

Application of Whole-Cell Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry for Rapid Identification and Clustering Analysis of *Pantoea* Species^{∇†}

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***Pantoea agglomerans* is an ecologically diverse taxon that includes commercially important plant-beneficial strains and opportunistic clinical isolates. Standard biochemical identification methods in diagnostic laboratories were repeatedly shown to run into false-positive identifications of *P. agglomerans*, a fact which is also reflected by the high number of 16S rRNA gene sequences in public databases that are incorrectly assigned to this species. More reliable methods for rapid identification are required to ascertain the prevalence of this species in clinical samples and to evaluate the biosafety of beneficial isolates. Whole-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) methods and reference spectra (SuperSpectrum) were developed for accurate identification of *P. agglomerans* and related bacteria and used to detect differences in the protein profile within variants of the same strain, including a ribosomal point mutation conferring streptomycin resistance. MALDI-TOF MS-based clustering was shown to generally agree with classification based on *gyrB* sequencing, allowing rapid and reliable identification at the species level.**

Pantoea agglomerans (20) is a ubiquitous plant-epiphytic bacterium that belongs to the family *Enterobacteriaceae*. While several strains are commercialized for biological control of plant diseases (23), the species also includes two phytopathogenic pathogens that carry distinctive virulence plasmids (32). *P. agglomerans* has a Jekyll-Hyde nature, being described also as an opportunistic human pathogen (30), which raises biosafety regulatory issues for the utilization of beneficial isolates (45). Clinical reports predominantly involve septicemia following penetrating trauma (16, 56) or nosocomial infections (14, 55). Clinical pathogenicity of this species has not been confidently confirmed (unfulfilled Koch's postulates). Infections attributed to *P. agglomerans* are typically of a polymicrobial nature involving patients affected by other diseases (14) and may represent secondary contamination of wounds. Standard clinical diagnostics and identification rely mainly on biochemical profiling analysis or alternatively on 16S rRNA gene sequencing, despite the inadequacy of these techniques for precise discrimination within the *Enterobacter* and *Pantoea* genera (5, 20, 39). Problems with correct identification have been observed for automated systems such as the API 20E (24, 39) and Vitek-2/GNI+ (39, 40) (both from bioMérieux) or the Phoenix (11, 38) and Crystal identification systems (40, 48) (both from BD Diagnostic Systems).

P. agglomerans is a composite taxon conglomerating former

Enterobacter agglomerans, *Erwinia milletiae*, and *Erwinia herbicola* strains. Accurate identification is complicated by the unsettled taxonomy of the “*P. agglomerans*-*E. herbicola*-*E. agglomerans*” complex (45). Recent analyses based on *gyrB* sequencing, multilocus sequence analysis (MLSA) (4), and fluorescent amplified fragment length polymorphisms (fAFLP) (45) indicate that strains belonging to *Enterobacter* or *Erwinia* archived in culture collections are often erroneously assigned to *P. agglomerans* and are likely also misidentified in clinical diagnostics. False classifications of environmental *P. agglomerans* strains as related *Pantoea* species, including human- or plant-pathogenic *P. ananatis*, are also common (45). Inadequate biochemical identification methods and uncertainty regarding current taxonomy are revealed also by the excessive number of 16S rRNA gene sequences incorrectly assigned to *P. agglomerans* that can be retrieved from GenBank (Fig. 1). Sequencing of housekeeping genes, MLSA, and fAFLP are labor-intensive, time-consuming, and impractical approaches as routine diagnostic tools.

Whole-cell matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (31) is an emerging technology for identification of bacteria (26, 46), fungi (17, 33), viruses (29, 51), insects (41), and helminths (42). MALDI-TOF MS-based identification can accurately resolve bacterial identity at the genus, species, and in some taxa subspecies levels (e.g., *Salmonella enterica* serovars, *Listeria* genotypes) (1, 18). Identity is based on unique mass/charge ratio (*m/z*) fingerprints of proteins, which are ionized using short laser pulses directed to bacterial cells obtained from a single colony embedded in a matrix. After desorption, ions are accelerated in vacuum by a high electric potential and separated on the basis of the time taken to reach a detector, which is directly proportional to the mass-to-charge ratio of an ion. This

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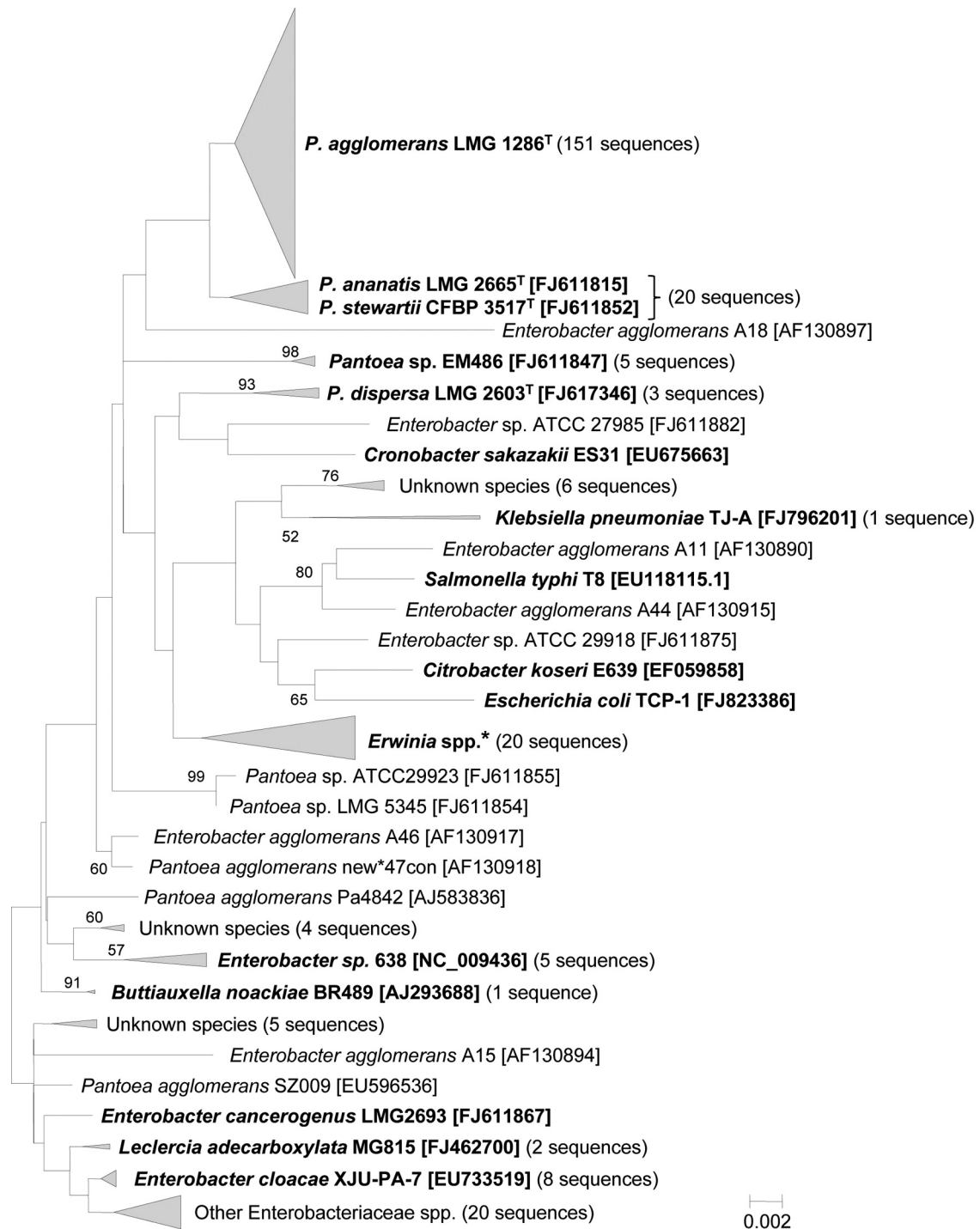


FIG. 1. Taxonomy of putative *P. agglomerans* isolates based on 16S rRNA gene sequences retrieved from GenBank under the currently accepted species name or under the old basonyms *Enterobacter agglomerans* and *Erwinia herbicola*. Out of a total of 331 complete or partial sequences found, 263 could be aligned over their 1,240-bp central region resulting in a minimum evolution tree. For the analysis, gaps and missing data were eliminated only in pairwise sequence comparisons, resulting in a total of 1,114 positions. Nodal supports were assessed by 1,000 bootstrap replicates. Only bootstrap values greater than 50% are shown. The scale bar represents the number of base substitutions per site. The number of "*P. agglomerans*" sequences clustering with a given reference strain is shown in parentheses. Reference strains and clades containing reference strains are marked in bold, and the corresponding accession numbers are indicated between brackets. For the genus *Erwinia* the following reference strains were used: *E. persicina* HK204 [NR_026049.1], *E. rhapontici* 2OP2 [FJ595873], *E. billingiae* Eb661 [AM055711], *E. tasmaniensis* Et2/99 [AM292080], and *E. amylovora* FAW 23482 [AY456711].

technique has been shown to deliver reproducible protein mass fingerprints starting from an aliquot of a single bacterial colony within minutes and without any prior separation, purification, or concentration of samples. Whole-cell MALDI-TOF MS is a reliable technique across broad conditions (e.g., different growth media, cell growth states), with limited variability in mass-peak signatures within a selected mass range ($2,000 < m/z < 20,000$) that does not affect reliability of identification (28, 31). MALDI-TOF MS profiles primarily represent ribosomal proteins, which are the most abundant cellular proteins and are synthesized under all growth conditions (47). MALDI-TOF MS identification profiles derived from several characterized strains for a given species are used to develop reference spectra (e.g., SuperSpectrum; AnagnosTec GmbH, Potsdam, Germany), and they include a subset of characteristic and reproducible markers. MALDI-TOF MS identification databases are currently available for a relatively wide range of clinical bacteria, and this method has become an accepted tool for routine clinical diagnostics due to enhanced simplicity, rapidity, and reliability. However, environmental bacteria, such as *Pantoea*, have not been widely evaluated using MALDI-TOF MS and are largely absent from identification databases, limiting the practical reach of this new technology.

Our objectives were to develop a robust method for rapid identification of *P. agglomerans* and related bacteria based on MALDI-TOF MS and to compare MALDI-TOF MS results against those obtained from a phylogenetic analysis based on *gyrB* sequencing as well as against biochemical identification methods.

MATERIALS AND METHODS

Screening and analysis of total available *Pantoea* 16S rRNA gene sequences.

The NCBI nucleotide database (<http://www.ncbi.nlm.nih.gov/nucleotide>) was scrutinized for 16S rRNA gene sequences of putative *P. agglomerans* isolates by searching for entries beneath the currently accepted species name and under the old basonyms *Enterobacter agglomerans* and *Erwinia herbicola*. Out of a total of 394 complete or partial sequences found, 263 were at least 1,240 bp long and were retained for the analysis together with 21 reference sequences of relevant species of *Enterobacteriaceae*. The resulting ClustalW (52) alignment was employed to construct a minimum evolution tree using the Molecular Evolutionary Genetics Analysis (MEGA) program, version 4.0 (50).

Bacterial strains. A collection of 53 strains received as *P. agglomerans*, a *Pantoea* sp., or *E. agglomerans* from research and culture collections and 20 reference strains belonging to closely related *Pantoea* species and other *Enterobacteriaceae* were compared (Table 1). The streptomycin-resistant, commercial biocontrol strain *Pantoea vagans* C9-1S (formerly *P. agglomerans* [45]) and a variant strain, *P. vagans* C9-1W, lacking the 530-kb pPag3 plasmid (49), were included to evaluate the sensitivity and robustness of MALDI-TOF MS. For standardized spectral acquisition and generation of "SuperSpectra," as determined by SARAMIS (spectral archive and microbial identification system) (AnagnosTec GmbH), bacteria were grown on LB agar at 28°C for 24 to 48 h. Tryptic soy agar (TSA) and Mueller-Hinton agar (MHA) were used in parallel to assess the influence of different media on species recognition. Alongside MALDI-TOF MS analysis, biochemical profiling and conventional Sanger sequencing of the *gyrB* gene were performed for all strains.

***gyrB* sequence analysis.** Amplification and sequencing of the *gyrB* gene were performed as described previously (45) by means of the HotStarTaq master mix kit (Qiagen, Basel, Switzerland) and the ABI PRISM BigDye Terminators version 1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA), respectively. Two degenerate primers were used to amplify and sequence a 970-bp region of the *gyrB* gene, *gyr*-320 (5'-TAARTTYGAYGAYAACTCYTAYAAAGT-3') and *rgyr*-1260 (5'-CMCCYTCCACCARGTAMAGTTC-3') (15). The phylogenetic tree was generated on the basis of a 740-bp fragment of the *gyrB* amplicon. DNA sequences were aligned with ClustalW (52). Sites presenting alignment gaps were excluded from analysis. The Molecular Evolutionary Genetics Analysis (MEGA) program, version 4.0 (50), was used to calculate evolutionary distances and to infer a tree based on the neighbor-joining (NJ) method using the maxi-

mum composite likelihood (MCL) model. Nodal robustness of the inferred tree was assessed by 1,000 bootstrap replicates. GenBank accession numbers for sequences used in this work were GU225728 and GU225729, FJ617346 to FJ617453, FJ617355 to FJ617459, FJ617361 to FJ617483, FJ617385 to FJ617486, FJ617389 to FJ617491, FJ617393 to FJ617496, FJ617398 to FJ617402, FJ617404 to FJ617405, FJ617404 to FJ617405, FJ617408 to FJ617413, FJ617416 to FJ617419, FJ617422, FJ617424, FJ617425 to FJ617427, EF988757 to EF988758, EF988768, and EU145275.

Biochemical identification. Automated biochemical identification of strains was performed using the Phoenix 100 ID/AST system (V5.66A) with NMC/ID-51 panels and the EpiCenter (V5.66A/V4.61A) microbiology data management system (BD Biosciences, Sparks, MD), following manufacturers' protocols. Each panel contained 45 substrates (Fig. 2) and included two fluorescent positive-control wells. Identification was determined using the Phoenix software by comparing the patterns of positive and negative reactions of individual samples with those of species contained in the commercial database. The current Phoenix database contains 90 genera, 324 species, and five CDC enteric groups.

MALDI-TOF MS spectrum acquisition. Cells from a single bacterial colony grown on LB agar for 24 h were transferred to a target spot of a steel target plate using a disposable loop, overlaid with 0.5 μ l of a 2,5-dihydroxybenzoic acid (DHB) matrix (AnagnosTec GmbH, Potsdam, Germany), and air dehydrated within 1 to 2 min at 24 to 27°C. Protein mass fingerprints were obtained using a MALDI-TOF mass spectrometry Axima confidence machine (Shimadzu-Biotech Corp., Kyoto, Japan), with detection in the linear, positive mode at a laser frequency of 50 Hz and within a mass range of 2,000 to 20,000 Da. Acceleration voltage was 20 kV, and the extraction delay time was 200 ns. A minimum of 20 laser shots per sample was used to generate each ion spectrum. For each bacterial sample, a total of 50 protein mass fingerprints were averaged and processed using the Launchpad version 2.8 software (Shimadzu-Biotech Corp.). For peak acquisition, the average smoothing method was chosen, with a smoothing filtering width of 50 channels. Peak detection was performed with the threshold-apex peak detection method using the adaptive voltage threshold which roughly follows the signal noise level, and subtraction of the baseline was set with a baseline subtraction filter width of 500 channels. For each sample, a list of the significant spectrum peaks was generated that included the *m/z* values for each peak, mass deviations, and signal intensity. Calibration was conducted for each target plate using spectra of the reference strain *Escherichia coli* K-12 (GM48 genotype). *E. coli* K-12 was deposited on each plate in two fixed positions, and the calibration was performed at the beginning of each plate acquisition. At the end, a second measurement of K-12 as a control was performed.

MALDI-TOF MS spectrum analysis and SuperSpectrum generation. Generated protein mass fingerprints were first imported in SARAMIS and analyzed using the following presetting parameters: mass range, from 2,000 to 20,000 Da; allowed mass deviation, 800 ppm. The spectra were related through cluster analysis by applying the single-link agglomerative algorithm of SARAMIS. Distance trees were compared to the neighbor-joining phylogenetic *gyrB* tree. As reference spectra for a rapid identification, SARAMIS uses so-called "SuperSpectra" consisting of taxon-specific biomarkers. SuperSpectrum generation was based on recovered mass signal markers with an absolute intensity of at least 200 mV included in the 2,000- to 20,000-Da mass range. To create the SuperSpectrum of a species, only the protein mass fingerprints of the strains clustering with the respective type strain in the *gyrB* tree were used (with species clustering indicated by the associated bracket). Using the SuperSpectrum tool, a subset of protein masses found in at least 90% of the strains of one species were selected and tested for their discriminatory power by comparing them to all of the database entries. Dependent on the amount of remaining species-identifying marker masses, each was given a numeric value in order to get a maximum total number of points not higher than 1,250. A set of 20 to 40 marker masses is normally sufficient to obtain a specific identification to the species level. The identification results obtained using MALDI-TOF MS were compared to those obtained using DNA sequencing combined with a BLAST similarity search and the outcome of the biochemical analysis using the Phoenix 100 ID/AST system.

Nucleotide sequence accession numbers. Sequences newly obtained as a result of this study were deposited in GenBank under accession numbers GU225728 and GU225729.

RESULTS AND DISCUSSION

Only 151 of 263 (i.e., 57%) 16S rRNA sequences retrieved from NCBI listed as belonging to *P. agglomerans* isolates clustered indeed with type strain *P. agglomerans* LMG 1286^T. The remaining ones could be assigned either to other *Pantoea* spp.

TABLE 1. Strains used in this study^b

Strain designation ^a	Received as	Origin	Host	Properties	Other designations or notes	Phoenix ^c	Reference
<i>Brenneria</i>							
LMG 2694 ^T	<i>B. nigrifluens</i>	United States	<i>Juglans regia</i>	Phytopathogen		No	57
LMG 2709 ^T	<i>B. rubrifaciens</i>	United States	<i>Juglans regia</i>	Phytopathogen		No	58
<i>Enterobacter</i>							
LMG 5336	<i>E. agglomerans</i>	United States	N/A	Clinical	<i>P. agglomerans</i> ATCC 29904	Yes	
LMG 5337	<i>E. agglomerans</i>	United States	Human	Clinical	<i>P. agglomerans</i> ATCC 29915	No	
LMG 5339	<i>E. agglomerans</i>	United States	Chicken	Veterinarianal	<i>P. agglomerans</i> ATCC 29917	Yes	
LMG 5341	<i>E. agglomerans</i>	United States	Human	Clinical	<i>P. agglomerans</i> ATCC 29919	Yes	
LMG 5345	<i>E. agglomerans</i>	United States	Human	Clinical	<i>P. agglomerans</i> ATCC 29923	Yes	
<i>Erwinia</i>							
CFBP 1232 ^T	<i>E. amylovora</i>	United Kingdom	<i>Pyrus communis</i>	Phytopathogen		(Yes)	2
CFBP 4342	<i>E. herbicola</i> pv. <i>gypsophylae</i>	Netherlands	<i>Gypsophila paniculata</i>	Phytopathogen		No	
CFBP 3618 ^T	<i>E. rhapontici</i>	United Kingdom	<i>Rheum rhabarbarum</i>	Environmental		No	27
CFBP 3622 ^T	<i>E. persicina</i>	Japan	<i>Lycopersicon esculentum</i>	Environmental		Yes	21
LMG 2613 ^T	<i>E. billingiae</i>	United Kingdom	<i>Pyrus communis</i>	Environmental		Yes	35
<i>Pantoea</i>							
ATCC 27981	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 865-74	Yes	
ATCC 27985	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 811-75	Yes	
ATCC 27987	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 1400-74	Yes	
ATCC 27988	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 1377-71	Yes	
ATCC 27989	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 1300-74	Yes	
ATCC 27990	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 1134-72	Yes	
ATCC 27991	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 358-74	Yes	
ATCC 27992	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 1269-74	Yes	
ATCC 27993	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 4-74	Yes	
ATCC 27994	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 3072-74	Yes	
ATCC 27995	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 4854-73	Yes	
ATCC 27996	<i>P. agglomerans</i>	United States	Insect	Environmental	CDC 5474-71	No	
ATCC 27998	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 1741-71	Yes	
ATCC 29001	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 164-75	Yes	
ATCC 29918	<i>P. agglomerans</i>	United States	Human	Clinical	CDC 4388-71	Yes	
C9-1	<i>P. agglomerans</i>	United States	Apple tree	Biocontrol	Wild-type	Yes	22
C9-1S	<i>P. agglomerans</i>	United States	Apple tree	Biocontrol	BlightBan C9-1 (Sm ^r)	No	22
C9-1W	<i>P. agglomerans</i>	United States	Apple tree	Biocontrol	ΔpPag3 variant of C9-1	No	49
CECT 4842	<i>P. agglomerans</i>	Sweden	Industrial	Environmental		Yes	
CIP 102343	<i>P. agglomerans</i>	Algeria	Human	Clinical		Yes	
CIP 102701	<i>P. agglomerans</i>	France	Human	Clinical		Yes	
CIP 82.100	<i>P. agglomerans</i>	Canada	Cereal plant	Environmental	ATCC 33243, type strain	No	27
CIP A181	<i>P. agglomerans</i>	France	Human	Clinical	of former <i>Erwinia herbicola</i>	Yes	
CPA-2	<i>P. agglomerans</i>	Spain	Apple tree	Biocontrol		No	37
E325	<i>P. agglomerans</i>	United States	Apple tree	Biocontrol	BloomTime	No	44
Eh1087	<i>P. agglomerans</i>	New Zealand	Apple tree	Biocontrol	Produces phenazine	No	25
Eh239	<i>P. agglomerans</i>	United States	Barley plant	Biocontrol		No	9
Eh252	<i>P. agglomerans</i>	United States	<i>Malus pumila</i>	Biocontrol		Yes	54
Eh318	<i>P. agglomerans</i>	United States	Apple tree	Biocontrol		No	59
Eh454	<i>P. agglomerans</i>	United States	Barley plant	Biocontrol		No	9
Eh460	<i>P. agglomerans</i>	United States	Barley plant	Biocontrol		No	9
EM13cb	<i>P. agglomerans</i>	Spain	Human	Clinical		Yes	
EM15cb	<i>P. agglomerans</i>	Spain	Human	Clinical		Yes	
EM17cb	<i>P. agglomerans</i>	Spain	Human	Clinical		Yes	
EM21cb	<i>P. agglomerans</i>	Spain	Human	Clinical		Yes	
EM22cb	<i>P. agglomerans</i>	Spain	Human	Clinical		Yes	
EM2cb	<i>P. agglomerans</i>	Spain	Human	Clinical		No	
EPS486	<i>P. agglomerans</i>	Spain	Apple tree	Environmental	EM486	Yes	
EPS595	<i>P. agglomerans</i>	Spain	<i>Pyrus communis</i>	Environmental	EM595	No	
EPS125	<i>P. agglomerans</i>	Spain	<i>Pyrus communis</i>	Biocontrol		No	3

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TABLE 1—Continued

Strain designation ^a	Received as	Origin	Host	Properties	Other designations or notes	Phoenix ^c	Reference
LMG 1286 ^T	<i>P. agglomerans</i>	Zimbabwe	Human	Clinical	Type strain: ATCC 27155 ^T , CECT 850 ^T	Yes	20
LMG 2557	<i>P. agglomerans</i>	United Kingdom	<i>Pyrus communis</i>	Environmental	NCCPB 1269	No	
LMG 2595	<i>P. agglomerans</i>	South Africa	<i>Allium cepa</i>	Environmental		No	
LMG 2941	<i>P. agglomerans</i>	N/A	<i>Malus sylvestris</i>	Environmental		No	
P10c	<i>P. agglomerans</i>	New Zealand	Apple tree	Biocontrol	BlossomBless	No	53
SC-1	<i>P. agglomerans</i>	United States	Cotton plant	Phytopathogen		No	34
VA21971	<i>P. agglomerans</i>	Switzerland	Human	Clinical		Yes	
ATCC 43348	<i>P. agglomerans</i> pv. <i>gypsophilae</i>	United States	<i>Gypsophila paniculata</i>	Phytopathogen	Crown gall disease	No	13
LMG 20103	<i>P. ananatis</i>	South Africa	Eucalyptus tree	Phytopathogen		No	
LMG 2665 ^T	<i>P. ananatis</i>	Brazil	<i>Ananas comosus</i>	Phytopathogen		Yes	36
LMG 2676	<i>P. ananatis</i>	United States	<i>Puccinia graminis</i> f. sp. <i>tritici</i>	Phytopathogen		No	43
LMG 5342	<i>P. ananatis</i>	United States	Human	Clinical	<i>P. ananatis</i> ATCC 29920	Yes	36
LMG 2603 ^T	<i>P. dispersa</i>	Japan	Soil	Environmental		No	20
LMG 2605	<i>P. dispersa</i>	Tanzania	<i>Vigna unguiculata</i>	Environmental		Yes	
LMG 2770	<i>P. dispersa</i>	United States	Human	Clinical		No	
CFBP 3614 ^T	<i>P. stewartii</i> subsp. <i>indologenes</i>	India	<i>Setaria italica</i>	Phytopathogen	LMG 2632	Yes	36
CFBP 3517 ^T	<i>P. stewartii</i> subsp. <i>stewartii</i>	United States	<i>Zea mays</i> var. <i>rugosa</i>	Phytopathogen	LMG 2715	Yes	36
LMG 24195	<i>P. vagans</i>	Uruguay	Eucalyptus tree	Phytopathogen	BCC 013		7
LMG 24196	<i>P. vagans</i>	Argentina	Eucalyptus tree	Phytopathogen	BCC 015	Yes	7
LMG 24199 ^T	<i>P. vagans</i>	Uganda	Eucalyptus tree	Phytopathogen	BCC 105 ^T		7
LMG 5343	<i>Pantoea</i> sp.	United States	Human	Clinical	<i>P. agglomerans</i> ATCC 29921	Yes	
<i>Tatumella</i>							
LMG 23359	<i>T. citrea</i>	Philippines	Pineapple plant	Environmental		Yes	8
LMG 22097	<i>T. punctata</i>	Japan	Orange tree	Environmental		Yes	8

^a ^T, type strain.

^b N/A, information not available.

^c Indicates whether or not Phoenix analysis has been performed for this strain. Parentheses indicate failure to grow using the standard Phoenix protocol.

(29 sequences), to the genus *Erwinia* (20 sequences), or to other taxa of the *Enterobacteriaceae* (62 sequences) or did not cluster with any of the chosen reference species or genera and did not produce significant matches with reliable 16S rRNA sequences at NCBI (i.e., blastn ≤ 97%) (Fig. 1). These results underscore the inadequacy of current biochemical and molecular identification methods employed in clinical diagnostics for *P. agglomerans* and the common use of obsolete taxonomy. Two ways incorrect sequences may appear in GenBank are blanket relocation to *P. agglomerans* of some species within the “*P. agglomerans*-*E. herbicola*-*E. agglomerans*” complex or a *posteriori* sequencing of isolates biochemically misidentified as *P. agglomerans*. The presence of such rogue data in the GenBank 16S rRNA gene database constitutes a potential pitfall for anyone trying to identify *P. agglomerans* only on the basis of 16S rRNA gene sequences.

Automated biochemical identification using the Phoenix 100 ID/AST system was less accurate than *gyrB* sequencing or MALDI-TOF MS and returned uncertain strain identification within *P. agglomerans* and related species. Of the 23 *Pantoea* strains analyzed, 19 were identified biochemically as *P. agglomerans* (Fig. 2), although only six of them could accurately be assigned to *P. agglomerans* using *gyrB* sequence analysis (Fig. 3). The Phoenix 100 ID/AST system was unable to separate the different species within the genus, as none of the other recognized *Pantoea* species is currently present in its database. Con-

versely, five strains belonging to other genera were incorrectly assigned to *P. agglomerans* using biochemical analysis: *Erwinia persicina* LMG 3622, *Enterobacter* sp. ATCC 27988 and ATCC 27991, *Tatumella punctata* LMG 22097, and *Tatumella citrea* LMG 23359, the latter two previously belonging to the former “Japanese species” of *Pantoea* (4, 8). For the first three strains this outcome is the consequence of imprecise biochemical profiling, as 29 out of 45 reactions within the NMC/ID-51 panel are allowed to deliver a variable result while still retaining the identification as *P. agglomerans* (Fig. 2). This is most evident in *Enterobacter* sp. ATCC 27988, where all 16 nonvariable reactions correspond to the Phoenix profile of *P. agglomerans* but where 14 out of the 29 reactions which allow a variable result are different between this strain and *P. agglomerans* LMG 1286^T. On the other hand, *Tatumella* strains LMG 22097 and LMG 23359 have biochemical profiles which are closely related to those of *Pantoea* and only recently have DNA-DNA hybridization and phenotypic tests allowed the transfer of these species to the genus *Tatumella* (8). One strain belonging to *P. agglomerans* (ATCC 27987) was incorrectly assigned biochemically to *Mannheimia haemolytica*, while *P. ananatis* ATCC 27996 and *Pantoea* sp. LMG 5343 were misidentified as *Vibrio cholerae* and *Cedecea davisae*, respectively. For all three strains the number of nonvariable reactions matching the Phoenix profile of *P. agglomerans* fell short of 15, which is apparently among the minimum prerequisites for a positive identification

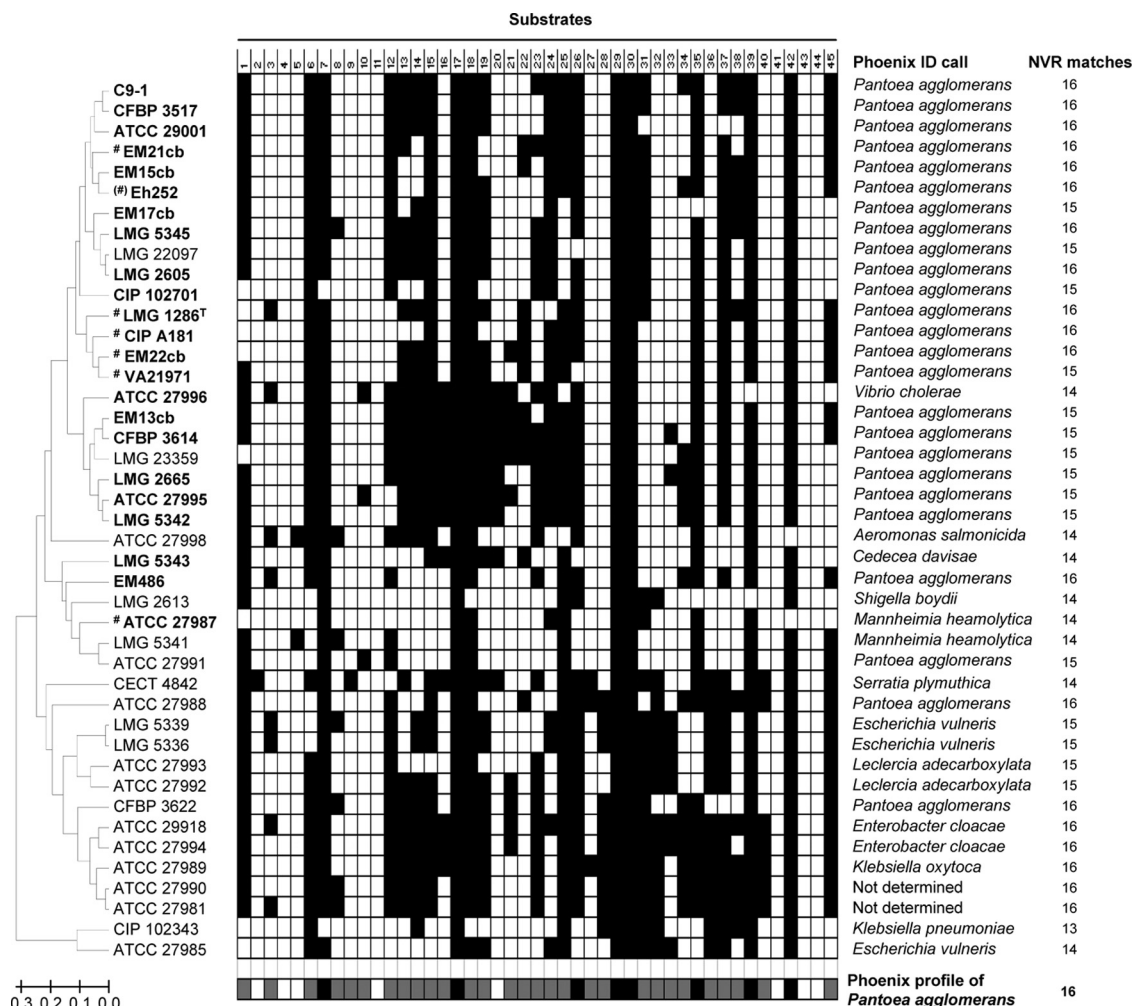


FIG. 2. PhoeniX 100 ID/AST system (BD Diagnostic Systems) results for a subset of 43 strains chosen among those considered in the study. A UPGMA dendrogram (on the left) was generated based on the binary biochemical data (shown in the middle) of the selected strains. Positive reactions are indicated by a black

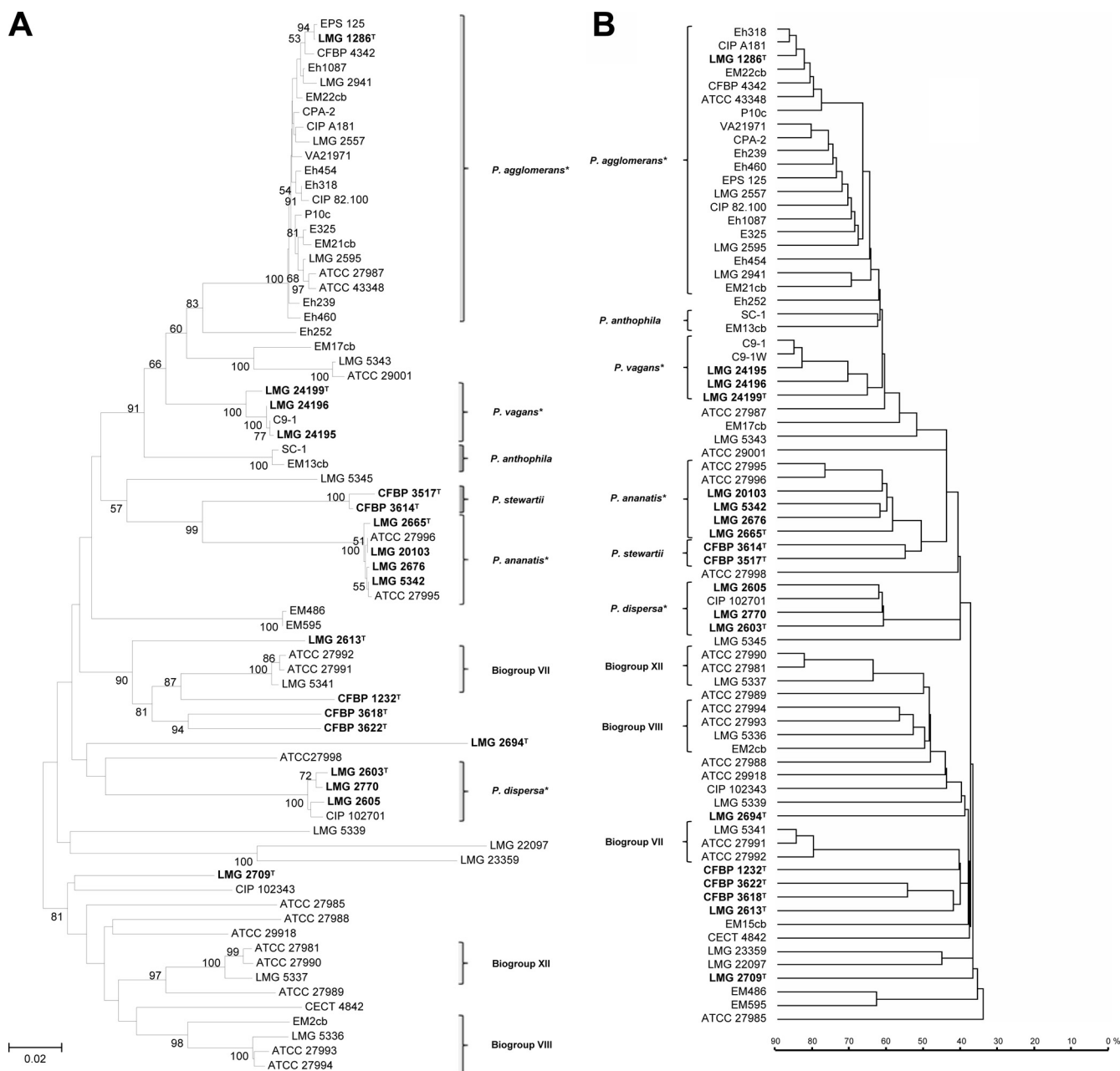


FIG. 3. Comparison between dendrograms derived from *gyrB* sequencing (A) and MALDI-TOF MS protein mass fingerprints (B). With the exception of the reference strains marked in bold, all other isolates were received as *P. agglomerans*, a *Pantoea* sp., or *E. agglomerans* from culture collections. The *gyrB* phylogenetic tree was generated on the basis of a 740-bp fragment of the *gyrB* amplicon using 1,000 bootstrap replicates of the neighbor-joining (NJ) method with the maximum composite likelihood (MCL) model without choosing any outgroup. Sites presenting alignment gaps were excluded from analysis. The MALDI-TOF MS dendrogram was constructed based on the protein mass fingerprint patterns of all analyzed strains using the single-link clustering algorithm implemented in SARAMIS. This algorithm is proprietary to the database provider and does not provide the option to perform bootstrap analysis. Correct clustering of each strain was confirmed by running at least four replicates per isolate, but, for clarity and conciseness, graphics were reduced to show a single representative spectrum per strain. The main clusters obtained in both phylogenetic trees were highly similar; thus, the two methods were comparable in discriminating *P. agglomerans* from other species and in recognizing previously misidentified strains. The diversity within the single species is apparently exaggerated by MALDI-TOF (deeper strain branches) and is not reflecting the actual phylogenetic distances. *, for *P. agglomerans*, *P. vagans*, *P. ananatis*, and *P. dispersa*, brackets indicate the isolates clustering with the respective type strain, which were used to create the SuperSpectrum of the corresponding species.

found to be intermediate, being offset from the *P. agglomerans* (sensu stricto) group when a mass range of 2 to 20 kDa was used (Fig. 3) but not when the lower limit of the mass range was raised to 3 kDa. ATCC 27987 was confirmed as *P. agglom-*

merans (sensu stricto) using fAFLP, although it must be noted that it was the only isolate genetically assigned to *P. agglomerans* for which the biochemical signature obtained with the Phoenix 100 ID/AST system was noticeably dissimilar from

<i>Pantoea agglomerans</i>	<i>Pantoea vagans</i>	<i>Pantoea ananatis</i>	<i>Pantoea dispersa</i>	<i>Pantoea agglomerans</i>	<i>Pantoea vagans</i>	<i>Pantoea ananatis</i>	<i>Pantoea dispersa</i>
		2424	2423		5687		
2448		2447	2447				5739
		2464					6055
		2767					6115
		3032		6199	6200		
		3073		6254	6255	6254	
			3098			6387	
		3124		6401	6402	6408	
			3169		6420		
			3253				6442
			3568				6511
		3573		6820			
		3622					7144
3630					7159	7152	
			3652	7180			7189
	3665			7245	7246	7249	
		3986		7264	7266	7271	
			4045				7284
	4173						7306
		4182	4182	7336	7337	7330	
			4227				7451
			4311		7474		
		4391	4392	7991	7993		
4419	4420				8020		
		4468			8125		
	4477						8250
			4544		8352		
			4592		8391		
			4611				8458
		4635	4635		8740		
4659							8803
			4724			8839	
4753	4753	4759	4758	8882	8885		8894
		4776				8939	
			4962		8958		
5018					9179		
			5142			9273	9273
		5172		9320			
		5380	5379			9390	
5396				9508	9510	9521	9519
			5453	9569	9569		
			5637		9973		
		5652		10286	10289		10285

FIG. 4. Characteristic masses (in daltons) recognized as markers for the identification of *P. agglomerans*, *P. vagans*, *P. ananatis*, and *P. dispersa*. Two or more masses are considered equivalent and are shown on the same line if their values deviate by less than 800 ppm from the average. The 800-ppm mass accuracy setting defining the spectral bin size was evaluated/validated by AnagnosTec GmbH. Even if the error margin in a data set analyzed on a single MALDI-TOF MS instrument is usually smaller (around 400 ppm), a larger bin size is necessary to allow, within the SARAMIS database, the comparison of data collected with different instruments in different laboratories. An 800-ppm mass accuracy still allows one to distinguish mass differences of 14 Da for proteins of 10 kDa, which is normally sufficient to detect single-amino-acid exchanges in proteins of that size. Masses shared by three or more *Pantoea* species are marked in bold.

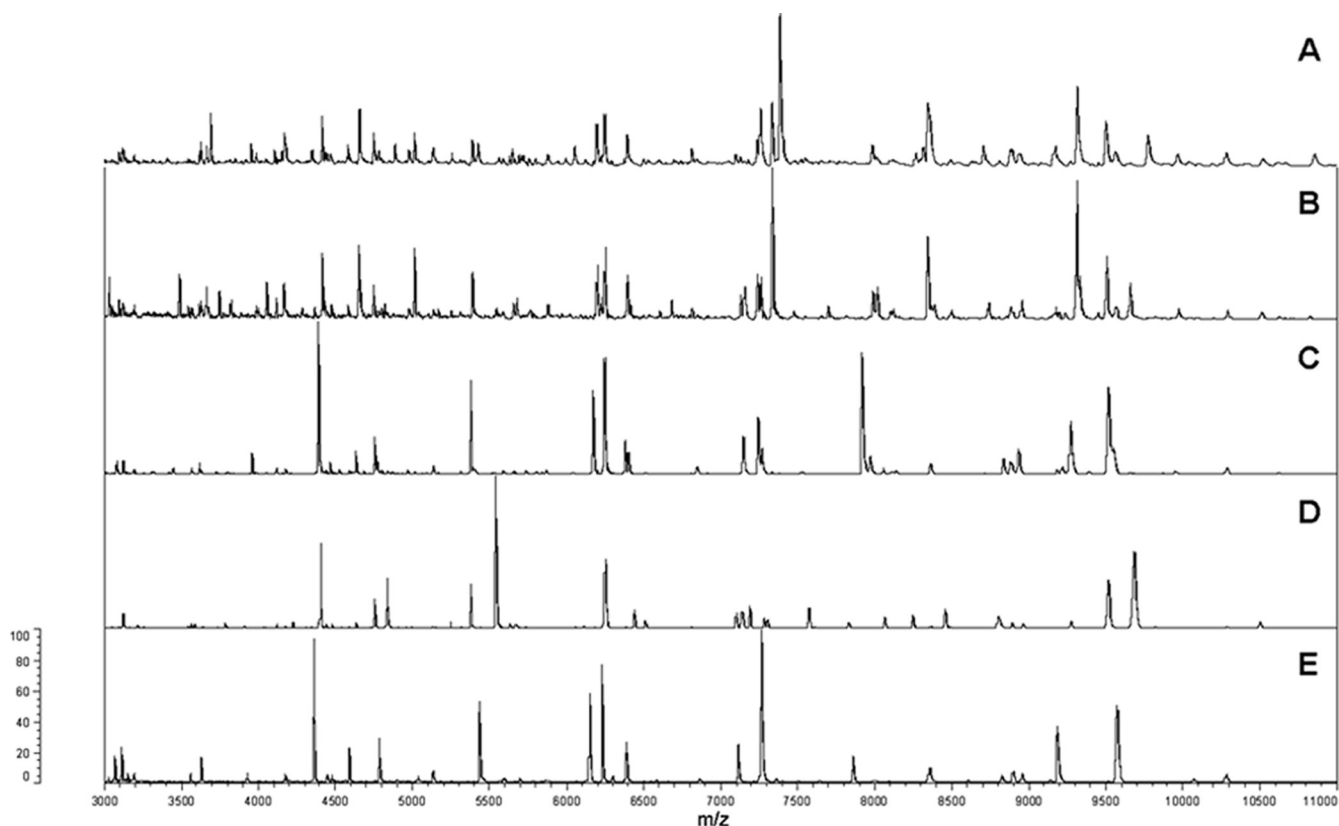


FIG. 5. MALDI-TOF MS protein mass fingerprints of representative strains belonging to different *Pantoea* species: (A) *P. agglomerans* LMG 1286^T, (B) *P. vagans* LMG 24199^T, (C) *P. ananatis* LMG 2676, and (D) *P. dispersa* LMG 2603^T. Similar and diverging marker masses for the identification of these *Pantoea* species are listed in Fig. 4. For comparison, the spectrum of strain LMG 5339 received from the BCCM/LMG collection under the incorrect name of *P. agglomerans* is shown at the bottom (E). Both *gyrB* sequencing and MALDI-TOF MS clearly demonstrate the misnaming of this strain. Relative intensities of ions (percentages) are shown on the y axis and the masses (in daltons) of the ions are shown on the x axis. The *m/z* values represent mass-to-charge ratios.

those of the other strains of the species, leading not only to a wrong automated identification based on the number of correct nonvariable reaction but also to an apparently incorrect clustering based on the overall reaction pattern (Fig. 2). The protein mass fingerprints of the 21 *P. agglomerans* (sensu stricto) strains and strains of *P. ananatis*, *P. dispersa*, and *P. vagans* were used to generate a SuperSpectrum with identifying mass peaks for each species (Fig. 4). A typical MALDI-TOF MS spectrum of *P. agglomerans* contained about 150 ion peaks between 2,000 and 20,000 Da, with the highest intensity peaks found between 4,000 and 11,000 Da. Comparison of these protein mass fingerprints defined a set of 21 markers present in at least 90% of all protein mass fingerprints for confident identification of *P. agglomerans*. While a subset of masses were shared by different *Pantoea* species, a combination of discriminatory signals provided a unique species-level signature (Fig. 4). MALDI-TOF MS spectra of strains having an identity that could not be genetically confirmed as *Pantoea* (e.g., LMG 5339) showed widely divergent profiles compared to strains confidently assigned to this genus (Fig. 5). These divergent signal patterns were reflected in both the MALDI-TOF MS and *gyrB* trees, with the related strains clustering well outside the genus *Pantoea* (Fig. 3). On the basis of 16S rRNA gene

sequencing, strain LMG 5339 showed 99.5% identity to *Buttiauxella agrestis* DSM 4586 (45).

MALDI-TOF MS analysis was able to discriminate strains within *Pantoea* and to segregate related strains into separate species/clades with the same level of accuracy as *gyrB* sequencing and more sensitively than either biochemical or 16S rRNA gene sequencing approaches. Clustering between strains within a group was not identical in the two methods, but these fluctuations at the subgroup level are inconsequential as long as the aim is to ensure that strains remain within a species. Misidentified strains previously grouped into *P. agglomerans* were accurately assigned to *P. ananatis*, *P. dispersa*, or the recently defined species *P. vagans* or to discrete clades following Brenner's biogroups (10). For example, strains could be assigned to biogroup VII (LMG 5336, ATCC 27993, ATCC 27994, and EM2cb), biogroup VIII (LMG 5341, ATCC 27991, and ATCC 27992), and biogroup XII (LMG 5337, ATCC 27981, and ATCC 27990) using both MALDI-TOF MS and *gyrB* sequencing (Fig. 3). Furthermore, MALDI-TOF MS was in agreement with *gyrB* sequencing regarding strains clustering together as *P. stewartii* (CFBP 3517 and CFPB3614), *P. anthophila* (SC-1 and EM13cb) or a probable novel *Pantoea* species (EM486 and EM595) (45).

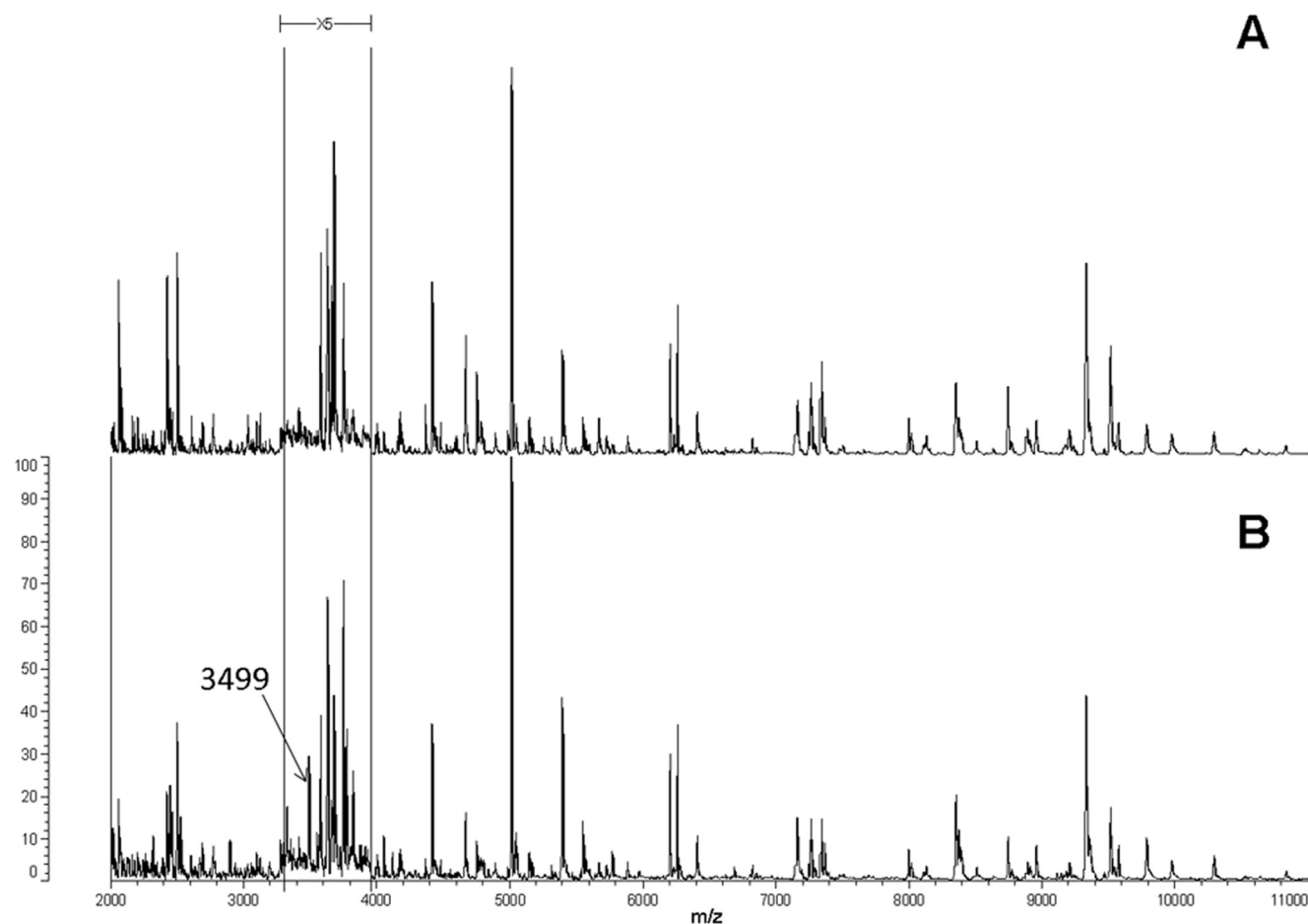


FIG. 6. Comparison of MALDI-TOF MS protein mass fingerprints of pigmentless *P. vagans* variant C9-1W (A) lacking the 530-kb plasmid pPag3 and the related wild-type strain *P. vagans* C9-1 (B). The masses (in daltons) of the ions are shown on the x axis. The m/z values represent mass-to-charge ratios. Relative intensities of ions (percentages) on the y axis are 5-fold exaggerated between 3,300 and 3,950 Da to highlight the disappearance of the 3,499-Da mass signal in C9-1W. Other masses missing from the plasmidless variant C9-1W were at 2,133, 2,140, 2,309, 2,396, 3,197, 3,552, 3,570, 4,295, and 4,850 Da, but most of the signals have low intensities and cannot easily be visualized in the protein profile. Marker masses for the identification of *P. vagans* are listed in Fig. 4.

Our standardized MALDI-TOF MS protocol analysis using strains grown on LB agar proved to deliver highly repeatable results, with only a few replicates that do not immediately cluster with the other measurements performed on the same isolate. Even so, plotting of all the replicates easily allows one to discard substandard measurements which, in this case, still cluster within the same species and hence would retain all prerequisites for successful identification at this taxonomical level (see Fig. S1 in the supplemental material). The use of alternative media such as TSA or MHA shows that the alteration of the growth parameters does have a certain influence on the composition of the mass spectra obtained. This is reflected in the merging of nearby strain measurements in the dendrogram and leads to a loss of resolution that does not allow the unambiguous recognition of each single strain anymore. However, all measurements within the same species are still kept in a tight cluster, thereby preserving the conditions for unequivocal identification at species level (see Fig. S2 in the supplemental material).

Identification using MALDI-TOF MS not only provided robust recognition but was also able to detect protein profile

differences within *P. vagans* C9-1 (4.88-Mb genome) resulting from a large genomic alteration such as curing of the 530-kb megaplasmid pPag3 (pigmentless variant C9-1W, 4.35-Mb genome) (49), as shown for instance for the loss of the 3,499-Da mass signal (Fig. 6). Other masses missing from the plasmid-cured variant C9-1W were at 2,133, 2,140, 2,309, 2,396, 3,197, 3,552, 3,570, 4,295, and 4,850 Da, while no peak was found exclusively in the wild type. Since distinctive marker signals for *P. vagans* (Fig. 4) were unaffected by the loss of the plasmid and the number of missing masses was relatively low, neither the species-level assignment of this variant nor its position in the MALDI-TOF MS dendrogram was altered with respect to the wild type (Fig. 3). This can be explained by the fact that the main signals in MALDI-TOF MS were reported to be ribosomal proteins (47), and thus only a fraction of masses disappears from the profile following the loss of pPag3. Indeed, a number of characteristic masses recognized as markers for the identification of the considered *Pantoea* species are compatible with the predicted masses of *Pantoea* sp. At-9b ribosomal proteins deposited in the UniProt database (<http://www.uniprot.org/>) within the allowed mass deviation of 800

TABLE 2. Compatibility of *Pantoea* spp. markers with the predicted masses of ribosomal *Pantoea* sp. At-9b proteins^a

<i>P. agglomerans</i>	Measured masses (Da)			<i>Pantoea</i> sp. At-9b			
	<i>P. vagans</i>	<i>P. ananatis</i>	<i>P. dispersa</i>	Ribosomal protein	Accession no. ^b	Predicted mass ^c	Modification ^d
		4,391	4,392	50S L36	C8QHD9	4,392	
4,419	4,420			50S L36	C8QHD9	4,392	+ acetyl
5,396				50S L34	C8Q9X2	5,396	
			5,453	50S L36	C8Q3U8	5,585	-met
		6,387		50S L33	C8QC26	6,372	+CH ₃
		6,387		L32	C8Q6B9	6,362	+acetyl
			6,442	L30	C8QHE2	6,574	-met
7,245	7,246	7,249		L29	C8QHF2	7,219	+acetyl
7,264	7,266	7,271		50S L35	C8Q6T8	7,269	
			7,284	50S L35	C8Q6T8	7,269	+CH ₃
7,991	7,993			50S L31	C8QBY0	7,957	+acetyl
8,882	8,885		8,894	L28	C8QC27	9,006	-met
	8,958			L27	C8QDH1	9,097	-met
	9,179			S16	C8QFQ0	9,174	
10,286	10,289		10,285	S19	C8QHF6	10,416	-met

^a Compatibility of the markers for the identification of *Pantoea* spp. (see Fig. 4) with the predicted masses of ribosomal proteins of *Pantoea* sp. At-9b deposited in the UniProt database (<http://www.uniprot.org/>) within the allowed mass deviation of 800 pm. Three single, common posttranslational modifications (N-terminal methionine loss, methylation, and acetylation) were considered to estimate the possible mass variations starting from the predicted mass of the protein in *Pantoea* sp. At-9b.

^b UniProt accession code.

^c Predicted mass without posttranslational modifications.

^d Modifications: -met = N-terminal methionine removal, -131 Da; +CH₃ = methylation, +14 Da; +acetyl = acetylation, +30 Da.

ppm (Table 2). Acquisition of the 135-kb virulence plasmid pPATH (32) also did not confound accurate identification of the phytopathogenic strain ATCC 43348 as *P. agglomerans* using MALDI-TOF MS. Moreover, replacement of the DHB matrix with sinapinic acid allowed us to identify the K43R (lysine to arginine) point mutation in 30S ribosomal protein

S12 (12) that confers streptomycin resistance in the commercial biocontrol strain *P. vagans* C9-1S (Fig. 7).

The primary drawback of MALDI-TOF MS for bacterial identification and diagnostics is the dearth of reference species in databases, which are limited primarily to clinical species while environmental bacteria such as *Pantoea* are largely absent. Unfortunately, there are at present no public repositories for diagnostic protein patterns, which are currently archived only within commercial databases such as that contained in SARAMIS. A further potential problem is the need of isolated colonies for MALDI-TOF MS analysis, a fact that may still represent a limitation for fastidious or slow-growing species (e.g., *Bordetella* sp., *Borrelia* sp., *Neisseria gonorrhoeae*, or *Mycoplasma* spp. in diagnostic laboratories). In such instances direct molecular or serological methods may still retain the advantage, although these methods often require some *a priori* assumption about the nature of the organism to be identified.

This study demonstrates both the accuracy and the simplicity of whole-cell MALDI-TOF MS for identification of the complex *P. agglomerans* group and related taxa. Strain identification using the unique protein profiles generated was achieved with minimal labor and materials and within a few minutes for MALDI-TOF MS sample preparation and analysis. This offers an attractive alternative to the relatively high investment required for single-locus validation, PCR amplification, and sequencing. Investment in a MALDI-TOF mass spectrometer is comparable to that needed for a 16-capillary DNA-sequencing machine, but it requires a fraction of the operating costs and consumables. We also demonstrated the application of MALDI-TOF MS for clustering analysis of *Pantoea*, almost equivalent to *gyrB* phylogenetic analysis. The unique ICMS fingerprints for *Pantoea* species developed in this study will facilitate more accurate and rapid

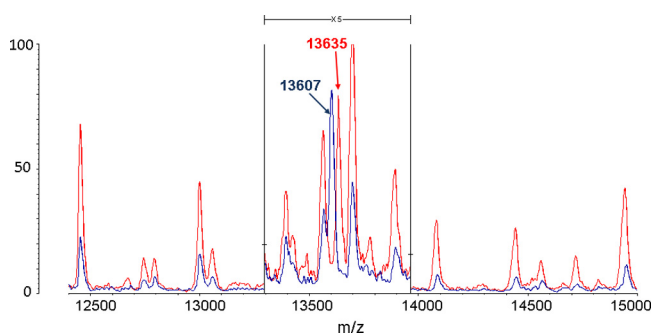


FIG. 7. MALDI-TOF MS identification of the spontaneous lysine-to-arginine mutation in 30S ribosomal protein S12 (encoded by the *rpsL* gene) leading to streptomycin resistance in commercial biocontrol strain *P. vagans* C9-1S (BlightBan C9-1). The 13,607-Da mass signal found in wild-type strain C9-1 (in blue) corresponds to the predicted mass of RpsL in *Pantoea* sp. At-9b (molecular mass = 13,737 Da; UniProt accession code, C8QDZ1), assuming the removal of the N-terminal methionine (-131 Da) and matrix-assisted protonation (+1 Da) following the laser pulse. In streptomycin-resistant strain C9-1S (in red) the peak is shifted at 13,635 Da, whereas the positions of the other peaks remain constant. This variation (+28 Da) corresponds precisely to the difference in molecular mass between arginine (molecular mass = 174.2 Da) and lysine (molecular mass = 146.2 Da). The masses (in daltons) of the ions are shown on the x axis. The *m/z* values represent mass-to-charge ratios. For improved clarity, the relative intensities of ions (percentages) on the y axis are 5-fold exaggerated between 13,300 and 13,970 Da.

identification of isolates from environmental and clinical samples using this technology.

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