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Elucidating Global Epidemiology of *Burkholderia multivorans* in Cases of Cystic Fibrosis by Multilocus Sequence Typing^{∇†}

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Burkholderia multivorans is a prominent *B. cepacia* complex (BCC) species causing infection in people with cystic fibrosis. Despite infection control measures being introduced to reduce the spread of BCC there is a continued emergence of infections by *B. multivorans*. Our objective was to analyze a global collection of *B. multivorans* isolates, comparing those from environmental and clinical sources with those from reported outbreaks. Multilocus sequence typing (MLST) was performed on 107 *B. multivorans* isolates to provide a detailed analysis of the global population biology of this species. MLST resolved 64 *B. multivorans* sequence types. Twelve of these were globally distributed and associated with human infection; two of these (ST-21 and ST-375) were also composed of environmental isolates. These global lineages included strains previously linked to large outbreaks (e.g., French epidemic clone ST-16). Though few environmental isolates of *B. multivorans* were available for analysis, of six strains identified, three were identical to strains recovered from cystic fibrosis (CF) infection. Although the ability of *B. multivorans* to cause CF outbreaks is known, our report here concerning the existence of globally distributed *B. multivorans* CF strains is a new observation for this emerging *B. cepacia* complex pathogen and suggests that certain strain types may be better adapted to human infection than others. Common transmission-associated risk factors were not obviously linked to the globally distributed strains; however, the overlap in strains recovered from water environments, industrial products, and human infection suggests that environmental sources may be an important reservoir for infection with *B. multivorans*.

Burkholderia multivorans is one of at least nine closely related gram-negative species that comprise the *B. cepacia* complex (BCC) (35). Originally these species were considered to be onion pathogens and were subsequently found to possess many beneficial properties for agricultural use (25). However, after more than two decades they have come to be widely known to cause problematic pulmonary infections in vulnerable individuals, particularly in people with cystic fibrosis (CF) (9).

B. multivorans and *Burkholderia cenocepacia* are the two predominant BCC species causing human infections, though bacteria from all currently defined BCC species have been cultured from CF sputum (19, 26, 28). However, most research has concentrated on *B. cenocepacia*, which is widely considered to be the most virulent BCC species, with much less information available on *B. multivorans*. Comparison of recent epidemiological surveys (4, 12, 28) with older studies (19, 26, 32) suggests that the proportion of BCC-infected CF patients in-

fecting with *B. multivorans* is rising relative to the proportions of infection with *B. cenocepacia* and the remaining BCC species, with prevalence in the United States ranging from 38% (28) to 51% (4) of BCC infections. This proportional rise is due to a decline in the total incidence of *B. cenocepacia* while *B. multivorans* acquisition has remained steady (D. Henry, D. Speert, and J. LiPuma, unpublished data). It is unclear why the incidence of *B. cenocepacia* has declined and that of *B. multivorans* has persisted, but it seems most likely that infection control measures are effective at limiting the interpatient spread of “epidemic” *B. cenocepacia* strains (12, 28). Furthermore, regional differences in *B. multivorans* recovery rates in cases of CF result in conflicting reports on the emergence and epidemiology of *B. multivorans* in this population (15, 26, 27, 34, 38).

The introduction of stringent infection control measures has been shown to reduce the incidence of patient-to-patient spread of *B. cenocepacia* (5, 26) and presumably has reduced interpatient spread of other BCC species. However, the continued emergence of *B. multivorans* strains (4, 28) suggests acquisition from other sources, such as the natural environment. Recent studies have found evidence that supports the potential for acquisition by humans of BCC, including *B. multivorans*, directly from natural environments (1, 14, 18).

The purpose of this study was to characterize the *B. mul-*

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tivorans population from clinical and environmental sources in order to evaluate strain distribution and propose mechanisms explaining its continued emergence in CF infection.

MATERIALS AND METHODS

Strains and culture. The 107 *B. multivorans* isolates examined in this study were drawn from the following collections: Cardiff University, Cardiff, Wales, United Kingdom (1–3, 22); the European *B. cepacia* Complex Referral Laboratory, Gent, Belgium (7, 36); the U.S. *B. cepacia* Research Laboratory and Repository, Ann Arbor, Michigan (19); and representatives of a published strain panel (8, 24). These collections contained isolates evaluated in previous molecular epidemiological studies (4, 8, 19, 24). All isolates from clinical sources were obtained with the consent of patients. Bacteria were cultured and identified using polyphasic taxonomic approaches as described previously (22). Isolates were all independently gathered and genetically typed by random amplified polymorphic DNA analysis (23), *recA* analysis (22, 36), repetitive extragenic palindromic PCR using a BOX A1R primer (4), or pulsed field gel electrophoresis (22) prior to inclusion in the study. All Laboratorium voor Microbiologie, Universiteit Gent (LMG), strains are available from the Belgium Coordinated Collections of Microorganisms (<http://bccm.belspo.be/>).

Multilocus sequence typing. To assess the genetic diversity of this species we examined isolates evaluated in previous molecular epidemiological studies (4, 8, 19, 24) that were (i) temporally diverse (distributed over a more than 40-year period from 1966 to 2006), (ii) geographically diverse (isolated in 12 countries on three continents), and (iii) from samples obtained from a range of natural, industrial, and clinical (isolates from CF and non-CF patients) environments. We carried out MLST analysis as previously described for the BCC by use of a single scheme for all BCC species (2). Each distinct (allele) sequence at each of seven genes 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 (ST) number was assigned. All sequences were deposited in the *Burkholderia cepacia* complex MLST Databases at <http://pubmlst.org/bcc/>. Novel sequence information for all seven loci was obtained for 107 *B. multivorans* isolates.

Analysis of MLST data. To construct gene trees of the concatenated sequences (2,773 bp) for the 64 *B. multivorans* STs, the neighbor-joining Jukes-Cantor method was used (MEGA v3; <http://www.megasoftware.net>). The significance of branching within the trees was evaluated by bootstrap analysis of 1,000 computer-generated trees. The STs within a clonal complex (CC) group were defined as having at least five MLST loci in common.

RESULTS

Assignment of allele and sequence types. For thorough evaluation of the *B. multivorans* species it was important to use a collection of strains identified as being diverse on the basis of analyses of *recA* sequences, *recA*-RFLP, repetitive extragenic palindromic-PCR using a BOX A1R primer, random amplified polymorphic DNA, and pulsed field gel electrophoresis profiles, primarily to obtain different STs. For each locus all alleles were found to be of identical lengths (2) for all *B. multivorans* isolates examined. Nucleotide sequence diversity was found at all seven loci as shown in Table 1. The numbers resolved from this BCC MLST scheme at each locus for *B. multivorans* ranged from 13 (*phaC*) to 43 (*gyrB*) alleles (mean = 28.0; Table 1).

Diversity of *B. multivorans* strains. In order to investigate the relationships and diversity of the *B. multivorans* isolates, two methods were used: concatenation and phylogenetic analysis of the nucleotide sequences for all seven MLST loci (2). We analyzed the concatenated sequences (2,773 bp) for the 64 *B. multivorans* STs alongside the 114 STs of all BCC species used to validate the BCC MLST scheme (2). *B. multivorans* was clearly resolved (100% bootstraps based on 1,000 randomizations) into a broad cluster distinct from all other BCC species (Fig. 1) concurrent with the identification of all isolates as representing *B. multivorans* prior to this study. The average concatenated nucleotide diversity among *B. multivorans* strains

TABLE 1. Allelic variation at each locus among 107 *B. multivorans* STs examined

| MLST locus | Allele size (bp) | No. of alleles |
|-------------|------------------|----------------|
| <i>atpD</i> | 443 | 23 |
| <i>gltB</i> | 400 | 33 |
| <i>gyrB</i> | 454 | 43 |
| <i>recA</i> | 393 | 29 |
| <i>lepA</i> | 397 | 27 |
| <i>phaC</i> | 385 | 13 |
| <i>trpB</i> | 301 | 28 |

was 1.0%, with the greatest diversity representing no more than 2.0% nucleotide divergence. This phylogenetic analysis identified a separate subgroup within *B. multivorans* comprised of STs 188, 194, 308, 320, 390, and 397 (Fig. 1), all of which demonstrated clear evidence of interspecies recombination events (see the supplemental material).

Global distribution and regional outbreaks of *B. multivorans* infections. In order to determine whether there are globally distributed *B. multivorans* strains in cases of CF infection we examined the prevalence of several isolates previously implicated in cases of patient-to-patient spread among isolates in a diverse global collection.

A globally distributed strain (ST-16) was found; among the isolates found in the global distribution was an isolate previously reported as being an epidemic strain causing patient-to-patient spread (31). This isolate, originally reported as part of another large *B. multivorans* outbreak in France among 22 CF patients that was caused by a strain of PCR ribotype X (31), was identified as ST-16 and was also found to have infected CF patients in Belgium (strain LMG14273), Australia (BCC0247), Canada (C6558), and New Zealand (SBL03-088 and SBL04-172) and a non-CF patient in the United States (CEP0600).

In addition to ST-16, a further 11 STs were identified among isolates found in more than one country over more than a decade: ST-17 (New Zealand and the United States), ST-18 (Canada and the United Kingdom), ST-21 (Canada and the United States), ST-24 (Canada and Brazil), ST-181 (Czech Republic and New Zealand), ST-190 (Canada and the United States), ST-195 (Canada and the United Kingdom), ST-198 (Canada and the United States), ST-270 (Canada and Belgium), ST-274 (Australia and New Zealand), and ST-375 (Belgium and Portugal). Note that the high representation of some countries in this list may be due largely to ascertainment bias based on disproportionate contribution of isolates to this study. Overall, half of the clonal complexes identified (CC1, CC4, CC5, and CC6) were clearly found to be distributed among different countries (including Canada, the United Kingdom, the United States, France, Portugal, Belgium, New Zealand, and Australia) on different continents. A further clonal complex (CC3) was found in clinical isolations in different countries on the same continent (Czech Republic and France).

For all cases of implied patient-to-patient spread examined, unique STs were found in each instance, with no further matches found within our global collection: ST-27 caused an outbreak among CF patients in Glasgow (38), ST-25 (previously named strain OBHM) was shared by U.S. patients (4), ST-180 caused multiple infections at a single treatment center

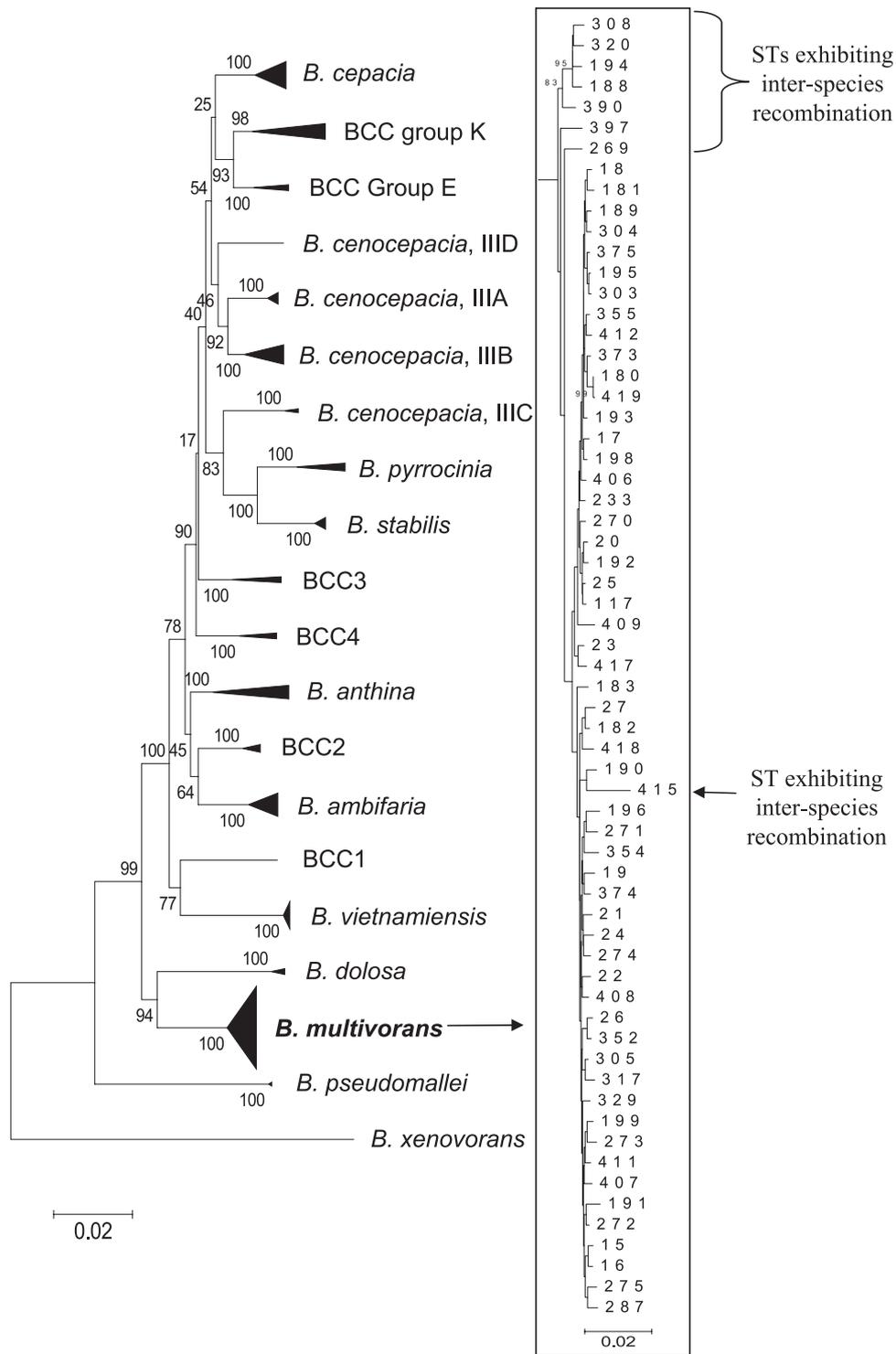


FIG. 1. Phylogenetic tree of concatenated sequence for the MLST alleles for *B. multivorans* STs in the context of other BCC species by use of the neighbor-joining Jukes-Cantor method, with STs exhibiting interspecies recombination among the MLST loci indicated.

(Prague, Czech Republic) among CF and non-CF patients, ST-179 (previously named strain TUL2) was shared by U.S. patients (4), ST-199 was shared by Canadian siblings with CF (26), ST-15 caused an outbreak among CF patients in South Wales (27), and ST-419 was found in an isolate causing an

outbreak among 32 patients in France with PCR ribotype F. Interestingly, ST-15 (the South Wales outbreak strain) was also found in a clonal complex (CC1; supplemental material) with ST-16 (the globally distributed strain also identified as being part of a French outbreak).

Identification of recombination. By comparing the allelic variations present within clonal complexes, it is possible to suggest whether the changes are due to point mutation (clonal evolution) or to recombination events. For example, when six alleles are identical between two STs in a clonal complex but one allele is different with respect to numerous nucleotides, this is likely the result of recombination. However, point mutations usually give rise to novel alleles, so wherever a change was identified and that allele was already commonly found in the MLST database, it was considered to have occurred through recombination (see the supplemental material). Among the eight clonal complexes identified, the allelic changes mostly appeared to be due to either intraspecies recombination or interspecies recombination, with only one change presumably by point mutation (see the supplemental material).

As the BCC MLST scheme incorporates at least nine species in a single approach it facilitates our identification of genetic exchange between MLST loci of different BCC species. Of the 15 alleles identified as having occurred through recombination the majority of these (8 alleles; 53%) appear to have arisen more specifically through interspecies recombination with other BCC species. In total, among the 64 *B. multivorans* STs, 12.7% (8 STs) exhibit alleles that are between 3.9% and 7.3% different from all other *B. multivorans* alleles and are either the same as or only one (0.3%) nucleotide divergent from alleles common to other BCC species (five alleles of *B. cenocepacia* [ET-12 lineage], two alleles from *B. stabilis*, and one allele from *B. cepacia*).

DISCUSSION

Typing methods for the unequivocal characterization of isolates are essential for global epidemiological and evolutionary analysis of bacterial pathogens. However, methodological differences in genotyping techniques have thus far limited the analysis of *B. multivorans* population genetics. Strain typing based on the comparison of DNA sequences rather than genome organizations or restriction fragments is a more reliable and an unambiguous indicator of strain identification. MLST has been shown to be an easily transferable, precise, and reproducible tool for typing BCC species (1, 2, 11, 20, 37). It is a simple tool that can offer a high level of strain identification without using polyphasic techniques. The sequence information is deposited in an online database resource accessible via the World Wide Web, which facilitates multicenter collaborative analysis in a way not previously possible. MLST is an important tool in assessing *B. multivorans* epidemiology, and it can be used to clearly identify widely distributed strains and assist in the global infection control of this pathogens. In the current study, MLST was for the first time able to demonstrate the characteristics of globally distributed *B. multivorans* CF strains and provide a unique insight into the population biology of this pathogen.

The prevalences of each BCC species in CF are not equal, with *B. cenocepacia* and *B. multivorans* being most prominent (19, 21, 25, 28). Historically, *B. cenocepacia* strains were responsible for the largest epidemics among CF communities in Canada (23, 32) and the United Kingdom (13) during the 1980s and 1990s before strict infection control measures were introduced (29). In addition, several *B. cenocepacia* strains

have reached an epidemic status within CF communities and are extremely virulent. Therefore, the issue of strain transmissibility is of major importance to individuals with CF and the treatment centers that provide their care. In contrast to *B. cenocepacia*, where direct patient-to-patient contact and socialization have been reported as the most probable mechanisms by which infections were spread (13, 26), the mode of transmission for *B. multivorans* infection has not been determined. Several reports have identified multiple patients in a specific area who acquired the same *B. multivorans* strain at the same time (4, 26, 27, 31, 38).

MLST concatenated sequence analysis, as predicted from a previous MLST study, (2), clearly distinguished the 64 *B. multivorans* STs from all other BCC species (100% bootstraps). The diversity seen among the *B. multivorans* STs corresponds to that seen in other large studies of the prevalence of *B. multivorans* strains in CF patients (26, 34).

Where spread of a *B. multivorans* strain had been reported, MLST corresponded in each instance to the original finding that a single strain type was responsible (see the supplemental material). No further infections with seven of these outbreak strains (ST-15, ST-25, ST-27, ST-117, ST-179, ST-199, and ST-419) have been reported or were found in this study; however, we were able to identify four independent ST-16 isolates (see the supplemental material) recovered from clinical infections outside of France, where the first CF-related outbreak of this strain was reported (30, 31). *B. multivorans* ST-16 was found in six countries on three continents; this represents the first reported globally distributed *B. multivorans* strain causing infections in individuals with and without CF. The global presence (see the supplemental material) and regional outbreaks (30, 31) of ST-16 suggest that this *B. multivorans* strain may represent a more transmissible CF lineage, such as those of *B. cenocepacia* ET-12 (25) and PHDC (6). There was no epidemiological evidence to link the spread of *B. multivorans* among patients of different geographic regions. Although ST-16 was originally identified as an epidemic strain in France (30, 31) and has been found to be the most common ST within isolates examined among different nonsibling patients in the Auckland area of New Zealand (C. Pope, unpublished data), it does not appear to have spread within the other four additional locations in which it has been recovered (see the supplemental material). This observation may reflect differences in hygiene policies and host susceptibilities or subtle genomic differences among isolates of this strain that may have profound effects upon transmissibility. A finding which adds weight to the notion that ST-16 is perhaps better adapted to human infection than other *B. multivorans* strains is that it is just a single-locus variant (*trpB*) of ST-15 and hence part of a clonal complex (CC1; see the supplemental material); thus, two closely related strain types were each associated with infection in multiple patients (27, 30, 31).

In total, 18.5% of *B. multivorans* strains (ST-16, ST-17, ST-18, ST-21, ST-24, ST-181, ST-190, ST-195, ST-198, ST-270, ST-274, and ST-375) were found to be dispersed across multiple countries. The observation that in the same locations, some genotypes remain unique to individual patients whereas other genotypes infect several patients is consistent with some strains having a higher degree of transmissibility, but transmission may not be via patient contact alone. Acquisition from

environmental sources should be considered (1), and the prevalence of certain strains among clinical isolates may also reflect the distribution of *B. multivorans* in contaminated products and local environments. The recent description of a major outbreak among CF patients linked to contaminated commercial saline solutions (10) highlights the fact that despite stringent infection control guidelines and the limited individual contact advocated by the CF community, contamination still provides a potential source of infection. MLST is highly suited to identifying such links between source, contamination, and infection (1, 20).

Though many unique STs were identified in isolates from CF patients, there was also evidence for *B. multivorans* strains having undergone recombination. An ability to exchange genetic material is of growing clinical interest and concern, as recombination of even a single gene could have profound effects, including increased resistance to antimicrobials, increased virulence, and potential vaccine immunity in the future. Recombination of the MLST loci could be found within strains from different geographic locations and was not limited to either just clinical or just environmental isolates. Though we were able to identify recombination only among the MLST genes it is unlikely that it is limited to these, and many other genes not involved in essential processes might recombine at a higher rate. Certainly systems exist in the BCC to facilitate recombination, with an extensive presence of insertion sequences (16), phages (33), and conjugative transfer genes and genomic islands (3). We have found evidence that as many as one in eight *B. multivorans* strains in this study appear to have recombined with strains from other species of the BCC.

A large proportion (62.5%) of the interspecies recombination of MLST loci found for clinical *B. multivorans* strains was identified as genetically indistinguishable from that seen with alleles of *B. cenocepacia* ET-12 strains. As *B. cenocepacia* ET-12 is mainly isolated from clinical settings and not from the natural environment we assume not only that these species occupy the same niche but also that genetic exchange is occurring within humans and not in the natural environment. Evidence for this comes from the fact that all the recombination with the ET-12 lineage was identified within samples isolated from sputum of CF patients (ST-188, ST-194, ST-308, and ST-320). In two of these cases (ST-308 and ST-320) an earlier sputum sample revealed a *B. multivorans* strain that appeared to represent the same strain prior to recombination (ST-199 and ST-317). This might imply that recombination has occurred within the CF patient and not simply that acquisition of a new closely related strain had occurred (37). However, the patients whose isolates showed the presence of ST-320 and ST-308 had no exposure to ET-12-infected patients, as it was absent from the hospital environment; in the case of ST-320, the hospital has been free of ET-12 for several years. So although it is difficult to identify the specifics of how these recombination events occurred, recombination still has an important, albeit undefined, role in the long-term evolution of pathogenicity for *B. multivorans* strains.

As *B. multivorans* is one of the two most prevalent BCC species in CF infections (along with *B. cenocepacia*) it is important to identify environmental and clinical reservoirs and the route of acquisition by patients. Though *B. multivorans* is infrequently isolated from the natural environment, MLST has

shown that identical STs can be isolated in nature and from clinical settings (ST-21 and ST-375). As the isolate for ST-375 (R-20526) was from a water source, this may explain the distribution of *B. multivorans* strains and their occurrence in geographically distinct regions (1). Furthermore, ST-373 (R-11581) was isolated from an industrial product. Our findings are consistent with the view that the use of BCC bacteria by agriculture and biotechnology industries represents a potential clinical risk to susceptible members of the community (17). We are currently seeking to evaluate more thoroughly different ecological niches such as water sources, industrial processes, and domestic products in order to identify whether any of them represents a high-risk source of *B. multivorans* for CF patients. The ability to carry out both strain differentiation and species identification in a single approach and then also to link globally distributed strains represents a major advance that will greatly enhance the clinical epidemiology of *B. multivorans* infection.

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