Comparative Genomic Analysis Reveals Potential Pathogenicity and Slow-growth Characteristics of genus Brevundimonas and Description of Brevundimonas pishanensis sp. nov.

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Transaction Report:

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Prof. Duochun Wang
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Re: Spectrum02468-21 (Comparative Genomic Analysis Reveals Potential Pathogenicity and Slow-growth Characteristics of genus Brevundimonas and Description of Brevundimonas pishanensis sp. nov.)

Dear Prof. Duochun Wang:

Thank you for submitting your manuscript to Microbiology Spectrum and apologies for the long delay, it was unusually difficult to find reviewers for your submission. Your manuscript has been reviewed by two experts, and I would now like you to modify your study in line with their comments below.

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Daria Van Tyne
Editor, Microbiology Spectrum

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Reviewer comments:

Reviewer #1 (Comments for the Author):

The authors performed comparative genomic analysis in 24 Brevundimonas species with a novel species they isolated in China. Phylogenetic analysis was performed using multiple approaches. Virulence/antibiotic associated genes were predicted using bioinformatics tools. Collectively, the evidence support that the newly isolated strain belongs to a novel species.

Major comments:
1. The authors grouped the different species into 5 "clades" using hierarchical clustering method based on pan genome diversity
which was based on gene presence and absence profile. However, the data seems to be contradictory to that from ANI and core genome tree. According to the core genome tree in Fig2A, TAR001 and TAR002 should not have been grouped into one same clade. Additionally, depending on the cutoff being used, the "clade" grouping could end up differently. The authors need to define the cutoff in the main text.

2. line 232: "... had a highly similar topology...": the reviewer disagrees with the conclusion. The phylogenetic tree from Fig 2A and Fig 2B differs at the clustering of TAR001/TAR002 and B. lutea NS26/B. aveniformis DSM17977.

3. Characterization of antibiotic resistance profile requires data support, such as MIC, which is missing in the main text. As for "slow growth", it is recommended the authors provide the doubling time.

Minor comments:
1. line 62: "tiny": it's better to use size in micro-meter instead of a generic "tiny".
2. line 66: "human beings": please specify location, eg on skin or colonized internally or opportunistic pathogen.
3. line 82: "few articles": please cite the related references.
4. line 219: "5 major clades": what's the cutoff value? what's the rational of using Fig 1C to define clades instead of Fig1B, 2A or 2B?
5. line 248: "data not shown": recommend to add this data to supplemental material.
6. line 291-304: why were different strains used in Fig 4A and 4B?
7. line 496: "two groups of ...": it is not clear which two groups are being discussed here. Was this data in the main text?

Reviewer #2 (Comments for the Author):

Really great work! I enjoyed reading this comprehensive manuscript. I have a couple of comments that I would like to be addressed.

Major:
1) Line 19: "Brevundimonas spp. have relatively small genomes (3.13±0.29 Mb)". Please note, bacteria can have a genome size between ~0.5 to ~14 Mbp. Please can you reword this as 3.13 Mbp is not relatively small when compared to other bacteria like Chlamydia spp. (~1.1Mbp). Also, could you please define what the +/- is representing? Standard deviation? Standard error of mean?

2) Line 109: Please change "The genome of strain CHPC 1.3453T was extracted" to "The DNA of strain CHPC 1.3453T was extracted"

3) Line 126: Was a reference used for QUAST? If so which reference was used?

4) Line 132: What alignment was performed? Muscle? ClustralW?

Figure 4: What does the axis represent? Please describe this in figure legend

Figure S2: What does the scale bar represent in the tree? How is the tree rooted? Midpoint? Outgroup? What do the numbers on the nodes represent? Bootstrap? How many replicates? When I refer back to the methods, I can see that 1,000 bootstrap replicated was done. Might be worth while to state this in the figure legend for readability.

Figure S4B: What does the scale bar represent in the tree? What does the axis represent? How is the tree rooted? Midpoint? Outgroup? What do the black dots on the nodes represent? What do the numbers on the nodes represent? Bootstrap? How many replicates? When I refer back to the methods, I can see that 1,000 bootstrap replicated was done. Might be worth while to state this in the figure legend for readability.

Minor:
1) Only a minor point, so please feel free to disregard this comment. I can appreciate that English may not be everyone's first language. Maybe the authors can revisit if they would like to use American (USA) English spellings or British English spellings. E.g., Faeces (British) is spelt as feces (USA) above. Bacteraemia (British) or Bacteremia (USA), diarrhoea (British) or diarrhea (USA), favourable (British) or favorable (USA)... ect.

2) Line 22: phylogenetic is spelt incorrectly

3) Line 32: Please change "a diarrhea patient" to "a patient(suffering with diarrhea)"

4) Line 34: Please define what MLSA is an abbreviation of and replace "as well as" with "and"

5) Line 68: Please change "human beings" to "human hosts"

6) Line 102: Please change "46-year-old Uygur (one of the Chinese ethnic minorities) man" to "46-year-old Uygur (one of the Chinese ethnic minorities) male"
7) Line 105: Quick note: I believe that LB stands for "Lysogeny broth", not Luria-Bertani medium- or variations of that. I could be wrong, but I am basing this comment from the original 1951 reference. Please note, Luria-Bertani is also described in the legend to Figure S5.

8) Line 107: Data availability. Please specify what data is available in GenBank The sequence read data? Or the assemblies? Please note, sequence read data is stored on the sequence read archive (SRA), not GenBank (Assemblies). Can you please refer to the BioProject number?

9) Line 114: Change "The genome DNA" to "The genomic DNA"

10) Line 145: Please define abbreviations when used first. I.e., digital DNA-DNA hybridisation (dDDH)

11) Line 259: Please change "with VFDB" to "with the VFDB"

12) Line 629: Please change "the gene kdsA and acpXL were detected in 95.8% (23/24)" to "the gene kdsA and acpXL were detected in 95.8% (n = 23/24)"

13) Line 272: Please change "The majority of the Brevundimonas species (87.5%, 21/24)" to "The majority of the Brevundimonas species (87.5%, n = 21/24)"

14) Line 294: What kind of alignment?

Staff Comments:

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Thank you for submitting your paper to Microbiology Spectrum.
Comparative Genomic Analysis Reveals Potential Pathogenicity and Slow-growth Characteristics of genus Brevundimonas and Description of Brevundimonas pishanensis sp. nov.

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ABSTRACT The genus Brevundimonas consists of Gram-negative bacteria widely distributed in environment and can cause human infections. However, the genomic characteristics and pathogenicity of Brevundimonas remain poorly studied. Here, the whole-genome features of 24 Brevundimonas type strains were described. Brevundimonas spp. have relatively small genomes (3.13±0.29 Mb) but high G+C contents (67.01±2.19 mol%). Two-dimensional hierarchical clustering divided those genomes into 5 major clades, in which Clade II and V contained nine and five species, respectively. Interestingly, phylogenetic analysis showed a one-to-one match between core- and accessory-genome, which suggests co-evolution of species within the genus Brevundimonas. The unique genes were annotated to biological functions like catalytic activity, translation and multi-substance metabolism, \textit{et al.} The majority of Brevundimonas spp. harbored virulence-associated genes \textit{icl}, \textit{tufA}, \textit{kdsA}, \textit{htpB} and \textit{acpXL}, which encoded isocitrate lyase, elongation factor, 2-dehydro-3-deoxyphospho-octonate aldolase, heat shock protein and acyl carrier protein, respectively. In addition, genomic islands (GIs) and phages/prophages were
identified within the *Brevundimonas* genus. Importantly, a novel *Brevundimonas*

species was identified from the feces of a patient (suffering with diarrhea) by the

analyses of biochemical characteristics, phylogenetic tree of 16S rRNA gene and

Multilocus sequence analysis (MLSA) sequences, and genomic data. The name

*Brevundimonas pishanensis* sp. nov. was proposed, with type strain CHPC 1.3453\(^T\)

(=GDMCC 1.2503\(^T\)= KCTC 82824\(^T\)). *Brevundimonas* spp. also showed obvious slow
growth compared with *Escherichia coli*. Our study reveals insights into genomic
characteristics and potential virulence-associated genes of *Brevundimonas* spp., and
provides a basis for further intensive study of the pathogenicity of *Brevundimonas*.

**IMPORTANCE** *Brevundimonas* spp., a group of bacteria from the family

*Caulobacteraceae* and associated with nosocomial infections, deserve widespread
attention. Our study elucidated genes potentially associated with the pathogenicity of
the *Brevundimonas* genus. We also described some new characteristics of
*Brevundimonas* spp., such as small chromosome size, high G+C content and
slow-growth phenotypes, which made the *Brevundimonas* genus as a good model
organism for in-depth studies of growth rate traits. Apart from the comparative
analysis of the genomic features of the *Brevundimonas* genus, we also reported a
novel *Brevundimonas* species, *Brevundimonas pishanensis*, from the feces of a patient
with diarrhea. Our study promotes the understanding of the pathogenicity
characteristics of *Brevundimonas* spp. bacteria.

**KEYWORDS** comparative genomics; *Brevundimonas*; pathogenicity; slow-growth;
new species proposed.

**INTRODUCTION**

Gram-negative, non-fermenting bacteria have raised increasing concern in clinical
practice, since they are one of the most common causes of nosocomial infection.
Among these, some are well known opportunistic pathogens associated with
hospital-acquired infections, for example, *Pseudomonas aeruginosa* (1),
*Acinetobacter baumannii* (2, 3), and *Enterococcus faecium* (4). *Brevundimonas* spp.
are relatively less known, but they are also opportunistic human pathogens potentially
related to hospital infections.

The genus *Brevundimonas*, first described by Segers et al. (5) in 1994, comprises a group of bacteria shared the basic microbiological characteristics, like Gram-negative, motile, tiny, non-fermenting, oxidase-positive, and aerobic or facultative anaerobic. There are currently 35 species within this genus (https://www.bacterio.net/genus/brevundimonas). A profusion of new members can be isolated from diverse sources, such as soil (6-9), lake or sea sediment (10-12), activated sludge (13), aquatic water (14, 15) and human hosts (16-19). In humans, *Brevundimonas* spp. were rarely isolated and occasionally caused disease in patients with impaired immunity (20). The most common and clinically relevant pathogenic species in the *Brevundimonas* genus are *B. vesicularis* (related to approximately 70% cases reported) and *B. diminuta* (related to more than 20% cases reported). Besides, cases of infection with *B. vancanneytii* were also reported (21). In the disease spectrum of the non-mixed infections caused by *Brevundimonas* spp., the common clinical manifestation ranked by the following, bacteraemia, septicaemia/sepsis, pneumonia/pleuritis, endocarditis, keratitis, and urinary tract infection (20). No case of *Brevundimonas* spp. isolated from the stool samples of patients with diarrhea has been reported so far.

At present, there are few literature reports on diseases caused by *Brevundimonas* spp., which makes the pathogenicity of *Brevundimonas* easily overlooked. Studies on the genetic characteristics of the *Brevundimonas* genomes are fewer. Some articles have underreported fragmented introduction to the whole-genome sequencing (WGS) of a certain *Brevundimonas* species. Comparative genomics of the *Brevundimonas* genus remained poorly studied. Few articles have checked the virulence genes and drug resistance genes in detail within the genus *Brevundimonas*. The lack of case reports worldwide may account for the lack of deep understanding of this genus. However, it is warranted to understand their pathogenicity and epidemiology, since the opportunistic infections caused by *Brevundimonas* spp. is under potential epidemic proportions.
In the present study, we explored the evolutionary features of *Brevundimonas* spp. based on WGS, and also performed a detailed comparative genomics analysis at the genus level. Meanwhile, we reported a novel species *Brevundimonas pishanensis* from the stool sample of a patient with diarrhea. Polyphasic approaches, involving biochemical analysis, 16S rRNA gene phylogenetic analysis, MLSA and whole-genome analysis, were performed to determine the taxonomic position of *Brevundimonas pishanensis* and to systematically describe its phenotypic and genetic characteristics.

**MATERIALS AND METHODS**

**Strain information.** Strain CHPC 1.3453$^T$ was recovered from the stool sample of a 46-year-old Uygur (one of the Chinese ethnic minorities) male, living in Pishan County of Hotan Prefecture, Xinjiang, China, on 27th November, 2018. The sample was collected under aseptic conditions. Then bacteria were isolated, purified and incubated in Luria-Bertani (LB) medium with 1% sodium chloride (NaCl, w/v) at 37°C for 24-48 h. In addition, 24 genomic sequences of *Brevundimonas* type strains (or representative strains) were used in this study, the data were available in GenBank as of June 2021 (https://www.ncbi.nlm.nih.gov/genbank/, see the Table S1).

**16S rRNA PCR and genome sequencing.** The DNA of strain CHPC 1.3453$^T$ was extracted by genomic DNA purification kit (Promega, USA) in accordance with the manufacturer’s instructions. PCR amplification was conducted with bacterial universal primers (27F and 1492R) (22, 23). The 16S rRNA gene sequencing and the whole genome sequencing were entrusted to RuiBiotech Co., Ltd. (Beijing, China) and Beijing Genomics Institute (BGI, Shenzhen, China) respectively. The genomic DNA was sequenced using Illumina HiSeq 2000 platform (Illumina Inc., San Diego, CA, USA) with a depth of 200× coverage. Library construction (paired-end reads sizes of 150 bp), genome sequencing, and data pipelining were performed by following the manufacturer’s protocols.

**Genome evaluation, assembly, and annotation.** FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) was used to evaluate the
raw sequence data of strain CHPC 1.3453^T, and the low-quality reads were filtered.
Then the clean data were assembled into contigs using SPAdes version 3.8.2 software
(24) with default parameters. After removing contigs < 500 bp, QUAST (version 4.6.3)
software (25) was applied to evaluate the genome assembly. Open reading frames
(ORFs) prediction and annotation were done by prodigal version 2.6.3 (26) and
Prokka version 1.13.3 (27) software.

**Phylogeny analysis.** For 16S rRNA gene phylogenetic analysis, the sequence of
strain CHPC 1.3453^T and the reference sequences of *Brevundimonas* spp. available
from GenBank (Table S2) were grouped together for alignment and comparison by
MEGA software version 7.0.21 (28). Phylogenetic trees were constructed by both
neighbour-joining (29) and maximum-likelihood (30) algorithms with 1,000 bootstrap
replications. For multilocus sequence analysis (MLSA), 5 housekeeping genes (*gyrB*,
*ppsA*, *recN*, *rpoC*, and *rpoD*) were selected and these gene sequences were extracted
from the genomes of strain CHPC 1.3453^T as well as other 23 type strains or
representative strains from the genus *Brevundimonas*. The maximum-likelihood (ML)
tree was reconstructed using PhyML version 3.1 (31) based on the above mentioned
sequences with 1,000 bootstraps.

**Comparative genomic analysis of *Brevundimonas* spp.** Orthologous gene clusters
from *Brevundimonas* spp. were identified using InParanoid v4.1 (32). The pairwise
homologous gene rate (PHGR) was calculated by the distribution of orthologous gene
clusters. A Perl script based on previous algorithm (33) was used to compute the
average nucleotide identity (ANI). The digital DDH (dDDH) value was calculated by
the Genome-to-Genome Distance Calculator (GGDC) web server
(http://ggdc.dsmz.de/). Pan genome analysis was performed by Roary pipeline (34)
with a relative relax cutoff of identity referring to the previous study (35). The
analysis of multiple genome alignments in the presence of genome collinearity was
done by MAUVE software version 2.4.0 (36). Genomic islands (GIs) were analyzed
using online tool IslandViewer (http://www.pathogenomics.sfu.ca/islandviewer/) with
default parameters. The phage/prophage sequences were predicted by PHASTER
(PHAge Search Tool Enhanced Release, http://phaster.ca). The identification of CRISPR-Cas system was done by CRISPRCasFinder (https://crisprcas.i2bc.paris-saclay.fr/CrisprCasFinder) while the detection of plasmid replicon by PlasmidFinder (http://www.genomicepidemiology.org/). The Venn diagram was drawn by R software (version 4.0.2).

**Virulence gene and antimicrobial resistance gene analysis.** The VFDB (Virulence Factor Database, http://www.mgc.ac.cn/VFs/) was used to predict and annotate the virulence genes. Protein sequences from the *Brevundimonas* strains were searched against the VFDB using BLASTp with identity ≥ 90%, length coverage ≥ 60%, and an E-value of 1e-5. The antimicrobial resistance genes were detected by ResFinder tool (https://cge.cbs.dtu.dk/services/ResFinder/) and were cross-compared with the Comprehensive Antibiotic Resistance Database (CARD, http://arpcard.mcmaster.ca). The BLASTp cutoff values were set as follow: E-value of 1e-5, identity ≥ 90%, and length coverage ≥ 60%.

**Growth curve test.** Strain CHPC 1.3453^T as well as the type strains of *B. vesicularis* (NBRC 12165^T), *B. diminuta* (ATCC 11568^T), *E. coli* (ATCC 25922^T) were incubated in LB medium at 37°C for 3-4 hours to the exponential phase to ensure maximum cell viability. Then 1.25× serial dilutions were made by LB medium, with the final dilution was 100-fold. The diluted bacterial cells were spread into a 96-well plate with the volume of 200 µl each. The change of OD values were detected using automated turbidity system Bioscreen C (Oy Growth Curves Ab Ltd., Raisio, Finland). Then a growth curve was drawn with the culture time as the abscissa and the logarithm of the number of bacteria or the growth rate as the ordinate.

**Identification of biochemical phenotype.** The biochemical reactions towards various substrates was determined by API 20NE and API 50CH systems (bioMérieux), according to the manufacturers’ instructions. Enzyme activities and other biochemical properties were conducted by API ZYM assay (bioMérieux). The biochemical phenotypes of strain CHPC 1.3453^T was compared with three type trains *B. diminuta* ATCC 11568^T, *B.vesicularis* NBRC 12165^T, and *B. halotolerans* MCS24^T in each
independent experiment.

**Antimicrobial susceptibility test.** The Antimicrobial susceptibility testing (AST) panel for aerobic Gram-negative bacilli (Shanghai Fosun Long March Medical Science Co., Ltd., China) was used to perform the drug susceptibility test using microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (37). The bacterial cell culture of strain CHPC 1.3453<sup>T</sup> was adjusted to 0.5 McF (McFarland) and then was spread onto antibiotic plates. After an incubation period of 18 to 20 hours, the results were interpreted. *E. coli* ATCC 25922<sup>T</sup> was used as a quality control.

**RESULTS**

**General features of Brevundimonas spp. genomes.** The average genome size of the 24 type stains (or representative strains) of *Brevundimonas* spp. was 3.13±0.29 Mb, ranging from 2.54 Mb (*B. halotolerans* MCS24<sup>T</sup>) to 3.75 Mb (*B. subvibrioides* ATCC 15264<sup>T</sup>). The average G+C content was 67.01±2.19 mol%, ranging from 60.8 mol% (*B. terrae* DSM 17329<sup>T</sup>) to 70.4 mol% (*B. viscosa* CGMCC 1.10683<sup>T</sup>). The genomes among the *Brevundimonas* genus were found to consist of approximately 3,131 protein-encoding genes on average. The draft genome size of the novel strain CHPC 1.3453<sup>T</sup> was 2,916,570 bp, with 17 assembled scaffolds, and N50 length at 474,365 bp. The G+C content of this strain was 61.6 mol%. And there were approximately 2,800 coding sequences (CDSs), and 47 tRNAs predicted in this genome. The annotation features in subsystems were shown in Fig. S1.

**Evolutionary conservation and diversity of Brevundimonas.** We initially studied the evolutionary status and genetic relationship of the genus *Brevundimonas* in the entire *Caulobacteraceae* family. The NJ-tree based on 346 single-copy homologous gene sequences demonstrated that the *Brevundimonas* species can be clearly distinguished from other genera (Fig. S2, Table S3). In an attempt to evaluate the conservation between different species within the *Brevundimonas* genus, we analyzed the pairwise homologous gene rate (PHGR) in the representative genomes of any two species. The violin plot (Fig. 1A) showed that the average PHGR for each single
species varied from 74.96% (B. bullata) to 48.37% (B. abyssalis). The PHGR of the
novel strain CHPC 1.3453\textsuperscript{T} ranged from 45.22% (B. denitrificans) to 89.52%  
(B. bullata), compared with the other 23 species within the genus Brevundimonas.

From the taxonomic perspective, our ANI analysis supported the species assignment
for the genus of Brevundimonas. The overall ANI values between any two
representative genomes, were under the classical boundary of 95% - 96% (33,38) for
an independent species or subspecies (Fig. 1B), except for two groups, i.e., B.
diminuta ATCC 11568\textsuperscript{T} - B. vancanneytii NCTC 9239, and B. abyssalis TAR-001\textsuperscript{T} - B.
denitrificans TAR-002\textsuperscript{T}. It was suggested that each group belongs to synonyms.

We analyzed the pan-genome diversity of Brevundimonas spp. by using the Roary
pipeline (Fig. 1C). A total of 17,194 genes were defined in 24 genomes, and 762 of
these genes were shared by all species, which formed a set of the so-called hard core
genes. Also, about 300 soft core genes were harbored by 95%-99% of the genomes.

Hard core genes together with soft core genes accounted for approximately
one-sixteenth of the pan genes. According to the gene presence or absence, the
complete hierarchical clustering of pan genes was divided into 5 major clades (Fig.
1C). Clade II contained the largest number of Brevundimonas species (n=9); followed
by Clade V (n=5), where the novel strain CHPC 1.3453\textsuperscript{T} was located. The conserved-
and pan- genes dilution curve demonstrated that the genomes of Brevundimonas spp.
were open and compatible (Fig. 1D). As the number of genomes increased, the
number of conserved genes tended to be stable, while the number of pan genes kept
increasing.

**Genetic structures of closely related members of Brevundimonas spp.** To further
reveal the genomic variation within the genus Brevundimonas, we constructed two
phylogenetic trees based on the set of core- and accessory- genome, respectively (Fig.
2A). Like the two-dimensional hierarchical clustering of pan-genome, the
core-genome phylogeny also reflected the tendency towards Clade I-V and formed
different evolutionary branches or clusters. Surprisingly, the phylogenetic tree based
on core genome had a highly similar topology compared to that based on accessory
genome, and the *Brevundimonas* spp. within the 5 clades also formed a one-to-one
mapping parallelism. This suggest that *Brevundimonas* spp. may have a relationship
of interactive co-evolution.

When the genome of the novel strain CHPC 1.3453\(^T\) was compared to the genomes of
other *Brevundimonas* spp., approximately hundreds of localized co-linear blocks
(LCBs) were estimated by MAUVE comparison (Fig. 2B, Fig. S3). *B. terrae* was the
most closely related species to the novel strain CHPC 1.3453\(^T\) in terms of genome
structure, with the smallest number of LCBs at 138, while *B. bullata* was the most
distantly related one, with the largest number of LCBs at 326. Comparing the genome
of CHPC 1.3453\(^T\) to that of *B. terrae*, 472,805 SNPs were calculated. The Venn
diagram (Fig. 2C) showed the shared and unique genes found in six *Brevundimonas*
genomes, and 432, 1278, 1205, 394, 1047, and 665 unique genes were identified in
each genome respectively. These unique genes were annotated by GO, COG and
KEGG, and many of genes were related to biological functions such as catalytic
activity, translation, multi-substance metabolism (like amino acid, nucleotide,
carbohydrate), etc. (data was not shown). Therefore, it is reasonable to speculate
*Brevundimonas* spp. genomes might undergo many sorts of rearrangements, since
massive LCBs indicate events relating to changes in genome structure, including
insertions, deletions, inversions, and translocations.

**Distribution of potential virulence-associated genes, antimicrobial resistance
genes, and mobile genetic elements.** Through comparison of the *Brevundimonas*
genomes with the VFDB, we found that *Brevundimonas* spp. shared
virulence-associated gene homologous to 20 species from different genus, with
*Brucella melitensis*, *Francisella tularensis*, *Legionella pneumophila* as the top three
species sharing the largest number of virulence-associated genes (Fig. 3A). The
virulence-associated genes in the novel strain CHPC 1.3453\(^T\) were sourced from the
above three species as well as *Mycobacterium tuberculosis* and *Helicobacter pylori*.
The majority of *Brevundimonas* spp. (including CHPC 1.3453\(^T\)) harbored the genes
*icl* (VFG001381), *tufA* (VFG046465), *kdsA* (VFG011414), *htpB* (VFG001855),
acpXL (VFG011430), which encoded isocitrate lyase, elongation factor Tu, 2-dehydro-3-deoxyphosphooctonate aldolase, heat shock protein HtpB, and acyl carrier protein, respectively. In particular, the gene kdsA and acpXL were detected in 95.8% (n = 23/24) Brevundimonas species, which were related to LPS with the function of adhesion and endotoxin (Fig. 3B).

The majority of the Brevundimonas species (87.5%, n = 21/24) were detected to carry one or more drug resistance genes (Table S4). B. diminuta and B. naejangsanensis harbored the most antimicrobial resistance genes, while other species, including the novel strain CHPC 1.3453T, harbored only one gene, namely an antibiotic efflux pump related gene adeF. Apart from adeF, B. diminuta contained a tetracycline resistance genes tet(C), while B. naejangsanensis contained a sulfonamide resistance gene sul2, as well as two tetracycline resistance genes tet(D) and tet(G) (Table S4).

We also analyzed the distribution of mobile genetic elements (MGEs, for example, genomic islands (GIs), plasmids, CRISPR-cas, phages and prophages) in 24 Brevundimonas species. The GIs were identified by three algorithms of IslandPath-DIMOB, SIGI-HMM and IslandPick, and the number of GIs ranged from 8 (B. aveniformis DSM 17977T) to 79 (B. vancanneytii NCTC 9239). Detailed information of the cross-species or the cross-genus GIs was listed in Table S5 and Table S6, respectively. Notably, the GIs from the novel strain CHPC 1.3453T, were highly homologous to GIs from B. subvibrioides ATCC 15264 (Accession number: CP002102.1) with 100% coverage and 100% identity. Meanwhile, 3 intact and 12 hypothetical phage/prophage sequences were detected by PHASTER (Table S7).

Strain CHPC 1.3453T was found to contain a complete phage sequence, which was genetically homologous to the sequence of PHAGE_Ralsto_RsoM1USA (Accession number: NC_049432). However, no plasmid replicon or CRISPR-cas related sequence was detected in the representative genomes of Brevundimonas genus.

Strain CHPC 1.3453T was identified as a novel species of the genus Brevundimonas. The 16S rRNA gene sequence (1,418 bp) of strain CHPC 1.3453T was aligned against the corresponding sequences of 33 known Brevundimonas species.
Strain CHPC 1.3453\textsuperscript{T} was demonstrated to share the highest similarity (98.53\%) to \textit{Brevundimonas terrae} KSL-145\textsuperscript{T} (DQ335215); followed by \textit{Brevundimonas diminuta} ATCC 11568\textsuperscript{T} (GL883089, 97.11\%) and \textit{Brevundimonas faecalis} CS20.3\textsuperscript{T} (FR775448, 95.34\%). The similarity values between the strain CHPC 1.3453\textsuperscript{T} and some \textit{Brevundimonas} spp. were slightly above 97\%, the threshold commonly suggested for species delineation (39). Phylogenetic analysis based on 16S rRNA gene sequences revealed that strain CHPC 1.3453\textsuperscript{T} belonged to the genus \textit{Brevundimonas}. MLSA method of 5 housekeeping genes (concatenated sequence in the order of \textit{gyrB-ppsA-recN-rpoC-rpoD}) was established for the determination of a more refined population structure among \textit{Brevundimonas} species. Strain CHPC 1.3453\textsuperscript{T} forming an independent and robust interspecies phylogenetic branch, was closely clustered with \textit{B. terrae}. Notably, strain CHPC 1.3453\textsuperscript{T} shared the highest sequence similarities (83.64\%) to \textit{B. terrae} DSM 17329\textsuperscript{T} in terms of MLSA sequence similarities (Fig. 4B), which was consistent with the results of 16S rRNA gene sequences analysis.

We further calculated the digital DDH (dDDH) and ANI value for the strain CHPC 1.3453\textsuperscript{T} and the closely related representative strains of the genus \textit{Brevundimonas} DSM 17329\textsuperscript{T} in terms of dDDH. In the view of Auch et al. (40), classical species-threshold for dDDH values was 70\%, clearly interpreting the differentiation between strain CHPC 1.3453\textsuperscript{T} and its closest relatives. Consistently, ANI values between strain CHPC 1.3453\textsuperscript{T} and other closely related type strains (or representative strains) of \textit{Brevundimonas} species ranged from 74.8\% to 78.2\%, lower than the 95\% - 96\% cutoff value for defining species threshold (33).

Cells of strain CHPC 1.3453\textsuperscript{T} were motile by a single polar flagellum. The strain formed circular, orange-yellow colonies with a diameter of 0.5-1.5 mm on LB agar and grey colonies on Blood agar at 35°C for 18-72 h, with translucent texture, round shape, and plump appearance (Fig. 5A, 5B). Strain CHPC 1.3453\textsuperscript{T} was small, short, rod-shaped, under high-resolution transmission electron microscopy (Fig. 5C). Cell
size was 1.2-4 μm in length and 0.4 μm in width. Phenotypic results showed that strain CHPC 1.3453T was Gram-stain-negative, oxidase-positive; it can grow on LB medium, Blood medium, BHI medium, or R2A agar; it was able to grow under a wide range of pH (pH 6-10, optimum pH 8) and NaCl tolerance concentrations (0-3%, w/v); it can grow at a temperature range of 15-42°C (it did not grow at 4°C); the optimum growth temperature was recommended to be 30-37°C; it was facultative aerobic. The strain was susceptible to amikacin, amoxicillin-clavulanate, ampicillin-sulbactam, cefepime, cefoperazone-sulbactam, cefoxitin, ceftazidine, ceftriaxone, cefuroxime, chloramphenicol, ciprofloxacin, colistin, ertapenem, fosfomycin, gentamicin, imipenem, levofloxacin, meropenem, minocycline, moxifloxacin, norfloxacin, piperacillin-tazobactam, tetracycline, tigecycline, and tobramycin but resistant to aztreonam, cefazolin, nitrofurantoin, and trimethoprim-sulfamethoxazole.

Members of *Brevundimonas* genus grow slowly on ordinary nutrient media (20), like *B. vesicularis* and *B. diminuta* (41). We conducted growth rate test for strain CHPC 1.3453T, with the type strains of *B. vesicularis* and *B. diminuta* as slow-growth controls, as well as *E. coli* ATCC 25922T as a fast-growth control (Fig. 5D). The growth rate of strain CHPC 1.3453T was approximately equivalent to that of *B. diminuta* ATCC 11568T; the bacterial cells colonized rapidly and the growth rate increased until a constant growth rate was achieved in the 12-16 h during the exponential phase; eventually, both strain CHPC 1.3453T and *B. diminuta* ATCC 11568T reached the saturation phase after 24 h; and both of them grew much more slower than *E. coli* ATCC 25922T, while faster than *B. vesicularis* NBRC 12165T.

To further determine the characteristics of strain CHPC 1.3453T, biochemical tests were performed. The results showed that β-glucosidase, D-xylose, inositol, D-melibiose, potassium 2-ketogluconate, and potassium 5-ketogluconate were assimilated, but the following substrates were not assimilated: L-tryptophane, D-glucose, arginine dihydrolase, urease, protease, 4-nitrophenyl-β-D-galactopyranoside, glucose, L-arabinose, D-mannitol, N-acetyl-D-glucosamine, D-maltose, potassium gluconate, capric acid, adipic acid,
malic acid, trisodium citrate, phenylacetic acid, glycerol, erythritol, D-arabinose, D-ribose, L-xylose, Methyl-β-D-xylopyranoside, D-galactose, D-fructose, D-mannose, L-sorbose, L-rhamnose, dulcitol, D-sorbitol, methyl-α-D-mannopyranoside, methyl-α-D-glucopyranoside, N-acetylglucosamine, amygdalin, arbutin, esculin ferric citrate, salicin, D-cellubiose, D-lactose (bovine origin), D-saccharose (sucrose), D-trehalose, inulin, D-melezitose, D-raffinose Amidon (starch), glycogen, xylitol, gentiobiose, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol. In API ZYM system assay, alkaline phosphatase, esterase (C4), leucine arylamidase, trypsin, acid phosphatase, and naphthol-AS-BI-phosphohydrolase were present, but esterase lipase (C8), lipase (C14), valine arylamidase, cystine arylamidase, α-chymotrypsin, α-galactosidase, β-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase were absent. Detailed information about the characteristics of strain CHPC 1.3453^T and type strains of other closely related species were listed in Table 2.

The above phenotypic, biochemical, phylogenetic, and genomic analysis clearly indicated strain CHPC 1.3453^T belongs to a novel species within the genus Brevundimonas. We propose the species name Brevundimonas pishanensis (pi.shan.en’sis. fem. adj. pishanensis, referring to Pishan County of Hotan Prefecture [pishan in Chinese], where the strain was recovered). The type strain is CHPC 1.3453^T (=GDMCC 1.2503^T = KCTC 82824^T) and the DNA G+C content is 61.6 mol%. The draft genome sequence of the strain has been deposited at the NCBI GenBank under accession number JAJKBG000000000.

DISCUSSION

In this study, we provide an extensive overview of the diverse genomic features of the genus Brevundimonas with major emphasis on pan-genome evolution, genetic structures and MGEs-related characterization (including potential virulence associated genes). We conducted a comparative analysis for the genomes of the type strains (or representative strains) of 24 Brevundimonas species, and analyzed the core- and accessory- genome sequences based on phylogenetic relationships. We also provide
an objective and comprehensive description of the potential virulence- and
drug-resistance genes carried by the *Brevundimonas* spp., which may be a basis for
the further exploration of their specific pathogenic mechanisms and drug resistance
risks. Furthermore, we reported a novel *Brevundimonas* species based on both
phenotypic and genomic analyses. It is the first *Brevundimonas* species recovered
from stool of a patient with diarrhea.
The G+C content of bacterial chromosomes varies from 17 mol% to 75 mol% (42),
which can serve as a basis for analyzing the relationships between bacterial species or
the origin of genes. Meanwhile, the genome size of most bacteria is positively
correlated with their G+C contents. The correlation value decreases at shorter genome
sizes, where there is a wider spread of G+C values (42). Within the genus
*Brevundimonas*, the variation of the genome size was smaller than that of the G+C
content (standard deviation of the genome size was smaller than that of the G+C
content). *B. subvibrioides* contained the largest genome size, followed by *B. bullata*,
*B. mediterranea*, and *B. nasdae*, while *B. viscosa* contained the highest G+C content,
followed by *B. fluminis* and *B. lenta*. However, the genus *Brevundimonas* is an
exception in terms of the relationship between the genome size and the G+C content.
The relatively small average genome size (3.13±0.29 Mb) of the *Brevundimonas*
genus was previously presumed to have a range of G+C content extend from 45% to
50% according to the correlation formula (43). In fact, we found that *Brevundimonas*
spp. have an average G+C content of up to 67%. It is interesting why *Brevundimonas*
spp. have small genome sizes but high G+C contents. One possible reason could be
the biases of codon usage (44). Genomic G+C content has been identified as the most
important determinant for codon usage (45). Despite synonymous amino acids,
unknown selective forces favoring GC-rich codons may influence the G+C content of
the genomes of *Brevundimonas* genus.
To gain further clarity on the interspecies relationships, we performed a pan-genome
analysis for the genomes of 24 *Brevundimonas* species. Most of these genomes shared
more than 1000 orthologous groups. From the perspective of evolution, a
high-resolution hierarchical clustering phylogram (Fig. 1C) based on the presence or absence of pan genes distinguished the 24 *Brevundimonas* spp. into different clustering branches, which was consistent with the results of ANI values. It should be noticed that *B. pishanensis*, a novel *Brevundimonas* species found in this study was evolutionarily closest to the *Brevundimonas* spp. in Clade V. Interestingly, the phylogentic analysis of the sequences of core-genome and accessory-genome showed a one-to-one match, which may suggest co-evolution of the core- and the accessory-genome of species within the genus *Brevundimonas* and equal roles played by the core- and the accessory-genome over the same period. One possible reason for their co-evolution may be horizontal gene transfer. For example, mobile elements have similar outcomes in the variations of core- or accessory-genome, and the probability of this occurrence is relatively consistent in each species (46). We then did a more refined genome structure comparison for several *Brevundimonas* spp. in Clade V. When the genomes were aligned, multiple scattered blocks were identified, suggesting that the genome structures of the novel *B. pishanensis* and other *Brevundimonas* spp. in Clade V were genetically distinct, which may due to a variety of genetic phenomena such as gene rearrangements, inversions, translocations, and insertions.

Opportunistic pathogens are constantly emerging and changing, with little or no apparent virulence, and these bacteria are mostly drug-resistant or multidrug-resistant (47-50). They are generally non-pathogenic in their indigenous locations, but when the homeostasis in human body is disrupted, they take advantage of the situation and invade the body, causing various diseases. This mainly happened in hospitalized and immunocompromised patients, leading to life-threatening infections with high mortality rates (51). Limited knowledge existed for the pathogenicity of the genus *Brevundimonas*. The majority of the currently known infections caused by *Brevundimonas* spp. were found to be accompanied by the patient's underlying diseases, for example, urinary tract infections (UTI) (52), bacteremia (16) and empyema (53) in clinical settings. Other co-infection and pseudo-outbreaks associated
with *Brevundimonas* spp. have also been rarely reported (52, 54). Despite intensive efforts, treatment strategies currently remained insufficient to eradicate such *Brevundimonas* spp. Infections (20). One aim driving this study was to understand the potential pathogenicity of *Brevundimonas* spp. by predicting the virulence gene profile, particularly the horizontal transfer of virulence genes associated with MGEs within species. We found that most *Brevundimonas* spp., except *B. terrae* DSM 17329T, carried five virulence genes (*icl*, *tufA*, *kdsA*, *htpB*, and *acpXL*). These virulence genes have homologues in species from other genus, such as *Mycobacterium tuberculosis*, and have been confirmed to associate with pathogenicity. For example, isocitrate lyase (ICL), an enzyme essential for the metabolism of fatty acids, plays a pivotal role in one of the fatty acid metabolism mechanisms for *Mycobacterium tuberculosis* persistence (55). Bhusal et al. (56) also demonstrated that ICL2 was critical for bacterial growth and virulence, which was mainly involved in the regulation of carbon fluxes in tricarboxylic acid cycle, glyoxylate shunt and methylcitrate cycle. Another example is the product encoded by *acpXL* gene, which acts as a donor of C28 fatty acid for lipid A (57) and involves in the transfer of tC28 fatty acid to lipid A precursor. Sharypova et al. proved that *acpXL* mutation indeed blocked the C28 acylation of lipid A (58). AcpXL could be a favourable substance to enhance the biochemical and infection phenotypes of Gram-negative bacteria (59).

Our analysis indicated that each *Brevundimonas* spp. contained a very similar virulence gene profile. However, only *B. vesicularis* and *B. diminuta* were clearly reported to be pathogenic, which accounted for nearly 90% of *Brevundimonas* infections. For drug resistance, *B. vesicularis* was reported to be associated with bacteremia and showed highly variable sensitivity to broad-spectrum antibiotics (60). *Brevundimonas* spp. were also influential factors in the spread of carbapenem resistance (61), which was the characterization of clinical environments. The novel strain *B. pishanensis* was found to harbor one antibiotic efflux pump related-gene *adeF*. No drug resistant genes were identified in the two *Brevundimonas* species causing human infections, *B. diminuta* and *B. vesicularis*. 
The growth rate of bacteria within the genus *Brevundimonas*, including the novel species *B. pishanensis*, was significantly lower than that of *E. coli*, especially in *B. vesicularis*. Bacterial growth is often system-wide linked, including gene expression, growth feedback, and proteome partition (62). It has been demonstrated that the growth rate can be served as a key parameter in assessing the overall bacterial growth (63, 64). Tight regulation of gene expression limits the waste of resources and energy, and specific genes or operons may be regulated by different mechanisms. Using *E. coli* as a paradigm, ppGpp, which is produced by two ppGpp synthetases RelA and SpoTacting, acts with DksA in a synergistic way, and becomes a major effector in bacterial growth rate regulation (65). Our study suggested that, when the environmental conditions are the same, *Brevundimonas* spp. may have increased ppGpp regulation ability, which then makes them have significantly slower growth rates than *E. coli*. Also, the nutritional and habitat conditions forced bacteria to exhibit a more diverse growth strategy of nutrient and metabolic versatility. The synergistic effect between ppGpp and DksA above was also a key effector for *E. coli* in the stringent response induced by nutrient starvation (66, 67). Some bacteria are already found to have a greater number and variety of genes encoding regulatory elements, as recently demonstrated that Gram-positive soil bacterium *Bacillus subtilis* and the Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* were able to accumulate a considerable abundance and diversity of transcriptional regulators, two-component systems and alternative σ factors under nutrient and habitat adverse conditions (68-70).

Next-generation sequencing (NGS), as an important milestone, has been widely applied in the identification of newly-isolated species (71). In our study, the values of both ANI and dDDH indicated that the genome of *B. pishanensis* CHPC 1.3453\(^T\) and those of taxonomically closely-related f *Brevundimonas* spp. showed low similarities, implying CHPC 1.3453\(^T\) could be a new species. Our further experiments also showed that CHPC 1.3453\(^T\) can be differentiated from other *Brevundimonas* species in the abilities to assimilate multiple biochemical substrates. Notably, Liu et al. used...
ANI and DDH data to identify 29 *Brevundimonas* taxa and found that at least 17 taxa should be assigned to novel *Brevundimonas* species (72). In this study, we did a comprehensive analysis for the *Brevundimonas* genomes. Still, two groups of *Brevundimonas* spp. were found to need correction and update of the taxonomy and classification. Two strains, *B. abyssalis* TAR-001<sup>T</sup> and *B. denitrificans* TAR-002<sup>T</sup>, had been mislabeled, and actually they belonged to the same species *B. abyssalis*.

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We have no conflict of interest.

**TABLE 1** Average Nucleotide Identity (ANI) and digital DNA–DNA hybridization (dDDH) between strain CHPC 1.3453<sup>T</sup> and closely related representative strains of the genus *Brevundimonas*

| Query genome | Reference genome      | Accession No. | dDDH (%) | Model CI         | Distance | ANI (%) |
|--------------|-----------------------|---------------|----------|------------------|----------|---------|
| CHPC 1.3453<sup>T</sup> | *B. bullata* HAMBI_262<sup>T</sup> | QLLC01        | 20.5     | [17.5 - 22.1%]  | 0.2      | 74.8    |
| CHPC 1.3453<sup>T</sup> | *B. diminuta* ATCC 11568<sup>T</sup> | ADU301        | 21.0     | [18.7 - 23.4%]  | 0.2      | 75.0    |
| CHPC 1.3453<sup>T</sup> | *B. naejangsanensis* DSM 23858<sup>T</sup> | ATXN01        | 20.4     | [17.2 - 21.8%]  | 0.2      | 75.0    |
| CHPC 1.3453<sup>T</sup> | *B. terrae* DSM 17329<sup>T</sup> | JAASQT01      | 21.7     | [18.2 - 22.8%]  | 0.2      | 78.2    |
| CHPC 1.3453<sup>T</sup> | *B. vancanneytii* NCTC 9239 | CP002102      | 20.7     | [19.5 - 24.1%]  | 0.2      | 75.1    |

Note: The dDDH value based on Formula 2 was calculated using the GGDC web server; ANI values were estimated using the web-based service ANI calculator (http://www.ezbiocloud.net/tools/ani). Model CI, model confidence interval.

**TABLE 2** Physiological and biochemical characteristics of strain CHPC 1.3453<sup>T</sup> and closely related species of the genus *Brevundimonas*

| Characteristics | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
|-----------------|---|---|---|---|---|---|---|---|
| Colony pigmentation | Yellow | Yellow | Orange/red | Light yellow | Greyish yellow | NA | Greyish yellow | Whitish yellow |
| Oxidase         | + | + | + | + | + | + | + | + |
| Urease          | - | - | - | - | + | NA | - | NA |
| Reduction of nitrates to nitrite | - | - | - | - | NA | w or - | NA | - |
| Reduction of nitrates to nitrogen | - | - | - | - | NA | w or - | NA | - |

**Assimilation of:**
| Substrate          | Glycerol | L-arabinose | D-galactose | D-glucose | D-maltose | D-cellobiose | Glycogen | Gentiose | D-tagatose | Arginine dihydrolase | β-glucosidase | Protease | N-Acetyl-D-Glucosamine |
|-------------------|----------|-------------|-------------|-----------|-----------|-------------|-----------|----------|------------|----------------------|---------------|-----------|------------------------|
|                   | -        | +           | -           | -         | -         | -           | +         | -        | w          | -                    | +             | +         |                        |
|                   | -        | w           | +           | -         | -         | w           | -         | w        | -          | -                    | -             | -         | -                      |
|                   | -        | w           | +           | -         | w         | -           | -         | w        | -          | -                    | -             | NA        | -                      |
|                   | -        | -           | +           | -         | -         | -           | -         | w        | -          | -                    | -             | NA        | -                      |
|                   | -        | -           | +           | -         | -         | w           | +         | -        | +          | -                    | -             | -         | +                      |
|                   | w        | w           | +           | +         | NA        | NA          | -         | NA       | -          | NA                   | -             | NA        | -                      |
|                   | -        | -           | -           | -         | -         | +           | +         | -        | -          | -                    | -             | +         | -                      |
|                   | -        | -           | -           | -         | -         | -           | +         | -        | +          | -                    | -             | -         | -                      |

**API ZYM reactions**

| Esterase (C 4)    | Esterase Lipase (C 8) | Leucine arylamidase | Valine arylamidase |
|-------------------|-----------------------|---------------------|--------------------|
| +                 | -                     | w                   | -                  |
| +                 | +                     | +                   | +                  |
| +                 | +                     | +                   | +                  |
| +                 | +                     | +                   | +                  |
| +                 | +                     | +                   | +                  |
| +                 | +                     | +                   | +                  |
| +                 | +                     | +                   | +                  |
| +                 | +                     | +                   | +                  |
| +                 | +                     | +                   | +                  |

**FIGURE LEGENDS**

**FIG 1** General genomic characteristics and diversity of *Brevundimonas* genus. (A) The pairwise homologous gene rate (PHGR) of 24 *Brevundimonas* spp. genomes. The bottom and top of the violin plots indicate 0.25 and 0.75 quantiles respectively. The dashes represent the median of each species in rainbow colors, and the bandwidth represents the density distribution. (B) Average Nucleotide Identity (ANI) among *Brevundimonas* members based on their WGS sequences. The colors from blue to red indicate a gradual increase in ANI values. (C) Pan genome diversity of *Brevundimonas* spp. for which a WGS is available. The two-dimensional hierarchical clustering of species and genes on the left is based on the presence (blue) or absence (grey) of genes. The red, yellow, green, blue, and brown on the right refer to the evolution *Clade I-V*, respectively. (D) Conserved- and pan- genes dilution curves of...
*Brevundimonas* genomes. The red boxplot represents the number of conserved genes, and the blue represents the number of pan genes.

**FIG 2** The evolutionary relationship of the novel *Brevundimonas* strain CHPC 1.3453ᵀ. (A) The core- and accessory- genome phylogeny of 24 *Brevundimonas* spp. strains. The colors of each species correspond to the colors of five different clades in the Fig. 1C. (B) The MAUVE comparison of CHPC 1.3453ᵀ and closely related members in the *Clade V* of pan genome clustering in Fig. 1C. (C) The Venn diagram of the shared and unique genes found in the novel strain CHPC 1.3453ᵀ and other closely related members in *Clade V*.

**FIG 3** The distribution of virulence-associated genes of *Brevundimonas* spp.. (A) Hierarchically clustering heatmap of 24 *Brevundimonas* spp. with respect to the virulence gene sources. The colors from blue to red represent the correlation frequency of virulence gene homology. (B) Hierarchically clustering heatmap of virulence-associated genes in 24 *Brevundimonas* spp. The colors represent the sequences-BLAST coverage values of virulence-associated genes.

**FIG 4** Phylogenetic trees of *Brevundimonas* species. (A) The neighbour-joining tree based on 16S rRNA gene sequences of the strain CHPC 1.3453ᵀ and other *Brevundimonas* spp. isolates. Bootstrap values below 70%, based on 1000 re-samplings, are not shown at branch nodes. Filled black circles at nodes indicate generic branches that are synchronously recovered by using neighbour-joining and maximum-likelihood algorithms. GenBank accession numbers of the 16S rRNA gene sequences are given in parentheses. Three strains, namely *Caulobacter fusiformis* ATCC 15257ᵀ, *Asticcacaulis excentricus* DSM 4724ᵀ, and *Sphingomonas adhaesiva* DSM 7418ᵀ, respectively, are served as outgroups. The horizontal bar represents 0.01 substitution per nucleotide site. (B) The maximum-likelihood tree based on 5 housekeeping gene concatenated sequences, in the order of
gyrB-ppsA-recN-rpoC-rpoD. Numbers at nodes indicate bootstrap values (percentage of 1000 replicates) greater than 70%. Filled black circles indicate generic branches that are also recovered by using neighbour-joining and maximum-likelihood algorithms. The horizontal bar represents 0.1 substitution per nucleotide site.

**FIG 5** Culture and morphology characteristics of strain CHPC 1.3453\textsuperscript{T}. (A) The growth of this strain after 18 h at 35°C on Blood Agar medium. (B) The morphology of single colony after 72 h growth at 35°C on Luria-Bertani (LB) medium. The bar represents a unit length of 10 millimeters. (C) The general morphology of a negatively-stained bacterial cell showed by high resolution transmission electron. The bar represents a unit length of 1000 nanometers. (D) Growth curves of strain CHPC 1.3453\textsuperscript{T}. Three strains, B. vesicularis NBRC 12165\textsuperscript{T} and B. diminuta ATCC 11568\textsuperscript{T} and E. coli ATCC 25922\textsuperscript{T}, were used controls.

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Manuscript number : Spectrum02468-21

Title: Comparative Genomic Analysis Reveals Potential Pathogenicity and Slow-growth Characteristics of genus Brevundimonas and Description of Brevundimonas pishanensis sp. nov.
Authors: Zhenzhou Huang, Keyi Yu, Yue Xiao, Yonglu Wang, Di Xiao, and Duochun Wang

Reviewer #1 (Comments for the Author):

The authors performed comparative genomic analysis in 24 Brevundimonas species with a novel species they isolated in China. Phylogenetic analysis was performed using multiple approaches. Virulence/antibiotic associated genes were predicted using bioinformatics tools. Collectively, the evidence support that the newly isolated strain belongs to a novel species.

Response:
Dear reviewer, we would like to thank you for your careful reading, helpful comments. We have carefully considered all comments and revised our manuscript accordingly.

Major comments:
1. The authors grouped the different species into 5 “clades” using hierarchical clustering method based on pan genome diversity which was based on gene presence and absence profile. However, the data seems to be contradictory to that from ANI and core genome tree. According to the core genome tree in Fig2A, TAR001 and TAR002 should not have been grouped into one same clade. Additionally, depending on the cutoff being used, the “clade” grouping could end up differently. The authors need to define the cutoff in the main text.

Response:
Thank you very much for your constructive suggestions. Defining clade in this study was performed using two approaches, including pan-genome variation and core-genome Maximum-likelihood method. The above methods get a consistent clade division, although they each have a corresponding cutoff value. Specifically, pan-genome analysis has a cutoff (based gene-presence/absence profile similarity) as 25.6%, while core-genome tree has a SNP-threshold of 500 SNPs for defining Brevundimonas genus into 5 clades. In core genome tree in Fig2A, it seems a bit misleading, because both the core- and accessory- genome trees only use the "Rectangular-Display only topology” visualization mode in the software, without the branch length. In fact, all the data (pan genome diversity, ANI, core- and accessory- genome phylogeny) consistently show that TAR001 and TAR002 are very closely related and should be grouped together. We have replaced the evolutionary trees (with branch length) in Figure 2A, and also marked the cutoff value of ‘clade division’ in the new manuscript.
2. line 232: "... had a highly similar topology...": the reviewer disagrees with the conclusion. The phylogenetic tree from Fig 2A and Fig 2B differs at the clustering of TAR001/TAR002 and B. lutea NS26/B. aveniformis DSM17977.

Response:
Thank you very much for your comments. The topology in Figure 2A seems to be a bit unclear, so that the reviewers have raised questions. To solve this problem, we changed the way the evolutionary tree displayed in the new Figure 2A and added a cutoff criterion for clade division.

3. Characterization of antibiotic resistance profile requires data support, such as MIC, which is missing in the main text. As for "slow growth", it is recommended the authors provide the doubling time.

Response:
Thanks very much for reviewer's comments. We have added the MIC value to the results of antibiotic resistance test (Line 338-349). We also provide the doubling time of bacterial growth (B. pishanensis sp. nov. = 75 min; B. diminuta = 81 min; B. vesicularis = 135 min; E. coli=27 min) in the new manuscript Line 360-364.

Minor comments:
1. line 62: "tiny": it's better to use size in micro-meter instead of a generic "tiny".

Response:
Thanks very much for pointing out the inappropriateness. We have made a correction in the new manuscript Line 64.

2. line 66: "human beings": please specify location, eg on skin or colonized internally or opportunistic pathogen.

Response:
Thanks very much for reviewer's comments. We have made a revision in the new manuscript Line 69.

3. line 82: "few articles": please cite the related references.

Response:
Thanks very much for reviewer's comments. we have cited the related references in the new manuscript in the Line 84.

References as follow:
16. Yang ML, Chen YH, Chen TC, Lin WR, Lin CY, Lu PL. 2006. Case report: infective endocarditis caused by Brevundimonas vesicularis. BMC INFECT DIS 6:179.
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4. line 219: "5 major clades": what's the cutoff value? what's the rational of using Fig 1C to define clades instead of Fig1B, 2A or 2B?

Response:
Thanks very much for reviewer’s comments. We have added a cutoff criterion for clade division in the new manuscript Line 225-226. Defining clade in this study was performed both pan-genome diversity, and core-genome Maximum-likelihood method. The above methods get a consistent clade division, although they each have a corresponding cutoff value. The clustering dendrogram was done by R (hclust function) using ‘complete’ parameter. Using pan-genome variation to divide clades, because pan-genome analysis not only includes information about the gene presence and absence profile of core genes, but also reflects the information of accessory-genes. It took into account the evolutionary differences of common sites, and also proved that the existence of some unique gene segments, which may be related to horizontal gene transfer. We also refer many recent publications [1-4] about “defining clade” based on pan-genome variation.

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5. line 248: "data not shown": recommend to add this data to supplemental material.

Response:
Thanks very much for reviewer’s comments. We have added this part of the data (GO, COG, KEGG annotation in the Fig. S4) and reword in the new manuscript Line 257-260.

6. line 291-304: why were different strains used in Fig 4A and 4B?

Response:
Thanks very much for reviewer’s comments. Fig 4A was done using all the 16S
rRNA gene sequences currently available on the GenBank. As a result, there are 33 sequences. As for the MLSA analysis in the Fig 4B, the sequence of each housekeeping gene was extracted from the whole genome sequence. As a result, there are currently only 24 (including the new species in this study) genome sequences.

7. line 496: “two groups of ...”: it is not clear which two groups are being discussed here. Was this data in the main text?
Response: Thanks very much for reviewer’s comments. Line 208-220: “The overall ANI values between any two representative genomes, were under the classical boundary of 95% - 96% (33,38) for an independent species or subspecies (Fig. 1B), except for two groups, i.e., B. diminuta ATCC 11568T - B. vancanneytii NCTC 9239, and B. abyssalis TAR-001T - B. denitrificans TAR-002T. It was suggested that each group belongs to synonyms.”
In order to avoid confusion for readers, we have re-declared the ‘two groups’ that appeared in the Discussion section Line 521-524.

Reviewer #2 (Comments for the Author):

Really great work! I enjoyed reading this comprehensive manuscript. I have a couple of comments that I would like to be addressed.
Response: Thanks very much for reviewer’s careful review and helpful suggestions. We have made point-by-point responses to the issues raised by the reviewers in the new manuscript.

Major:
1) Line 19: “Brevundimonas spp. have relatively small genomes (3.13(plus minus)0.29 Mb)”. Please note, bacteria can have a genome size between ~0.5 to ~14 Mbp. Please can you reword this as 3.13 Mbp is not relatively small when compared to other bacteria like Chlamydia spp. (~1.1Mbp). Also, could you please define what the +/- is representing? Standard deviation? Standard error of mean?
Response: Thanks very much for reviewer’s comments. Firstly, our data following the normal distribution, ‘+/-’ is ‘Mean ± Standard deviation’. As the reviewer pointed out, bacteria can have a genome size between ~0.5 to ~14 Mbp. Brevundimonas spp. was subordinate to Family Caulobacteraceae, Order Caulobacterales. We have
done a statistics for the size of the Family Caulobacteraceae bacterial genome larger to be 4.49±0.83 Mb (t test, \( P < 0.001 \)). Therefore, we determined that the bacterial genome size of Brevundimonas spp. is relatively small in the whole Family Caulobacteraceae bacteria. We have made a supplementary note in the Line 19 and ‘Result’ section of the new manuscript Line 196-198.

2) Line 109: Please change "The genome of strain CHPC 1.3453T was extracted" to "The DNA of strain CHPC 1.3453T was extracted"
Response:
Thanks very much for reviewer’s comments. We have changed ‘the genome’ to ‘the DNA’ in the new manuscript Line 112.

3) Line 126: Was a reference used for QUAST? If so which reference was used?
Response:
Thanks very much for reviewer’s comments. The published paper, which citation format is “Gurevich A, Saveliev V, Vyahhi N, Tesler G. 2013. QUAST: quality assessment tool for genome assemblies. BIOINFORMATICS 29:1072-1075” has been cited as Reference No.25 in the new manuscript Line 127.

4) Line 132: What alignment was performed? Muscle? ClustralW?
Response:
Thanks very much for reviewer’s comments. The alignment of core genome sequences was aligned by Roary software automatically; while the alignment of 16S rRNA gene and multilocus sequence analysis (MLSA), we used the ‘ClustralW module’ that comes with the MEGA software version 7.0.21.

Figure 4: What does the axis represent? Please describe this is figure legend
Response:
Thanks very much for pointing out the inappropriateness. The axis represents the length of evolutionary branches. At present, we have used the horizontal bar as a tree scale in the figure; and to avoid redundancy, we have removed the axis in the new version of the Figure 4.

Figure S2B: What does the scale bar represent in the tree? How is the tree rooted? Midpoint? Outgroup? What do the numbers on the nodes represent? Bootstrap? How many replicates? When I refer back to the methods, I can see that 1,000 bootstrap replicated was done. Might be worth while to state this in the figure legend for readability.
Response:
Thanks very much for reviewer’s comments. In the Figure S2, they are not the scale bars. The true scale is located at the bottom of this image. Those colored horizontal lines represent different genus in the Family Caulobacteraceae. Those non-Brevundimonas genus genomes served as outgroups in the phylogenetic tree, which helps to root the tree and helps to locate the evolutionary position of Brevundimonas genus in the Family Caulobacteraceae. Bootstrap values over 70%,
based on 1000 resamplings, are shown at branch nodes. We have revised it in the ‘Materials and Methods’ section and also marked in the figure legend of Figure S2.

Figure S4B: What does the scale bar represent in the tree? What does the axis represent? How is the tree rooted? Midpoint? Outgroup? What do the black dots on the nodes represent? What do the numbers on the nodes represent? Bootstrap? How many replicates? When I refer back to the methods, I can see that 1,000 bootstrap replicated was done. Might be worth while to state this in the figure legend for readability.

Response:
Thanks very much for reviewer’s comments. This question is a duplicate of the above, and we have revised it according to the reviewers’ good suggestions. We are very grateful to the reviewer.

Minor:
1) Only a minor point, so please feel free to disregard this comments. I can appreciate that English may not be everyones first language. Maybe the authors can revisit if they would like to use American (USA) English spellings or British English spellings. E.g., Faeces (British) is spelt as feces (USA) above. Bacteraemia (British) or Bacteremia (USA), diarrhoea (British) or diarrhea (USA), favourable (British) or favorable (USA)... ect.

Response:
Thanks very much for reviewer’s suggestions for revisions in the language. We carefully revisited the wording habits in the manuscript, and switched to the American English language style uniformly.

2) Line 22: phylogenetic is spelt incorrectly
Response:
Thanks very much for pointing out the inappropriateness. We have corrected the spelling of this word in the new manuscript Line 23.

3) Line 32: Please change "a diarrhea patient" to "a patient(suffering with diarrhea)"
Response:
Thanks very much for reviewer’s suggestions. We changed to “suffering from diarrhea” in the Line 32 and other place throughout new manuscript.

4) Line 34: Please define what MLSA is an abbreviation of and replace "as well as" with "and"
Response:
Thanks very much for reviewer’s comments. MLSA is the abbreviation of ‘multilocus sequence analysis’; For the first occurrence of abbreviations, we define all their full name throughout the new manuscript. According to the reviewer’s suggestion, we replace "as well as" with "and" in the manuscript Line 34.
5) Line 68: Please change "human beings" to "human hosts"

Response:
Thanks very much for reviewer’s suggestions. We have made a revision in the new manuscript Line 69.

6) Line 102: Please change "46-year-old Uygur (one of the Chinese ethnic minorities) man" to "46-year-old Uygur (one of the Chinese ethnic minorities) male"

Response:
Thanks very much for reviewer’s suggestions. We have made a revision in the new manuscript Line 104.

7) Line 105: Quick note: I believe that LB stands for "Lysogeny broth", not Luria-Bertani medium- or variations of that. I could be wrong, but I am basing this comments from the original 1951 reference. Please note, Luria-Bertani is also described in the legend to Figure S5

Response:
Thanks very much for reviewer’s good suggestions. Lysogeny broth (LB), a nutritionally rich medium, is primarily used for the growth of bacteria. It is also known as Luria broth or Luria-Bertani broth or Lennox broth. Though the name ‘Luria-Bertani broth’ is very widely used. The acronym ‘LB’ has been variously interpreted, perhaps flatteringly, but incorrectly, as Luria broth, Lennox broth, or Luria Bertani medium. For the historical record, the abbreviation ‘LB’ was intended to stand for “lysogeny broth”. Therefore, we have made a correction in the new manuscript Line 107-108, in the legend to Figure S5 Line 586.

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8) Line 107: Data availability. Please specify what data is available in GenBank The sequence read data? Or the assemblies?
Please note, sequence read data is stored on the sequence read archive (SRA), not GenBank (Assemblies). Can you please refer to the BioProject number?
Response:
Thanks very much for reviewer’s comments. We have uploaded the assembled genome sequence of novel species (Brevundimonas pishanensis sp. nov.) to NCBI (Assembly database) and obtained the BioProject number PRJNA780817 and Assembly number JAJKBG000000000. We also marked both numbers in the manuscript (line 393).

9) Line 114: Change “The genome DNA” to “The genomic DNA”
Response:
Thanks very much for reviewer’s suggestions. We have made a revision in the new manuscript Line 112.

10) Line 145: Please define abbreviations when used first. I.e., digital DNA-DNA hybridization (dDDH)
Response:
Thanks very much for reviewer’s suggestions. For the first occurrence of abbreviations, we define all their full name throughout the new manuscript.

11) Line 259: Please change "with VFDB" to "with the VFDB"
Response:
Thanks very much for reviewer’s suggestions. We have made a revision in the new manuscript Line 266.

12) Line 629: Please change "the gene kdsA and acpXL were detected in 95.8% (23/24)" to "the gene kdsA and acpXL were detected in 95.8% (n = 23/24)"
Response:
Thanks very much for reviewer’s comments. We have made a revision in the new manuscript Line 277.

13) Line 272: Please change "The majority of the Brevundimonas species (87.5%, 21/24)" to "The majority of the Brevundimonas species (87.5%, n = 21/24)"
Response:
Thanks very much for reviewer’s comments. We have made a revision in the new manuscript Line 279.

14) Line 294: What kind of alignment?
Response:
Thanks very much for reviewer’s comments. For phylogenetic analysis based on
16S rRNA gene sequences and MLSA concatenated sequence (in the order of gyrB-ppsA-recN-rpoC-rpoD), we used the ‘ClustalW module’ that comes with the MEGA software version 7.0.21 to align multiple gene sequences. On this basis, we then built an evolutionary tree by MEGA software.
March 26, 2022

Prof. Duochun Wang
National Institute for Communicable Disease Control & Prevention, China CDC
Changbai Road 155, Changping
Beijing 102206
China

Re: Spectrum02468-21R1 (Comparative Genomic Analysis Reveals Potential Pathogenicity and Slow-growth Characteristics of genus Brevundimonas and Description of Brevundimonas pishanensis sp. nov.)

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Supplemental Material: Accept