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Expanded Multilocus Sequence Typing for *Burkholderia* Species▽†

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PCR primers targeting loci in the current *Burkholderia cepacia* complex multilocus sequence typing scheme were redesigned to (i) more reliably amplify these loci from *B. cepacia* complex species, (ii) amplify these same loci from additional *Burkholderia* species, and (iii) enable the use of a single primer set per locus for both amplification and DNA sequencing.

The multilocus sequence typing (MLST) scheme for the *Burkholderia cepacia* complex has provided important insights into the population dynamics, diversity, and recombination events in this group of opportunistic pathogens (1–3, 7). It has also been effective in identifying previously misclassified strains and has proved useful in the recent identification of seven novel species in the *B. cepacia* complex (9, 10). This expansion of the *B. cepacia* complex and our growing appreciation that other *Burkholderia* species, particularly *B. gladioli*, are also involved in human infection, provide an opportunity to expand the capacity of the current MLST scheme. We thus sought to redesign the primers targeting the loci in the current MLST scheme to (i) more reliably amplify these seven loci from all 17 *B. cepacia* complex species; (ii) amplify these loci from additional *Burkholderia* species, including *B. gladioli* and as yet unclassified *Burkholderia* species; and (iii) enable the use of a single primer set per locus for both amplification and DNA sequencing.

We aligned all seven loci (*atpD*, *gltB*, *gyrB*, *lepA*, *phaC*, *recA*, and *trpB*) in the current MLST scheme from *Burkholderia* strains for which complete genome sequences were available in the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi) or Joint Genome Institute (http://www.jgi.doe.gov/genome-projects/) database. These strains included *Burkholderia ambifaria* AMMD, *B. cenocepacia* J2315, *B. cenocepacia* AU1054, *B. cenocepacia* PC184, *B. multivorans* ATCC 17616, *B. vietnamiensis* G4, *B. dolosa* AU0158, *B. xenovorans* LB400, *B. phymatum* STM815, *B. phytofirmans* PsJN, *B. mallei* ATCC 23344, *Burkholderia* sp. strain 383, and *Burkholderia* sp. strain H160. Open reading frames were aligned by using MegAlign (DNASTAR Inc., Madison, Wis.), and *Burkholderia* genus-level primers were designed to amplify sequences that include the regions amplified by the original MLST primers (3) (Table 1).

To test the utility and range of the new primers, we performed MLST analysis on strains representing multiple *Burkholderia* species. Strains were obtained from the strain collections of the *Burkholderia cepacia* Research Laboratory and Repository (University of Michigan, Ann Arbor, MI) and Cardiff University (Cardiff, Wales, United Kingdom). Bacteria were cultured and lysed as described previously (4). Briefly, a single bacterial colony was suspended in 20 μl of lysis buffer containing 0.25% (vol/vol) sodium dodecyl sulfate and 0.05 N NaOH. After the suspension was heated for 15 min at 95°C, 180 μl of high-performance liquid chromatography-grade H_2O was added, and the bacterial lysate suspension was stored at 4°C. Amplification of targeted DNA was performed in 25-μl reaction mixture volumes containing a final concentration of 2 mM $MgCl_2$, 20 mM Tris-HCl, 50 mM KCl, 250 μM of each deoxynucleoside triphosphate (ISC Bioexpress, Kaysville, UT), 0.4 μM of each primer, 1 M betaine (Sigma-Aldrich, St. Louis, MO), 10% dimethyl sulfoxide (Sigma-Aldrich), 2 U *Taq* polymerase (Invitrogen, Carlsbad, CA), and 2 μl of bacterial lysate. Amplification was performed with a PTC-100 (MJ Research Inc., Waltham, MA) thermocycler. After an initial denaturation step of 2 min at 95°C, 30 PCR cycles were completed, each PCR cycle consisting of 30 s at 94°C, 30 s at the appropriate annealing temperature (Table 1), and 60 s at 72°C, followed by a final extension step of 5 min at 72°C. Sequence analysis and editing of the amplified DNA were performed as previously described (8).

Each distinct sequence (allele) at each of the seven loci was assigned a unique arbitrary number (allele type). For each allelic profile (considered to be isogenic when they were indistinguishable at all seven loci), a unique arbitrary sequence type number was assigned. All sequences were deposited in the *Burkholderia cepacia* complex MLST database at http://pubmlst.org/bcc. Pairwise analysis using Clustal V (DNAStar) was employed to construct gene trees of the concatenated sequences of all distinct sequence types.

To confirm that the new primers more reliably amplify MLST loci from *B. cepacia* complex strains, we successfully amplified 43 loci (7 *atpD* loci, 10 *gltB* loci, 17 *gyrB* loci, 2 *lepA* loci, 3 *phaC* loci, 2 *recA* loci, and 2 *trpB* loci) from 25 *B. cepacia* complex strains that previously had failed to amplify with the primers and PCR conditions described for the original *B.*

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TABLE 1. Oligonucleotide primer sequences and annealing temperatures for the amplification and sequencing of seven MLST loci for *Burkholderia* species

Gene	Amplicon size (bp)	Primer sequence (5′→3′)	Annealing temp (°C)
atpD	756	ATGAGTACTRCTGCTTTGGTAG AAGG CGTGAAACGGTAGATGTT GTCG	56
gltB	652	CTGCATCATGATGCGCAAGTG CTTGCCGCGGAARTCGTTGG	58
gyrB	738	ACCGGTCTGCAYCACCTCGT YTCGTTGWARCTGTCGTTCCA CTGC	60
lepA	975	CTSATCATCGAYTCSTGGTTCG CGRTATTCCTTGAACTCGTA RTCC	55
phaC	525	GCACSAGYATYTGCCAGCG CCATSTCSGTRCCRATGTAGCC	58
recA	704	AGGACGATTCATGGAAGA WAGC GACGCACYGAYGMRTAGA ACTT	58
trpB	787	CGCGYTTCGGVATGGARTG ACSGTRTGCATGTCCTTGTCG	58

cepacia complex MLST scheme (3) (see Table S1 in the supplemental material). To assess the utility of the new primers at the genus level, we amplified all seven loci from 80 strains representing 36 named *Burkholderia* species (including 16 of the 17 *B. cepacia* complex species) as well as another 14 *Burkholderia* strains that remain unclassified with respect to species (see Table S2 in the supplemental material). In all cases, the new genus-level primer sets provided PCR products of the expected size that were confirmed as the intended target by DNA sequence analyses.

The original *B. cepacia* complex MLST scheme was designed at a time when the *B. cepacia* complex was comprised of nine species and the complete genome sequences of only three *B. cepacia* complex strains were available (3). The *B. cepacia* complex now includes 17 distinct species, and a total of 68 *Burkholderia* strains have had or are in the process of having their genome sequences determined; at least 16 of these strains represent species within the *B. cepacia* complex. This expanded *Burkholderia* taxonomy and the marked increase in *Burkholderia* genome sequence information have provided an opportunity to address shortcomings in the original MLST methodology. Most problematic is that the original PCR primers fail to amplify the intended target from a significant minority of *B. cepacia* complex strains. Indeed, we have found that approximately 10% of *B. cepacia* complex strains analyzed included at least one locus that could not be amplified by using the original MLST primers (data not shown). Commonly, such failures involve strains from the more recently described *B. cepacia* complex species; however, loci in strains belonging to the older *B. cepacia* complex species (e.g., *B. multivorans* and *B. cenocepacia*) have also failed to amplify with these primers. The original *B. cepacia* complex MLST method also employed two sets of oligonucleotide primers per locus: one pair to amplify the target locus and a second internal nested pair for DNA sequencing. This approach is advantageous in allowing greater flexibility in designing sequencing primers when the genetic diversity of the targeted loci is unclear. However, the greater *Burkholderia* genome sequence data now available allowed the design of a single set of primers for both amplification and sequencing.

The MLST primers described herein were specifically designed to amplify sequences that include the regions amplified by the original MLST primers. Therefore, the new primers provide results that are entirely compatible with the current *B. cepacia* complex MLST scheme. These primers were also designed to amplify larger regions (50 bp to 200 bp) of DNA flanking the loci included in the original MLST scheme. This allows a single PCR per locus to amplify a region with enough flanking DNA to provide high-quality sequence read coverage of the target of interest. The redesigned primers and PCR conditions also improve the performance of MLST for *B. cepacia* complex species; we observed no loci that failed to amplify with the new primers and PCR conditions, including several from a set of 25 *B. cepacia* complex strains that failed to amplify with the original *B. cepacia* complex MLST primers. A potential disadvantage of the new primers and PCR conditions, however, is that since not all loci are amplified with the same annealing temperatures (Table 1), complete MLST analysis cannot be performed with a single PCR run.

The use of degenerate primers allowed expansion of the *B. cepacia* complex MLST scheme to include analysis of other *Burkholderia* species, including the clinically relevant species *B. gladioli*, *B. mallei*, and *B. pseudomallei*, as well as species important in agriculture and the environmental sciences, such as *B. fungorum*, *B. glumae*, and *B. plantarii*. Given their degenerate design, however, it is unlikely that these primers are specific only for *Burkholderia* species; further testing of a broader range of related genera is required in this regard. MLST analysis of *B. mallei* and *B. pseudomallei* may also be performed by using a previously described scheme that includes two of the seven target genes (*gltB* and *lepA*) in the *B. cepacia* complex MLST scheme (5).

We included in our analysis four *Burkholderia* strains that previous analyses, including *recA* and 16S rRNA gene sequencing, indicated were members of the *B. cepacia* complex but could not be placed into any of the 17 named species in this group. MLST analysis of these strains (AU0141, AU7004, BC0021, and AU3207; Fig. 1) supports their taxonomic position within the *B. cepacia* complex and their status as being currently unclassified with respect to species. We also included four strains (AU9801, AU15822, AU12613, and AU13858; Fig. 1) that were recovered from patient specimens and previously identified to the *Burkholderia* genus level by 16S rRNA-directed PCR analyses (6). MLST analysis of strains AU9801, AU15822, AU12613, and AU13858 provided tentative assignment as *Burkholderia plantarii*, *B. tropica*, *B. fungorum*, and *B. caledonica*, respectively. These species are rarely recovered from infected humans, and all were initially misidentified by commercial phenotype-based identification systems. The analysis of clinical isolates representing potentially novel *B. cepacia* complex species and *Burkholderia* species that are rarely en-

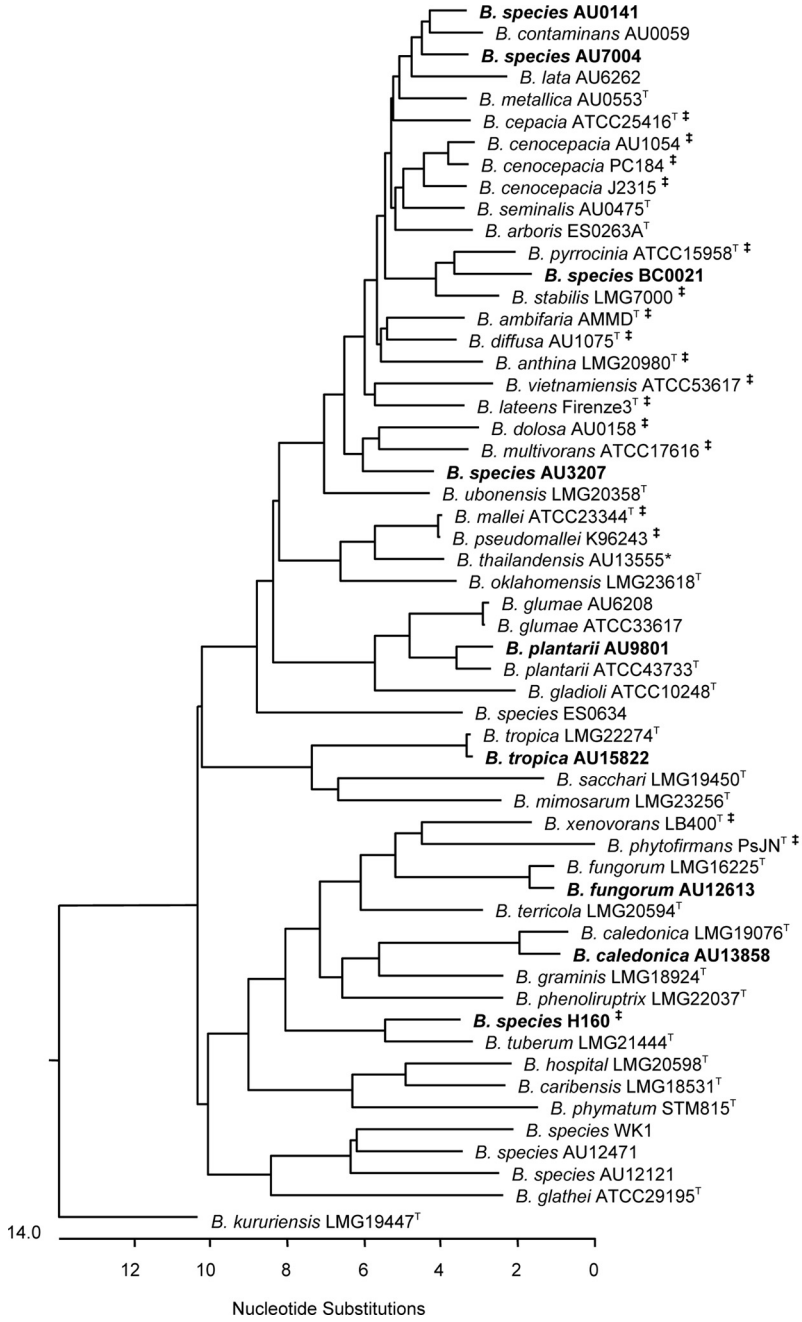

FIG. 1. Phylogenetic tree of concatenated nucleotide sequences from the seven MLST loci, generated by using Clustal V. *Burkholderia* strains included are representatives of the strain set analyzed as part of this study (see supplemental material) or strains for which genome sequence data were available in public databases (indicated by ‡ symbol). *Burkholderia* strains in bold type are discussed in the text. Strains belonging to various *Burkholderia* species and *Burkholderia* sp. strains are shown.

countered from human specimens highlights the utility and expanded capacity of the revised MLST scheme.

Finally, our study also revealed a potential limitation to the *Burkholderia* MLST scheme that has not been described in previous studies. Amplification and sequence analysis of the *phaC* locus in *Burkholderia* sp. strain H160 suggested the presence of two copies of this gene. In silico analysis of the draft genome sequence of this strain (currently being sequenced by the Joint Genome Institute [http://genome.jgi-psf.org]) confirmed the presence of two *phaC* homologues with 83% sequence identity, one on each of the two replicons of this genome. The presence of multiple copies, which may not be 100% identical, of any MLST locus confounds the interpretation of results and could limit the utility of this method for epidemiologic and population genetic studies. This finding also highlights a limitation of applying MLST analysis to genetically plastic bacterial species in which large-scale genetic rearrangements, which may or may not be apparent by MLST, are

possible. Depending on the questions being addressed, other genotyping methods that provide a more comprehensive assessment of the whole bacterial genome may complement MLST analysis.

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