sand, referring to the isolation of strains from sandy soil).

After 2 days of incubation at 20 °C on 10-fold-diluted TSB agar, colonies are flat, translucent and whitish with a yellowish central part and 3–7 mm in diameter with a granular-structured periphery (colony type II) (De Boer *et al.*, 2004). Cells exhibit oxidase and weak catalase activity. The *nifH* gene, required for nitrogen fixation, is not detected by PCR-based methods. Carbon-source utilization is presented in Table 2. *C. arenae* can be differentiated from *C. fungivorans* and *C. pratensis* by the inability to assimilate trehalose and the lack of *β*-galactosidase activity.

The type strain is Ter10^T (=LMG 23964^T =CCUG 54727^T). It has a DNA G+C content of 59 mol% and was isolated from (semi-)natural grassland in the Netherlands in 1998.

Description of *Collimonas pratensis* sp. nov.

Collimonas pratensis (pra.ten′sis. L. fem. adj. *pratensis* growing in a meadow, referring to the isolation of strains from grassland).

Table 2. Physiological characterization of *C. fungivorans* and the novel strains

Clusters B and C correspond to *C. fungivorans*. Clusters A and D correspond to the novel species *C. arenae* sp. nov. and *C. pratensis* sp. nov., respectively. +, Positive; w, weakly positive; −, negative; d, strain-dependent, with reaction for the type strain in parentheses. All strains characterized in this study were positive for utilization of bromosuccinic acid, D-fructose, D-galactose, α-D-glucose[a]*, L-asparagine, D-glucuronic acid, L-glutamic acid, D-mannitol[a], β-hydroxybutyric acid and malic acid[a]. All strains were also positive for production of alkaline phosphatase[b], leucine arylamidase[b], acid phosphatase[b] and naphthol-AS-BI-phosphohydrolase[b]. All strains were negative for utilization of i-erythritol, melibiose, α-cyclodextrin, methyl β-D-glucoside, itaconic acid, raffinose, thymidine, gentiobiose, L-rhamnose, L-phenylalanine, phenylethylamine, putrescine, sucrose, 2,3-butanediol, adonitol, α-D-lactose, lactulose, maltose, sebacic acid, DL-carnitine, glucose 1-phosphate and γ-hydroxybutyric acid. All strains were also negative for production of cystine arylamidase[b], β-glucuronidase[b], α-glucosidase[b], α-fucosidase[b], assimilation of adipic acid[a] and phenylacetic acid[a], indole production[a], D-glucose fermentation[a] and arginine dihydrolase[a]. All strains were weakly positive for production of esterase[b], esterase lipase[b] and valine arylamidase[b].

Characteristic*	Clusters B and C	Cluster A	Cluster D
Assimilation of:			
Urocanic acid	d (+)	−	d (+)
Succinamic acid	−	d (−)	d (w)
Hydroxy-L-proline	−	−	d (−)
Inosine	d (+)	+	d (+)
L-Fucose	+	+	d (+)
Uridine	+	+	d (+)
Formic acid	+	+	d (+)
L-Ornithine	−	d (−)	−
α-Ketovaleric acid	d (−)	d (−)	−
D-Alanine	d (w)	d (−)	−
D-Galacturonic acid	−	d (+)	d (−)
L-Proline	+	+	d (+)
myo-Inositol	+	+	d (+)
D-Gluconic acid	+	d (+)	d (+)
Malonic acid	d (−)	−	−
2-Aminoethanol	d (+)	−	−
Trehalose	+	−	+
D-Glucosaminic acid	+	d (+)	d (+)
Propionic acid	d (+)	+	d (w)
D-Serine	d (w)	−	−
L-Aspartic acid	+	+	d (+)
Glycerol	+	+	d (+)
L-Arabinose[a]	d (+)	+	d (+)
Xylitol	d (+)	−	d (+)
α-Hydroxybutyric acid	d (w)	+	d (−)
D-Saccharic acid	+	+	d (+)
DL-α-Glycerol phosphate	−	d (−)	d (−)
D-Arabitol	d (+)	+	+
Cellobiose	d (−)	−	−
Succinic acid	+	+	d (+)
Glucose 6-phosphate	−	d (+)	d (−)
D-Mannose[a]	d (+)	d (w)	d (+)
Potassium gluconate[a]	d (w)	d (+)	d (w)
Capric acid[a]	−	d (−)	−
Trisodium citrate[a]	d (+)	d (−)	+
Production of:			
Lipase[b]	w	d (w)	+
Trypsin[b]	d (+)	d (w)	d (w)
α-Chymotrypsin[b]	d (+)	d (w)	d (w)
α-Galactosidase[b]	−	−	d (w)
β-Galactosidase[b]	+	−	+
α-Mannosidase[b]	d (w)	−	d (−)
β-Glucosidase[b]	d (−)	−	d (−)
Protease[a]	d (+)	d (+)	d (w)

*Results were obtained with the Biolog GN test system unless indicated as follows: *a*, API 20NE; *b*, API ZYM.

After 2 days of incubation at 20 °C on 10-fold-diluted TSB agar, colonies are small, glossy and whitish, 1–3 mm in diameter (colony type III) (De Boer *et al.*, 2004). One isolate (R-35518) produces a purple pigment, which deviates from the general genus description (De Boer *et al.*, 2004). Cells exhibit oxidase but no or weak catalase activity. The *nifH* gene required for nitrogen fixation is not detected by PCR-based methods. Carbon-source utilization and enzyme production are given in Table 2 and indicate a strong phenotypic flexibility within the species. *C. pratensis* can be differentiated from *C. fungivorans* by its colony morphology and its pronounced lipase activity. Additionally, comparison between the type strains of *C. pratensis* and *C. fungivorans* shows more differences in use of carbon substrates and production of enzymes (Table 2). Differentiation of *C. pratensis* from *C. arenae* is discussed above.

The type strain is Ter91^T (=LMG 23965^T =CCUG 54728^T), which has a DNA G+C content of 59 mol% and was isolated from (semi-)natural grassland in the Netherlands.

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