Whole-Genome Sequencing-Based Re-Identification of Pseudomonas putida/fluorescens Clinical Isolates Identified by Biochemical Bacterial Identification Systems

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ABSTRACT The genus Pseudomonas, a complex Gram-negative genus, includes species isolated from various environments, plants, animals, and humans. We compared whole-genome sequencing (WGS) with clinical bacteriological methods and evaluated matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) to identify Pseudomonas species. Clinical isolates (N = 42) identified as P. putida or P. fluorescens by a bacterial identification system based on biochemical properties were reexamined by another identification system based on biochemical properties, two systems based on MALDI-TOF MS, and WGS. WGS revealed that 30 of the 42 isolates belonged to one of 14 known Pseudomonas species, respectively. The remaining 12 belonged to one of 9 proposed novel Pseudomonas species, respectively. MALDI-TOF MS analysis showed that the 9 novel species had unique major peaks. These results suggest that WGS is the optimal method to identify Pseudomonas species and that MALDI-TOF MS may complement WGS in identification. Based on their morphologic, physiologic, and biochemical properties, we propose nine novel Pseudomonas species.

IMPORTANCE Most of the clinical isolates, identified as P. putida or P. fluorescens, were misidentified in clinical laboratories. Whole-genome sequencing (WGS) revealed that these isolates belonged to different Pseudomonas species, including novel species. WGS is a gold-standard method to identify Pseudomonas species, and MALDI-TOF MS analysis has the potential to complement WGS to reliably identify them.

KEYWORDS Pseudomonas, human pathogen, re-identification, whole-genome sequencing, MALDI-TOF MS

Pseudomonas is a complex Gram-negative genus (1) which includes 270 species in the List of Prokaryotic names with Standing in Nomenclature (LPSN) (http://www.bacterio.net). Several Pseudomonas species cause opportunistic infections in humans, including P. aeruginosa (1, 2), P. alcaligenes (3), P. asiatica (4), P. fluorescens (3), P. juntendi (5), P. mendocina (6), P. monteilii (7), P. mosseli (3), P. otitidis (8), P. putida (3), P. stutzeri (3), P. tohonis (9) and P. yangonensis (10). Multidrug-resistant P. aeruginosa isolates have been spreading worldwide (2), with resistance also observed in Pseudomonas species, P. asiatica (4), P. juntendi (5), P. monteilii (7), P. putida (11) and P. yangonensis (10).

Since P. putida and P. fluorescens groups include 30 and 41 species, respectively, and have 16S rRNA sequences with ≥97% similarity (12, 13); isolates of these groups are often misidentified (14). Recent re-identification of P. putida strains in GenBank...
using average nucleotide identity (ANI) (15) and digital DNA-DNA hybridization (dDDH) analysis (16) based on their whole genome sequences found that, except for the type strain, many had been incorrectly identified as P. putida (14).

In clinical laboratories, bacteria are routinely identified by automated bacterial identification systems, including the MicroScan WalkAway system (Beckman Coulter, La Brea, CA) and the Vitek 2 system (bioMérieux, Marcy-l’Étoile, France), based on biochemical properties. Recently, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) low-cost rapid systems, including Vitek MS (bioMérieux) and MALDI Biotyper (Bruker, Billerica, MA), have been adopted for bacterial identification (17). In contrast, whole-genome sequencing (WGS) is not routinely used due to its high cost and complicated procedures. Carbapenem-resistant isolates of P. asiatica, a recently proposed Pseudomonas species, had been identified as P. putida spreading in hospitals in Myanmar (4). In this study, we re-identified clinical isolates which had been identified as P. putida or P. fluorescens in clinical laboratories using WGS, and analyzed whole proteins of these isolates using MALDI-TOF MS.

RESULTS

Bacterial identification using automated systems routinely used in clinical laboratories. Of 42 isolates identified as P. putida/fluorescens using Microscan Walkaway, Vitek 2 and MALDI Biotyper re-identified 41, and Vitek MS re-identified 38 (Table 1).

Identification based on WGS. ANI and/or dDDH analysis identified 30 of the 42 isolates as known Pseudomonas species (Table S3). WGS, however, was unable to identify the remaining 12 (Table S3). Comparisons of these 12 isolates with each other by ANI and dDDH analysis showed they belonged to one of 9 different species, respectively (Table S4). Based on morphologic, physiologic, and biochemical properties, we propose that the novel species be named P. sputi sp. nov. (BML-PP014T), P. pseudonitroreducens sp. nov. (BML-PP015T, BML-PP034, and BML-PP043), P. parasilichuanensis sp. nov. (BML-PP020T), P. paraglycinae sp. nov. (BML-PP023T), P. ceruminis sp. nov. (BML-PP028T), P. parakoreensis sp. nov. (BML-PP030T), P. pharyngis sp. nov. (BML-PP036T), P. urethralis sp. nov. (BML-PP042T), and P. fauclium sp. nov. (BML-PP048T and BML-PP049).

Bacterial identification systems compared with ANI and dDDH analysis. Of the 42 isolates, all four identification systems identified BML-PP041 as P. putida (Table 1). Microscan Walkaway, Vitek 2, and Vitek MS correctly identified BML-PP041, but incorrectly identified or did not identify the other 41 isolates (Table 1). MALDI Biotyper correctly identified 10 isolates but incorrectly identified or did not identify 32 isolates (Table 1).

Phylogenetic analysis. Of 12 isolates belonging to the novel Pseudomonas species, 3 belonged to the P. aeruginosa group, 4 to the P. fluorescens group, and 5 to the P. putida group (Fig. 1).

Biochemical and fatty acid properties of the novel type strains. Morphologic, physiologic, and biochemical properties of the 9 novel type strains are listed in Table S5 in the supplemental material, with descriptions in the supplemental manuscript. The major fatty acids were C16:0 (22.5 to 42.3%), summed feature 3 (C16:1ω7c/C16:1ω6c; 12.0 to 40.3%), and summed feature 8 (C18:1ω7c/C18:1ω6c; 7.9 to 26.0%) (Table S6).

MALDI-TOF MS analysis. The MALDI-TOF MS profiles of the 12 isolates belonging to novel species were compared with profiles of known type strains close to the novel type strains, based on a phylogenetic tree (Fig. 1). The profiles of these novel type strains differed from those of known type strains close to them, whereas the profiles of isolates belonging to the same novel species were almost identical to each other (Fig. 2 and Fig. S1). Compared with the type strain P. nitroreducens, the three strains of P. pseudonitroreducens sp. nov. had two specific peaks at 7.691 and 8.042 m/z (Fig. 2A). Compared with the type strain P. plecoglossicida, the three novel species had unique peaks: at 9.235 and 10.255 m/z for P. ceruminis sp. nov. (BML-PP028T); 9.251, 9.618, and 9.901 m/z for P. urethralis sp. nov. (BML-PP042T); and 9.115, 9.574, and 9.859 m/z for P. fauclium (BML-PP048T and BML-PP049) (Fig. 2B). MALDI-TOF MS detected three unique
### TABLE 1
Identification results with commercial identification platforms and ANI/dDDH analysis

| Isolate          | Commercial identification platforms                                                                 | MALDI Biotyper       | Vitek 2         | ANI and dDDH analysis |
|------------------|-------------------------------------------------------------------------------------------------------|----------------------|------------------|------------------------|
| BML-PP010        | P. putida/fluorescens Aeromonas salmonicida                                                           | P. marginalis        | P. fluorescens   | P. carnis              |
| BML-PP011        | P. putida/fluorescens P. fluorescens/P. stutzeri                                                      | P. rhodesiae         | P. fluorescens   | P. rhodesiae           |
| BML-PP012        | P. putida/fluorescens P. fluorescens                                                                 | P. cedrina           | P. fluorescens   | P. carnis              |
| BML-PP013        | P. putida/fluorescens P. fluorescens                                                                 | P. viridiflava       | Unidentified organism | P. qingdaonensis       |
| BML-PP014        | P. putida/fluorescens Acinetobacter haemolyticus                                                     | P. koreensis         | P. fluorescens   | P. carnis              |
| BML-PP015        | P. putida/fluorescens P. aeruginosa                                                                 | P. nitroreducens     | P. aeruginosa    | P. pseudonitroreducens sp. nov. |
| BML-PP016        | P. putida/fluorescens P. fluorescens/Aeromonas salmonicida/Acinetobacter haemolyticus                | P. poae              | P. fluorescens   | P. carnis              |
| BML-PP017        | P. putida/fluorescens Acinetobacter haemolyticus                                                     | P. koreensis         | P. fluorescens   | P. atacamensis         |
| BML-PP018        | P. putida/fluorescens P. putida                                                                      | P. fulva             | P. putida        | P. florua              |
| BML-PP019        | P. putida/fluorescens P. fluorescens/P. putida                                                        | P. protegens         | P. fluorescens   | P. protegens           |
| BML-PP020        | P. putida/fluorescens P. putida                                                                      | P. putida            | P. putida        | P. juntendi            |
| BML-PP021        | P. putida/fluorescens P. putida                                                                      | P. monteilii         | P. putida        | P. asiatica            |
| BML-PP022        | P. putida/fluorescens P. putida                                                                      | P. monteilii         | P. putida        | P. asiatica            |
| BML-PP023        | P. putida/fluorescens P. fluorescens/Acinetobacter haemolyticus                                      | P. koreensis         | P. fluorescens   | P. paraglycinae sp. nov. |
| BML-PP024        | P. putida/fluorescens P. aeruginosa                                                                 | P. koreensis         | P. fluorescens   | P. glycinae            |
| BML-PP025        | P. putida/fluorescens P. fluorescens                                                                 | P. corrugeta         | P. fluorescens   | P. protegens           |
| BML-PP026        | P. putida/fluorescens P. putida                                                                      | P. monteilii         | P. putida        | P. juntendi            |
| BML-PP027        | P. putida/fluorescens P. aeruginosa/P. fluorescens/P. putida                                        | Unidentified organism | Unidentified organism | P. qingdaonensis       |
| BML-PP028        | P. putida/fluorescens Burkholderia gladioli                                                          | P. pleaglossicida    | P. putida        | P. ceruminis sp. nov.  |
| BML-PP029        | P. putida/fluorescens P. stutzeri                                                                    | P. otitidis          | Unidentified organism | P. otitidis           |
| BML-PP030        | P. putida/fluorescens P. aeruginosa                                                                 | P. aeruginosa        | P. fluorescens   | P. paraglycinae sp. nov. |
| BML-PP031        | P. putida/fluorescens P. fluorescens                                                                 | P. rhodesiae         | P. fluorescens   | P. rhodesiae           |
| BML-PP033        | P. putida/fluorescens P. aeruginosa/P. putida                                                        | P. otitidis          | Unidentified organism | P. otitidis           |
| BML-PP034        | P. putida/fluorescens P. mendocina                                                                   | P. nitroreducens     | P. aeruginosa    | P. pseudonitroreducens sp. nov. |
| BML-PP035        | P. putida/fluorescens Unidentified organism                                                            | P. rhodesiae         | P. fluorescens   | P. carnis              |
| BML-PP036        | P. putida/fluorescens P. fluorescens/Acinetobacter haemolyticus                                      | P. atofoamaus        | P. fluorescens   | P. pharyngis sp. nov.  |
| BML-PP037        | P. putida/fluorescens P. stutzeri                                                                    | Pseudomonas sp.      | P. putida        | P. tohanis             |
| BML-PP038        | P. putida/fluorescens P. fluorescens/P. fluorescens/Aeromonas salmonicida/Acinetobacter haemolyticus | P. fluorescens       | P. fluorescens   | P. carnis              |
| BML-PP039        | P. putida/fluorescens P. fluorescens                                                                 | P. koreensis         | P. fluorescens   | P. glycinae            |
| BML-PP040        | P. putida/fluorescens P. fluorescens/P. fluorescens/Acinetobacter haemolyticus                       | P. fluorescens       | P. fluorescens   | P. lactis              |
| BML-PP041        | P. putida/fluorescens P. putida                                                                      | P. putida            | P. putida        | P. putida              |
| BML-PP042        | P. putida/fluorescens P. putida                                                                      | P. putida            | P. putida        | P. urethrais sp. nov.  |
| BML-PP043        | P. putida/fluorescens P. aeruginosa                                                                  | P. nitroreducens     | P. aeruginosa    | P. pseudonitroreducens sp. nov. |
| BML-PP044        | P. putida/fluorescens P. fluorescens                                                                 | P. masselli          | P. putida        | P. masselli            |
| BML-PP045        | P. putida/fluorescens P. aeruginosa/P. fluorescens/P. mendocina                                      | Pseudomonas sp.      | P. putida        | P. tohanis             |
| BML-PP046        | P. putida/fluorescens Acinetobacter haemolyticus                                                     | P. fulva             | P. putida        | P. fulva               |
| BML-PP047        | P. putida/fluorescens P. putida                                                                      | P. monteilii         | P. putida        | P. juntendi            |
| BML-PP048        | P. putida/fluorescens P. fluorescens                                                                 | P. putida            | P. putida        | P. faucium sp. nov.    |
| BML-PP049        | P. putida/fluorescens P. aeruginosa/P. fluorescens                                                    | P. putida            | P. putida        | P. faucium sp. nov.    |
| BML-PP050        | P. putida/fluorescens P. aurigenosa                                                                    | Pseudomonas sp.      | P. alcaligenes  | P. tohanis             |
| BML-PP051        | P. putida/fluorescens P. putida                                                                      | P. monteilii         | P. putida        | P. juntendi            |
| BML-PP052        | P. putida/fluorescens P. aeruginosa/P. fluorescens                                                    | P. masselli          | P. putida        | P. masselli            |

*Bacteria identification results by ANI/dDDH analysis and automated systems. Agreements with ANI/dDDH analysis are shown in gray.
major peaks for *P. paraglycinae* sp. nov., four for *P. parakoreensis* sp. nov., four for *P. parasichuanensis* sp. nov., two for *P. pharyngis* sp. nov., and one for *P. sputi* sp. nov. (Fig. S1).

**Drug susceptibility testing and drug-resistance genes.** As shown by colistin susceptibility in Table 2, three strains of *P. protegens*, including the type strain, were highly resistant to colistin, with MICs of 512 to 4,096 µg/mL; and six strains of *P. carnis*, including the type strain, and a *P. lactis* isolate were moderately resistant to colistin, with MICs of 8 to 64 µg/mL. The remaining isolates were susceptible to colistin (Table 2). *P. protegens* and *P. carnis*, for which the type strains had been isolated from soil and pork, respectively (18, 19), may be intrinsically resistant to colistin. Most of the 42 isolates were sensitive to other antimicrobial agents, except for aztreonam (Table S7).

Assessments of other drug-resistance genes harbored by these 42 isolates showed that 3 of them (BML-PP029, BML-PP030, and BML-PP033) harbored known acquired drug-resistance genes, whereas the remaining 39 isolates did not. Two *P. otitidis* isolates...
(BML-PP029 and BML-PP033) harbored bla\textsubscript{POM-1}–like genes with 98.3 to 98.8% identity, and one \textit{P. parakoreensis} sp. nov. isolate (BML-PP030\textsuperscript{T}) harbored \textit{aadA6} (Table S8).

**DISCUSSION**

Improvements are required in automated bacterial identification systems for clinical isolates of \textit{Pseudomonas}, especially isolates belonging to the \textit{P. putida} and \textit{P. fluorescens} groups. This study demonstrated that these automated systems performed poorly for identifying isolates belonging to these groups. Specifically, of the 264 \textit{P. aeruginosa} strains deposited in GenBank, 259 (98%) were correctly identified as \textit{P. aeruginosa}, whereas all 28 strains deposited as \textit{P. fluorescens} and all 35 deposited as \textit{P. putida} had been incorrectly identified (14). In addition, two clinical isolates (BML-PP029 and BML-PP033) of \textit{P. otitidis} were incorrectly identified or not identified by the automated systems (Table 2), although \textit{P. otitidis} is a clinically important species belonging to the \textit{P. aeruginosa} group. These results indicate that \textit{Pseudomonas} bacteria should be identified using ANI and dDDH analyses.

At present, these WGS-based identification analyses cannot be adapted for use in clinical laboratories. Bacterial identification systems using MALDI-TOF MS are required to correctly identify clinical isolates of \textit{Pseudomonas} species (17). The strains belonging to the nine novel species had unique major MALDI-TOF MS peaks compared with the type strains of closely related species. Our results suggest that MALDI-TOF MS analysis is able to identify \textit{Pseudomonas} species; nevertheless, they have 16S rRNA sequences with $\geq$97% similarity to each other.

Our findings strongly suggest the necessity of using up-to-date databases of bacterial species, especially \textit{Pseudomonas} species, in automated bacterial identification systems. If bacterial strains collected by individual researchers are used in comparisons, their whole genome sequences should be determined, and the species identified using ANI and dDDH.

Some \textit{Pseudomonas} species are likely intrinsically resistant to colistin/polymyxin. For example, all strains of \textit{P. protegens} and \textit{P. camis} tested in this study, including seven
clinical isolates and the two type strains, were resistant to colistin. Other clinically important species known to be intrinsically resistant to colistin/polymyxin include *Burkholderia* spp., *Proteus mirabilis*, *Serratia marcescens*, and *Yersinia* spp (20). Epidemiological and bacteriological studies are needed to clarify whether these *Pseudomonas* spp. have intrinsic resistant to colistin/polymyxin.

This study has some limitations, including the following: (i) the quantity of tested isolates may have been too small to obtain reliable species-specific peaks of MALDI-TOF MS, (ii) a lack of clinical information about the isolates limits estimations of the species’ clinical significance, and (iii) besides *P. fluorescens* and *P. putida*, it is necessary
to clarify whether other Pseudomonas species besides P. aeruginosa may be isolated from human samples or associated with pathogenesis in humans.

**Conclusion.** Of 42 isolates previously identified as P. putida or P. fluorescens by a bacterial identification system, only 1 was identified as P. putida by four automated identification systems. The 42 isolates included 9 novel Pseudomonas species, which we proposed here. This study indicates that WGS may be the most reliable method for identifying Pseudomonas species, and that MALDI-TOF MS analysis has the potential to complement WGS to reliably identify novel species. However, even up-to-date data-bases must be treated with caution since there will always be some lag between discovery and valid documentation of novel species.

**MATERIALS AND METHODS**

**Bacterial identification using automated systems.** We re-identified 42 isolates, previously identified as P. putida or P. fluorescens by the MicroScan WalkAway system (see Table 3 and Fig. 3 for source details), using the MALDI Biotyper, Vitek 2, and Vitek MS identification systems.

**DNA extraction and WGS.** Genomic DNA was extracted using DNeasy Blood and Tissue kits (Qiagen, Hilden, Germany). Genomic libraries were prepared using Nextera XT DNA kits (Illumina, San Diego, CA). Paired-end sequencing was performed using MiSeq Reagent Kits v3 (600-cycle). The sequence reads were
quality-trimmed using CLC Genomics Workbench v11 (Qiagen) with the following parameters: quality limit = 0.05, number of 5'-terminal nucleotides to remove = 10, number of 5'-terminal nucleotides to remove = 15, and discarded reads below length = 50, and assembly of the trimmed reads was performed using shovill v1.1.0 with default settings.

**Species identification based on whole genome sequences.** The 42 isolates were re-identified using ANI and dDDH by comparing their whole genome sequences with those of type strains belonging to genus *Pseudomonas*. ANI and dDDH values were determined by the OrthoANIu algorithm (21) and the Genome to Genome Distance Calculator (GGDC) v2.1 (http://ggdc.dsmz.de/ggdc.php [16]), respectively. In accordance with the International Journal of Systematic and Evolutionary Microbiology (22), the cutoff values of ANI and dDDH between each isolate and the type strain belonging to a species were defined as 95% and 70%, respectively. Isolates not identified by ANI and dDDH were re-analyzed using the Type (Strain) Genome Server (TYGS) (https://tygs.dsmz.de/).

**Drug susceptibility testing.** MICs of drugs against the 42 isolates were determined by microdilution method and interpreted according to CLSI guidelines (M100-S25) (23). Antimicrobial agents were 2-fold diluted in Mueller-Hinton broth (Becton Dickinson, Sparks, MD) at concentrations ranging from 0.0078 to 16 µg/mL for ciprofloxacin and levofloxacin, 0.25 to 4,096 µg/mL for colistin, and 0.25 to 512 µg/mL for the others.

**Drug-resistance genes.** Assembled genome sequences were searched for genes associated with drug resistance using ResFinder v4.1 (https://cge.cbs.dtu.dk/services/ResFinder/) (24).

**Phylogenetic analysis.** Phylogenetic analysis was performed using kSNP3 v3.1 software, with a k-mer length of 31 (25). A neighbor-joining phylogenetic tree was estimated based on pan-genome 18,432,899 single-nucleotide polymorphisms from genomes, which included recombinant sites, of the 12 isolates classified as new species and the 81 type strains of *Pseudomonas*. The accession numbers for these genome data are listed in Tables S1 and S2.

**MALDI-TOF MS analysis.** Whole bacterial proteins were analyzed using MALDI-TOF MS, as described previously (26). Cell lysates were mixed with a sinapinic acid matrix solution. MALDI mass spectra were acquired in the range of 2,000 to 30,000 m/z in positive-ion linear mode by averaging 1,000 laser shots using an AXIMA Performance (Shimadzu/Kratos, UK) equipped with a pulsed N2 laser (λ = 337 nm). Mass calibration was performed using adrenocorticotropic hormone 18 to 39 ([M + H]⁺, 2,466.7 m/z) and myoglobin ([M + 2H]²⁺, 8,476.8 m/z) as marker proteins of external calibration.

**Biochemical properties and fatty acids contents of new species.** Biochemical tests were performed using API 20NE (bio Mérieux), API ZYM kits (bio Mérieux) and GN3 MicroPlates (Biolog, Hayward, CA), according to the manufacturers’ instructions. The morphology and dimensions of cells grown for 24 h at 30°C on lysogeny broth (LB) agar (Becton, Dickinson and Co., Franklin Lakes, NJ) were determined by scanning electron microscopy (S4800, Hitachi, Tokyo, Japan). Gram staining was performed as described (26). Fluorescent pigments were detected with King’s A and B agar (Elken Chemical Co., Ltd., Tokyo, Japan). Physiological tests, including growth at different temperatures (4°C to 44°C at intervals of 4.0°C), pH (pH of 5 to 10, at intervals of 0.5 pH), and NaCl concentrations (1% to 10% [wt/vol], at intervals of 1%) were performed in LB (Becton Dickinson), as described (26). Catalase and oxidase activities were determined using 3% (vol/vol) hydrogen peroxide and Kovács’ reagent, respectively. Fatty acids contents of isolates were analyzed using the Sherlock Microbial Identification (MIDI) system (v6.0) as described (26). Bacterial strains were cultured on tryptic soy broth agar (30 g · L⁻¹ tryptic soy broth, 15 g · L⁻¹ agar; Becton Dickinson) for 1 day at 30°C, a culture condition frequently used to analyze fatty acids in *Pseudomonas* novel species (26, 27).

**FIG 3** Geographic distribution of the 42 clinical isolates in Japan. The 42 isolates named in Table 3 came from the 20 prefectures shown in the map.
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