Precise Species Identification for *Enterobacter*: a Genome Sequence-Based Study with Reporting of Two Novel Species, *Enterobacter quasiroggenkampii* sp. nov. and *Enterobacter quasimori* sp. nov.

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**ABSTRACT** The genus *Enterobacter* comprises common pathogens and has a complicated taxonomy. Precise taxonomic assignment lays a foundation for microbiology. In this study, we updated the *Enterobacter* taxonomy based on robust genome analyses. We found that all *Enterobacter* subspecies assignments were incorrect. *Enterobacter cloacae* subsp. dissolvens and *Enterobacter hormaechei* subsp. hoffmannii are species (*Enterobacter dissolvens* and *Enterobacter hoffmannii*, respectively) rather than subspecies. *Enterobacter xiangfangensis*, *Enterobacter hormaechei* subsp. oharae, and *Enterobacter hormaechei* subsp. steigerwaltii are not *Enterobacter hormaechei* subspecies but belong to the same species (*Enterobacter xiangfangensis*). *Enterobacter timonensis* should be removed to *Pseudenterobacter*, a novel genus. We then reported two novel species, *Enterobacter quasiroggenkampii* and *Enterobacter quasimori*, by genome- and phenotype-based characterization. We also applied the updated taxonomy to curate 1,997 *Enterobacter* genomes in GenBank. Species identification was changed following our updated taxonomy for the majority of publicly available strains (1,542, 77.2%). The most common *Enterobacter* species was *E. xiangfangensis*. We identified 14 novel tentative *Enterobacter* genomspecies. This study highlights that updated and curated taxonomic assignments are the premise of correct identification.

**IMPORTANCE** *Enterobacter* species are major human pathogens. Precise species identification lays a foundation for microbiology, but the taxonomy of *Enterobacter* is complicated and confusing. In this study, first, we significantly updated the taxonomy of *Enterobacter* by rigorous genome analyses and found that all subspecies assignments of *Enterobacter* were incorrect. Second, we characterized and reported two novel *Enterobacter* species with clinical significance. Third, we curated 1,997 *Enterobacter* genome sequences deposited in GenBank and found that the species identification of most *Enterobacter* strains needed to be corrected. Fourth, we found that the most common *Enterobacter* species seen in clinical samples is *Enterobacter xiangfangensis* rather than *Enterobacter cloacae*. Fifth, we identified 14 tentative novel *Enterobacter* and 18 tentative novel non-*Enterobacter* species. This study highlights that updated and curated taxonomic assignments are the premise of correct species identification. We recommend that future *Enterobacter* studies need to use the updated taxonomy to avoid misleading information.

Citation Wu W, Feng Y, Zong Z. 2020. Precise species identification for *Enterobacter*: a genome sequence-based study with reporting of two novel species, *Enterobacter quasiroggenkampii* sp. nov. and *Enterobacter quasimori* sp. nov. *mSystems* 5:e00527-20. https://doi.org/10.1128/mSystems.00527-20.

Editor Rup Lal, University of Delhi
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Received 9 June 2020
Accepted 20 July 2020
Published 4 August 2020
Enterobacter is a genus of Gram-negative, non-spore-forming bacteria of the family Enterobacteriaceae and is close to the genera Leclercia and Lelliottia (1). Enterobacter is widely distributed in nature and is a well-known pathogen for plant diseases (2). In addition, Enterobacter is also part of the commensal microflora of the human gut (3, 4). A few Enterobacter species, e.g., Enterobacter cloacae, Enterobacter asburiae, and Enterobacter hormaechei, are common pathogens of human infections, particularly hospital-acquired infections (5). Precise species and subspecies assignation of bacterial isolates lays a foundation for understanding the epidemiology, pathogenesis, and microbiological features of bacteria and has important implications for diagnosis, treatment, prognosis, and prevention. Clinical microbiology laboratories commonly use phenotype-based tests including automated microbiology systems such as Vitek II and matrix-assisted laser desorption ionization–time of flight mass spectrum (MALDI-TOF) for species identification, which usually identify Enterobacter clinical isolates as E. cloacae or sometimes as E. asburiae, E. hormaechei, or Enterobacter kobei. However, it is known that phenotype-based tests cause misidentification of Enterobacter and are unreliable for precise species identification (3). DNA-DNA hybridization (DDH) with a ≥70% cutoff has long been used as the “gold standard” for species delineation (6), but DDH is error-prone and has low reproducibility. 16S rRNA gene sequence identity has therefore been used as a proxy of DDH. However, it is well known that analysis on 16S rRNA gene sequence is insufficient for accurate bacterial species assignation (7). As the cost has been massively reduced, whole-genome sequencing has been increasingly used in clinical microbiology laboratories, which allows precise species identification (8). The pairwise average nucleotide identity (ANI) with a ≥96% cutoff and in silico DNA-DNA hybridization (isDDH, also called digital DDH [dDDH]) with a ≥70.0% cutoff mimic traditional DDH and have been widely used for precise species identification (9–11).

Updated and curated taxonomic assignment is the premise of precise species identification, but the taxonomy of Enterobacter is complicated by the fact that many species that used to belong to Enterobacter have been moved out to other genera. For instance, Enterobacter aerogenes, Enterobacter agglomerans, and Enterobacter cowanii have been moved to the genus Klebsiella, Pantoea, and Kosakonia, respectively (4, 12, 13). Until now, the Enterobacter genus has been comprised of 19 species plus 6 subspecies with validly published names (Table 1) (14). Genome sequences of type strains of all Enterobacter species and subspecies except Enterobacter cloacae subsp. dissolvens are available. However, previous studies have found that the species and subspecies assignation within the genus Enterobacter is problematic (15). In particular, subspecies assignments within Enterobacter have been defined based on low-resolution analytical methods (16, 17) and may need to be carefully examined (18). For instance, Enterobacter xiangfangensis has been validly published as a species (19) but a recent study has proposed it as a subspecies of E. hormaechei (Enterobacter hormaechei subsp. xiangfangensis) based on an in silico analysis (18). This causes confusion, and each taxon should bear only one correct assignation (20).

In this study, we performed whole-genome sequencing for the type strain and report here that E. cloacae subsp. dissolvens is actually an independent species rather than a subspecies of E. cloacae. We then performed genome-based comparison and a phylogenetic analysis to clarify the exact taxonomic positions of the subspecies of E. hormaechei. We found that E. hormaechei and E. xiangfangensis are indeed different species, while E. hormaechei subsp. steigerwaltii and E. hormaechei subsp. oharae are later synonyms of E. xiangfangensis. In addition, E. hormaechei subsp. hoffmannii is a species rather than a subspecies. We also found that Enterobacter timonensis should be removed to a novel genus with the proposed name Pseudenterobacter. We also identified and characterized two novel Enterobacter species, which were distinct from
### TABLE 1 Classification and nomenclature of the genus *Enterobacter* as of April 2020

| Species | Type strain | Accession no. or current species name |
|---------|-------------|-------------------------------------|
| *Enterobacter asburiae*<sup>a</sup> | JCM 6051 | CP011863 |
| *Enterobacter bugandensis* | EB-247 | FYB00000000 |
| *Enterobacter cancerogenus* | ATCC 35316 | ERR1854846 |
| *Enterobacter chengduensis* | WCHEC1-C4 | MTSS00000000 |
| *Enterobacter chuandaensis* | 090028 | QZCS00000000 |
| *Enterobacter cloacae* | ATCC 13047 | CP001918 |
| *E. cloacae* subsp. cloacae | ATCC 13047 | CP001918 |
| *E. cloacae* subsp. dissolvens | ATCC 23373 | WJWQ00000000<sup>d</sup> |
| *Enterobacter hormaechei* | ATCC 49162 | MKEQ00000000 |
| *E. hormaechei* subsp. hormaechei | ATCC 49162 | MKEQ00000000 |
| *E. hormaechei* subsp. hoffmannii | DSM 14563 | CP017186 |
| *E. hormaechei* subsp. oharae | DSM 16687 | CP017180 |
| *E. hormaechei* subsp. steigerwaltii | DSM 16691 | CP017179 |
| *Enterobacter huaxiensis* | 090008 | QZCT00000000 |
| *Enterobacter kobei* | ATCC BAA-260 | CP001918 |
| *Enterobacter ludwigii* | EN-119 | CP017181 |
| *Enterobacter mori*<sup>b</sup> | LMG 25706 | GL890773 |
| *Enterobacter oligotrophica* | CCA6 | AP019007 |
| *Enterobacter quasihormaechei* | WCHEs120003 | SJO00000000 |
| *Enterobacter rosenkampii* | DSM 16690 | CP017184 |
| *Enterobacter sicauanensis* | WCHEC1597 | POVL00000000 |
| *Enterobacter soli*<sup>c</sup> | ATCC BAA-2102 | LXS00000000 |
| *Enterobacter timonensis* | mt20 | FCP00000000 |
| *Enterobacter wuouensis* | WCHEs120002 | SJO00000000 |
| *Enterobacter xiangfangensis*<sup>e</sup> | LMG 27195 | CP017183 |

**Species rejected (n = 4)**

- *Enterobacter muelleri*<sup>a</sup> | JM-458 | *Enterobacter asburiae* |
- *Enterobacter siamensis*<sup>d</sup> | C2361 | *Enterobacter mori* |
- *Enterobacter tabaci*<sup>b</sup> | YIM Hb-3 | *Enterobacter cancerogenus* |
- *Enterobacter taylorae*<sup>d</sup> | ATCC 35317 | *Enterobacter massiliensis* |

**Species listed in LPSN but moved out of**

- *Enterobacter aerogenes* | ATCC 13048 | Klebsiella aerogenes |
- *Enterobacter agglomerans* | ATCC 27155 | Pantoea agglomerans |
- *Enterobacter amnigenus* | ATCC 33072 | Lelliottia amnigena |
- *Enterobacter arachidis* | KCTC 22375 | Kosakonia arachidis |
- *Enterobacter cowanii* | CCUG 45998 | Kosakonia cowanii |
- *Enterobacter gergoviae* | ATCC 33028 | Pluralibacter gergoviae |
- *Enterobacter helveticus* | JCM 16470 | Cronobacter helveticus |
- *Enterobacter intermedius* | ATCC 33110 | Riuvera intermedia |
- *Enterobacter massiliensis* | JC163 | Metakosakonia massiliensis |
- *Enterobacter mimipressuralis* | CIP 104980 | Lelliottia mimipressuralis |
- *Enterobacter oryzae* | DSM 24251 | Kosakonia oryzae |
- *Enterobacter oryzendophyticus* | LMG 26432 | Kosakonia oryzendophytica |
- *Enterobacter oryziphilus* | LMG 26429 | Kosakonia oryziphila |
- *Enterobacter pulvisci* | DSM 19144 | Cronobacter pulvisci |
- *Enterobacter pyrinius* | ATCC 49851 | Pluralibacter pyrinius |
- *Enterobacter radicincitans* | CIP 108468 | Kosakonia radicincitans |
- *Enterobacter sacchari* | CGMCC 1.12102 | Kosakonia sacchari |
- *Enterobacter sakazakii* | ATCC 29544 | Cronobacter sakazakii |
- *Enterobacter taylorae* | DSM 18397 | Cronobacter taylorae |

<sup>a</sup>*Enterobacter muelleri* is a later synonym of *Enterobacter asburiae* (42).
<sup>b</sup>*Enterobacter tabaci* (type strain YIM Hb-3) is a later synonym of *Enterobacter mori* (15).
<sup>c</sup>*The species status of* Enterobacter xiangfangensis *has been proposed as a subspecies of* Enterobacter hormaechei *rather than a valid species (18). However, its type strain has only 94.48% ANI and 60.0% isDDH with* E. hormaechei *type strain ATCC 49162*<sup>f</sup> (GenBank accession no. MKEQ00000000). Therefore, it is clear that* E. xiangfangensis *and* E. hormaechei *are two different species.
<sup>d</sup>*The genome sequencing was performed in the present study.
<sup>e</sup>*Enterobacter siamensis* is rejected as the 16S rRNA sequence of its type strain available in collections does not match its record in GenBank (43).
<sup>f</sup>*Enterobacter taylorae* is a later synonym of *Enterobacter cancerogenus* (44).
Enterobacter dissolvens subsp. hormaechei species each appeared to form a distinct branch. The ANI values between the strain steigerwaltii sequences were identical to those of strain ATCC 23373T previously deposited in atpD GC content. No contamination was identified in the genomes. The species (below the 96% ANI cutoff to define a bacterial species (fore proposed that /
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strated that the type strains of Enterobacter xiangfangensis that formed a clade, which was distinct from all other Enterobacter strains with whole-genome sequences available are not E. cloacae but E. xiangfangensis. We also found that there are 14 tentative novel Enterobacter species based on genome analysis, which need to be further studied using phenotype-based methods to establish their species status.

**RESULTS**

*E. cloacae* subsp. *dissolvens* is a species rather than a subspecies and should be renamed *Enterobacter dissolvens*. Whole-genome sequencing for strain ATCC 23373T generated 2,908,248 reads and 0.87 gigabases, which were assembled into a 4.84-Mb draft genome containing 51 contigs >200 bp in length (N50 415,836 bp) with a 55.16% GC content. No contamination was identified in the genomes. The gyrB, rpoB, infB, and atpD sequences were identical to those of strain ATCC 23373T previously deposited in GenBank (accession no. JX424979, JX425238, JX425108, and JX424849, respectively), suggesting that this strain was indeed strain ATCC 23373T. The ANI value between strain ATCC 23373T and E. cloacae subsp. cloacae ATCC 13047T (GenBank accession no. CP0001918) was 94.79% (ATCC 13047T versus ATCC 23373T) or 94.92% (vice versa), below the 96% ANI cutoff to define a bacterial species (9). The isDDH value between the type strains was 62.0%, lower than the 70.0% cutoff to define a bacterial species (10). Both ANI and isDDH analyses indicate that *E. cloacae* subsp. *dissolvens* should be considered a species different from *E. cloacae* subsp. *cloacae*. In addition, the ANI and isDDH values between strain ATCC 23373T and type strains of all other Enterobacter species are <95% and <70%, respectively (see Table S1 in the supplemental material). We therefore proposed that *E. cloacae* subsp. *dissolvens* should be elevated to the species level as *Enterobacter dissolvens* sp. nov. (type strain ATCC 23373T = CIP 105586T = JCM 6049T = LMG 2683T).

*Enterobacter xiangfangensis* is not a subspecies of *E. hormaechei*. The core gene-based phylogenomic tree (see Fig. S1 in the supplemental material) demonstrated that the type strains of *E. xiangfangensis* and other *E. hormaechei* subspecies formed a clade, which was distinct from all other Enterobacter species. This suggests that *E. xiangfangensis* and the *E. hormaechei* subspecies are indeed closely related. Within this *E. hormaechei* clade, *E. hormaechei* subsp. oharae, *E. hormaechei* subsp. steigerwaltii, and *E. xiangfangensis* were clustered together, while the other two subspecies each appeared to form a distinct branch. The ANI values between the strain *E. hormaechei* subsp. *hormaechei* ATCC 49162T, which is also the type strain of the species *E. hormaechei*, and the type strains of other subspecies and *E. xiangfangensis* range from 94.13% to 94.79% (Table 2), which are below the 96% ANI cutoff to define a bacterial species (9). The isDDH value between *E. hormaechei* subsp. *hormaechei* ATCC 49162T and the type strains of other subspecies and *E. xiangfangensis* ranges from 58.0% to 62.5% (Table 2), also lower than the 70% cutoff to define a bacterial species. Both ANI

| TABLE 2 | The ANI and isDDH values between type strains of *Enterobacter hormaechei* subspecies |
| “Subspecies” | ANI/isDDH, %, for “subspecies”: |
| | hormaechei | hoffmannii | oharae | steigerwaltii | xiangfangensis |
| hormaechei | 94.09/58.0 | 94.79/62.5 | 94.71/61.7 | 94.47/60.0 |
| hoffmannii | 94.13/58.0 | 95.59/66.9 | 95.60/66.5 | 95.71/66.6 |
| oharae | 94.79/62.5 | 95.69/66.9 | 97.38/80.8 | 97.01/76.2 |
| steigerwaltii | 94.56/61.7 | 95.39/66.5 | 97.16/80.8 | 96.62/75.8 |
| xiangfangensis | 94.48/60.0 | 95.61/66.6 | 96.88/76.2 | 96.84/75.8 |

*Subspecies* and strains: *E. hormaechei* subsp. *hormaechei* ATCC 49162T; *E. hormaechei* subsp. *hoffmannii* DSM 14563T; *E. hormaechei* subsp. *oharae* DSM 16687T; *E. hormaechei* subsp. *steigerwaltii* DSM 16691T; *E. xiangfangensis* LMG 27195T. Pairwise ANI and isDDH values above the cutoff to define a bacterial species are highlighted in bold.
and isDDH analyses clearly indicate that three other subspecies (E. hormaechei subsp. steigerwaltii, E. hormaechei subsp. oharae, and E. hormaechei subsp. hoffmannii) and E. xiangfangensis actually do not belong to E. hormaechei and should not be considered subspecies of E. hormaechei.

**Enterobacter hormaechei subsp. oharae and Enterobacter hormaechei subsp. steigerwaltii are not subspecies of Enterobacter hormaechei but are later synonyms of Enterobacter xiangfangensis.** Pairwise ANI values among type strains of E. hormaechei subsp. oharae (strain DSM 16687T), E. hormaechei subsp. steigerwaltii (DSM 16691T), and E. xiangfangensis (LMG 27195T) were all ≥96.62%, and the pairwise isDDH values of the three strains were all ≥75.8% (Table 2). Both the ANI and isDDH values among the three strains were well above the cutoffs to define a bacterial species, indicating that the three type strains belong to a common species. The fact that ANI and isDDH values among E. xiangfangensis, E. hormaechei subsp. oharae, and E. hormaechei subsp. steigerwaltii are above the cutoff to define bacterial species has also been noticed before (18) and is used as the evidence that E. xiangfangensis is a subspecies of E. hormaechei (18). As demonstrated above, E. hormaechei subsp. oharae and E. hormaechei subsp. steigerwaltii do not belong to E. hormaechei in fact. Therefore, the >96% ANI and >70% isDDH values between E. xiangfangensis and E. hormaechei subsp. oharae or E. hormaechei subsp. steigerwaltii cannot be used as the evidence to reject the species status of E. xiangfangensis but provide the proof that the three “subspecies” actually belong to a common species.

**Enterobacter hormaechei subsp. hoffmannii is not a subspecies of Enterobacter hormaechei but is a novel species.** The ANI values between the type strain of E. hormaechei subsp. hoffmannii (DSM 14563T) and type strains of E. hormaechei subsp. oharae, E. hormaechei subsp. steigerwaltii, and E. xiangfangensis range from 95.59% to 95.71% (Table 2), which fall into the 95 to 96% inconclusive zone of defining a bacterial species (9, 21). The isDDH value between E. hormaechei subsp. hoffmannii strain DSM 14563T and the type strains of E. hormaechei subsp. oharae, E. hormaechei subsp. steigerwaltii, and E. xiangfangensis ranges from 66.5% to 66.9% (Table 2), lower than the 70% cutoff to define a bacterial species. Therefore, E. hormaechei subsp. hoffmannii is a novel Enterobacter species rather than a subspecies of any known Enterobacter species, and we propose the species name as Enterobacter hoffmannii.

**Enterobacter timonensis should be removed to a novel genus with the proposed name Pseudenterobacter.** The core gene-based phylogenomic tree of the family Enterobacteriaceae (Fig. 1) and that of the genus Enterobacter and closely related genera (Fig. 2) demonstrated that E. timonensis forms an independent branch, which is well separated from all other Enterobacter species by species of the genera Leclercia and Lelliottia. The ANI values between the type strain of E. timonensis and those of all other Enterobacter species are <85% (82.03 to 83.78%, Table S1), while the values between type strains of other Enterobacter species are >85%. Correspondingly, the isDDH values between the type strain of E. timonensis and those of all other Enterobacter species are <30% (24.7 to 26.3%, Table S1), while the values between type strains of other Enterobacter species are >30%. The above findings suggest that E. timonensis does not belong to the genus Enterobacter. The ANI and isDDH values for the type strain of E. timonensis and those of Leclercia and Lelliottia species are <85% and <30%, respectively. The phylogenomic trees (Fig. 1 and 2) demonstrated that E. timonensis is also distinct from Leclercia and Lelliottia species. Therefore, it is evident that E. timonensis does not belong to the genus Leclercia nor Lelliottia but to a novel genus. As it is closely related to Enterobacter, we propose the genus name Pseudenterobacter (Pseud.en.te.ro-bac-ter. Gr. adj. pseudês false; N.L. masc. n. Enterobacter a bacterial generic name; N.L. fem. n. Pseudenterobacter, a genus falsely [or incorrectly] classified in Enterobacter). E. timonensis should therefore be renamed Pseudenterobacter timonensis.

**Two Enterobacter strains from blood represent a novel species, named Enterobacter quasiroggenkampii sp. nov.** Strains WCHECL1060T and 0900040 were both identified as E. cloacae by Vitek II. The two strains had very different genomic fingerprints obtained by macrorestriction analysis (see Fig. S2 in the supplemental material).
FIG 1 A phylogenetic tree based on the concatenated nucleotide sequence of core genes of Enterobacter quasimori strain 090044, Enterobacter quasiroggenkampii strains WCH11060, and 090040, (Continued on next page)
The 16S rRNA gene sequence of the two strains shared 99.61% identity (6 bases mismatch) and was 99% identical to those of type strains of a few *Enterobacter* species including *E. asburiae*, *E. cloacae*, *E. hormaechei*, *E. kobei*, and *E. ludwigii*.

The draft whole-genome sequence of strain WCHECL1060\(^T\) has been reported by us before \(^22\), and its 4.8-Mb draft genome was assembled from 1.7 gigabases into 21 contigs \(\geq 200\) bp in length \((N_{\text{contig}} 714,400 \text{ bp})\) with a 55.68% GC content. For strain 090040, 4,788,302 reads and 1.73 gigabases were generated, which were assembled into a 4.9-Mb draft genome containing 30 contigs \(\geq 200\) bp in length \((N_{\text{contig}} 515,146 \text{ bp})\) with a 55.69% GC content. No contamination was identified for the genomes of WCHECL1060\(^T\) and 090040. The ANI value between strains WCHECL1060\(^T\) and 090040 was 98.4% \((\text{Table 3})\). In contrast, the ANI values between the two strains and type strains of all known *Enterobacter* species were \(\leq 96\%\) and the highest value \((95.37\%/95.30\%,\ \text{respectively})\) was seen with *E. roggenkampii* DSM 16690\(^T\) \((\text{Table 3} \text{ and Table S1})\).

The isDDH value between strains WCHECL1060\(^T\) and 090040 was 88% \((\text{Table 3})\).

| Strain or Isolate | Accession no. |
|-------------------|--------------|
| WCHECL1060\(^T\)  | CP017183     |
| 090040            | RXRX0000000  |

FIG 1 Legend (Continued)

**FIG 2** A more precise phylogenetic tree based on the concatenated nucleotide sequence of core genes of tentative taxa and type strains of genera *Enterobacter*, *Leclercia*, and *Lelliottia* (listed in Tables 5 and 7 and Data Set S1). Strains and their nucleotide accession numbers are listed alongside the names of species. For species and subspecies with names that need to be revised as suggested in this study, the revised names are shown first, and the current names are shown after the slash. The tree was inferred using the maximum likelihood method under the GTRGAMMA model with a 1,000-bootstrap test, and branches with support over 50% are indicated by gradients. Bar, value indicates the nucleotide substitutions per site.

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whereas isDDH values between the two strains and type strains of all known *Enterobacter* species were 64.8%/65.2%, respectively (with *E. roggenkampii* DSM 16690T), or lower (Table 3 and Table S1), which were below the 70% cutoff to define a bacterial species. Therefore, the ANI and isDDH analyses clearly suggest that the two strains represent a novel species of the genus *Enterobacter*.

Biochemical characteristics between strains WCHECL1060T and 090040 and type strains of other *Enterobacter* species are shown in Table 4. For both strains, growth occurs at 4 to 37°C with optimal growth at 35 and 37°C, but not at 45 or 50°C. Cells grow at 35°C in the presence of 0 to 9% (wt/vol) NaCl in tryptic soy broth (TSB). Both strains were positive for the catalase test but negative for oxidase activity. Cells of the two strains are Gram negative, motile, non-spore-forming, facultatively anaerobic, and rod shaped. Colonies are circular, white, translucent, raised, and smooth after 24 h of incubation at 35°C on nutrient agar. Acid is produced from glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, sucrose, melibiose, amygdalin, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, D-sorbitol, potassium 2-ketogluconate, and methyl-α-D-glucopyranoside but not erythritol, L-xylose, D-adonitol, D-arabinose, potassium gluconate, and methyl-α-D-mannopyranoside. Both strains have a positive reaction for β-D-galactosidase, arginine dihydrolase, and ornithine decarboxylase but are negative for lysine decarboxylase, deaminase, and gelatinase. Both are also negative for urease activity and indole production but positive for the Voges-Proskauer reaction. Both strains can utilize citrate but do not produce H₂S. The two strains can be differentiated from all other *Enterobacter* species by their ability to ferment inositol, D-sorbitol, and melibiose but not potassium glucuronate, L-fucose, and methyl-α-D-mannopyranoside.

The comparison of the fatty acid profiles of the strains WCHECL1060T and 090040 and type strains of other *Enterobacter* species are shown in Table S2 in the supplemental material. Although the proportions of the fatty acids were slightly different, the major cellular fatty acids of strains WCHECL1060T and 090040 were C₁₆:₀, C₁₇:₀ cyclo, and C₁₈:₁ω₇c which were consistent with those of other *Enterobacter* species. The antimicrobial susceptibility profiles and antimicrobial resistance genes of the two strains are described in the supplemental material (Text S1 and Table S3).

### Table 3: Average nucleotide identity, in silico DNA-DNA hybridization, and percentage of conserved proteins values between strains WCHECL1060T, 090040, and 090044T and the type strain of species belonging to the genus *Enterobacter*

| Species and/or strain | Accession no. | ANI/isDDH, %, for strain: |
|----------------------|--------------|---------------------------|
|                      | WCHECL1060T  | 090040                    | 090044T                      |
| *E. asburiae* ATCC 35953T | CP011863     | 93.25/51.7                | 93.01/51.8                  | 90.07/40.9 |
| *E. bugandensis* EB-247T | FYBI000000000 | 91.02/43.1                | 90.62/43.2                  | 88.95/38.0 |
| *E. cancerogenus* ATCC 33241T | ERR1854846    | 86.39/31.5                | 85.70/31.6                  | 86.33/32.3 |
| *E. chengduensis* WCHECL-C4T | MT50000000000 | 92.19/49.3                | 92.02/49.3                  | 89.10/38.7 |
| *E. chuaenaedis* 090028T | QZCS500000000 | 90.49/42.7                | 90.60/42.9                  | 89.25/38.5 |
| *E. cloacae* ATCC 13047T | CP001918     | 88.28/35.8                | 87.53/35.8                  | 87.30/34.4 |
| *E. dissolvens* ATCC 23373T | WJWQ000000000 | 87.92/35.9                | 87.94/35.9                  | 87.52/34.5 |
| *E. hoffmannii* DSM 14563T | CP0171186    | 86.91/33.5                | 86.93/33.7                  | 87.76/34.8 |
| *E. hormaechei* ATCC 49162T | MKEQ000000000 | 87.51/33.7                | 86.90/33.8                  | 87.77/35.0 |
| *E. huxiensis* 090008T | QZCT000000000 | 87.36/34.5                | 87.20/34.6                  | 88.72/37.6 |
| *E. kobel DSM 13645T* | CP011863     | 90.26/40.6                | 89.72/40.7                  | 88.19/36.1 |
| *E. ludwigii* EN-119T | CP017279     | 88.03/34.9                | 87.70/35.0                  | 86.91/33.0 |
| *E. mori* LMG 25706T | AEXB000000000 | 88.98/37.3                | 88.15/37.4                  | 95.32/66.8 |
| *E. oligotrophica* CCA6T | AP019007     | 84.62/28.4                | 84.58/28.4                  | 87.81/34.9 |
| *E. quasihormaechei* WCHE120003T | SJON000000000 | 86.97/33.6                | 87.80/33.8                  | 87.63/34.9 |
| *E. rogenkampii* DSM 16690T | CP0171184    | 95.37/64.8                | 95.30/65.2                  | 89.62/39.8 |
| *E. sicaenaensis* WCHECL1597T | POVL000000000 | 91.10/44.7                | 90.82/44.8                  | 88.07/36.4 |
| *E. soli* ATCC BAA-2102T | LXS000000000 | 85.93/30.8                | 85.21/30.7                  | 85.70/31.0 |
| *E. wuouensis* WCHE120002T | SJOO000000000 | 87.62/35.7                | 88.38/35.8                  | 88.91/38.1 |
| *E. xiangfangensis* LMG 27195T | CP017183     | 87.54/34.0                | 87.09/34.0                  | 87.74/35.0 |
| *Pseudenterobacter timonensis* mt20T | FCOP000000000 | 82.03/25.9                | 82.20/26.0                  | 82.41/25.8 |
| WCHECL1060T | LFDQ000000000 | 98.40/88.0                | 89.68/40.2                  | 89.65/40.3 |
| 090044T | RXSJ000000000 | 89.57/40.2                | 89.65/40.3                  |
The results presented here indicate that two strains represent a novel species within the genus Enterobacter, which is clearly distinct from all known Enterobacter species. As it is most closely related to E. rogenkampii in whole-genome analysis, we propose the name Enterobacter quasiroggenkampii sp. nov. (qua.si.ro.gen.kamp.i; L. adv. quasi nearly, almost; N.L. gen. n. rogenkampii of Roggenkamp, and a specific epithet in the genus Enterobacter; N.L. gen. n. quasiroggenkampii almost rogenkampii) for this species with WCHECL1060T (= GDMCC 1.1742T = KCTC 529922) as the type strain.

An Enterobacter strain from blood represents another novel species, named Enterobacter quasimori sp. nov. Strain 090044T was identified as E. cloacae by Vitek II. The 16S rRNA gene sequence of the strain was 99% identical to those of type strains of a few Enterobacter species including E. asburiae, E. bugandensis, E. hormaechaei, E. kobei, and E. ludwigii. Whole-genome sequencing for strain 090044T generated 4,498,239 reads and 1.35 gigabases, which were assembled into a 4.71-Mb draft genome containing 53 contigs ≥200 bp in length (N50 291,547 bp) with a 55.76% GC content. No contamination was identified. The ANI values between strain 090044T and type strains of all known Enterobacter species and WCHECL1060T were <96%, and the highest value (95.32%) was seen with E. mori LMG 25706T (Table 3 and Table S1). The isDDH values between strain 090044T and type strains of all known Enterobacter species and WCHECL1060T were <70%, and the highest value (66.8%) was seen with E. mori LMG 25706T (Table 3 and Table S1). Therefore, based on the ANI and isDDH analyses, it is evident that the strain represents a novel species of the genus Enterobacter.

For strain 090044T, growth occurs at 4 to 37°C with optimal growth at 35 and 37°C, but not at 45 or 50°C. Cells grow at 35°C in the presence of 0 to 9% (wt/vol) NaCl in TSB. They are positive for the catalase test but negative for oxidase activity. Cells of strain
090044T are Gram negative, motile, non-spore-forming, facultatively anaerobic, and rod shaped. Colonies are circular, white, translucent, raised, and smooth after 24 h of incubation at 35°C on nutrient agar. Acid is produced from glycerol, L-arabinose, D-ribose, D-xylose, D-galactose, D-glucose, sucrose, melibiose, amygdalin, D-fructose, D-mannose, L-rhamnose, inositol, D-mannitol, D-sorbitol, dulcitol, D-turanose, potassium 2-ketogluconate, and methyl-\(\beta\)-D-glucopyranoside but not erythritol, L-xylose, D-adonitol, D-arabinose, potassium gluconate, and methyl-\(\beta\)-D-mannopyranoside. Strain 090044T has a positive reaction for \(\beta\)-galactosidase, arginine dihydrolase, and ornithine decarboxylase but is negative for lysine decarboxylase, deaminase, and gelatinase. It is also negative for urease activity and indole production but positive for the Voges-Proskauer reaction. It can utilize citrate but does not produce H\(_2\)S. It is catalase positive and oxidase negative. Strain 090044T can be differentiated from other Enterobacter species and WCHECL1060T by its ability to ferment inositol, D-sorbitol, dulcitol, D-turanose, and melibiose but not potassium gluconate, L-fucose, and methyl-\(\alpha\)-D-mannopyranoside. The major cellular fatty acids of strain 090044T were C\(_{16:0}\), C\(_{17:0}\) cyclo, and C\(_{18:1}\)\(\omega\)7c, which were consistent with those of other Enterobacter species (Table S2). The antimicrobial susceptibility profile and antimicrobial resistance genes of the strain are described in the supplemental material (Text S1 and Table S3).

The results presented here indicate that strain 090044T represents a novel species within the genus Enterobacter. As it is most closely related to E. quasimori in whole-genome analysis, we propose the name Enterobacter quasimori sp. nov. (qua.s.i.mo.ri; L. adv. quasi nearly, almost; N.L. gen. n. mori of Zhu, and a specific epithet in the genus Enterobacter; N.L. gen. n. quasimori almost mori) for this species with 090044T (\(=\) GDMCC 1.1735\(^T\) = JCM 33940\(^T\)) as the type strain.

Most Enterobacter genomes in GenBank need to be curated for precise species identification. Based on the above findings, the taxonomy of Enterobacter should be updated to comprise 22 species at present (Table 5). There were 1,997 Enterobacter strains with genomes deposited in GenBank, and the species identification is required to be curated for most \((n = 1,542, 77.2\%)\) of these strains in four scenarios. First, among

| Species (\(n = 22\)) | Type strain | Accession no. |
|----------------------|-------------|---------------|
| Enterobacter asburiae\(^a\) | JCM 6051 | CP011863 |
| Enterobacter bugandensis | EB-247 | FYB00000000 |
| Enterobacter cancerogenus | ATCC 35316 | ERR185486 |
| Enterobacter chengduensis | WCHECI-C4 | MTS00000000 |
| Enterobacter chuanduensis | 090028 | QZCS00000000 |
| Enterobacter cloacae | ATCC 13047 | CP001918 |
| Enterobacter dissolvens\(^b\) | ATCC 23373 | WJWQ00000000 |
| Enterobacter hoffmannii\(^c\) | DSM 14563 | CP017186 |
| Enterobacter hormaechei | ATCC 49162 | MKEQ00000000 |
| Enterobacter huxiensis | 090008 | QZCT00000000 |
| Enterobacter kobei | ATCC BAA-260 | CP017181 |
| Enterobacter ludwigii | EN-119 | CP017279 |
| Enterobacter mori\(^d\) | LMG 25706 | GLB90773 |
| Enterobacter oligotrophica | CCA6 | AP019007 |
| Enterobacter quasihormaechei | WCHEs120003 | SJO00000000 |
| Enterobacter quasimori | 090044 | RXXR00000000 |
| Enterobacter quasirogenkampii | WCHECL1060 | LFDQ00000000 |
| Enterobacter rogenkampii | DSM 16690 | CP017184 |
| Enterobacter sichenensis | WCHECI1597 | PVLO00000000 |
| Enterobacter soli | ATCC BAA-2102 | LXS00000000 |
| Enterobacter wuhouensis | WCHEs120002 | SJO00000000 |
| Enterobacter xiangfangensis\(^e\) | LMG 27195 | CP017183 |

\(^a\)Enterobacter muelleri is a later synonym of Enterobacter asburiae (42).  
\(^b\)Previously known as Enterobacter cloacae subsp. dissolvens.  
\(^c\)Previously known as Enterobacter hormaechei subsp. hoffmannii.  
\(^d\)Enterobacter tabaci is a later synonym of Enterobacter mori (15).  
\(^e\)Enterobacter hormaechei subsp. sharae and Enterobacter hormaechei subsp. steigerwaltii are later synonyms of Enterobacter xiangfangensis.
1,997 *Enterobacter* strains with genomes deposited in GenBank, 1,960 were indeed *Enterobacter* strains but 37 did not belong to the genus *Enterobacter*. Five strains did not even belong to the family *Enterobacteriaceae* but rather belonged to the genus *Pantoea* of the family *Erwiniaeeae* (n = 4) or the genus *Serratia* of the family *Yersiniaceae* (n = 1; Data Set S2). Thirty strains belonged to other species of the family *Enterobacteriaceae*, among which 7 belonged to known species including *Atlantibacter subterrae*, *Citrobacter portucalensis*, *Escherichia coli*, *Klebsiella aerogenes*, and *Klebsiella pneumoniae*, while 23 strains could not be assigned to known species. We found the 23 strains actually belonged to 18 novel unnamed species, which are tentatively assigned to taxons A to R here (Table S4). Two belong to *E. timonensis*, which should be removed to the genus *Pseudenterobacter*. Second, of the 1,960 *Enterobacter* strains, 155 strains were only labeled as *Enterobacter* spp. (n = 117), *E. cloacae* complex (n = 34), or *Enterobacter* genomosp. (n = 4) but were not assigned to the species level (Data Set S2). Third, species were misidentified for 481 *Enterobacter* strains, most (n = 460) of which were labeled as *E. cloacae* but actually belonged to other *Enterobacter* species. Fourth, there were 869 strains whose species identification needs to be updated according to the findings in this study. In particular, only 80 (14.8%) out of the 540 genomes labeled as *Enterobacter* spp. were actually labeled as *Enterobacter* spp. (n = 117), *E. cloacae* complex (n = 34), or *Enterobacter* genomosp. (n = 4) but were not assigned to the species level (Data Set S2). Third, species were misidentified for 481 *Enterobacter* strains, most (n = 460) of which were labeled as *E. cloacae* but actually belonged to other *Enterobacter* species. Fourth, there were 869 strains whose species identification needs to be updated according to the findings in this study. In particular, only 80 (14.8%) out of the 540 genomes labeled as *Enterobacter* spp. were actually labeled as *Enterobacter* spp. (n = 117), *E. cloacae* complex (n = 34), or *Enterobacter* genomosp. (n = 4) but were not assigned to the species level (Data Set S2).

After curation of precise species identification, among the 1,960 *Enterobacter* strains, half (n = 994, 50.7%) actually belonged to *E. xiangfangensis*, while *E. hoffmannii* is the second most common species with 287 strains (14.7%; Table 6), followed by *E. asburiae* (n = 116, 5.9%) and *E. roggenkampii* (n = 112, 5.7%). However, there were 60 (3.1%) strains that could not be assigned to any known *Enterobacter* species. Instead, the 60 strains can be assigned to 14 potentially novel *Enterobacter* species, which are unnamed as they have not been characterized by phenotype methods. The 14 potentially novel *Enterobacter* species were assigned taxons 1 to 14 here (Table 7). There were 1,496 strains from human specimens. Among strains from human, *E. xiangfangensis* was still the most common species (805/1,496, 53.8%; Table 6) and *E. hoffmannii* was the second most common (251/1,496, 17.2%). Although the selection of bacterial strains is usually biased for genome sequencing, the common identification of the two *Enterobacter* species from human specimens is unlikely to be a coincidence. The reasons why isolates of the two *Enterobacter* species are commonly recovered from human specimens warrant further studies.

**DISCUSSION**

In this study, we first updated the taxonomy of the genus *Enterobacter* and modified the taxonomic assignments for *E. timonensis* and the subspecies of *E. cloacae* and *E. hormaechei* by genome analyses and also reported two novel species, which were characterized by both genome- and phenotype-based methods. We then applied the updated taxonomy assignments to curate genome sequences deposited in GenBank with the label of *Enterobacter* and found that the species identification of most *Enterobacter* strains with genome sequences available needed to be corrected.

We found that all subspecies assignments in the genus *Enterobacter* were incorrect and their use should be discontinued. Genetic clustering of the hsp60 (a housekeeping gene) sequence has been used as the premise for assigning *E. hormaechei* subsp. *hormaechei*, *E. hormaechei* subsp. *oharae*, and *E. hormaechei* subsp. *steigerwaltii* (16, 17). However, determining taxonomic assignment using a single-gene-based approach has omitted valuable information available from the rest of the genome and potentially led to unreliable conclusions about taxonomic positions. Such subspecies assignment should be rigorously reexamined based on analysis of whole-genome sequences. Indeed, on the basis of whole-genome-based analysis, it becomes evident that the subspecies of *E. hormaechei* actually belong to three species. *E. xiangfangensis* is not a subspecies of *E. hormaechei* but an independent species, while *E. hormaechei* subsp. *steigerwaltii* and *E. hormaechei* subsp. *oharae* belong to the same species as *E. xiang-
fangensis. *E. hormaechei* subsp. *hoffmannii* is a novel species, *E. hoffmannii*. Whole-genome-based analysis also reveals that *E. cloacae* subsp. *dissolvens* is actually a species, *E. dissolvens*, rather than a subspecies of *E. cloacae*. The above findings also highlight that the assignment of subspecies should be prudent as there is no general guideline for defining subspecies using genome data (23) and subspecies assignment

### TABLE 6 Species distribution of 1,960 *Enterobacter* strains with genome sequences available in GenBank

| Proposed species                  | No. all sources | No. human strains |
|-----------------------------------|-----------------|-------------------|
| *Enterobacter asburiae*           | 116             | 78                |
| *Enterobacter bugandensis*        | 55              | 42                |
| *Enterobacter cancerogenus*       | 14              | 3                 |
| *Enterobacter chengduensis*       | 5               | 5                 |
| *Enterobacter chuandoensis*       | 2               | 1                 |
| *Enterobacter cloacae*            | 86              | 60                |
| *Enterobacter dissolvens*         | 15              | 7                 |
| *Enterobacter hoffmannii*         | 287             | 251               |
| *Enterobacter hormaechei*         | 13              | 8                 |
| *Enterobacter huaxiensis*         | 2               | 2                 |
| *Enterobacter kobei*              | 97              | 72                |
| *Enterobacter ludwigii*           | 55              | 28                |
| *Enterobacter mori*               | 9               | 5                 |
| *Enterobacter oligotrophica*      | 1               | 6                 |
| *Enterobacter quasihormaechei*    | 9               | 5                 |
| *Enterobacter quasiroggenkampii*  | 8               | 0                 |
| *Enterobacter roggenkampii*       | 112             | 75                |
| *Enterobacter sichuanensis*       | 15              | 12                |
| *Enterobacter soli*               | 4               | 0                 |
| *Enterobacter wuhouensis*         | 1               | 1                 |
| *Enterobacter xiangfangensis*     | 994             | 805               |
| Taxon 1                           | 2               | 0                 |
| Taxon 2                           | 10              | 3                 |
| Taxon 3                           | 8               | 2                 |
| Taxon 4                           | 14              | 11                |
| Taxon 5                           | 4               | 4                 |
| Taxon 6                           | 2               | 1                 |
| Taxon 7                           | 1               | 0                 |
| Taxon 8                           | 8               | 4                 |
| Taxon 9                           | 2               | 0                 |
| Taxon 10                          | 3               | 2                 |
| Taxon 11                          | 1               | 0                 |
| Taxon 12                          | 1               | 1                 |
| Taxon 13                          | 2               | 0                 |
| Taxon 14                          | 2               | 2                 |
| Total                             | 1,960           | 1,496             |

### TABLE 7 Tentative taxon assignments for novel, unnamed *Enterobacter* species

| Taxon Accession no. | Reference straina | Closest species            | ANl, % | DDH, % |
|---------------------|-------------------|----------------------------|--------|--------|
| 1 AYJG000000000     | MGH 24            | *E. asburiae*              | 95.403 | 63.70  |
| 2 AZUB000000000     | DC4               | *E. quasiroggenkampii*     | 94.884 | 58.70  |
| 3 JDWG000000000     | JD8715            | *E. asburiae*              | 90.937 | 42.00  |
| 4 JZXZ000000000     | 44246             | *E. chengduensis*          | 95.521 | 64.40  |
| 5 LECZ000000000     | GN03164           | *E. asburiae*              | 92.904 | 49.70  |
| 6 CP014280          | MBRL1077          | *E. bugandensis*           | 95.532 | 63.70  |
| 7 QBJD000000000     | RIT 418           | *E. wuhouensis*            | 87.641 | 52.10  |
| 8 QMCS000000000     | 148H3             | *E. quasiroggenkampii*     | 94.463 | 56.50  |
| 9 QQXP000000000     | 9-2               | *E. asburiae*              | 90.268 | 40.30  |
| 10 PXJY000000000    | CRE81             | *E. bugandensis*           | 94.295 | 55.90  |
| 11 VRK500000000     | TN152             | *E. asburiae*              | 95.255 | 62.50  |
| 12 QYOF000000000    | T0143A.B-3        | *E. xiangfangensis*        | 95.989 | 66.80  |
| 13 JAALAZ000000000  | M-7-X3            | *E. xiangfangensis*        | 94.957 | 60.10  |
| 14 FJXR000000000    | e1252             | *E. asburiae*              | 95.660 | 65.00  |

aThe strain with genome sequence deposited in GenBank at the earliest date was selected as the reference strain.
requires rigorous studies. These studies should include large-scale properly designed investigations on clinical significance such as host specificity of these bacteria to examine the rationale why subspecies should be created and separately recognized (23) and to avoid unnecessary confusion or even chaos.

We also found that most genomes labeled as *E. cloacae* and *E. hormaechei* are not correctly identified to the species level. The incorrect identification may be due to different reasons. Of note, the ≥95% ANI cutoff alone is widely used for species assignment, but such a cutoff is unable to resolve closely related species (24). Previous studies have corroborated that the stringent ≥96% ANI cutoff is more accurate with better correlation with the 70% DDH cutoff (11) but also highlight that species assignment based on a single algorithm may not be robust. In this study, we employed both ANI with a ≥96% ANI cutoff and isDDH for robust species assignment. In addition, for *E. cloacae*, phenotype-based tests used in clinical microbiology laboratories commonly identify *Enterobacter* clinical isolates as *E. cloacae* as evidenced by the misidentification of strains WCHECL1060\(^T\), 090040, and 090044\(^T\) by Vitek II. In contrast, for *E. hormaechei*, incorrect identification was mainly due to incorrect subspecies assignments as discussed above. This highlights that updated and curated taxonomic assignments are the premise of correct and precise species identification. We suggest that future studies on *Enterobacter* need to consider the correct species and subspecies identification to provide robust results while avoiding misleading information.

We report two novel *Enterobacter* species here and found that there were 14 tentative novel *Enterobacter* species and 18 tentative non-*Enterobacter* species of the family *Enterobacteriaceae*, which are clearly listed in the study. This invites more studies on these tentative species by both genome- and phenotype-based methods to establish their species status and to propose appropriate species names. Such studies will further reveal the complicated taxonomy of *Enterobacter*, a genus of bacterial species with clinical significance.

**Conclusions.** All subspecies assignments in the genus *Enterobacter* were incorrect, and their use should be discontinued. *E. cloacae* subsp. *dissolvens* is a species and should be renamed *E. dissolvens*. *E. xiangfangensis* is not a subspecies of *E. hormaechei*, while *E. hormaechei* subsp. *oharae* and *E. hormaechei* subsp. *steigerwaltii* are not subspecies of *E. hormaechei* but belong to the same species of *E. xiangfangensis*. *E. hormaechei* subsp. *hoffmannii* is a species and should be renamed as *E. hoffmannii*. *E. timonensis* should be removed to *Pseudeenterobacter*, a novel genus. Two novel *Enterobacter* species, *E. quasiroogenkampii* and *E. quasimori*, were identified. *E. quasiroogenkampii* can be distinguished from all known *Enterobacter* species by its ability to ferment inositol, D-sorbitol, and melibiose but not potassium gluconate, L-fucose, and methyl-α-D-mannopyranoside. *E. quasimori* can be distinguished from all known *Enterobacter* species by its ability to ferment inositol, D-sorbitol, dulcitol, D-turanose, and melibiose but not potassium gluconate, L-fucose, and methyl-α-D-mannopyranoside. The species identifications for most *Enterobacter* strains with genomes deposited in GenBank are required to be curated. The most common *Enterobacter* species seen in clinical samples appears to be *E. xiangfangensis*. Fourteen novel tentative *Enterobacter* genome species were also found and warrant further phenotype-based characterizations.

**MATERIALS AND METHODS**

**Strain and initial species identification.** The type strain of *E. cloacae* subsp. *dissolvens* ATCC 23373\(^T\) was obtained from the Guangdong Microbial Culture Collection Center (http://www.gdmcc.net/). Three nonduplicated clinical strains, WCHECL1060\(^T\), 090040, and 090044\(^T\), were all recovered from the blood culture of three different patients with fever as part of routine patient care at West China Hospital of Sichuan University, Chengdu, China, in 2014 or 2016. This study has been approved by the Ethical Committee of West China Hospital, and the informed consent was waived as this study was to retrospectively characterize bacterial strains that were collected as part of routine patient care.

Initial species identification was performed using the Vitek II automated system (bioMérieux, Marcy l’Étoile, France). The 16S rRNA gene sequences of the three strains were obtained as described previously (25) and were compared using a pairwise nucleotide sequence alignment tool (https://www.ezbiocloud.net/tools/pairAlign) using Myers and Miller’s algorithm (26). As strains WCHECL1060\(^T\) and 090040...
belonged to the same species, they were subjected to pulsed-field gel electrophoresis by XbaI macrorestriction, which was performed as described previously (27), to determine their clonal relatedness.

Whole-genome sequencing. We have reported the draft genome of strain WCHEL1060\(^T\) before (22). Genomic DNA of E. cloacae subsp. dissolvens ATCC 23373\(^T\), strain 090040, and strain 090044\(^T\) was prepared using the QiAamp DNA minikit (Qiagen, Hilden, Germany), and DNA sequencing libraries were prepared using the NEBNext Ultra II DNA Library Prep kit for Illumina (NEB, Ipswich, MA, USA). Whole-genome sequencing was performed using the HiSeq 2500 Sequencer (Illumina, San Diego, CA, USA) with the 150-bp paired-end protocol and about 200× coverage. Reads were trimmed using Trimmomatic v0.39 (28) under the default setting and were then assembled into contigs using SPAdes v3.11.1 (29) under careful mode. Genome completeness and contamination were examined using CheckM v1.0.18 (30). The genome sequences were reported following recommendations of standards for describing a new taxonomy (23).

Phylogenetic analysis of the genus Enterobacter based on core genes. Whole-genome sequences of the type strains of all species and subspecies within the genus Enterobacter and all other species of the family Enterobacteriaceae (listed in Data Set S1 in the supplemental material) were retrieved from the NCBI database. A core genome phylogenetic tree based on concatenated sequences of core genes was constructed as described previously (31). Prokka v1.12 (32) was used to annotate these genome sequences, and orthologues of these strains were identified using OrthoFinder v2.26 (33) to represent the core genome of these Enterobacteriaceae strains. The gene sequences were aligned and concatenated using MAFFT v7.313 (34) and AMAS v0.98 (35), which were then used to infer a phylogenomic tree using RAxML v8.2.12 (36) with GTR plus gamma distribution and a 1,000-bootstrap test.

Determination of overall genome relatedness. The pairwise average nucleotide identity (ANI) and in silico DNA-DNA hybridization (isDDH) between strains ATCC 23373\(^T\), WCHEL1060\(^T\), 090040, and 090044\(^T\) and the type strain of Enterobacter species and subspecies were determined using the JSpecies program based on BLAST (jspecies.ribohost.com) (37) and GGDC (formula 2) (10), respectively. A 70.0% isDDH (9, 10) or a ≥96% ANI (9) value was used as the cutoff to define a bacterial species.

Phenotypic characterization for strains of two novel species. Motility was examined using a CX21FS1 light microscope (Olympus, Tokyo, Japan). The Gram-staining reaction was performed as described previously (38). Growth at different temperatures (4, 15, 20, 25, 30, 35, 37, 45, and 50°C), at different pH values (3.0 to 12.0, at intervals of 1.0 pH unit), and at various salt concentrations (0 to 10% [wt/vol] NaCl) was determined in 15-ml test tubes containing 3 ml tryptic soy broth (TSB; Hopebio, Qingdao, China) after incubation for 2 days in a thermostatically controlled water bath as described previously (39). Anaerobic growth was performed by incubating cultures on nutrient agar for 7 days in an anaerobic bag (bioMérieux). Biochemical characteristics of the three strains were determined using the API 20E kit and API 50CH kit according to the manufacturer’s instructions (bioMérieux). Catalase activity was examined by bubble formation after dropping 3% (vol/vol) H\(_2\)O\(_2\) on fresh biomass grown for 24 h on nutrient agar. Oxidase activity was determined using oxido-reagent (bioMérieux). All tests were carried out by incubating at 35°C unless indicated otherwise.

Analysis of whole-cell fatty acids for strains of two novel species. Whole-cell fatty acids of strains WCHEL1060\(^T\), 090040, and 090044\(^T\) were analyzed by Guangdong Institute of Microbiology (Guangzhou, Guangdong, China) as described previously (40).

Antimicrobial susceptibility and antimicrobial resistance genes of strains of two novel species. In vitro antimicrobial susceptibility tests were performed by Vitek II using broth microdilution. In addition, MICs of colistin, imipenem, and meropenem were also determined using the microdilution broth method of the Clinical and Laboratory Standards Institute (CLSI) (41). Breakpoints defined by CLSI (41) were applied except for tigecycline, for which breakpoints defined by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; http://www.euCAST.org/) were used. Antimicrobial resistance genes of clinical strains WCHEL1060\(^T\), 090040, and 090044\(^T\) were identified from genome sequences using the ABRicate program v1.0.1 (https://github.com/tseemann/abricate) to query the ResFinder database (http://www.genomiccepideomiology.org/), accessed 16 April 2020).

Curation of species identification for Enterobacter genome species in GenBank. We used taxid547 [Organism:exp] AND "latest refseq" [filter] to search NCBI GenBank and found 1,997 genome sequences labeled Enterobacter (Data Set S2 in the supplemental material, accessed 16 April 2020). All of the 1,997 sequences were retrieved and were then subjected to precise species identification using ANI and isDDH as described above. Strains that have a <70% isDDH value and a <96% ANI value with any known Enterobacter species are likely to belong to a novel species, which is temporarily assigned a taxon here as the establishment of a novel species requires phenotypic characterizations in addition to genome analysis.

Data availability. The draft whole-genome sequences of strains ATCC 23373\(^T\), WCHEL1060\(^T\), 090040, and 090044\(^T\) have been deposited into DDBJ/EMBL/GenBank under accession numbers WJWQ00000000, LFQO00000000, RX5000000000, and RRXR00000000, respectively. Whole-genome sequences of the type strains of all species and subspecies within the genus Enterobacter and all other species of the family Enterobacteriaceae retrieved from the NCBI database are listed in Data Set S1. The 1,997 genome sequences labeled as Enterobacter in GenBank (accessed 16 April 2020) are listed in Data Set S2.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TEXT S1, DOCX file, 0.02 MB.

FIG S1, TIF file, 2.7 MB.
ACKNOWLEDGMENTS

We are grateful to Liang Huang and Yi Xie at West China Hospital for collecting clinical isolates.

The work was supported by grants from the National Natural Science Foundation of China (project no. 81772233, 81661130159, and 81861138055), West China Hospital of Sichuan University (1.3.5 project for disciplines of excellence, project no. ZYYS08006, and grant no. 312190022), and the Newton Advanced Fellowship, Royal Society, UK (NA150363).

There is no conflict of interest for all authors.

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