Dickeya solani sp. nov., a pectinolytic plant-pathogenic bacterium isolated from potato (Solanum tuberosum)

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Pectinolytic bacteria have been recently isolated from diseased potato plants exhibiting blackleg and slow wilt symptoms found in a number of European countries and Israel. These Gram-negative, motile, rods were identified as belonging to the genus Dickeya, previously the Pectobacterium chrysanthemi complex (Erwinia chrysanthemi), on the basis of production of a PCR product with the pelADE primers, 16S rRNA gene sequence analysis, fatty acid methyl esterase analysis, the production of phosphatases and the ability to produce indole and acids

Abbreviations: ANI, average nucleotide identity; IGS, intergenic spacer; MLSA, multilocus sequence analysis; PFGE, pulsed field gel electrophoresis.

The GenBank/EMBL accession numbers for the sequences of the intergenic spacer region (IGS) and housekeeping genes of the species of the genus Dickeya used for the multilocus sequence analysis are: KC844728–KC844754 (IGS), KC844485–KC844511 (dnaX), KC844512–KC844538 (dnaN), KC844539–KC844565 (fusA), KC844566–KC844592 (gaps), KC844593–KC844619 (gyrA), KC844620–KC844646 (purA), KC844647–KC844673 (recA), KC844674–KC844700 (gpdB), KC844701–KC844727 (rpoS). The GenBank/EMBL accession numbers for the 16S rRNA gene sequences of D. solani IPO 2222T and IPO 3295 are KF639914 and KF639915, respectively. The GenBank/EMBL accession numbers for the whole-genome sequences of the members of the genus Dickeya used in this study for the average nucleotide identity analysis are: D. solani IPO 2222T, AONU00000000; D. solani GBBC 2040, AONX00000000; D. solani MK10, AOO0P00000000; D. solani MK16, AOOQ00000000; D. dianthicola NCPPB 453T, AOOB00000000; D. dianthicola GBBC 2039, AOOM00000000; D. dianthicola IPO 980, AOOQ00000000; D. dianthicola NCPPB 3534, AOOK00000000; D. dadantii subsp. dadantii NCPPB 898T, AOOE00000000; D. dadantii subsp. dieffenbachiae NCPPB 2976T, AOGO00000000; D. dadantii subsp. dadantii NCPPB 3537, AOOL00000000; D. dadantii subsp. dadantii CFBP3855, NC014500.

A set of 15 supplementary figures and three supplementary tables are available with the online version of this paper.
from α-methylglucoside. Differential physiological assays used previously to differentiate between strains of *E. chrysanthemi*, showed that these isolates belonged to biovar 3. Eight of the isolates, seven from potato and one from hyacinth, were analysed together with 21 reference strains representing all currently recognized taxa within the genus *Dickeya*. The novel isolates formed a distinct genetic clade in multilocus sequence analysis (MLSA) using concatenated sequences of the intergenic spacer (IGS), as well as *dnaX*, *recA*, *dnaN*, *fusA*, *gapA*, *purA*, *rplB*, *rpoS* and *gyrA*. Characterization by whole-cell MALDI-TOF mass spectrometry, pulsed field gel electrophoresis after digestion of whole-genome DNA with rare-cutting restriction enzymes, average nucleotide identity analysis and DNA–DNA hybridization studies, showed that although related to *Dickeya dadantii*, these isolates represent a novel species within the genus *Dickeya*, for which the name *Dickeya solani* sp. nov. (type strain IPO 2222T = LMG25993T = NCPPB4479T) is proposed.

Recent studies have shown that *Pectobacterium chrysanthemi* (Hauben et al., 1998) is a highly diverse genus, with at least 15 distinct species and biovars, including *P. chrysanthemi* biovar 3. These attempts culminated in the reclassification of the taxon into six genomic species within the novel genus, *Dickeya*, i.e. *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. dieffenbachiae*, *D. paradisiaca* and *D. zeae* (Samson et al., 2005). Subsequent revision of this group led to the further reclassification of *D. dieffenbachiae* as a subspecies of *D. dadantii* (Brady et al., 2012).

Recently, pectinolytic Gram-stain-negative bacteria have been isolated from potatoes grown in Europe and Israel showing symptoms of blackleg and slow wilt (Toth et al., 2011). The bacteria were identified as belonging to the genus *Dickeya* on the basis of a positive reaction in a PCR assay with primers specific for *pelADE* (Nassar et al., 1996), the production of phosphatases and the ability to produce acids from α-methylglucoside (Hyman et al., 1998). Differential physiological assays assigned these strains to *D. dadantii* subsp. *dadantii* and *D. zeae*; previously *E. chrysanthemi* biovar 3. However, strains forming a distinct clade based on the 16S–23S rRNA gene intergenic spacer (IGS) sequence (group I) (Laurila et al., 2008), *dnaX* (clade IV) (Ślawnik et al., 2009) and *recA* (clade DUC-1) (Parkinson et al., 2009) and distinct from other species of the genus *Dickeya* on the basis of repetitive element PCR (REP-PCR) (Ślawnik et al., 2009) were observed. They were tentatively and informally named ‘*Dickeya solani*’ at that time to assist phytopathologists and policy makers across Europe and beyond (Toth et al., 2011). ‘*D. solani*’ is currently the predominant bacterial potato pathogen in many European countries (Toth et al., 2011) and has also been isolated from hyacinth (Ślawnik et al., 2009) and iris (J. Van Doorn, The Netherlands, personal communication).

Eight novel potato and hyacinth isolates from six different countries and a panel of reference strains comprising at least two strains from each of the currently recognized taxa within the genus *Dickeya* were studied (Table S1, available in IJSEM Online).

Template DNA from all strains of members of the genus *Dickeya*, including the strains designated as ‘*D. solani*’, yielded a PCR product with the *pelADE* primers (Nassar et al., 1996) with the exception of *D. paradisiaca* strains IPO 2127 and 2128. Phylogenetic analysis of 16S rRNA gene sequences grouped the strains with type strains of various species of the genus *Dickeya* and separated them from species of the genus *Pectobacterium* (Fig. S1). Fatty acid methyl ester (FAME) profile analysis performed according to the protocol of Stead et al. (1992) also confirmed that the eight isolates belonged to the genus *Dickeya* (Fig. S2). *C16:0*, *C14:0/iso-C16:1* and *C18:1ω7c* are the predominant fatty acid, with significant amounts of *C12:0*, *C14:0* and *C16:0/iso-C16:1* (data not shown). FAME analysis, however, was unable to differentiate between species of the genus *Dickeya*.

A motility assay, performed in soft agar as previously described (Czajkowski et al., 2012) indicated that all eight strains classified as ‘*D. solani*’ were motile. Cells were rod-shaped with a maximum size of 0.9 × 2.0 μm as determined by transmission electron microscopy (de Freitas Neto et al., 2013) (Fig. S3). All strains classified as ‘*D. solani*’ reacted strongly with antibodies (100-fold diluted) against serogroup O1 by indirect immunofluorescence (Van Vuurde et al., 1983).

All strains classified as ‘*D. solani*’ were Gram-stain-negative, determined using a commercial kit supplied by Becton Dickinson, produced phosphatases and indole, determined using the method of Hyman et al. (1998) and belonged to biovar 3 according to the methods described by Ngwira & Samson (1990) and Samson et al. (1987, 2005), as reported previously by Ślawnik et al. (2009) (Table S2). The other strains isolated from potatoes studied here were found to belong to biovar 1 (IPO 3327), biovar 3 (IPO 3329 and IPO 3332), biovar 6 (IPO 3330) or biovar 7 (IPO 3331 and IPO 980).

Whole-cell MALDI-TOF MS spectral analysis and SuperSpectrum generation were done on strains grown on
Tryptic Soy Agar (TSA) plates at 28 °C for 24 h. Protein mass fingerprints were obtained using a MALDI-TOF MS Axima Confidence machine (Shimadzu Biotech) as described in Vanderoemme et al. (2013). All strains classified as ‘D. solani’ clustered tightly, showing similarity to D. dadantii subsp. dadantii and D. dadantii subsp. dieffenbachiae, but were distantly related to D. dianthicola (Fig. 1). Biomarker mass patterns were calculated for the clade of strains classified as ‘D. solani’ strains using the Spectral Archive And Microbial Identification System (SARAMIS; AnagnosTec) SuperSpectrum tool as described by Rezzonico et al. (2010). A set of 24 masses were discriminatory for the members of the ‘D. solani’ clade and used as a SuperSpectrum for automated identification of strains that are members of biovar 3 (Fig. S4). In Table S2, the phenotypic characteristics used to differentiate strains classified as ‘D. solani’ from other species of the genus Dickeya are summarized.

The PCR amplification reactions for MLSA, were carried out using primers listed in Table S3. For dnaN, fusA, gapA, purA and rplB, PCR amplicons were initially generated using external primers. PCR amplification and sequencing have been described previously for recA (Waleron et al., 2002a), dnaX (Sławiak et al., 2009), rpoS (Waleron et al., 2002b) and IGS sequences (Fessehaie et al., 2002). Primers based on conserved regions of gyrA, derived from whole genomes of members of the genus Dickeya present in the GenBank, were designed using the clustal w Multiple Alignment Program (Larkin et al., 2007). The results were analysed using ‘Sequencing Analysis’ (Applied Biosystems) software. The alignments were created using Lasergene-SeqMan 7.0.0 (DNASTAR), forward and reverse sequences were assembled and refined by eye according to the consensus. Sequences of ten different housekeeping genes were aligned using CLUSTAL w (Larkin et al., 2007). Phylogenetic trees were generated for each gene (Figs S5–S14) and for the concatenated sequence data (Fig. 2) using the maximum-likelihood (ML) method (MEGA5; Centre for Evolutionary Functional Genomics) (Tamura et al., 2011). Gaps or missing data in alignment were excluded when site coverage was below 95 %. Bootstrap percentages were calculated with 1000 replicates. In the MLSA analysis using concatenated sequences strains classified as ‘D. solani’ were recovered in a homogeneous clade most closely related to D. dadantii subsp. dadantii and D. dadantii subsp. dieffenbachiae (Fig. 2) with D. dianthicola and D. paradisiaca, more distant from this group. Individual phylogenetic trees showed that strains classified as ‘D. solani’ displayed similar or identical clustering in most of these analyses (Figs S5–S14).

Pulsed field gel electrophoresis (PFGE) after restriction digestion by either XbaI or I-CeuI (New England BioLabs)

**Fig. 1.** Dendrogram derived from whole-cell MALDI-TOF MS protein mass fingerprints. Protein mass fingerprint patterns obtained after running independently at least four replicates per strain at two different evaluation times were reduced to a consensus spectrum in which peaks were reproducible in at least half of the measurements. The tree was constructed using the UPGMA clustering algorithm with Dice coefficient. Similarity distances are displayed as percentage values and bootstrap values calculated for 1000 reiterations are indicated.
was performed as described by Kleitman et al. (2008), whilst omitting incubation of the agarose plugs in lysozyme solution. Banding patterns were analysed by Dice coefficient, with a 1.0–1.5% tolerance window, clustering was achieved by UPGMA using the Molecular Analysts Fingerprinting II software, version 3 (Bio-Rad). All strains classified as ‘D. solani’ were recovered in a tight, distinct group well separated from all other taxa (Fig. S15).

Genomic DNA was extracted according to a procedure described by Wilson (1987) with modifications, for DNA–DNA hybridization. Hybridizations were conducted in quadruplicate in the presence of 50% formamide at 46.5°C using a method described by Ezaki et al. (1989), with mean values reported. The percentage of DNA–DNA relatedness in hybridization studies was highest (72%) between D. solani LMG 25993 (IPO 2222T) and D. dadantii subsp. dadantii LMG 25991T (IPO 2120T) (Table 1). A high degree of relatedness was also found between LMG 25991T (IPO 2222T) and strains of D. dianthicola LMG 25731T (IPO 2116T) and LMG 25731 (IPO 2116), with values of 69% and 64%, respectively. However, relatively low percentages were found with D. zeae LMG 2505T (IPO 2131T) and D. chrysanthemi LMG 2804T (IPO 2118T), with 43% and 46%, respectively.

The pairwise ANI values were determined using the draft genome sequences of members of the genus Dickeya.
Table 1. Average percentage DNA–DNA relatedness between Dickeya solani sp. nov. and members of the genus Dickeya as determined in DNA–DNA hybridization studies

Strains: 1, D. dianthicola LMG 2485T; 2, D. dianthicola LMG 25731; 3, D. solani LMG 25993T; 4, D. dadantii subsp. dadantii LMG 25991T; 5, D. zae LMG 2505T; 6, D. chrysanthemi, LMG 2804T. Hybridizations were performed in quadruplicate.

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Table 2. Pairwise ANI values for draft genomes of strains of members of the genus Dickeya

Strains: 1, D. dianthicola GBCC2039; 2, D. dianthicola NCPP453T; 3, D. dianthicola NCPPB3534; 4, D. dianthicola IPO980; 5, D. dadantii subsp. dieffenbachiae NCPPB2976T; 6, D. dadantii subsp. dadantii NCPPB3537; 7, D. dadantii subsp. dadantii NCPPB898T; 8, D. dadantii subsp. dadantii NCO145009; 9, D. solani GBCC2040; 10, D. solani IPO2222T; 11, D. solani MK10; 12, D. solani MK16. Bold type indicates above the threshold for assigning strains to separate species.

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DNA–DNA hybridization and ANI analysis gave conflicting indications as to whether ‘D. solani’ should be recognized at or below the species level, with DNA–DNA hybridization values exceeding the 70 % cut-off point for species delineation (Wayne et al., 1987), in comparison with D. dadantii. In contrast, the ANI values between strains classified as ‘D. solani’ and D. dadantii and D. dianthicola, were 0.94 and 0.92, respectively, falling below the recommended boundary for species delineation of 0.95–0.96 (Richter & Rosselló-Mora, 2009). As all other characterization methods applied here show good separation between strains classified as ‘D. solani’ and D. dadantii. It was therefore concluded that there was a sufficient difference between strains classified as ‘D. solani’ and D. dadantii to merit description at the species level. A similar approach has been taken to describe the pith necrosis pathogen of tomato, Pseudomonas mediterranea (Catara et al., 2002), where although DNA–DNA hybridization values greater than 70 % were observed between some strains of this taxon and its close relatives, the weight of evidence from other phenotypic or molecular characterizations methods was judged sufficient to merit description of the taxon as a separate species and not a subspecies of its close relative Pseudomonas corrugata.

Although strains classified as ‘D. solani’ studied here were obtained from different geographical regions and hosts they were consistently recovered as a homogeneous cluster by MLSA and PFGE, to the extent that they can be viewed as a single clone. It is not uncommon for highly specialized pathogens that exist in close association with their host to show a high degree of clonality (Tibayrenc & Ayala, 2012),...
either as a result of their recent emergence or their lack of opportunity for genetic exchange (Spratt, 2004). This is especially true for Mycobacterium tuberculosis (Sreevatsan et al., 1997), Bacillus anthracis (Kolsto et al., 2009), Burkholderia mallei (Godoy et al., 2003) and Yersinia pestis (Achtman et al., 1999). This is particularly the case with Burkholderia mallei and Burkholderia pseudomallei where DNA–DNA hybridization values between strains of the two species exceed 75% (Rogul et al., 1970). However, important differences in their biochemical characteristics, clinical symptoms and epidemiology warrant continued recognition of these taxa as two distinct species (Gevers et al., 2005). A similar approach is therefore proposed here in order to recognize the essentially clonal ‘D. solani’, as a distinct species, separate from its close relative D. dadiantii.

Description of Dickeya solani sp. nov.

Dickeya solani (so.la’ni N.L. gen. neut. n. solani of Solanum, of the potato).

The bacteria are Gram-stain-negative, motile rods belonging to serogroup O1, C16:1ω7c/C16:1ω6c, C16:0 and C18:1ω7c are the predominant fatty acids, with significant amounts of C12:0, C14:0 and C14:0 3-OH/iso-C16:1. Produces phosphatases and is able to produce indole and acids from α-arabinose, mannitol, melibiose and raffinose but not from 5-ketogluconate or inulin. Pectinolytic and can rapidly degrade potato tissue. Can be readily distinguished from other members of the genus Dickeya by using concatenated sequences of the intergenic spacer region (IGS) and of dnaA, recA, dnaN, fusA, gapA, purA, rplB, rpoS or gyrA, by MALDI-TOF mass spectrometry and PFGE.

The type strain IPO 2222T (=LMG25993T, NCIBP4479) was isolated from potato (Solanum tuberosum) grown in The Netherlands in 2007.

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References


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