Multilocus Restriction Typing: A Novel Tool for Studying Global Epidemiology of Burkholderia cepacia Complex Infection in Cystic Fibrosis

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Burkholderia cepacia complex infections contribute significantly to mortality and morbidity in persons with cystic fibrosis (CF). The aim of this study was to evaluate the use of a novel typing method, multilocus restriction typing (MLRT), for investigation of the global epidemiology of B. cepacia complex genomovar III, the species most commonly encountered in CF. In the MLRT method, variation at several loci is indexed by restriction analysis of polymerase chain reaction–amplified genes. Data obtained by MLRT and pulsed-field gel electrophoresis analysis of a large number of B. cepacia genomovar III isolates (including isolates belonging to epidemic lineages and environmental isolates) show a strong correlation. MLRT extends the utility of isolate genotyping by allowing comparisons of isolates collected in studies of larger scale (both temporal and spatial). The portability of MLRT data will facilitate comparison of data obtained in different laboratories. In addition, data obtained with MLRT can be used in studies of bacterial population structure.

The Burkholderia cepacia complex is comprised of 8 genomic species: B. cepacia genomovars I, III, and VI, B. multivorans, B. stabilis, B. vietnamiensis, B. ambifaria, and B. pyrrociniua [1]. B. cepacia complex infections in immunocompetent patients occur only sporadically, although nosocomial infections have been reported, and they are often caused by contaminated disinfectants and anesthetic solutions [2]. B. cepacia complex organisms are important opportunistic pathogens in persons with cystic fibrosis (CF) [2, 3]. In CF, B. cepacia complex strains can spread between patients within the hospital or through social contact outside the hospital. Infection has a significant impact on rates of morbidity and mortality. Therefore, strict infection-control measures are recommended to reduce patient-to-patient spread [2, 3].

Although all members of the B. cepacia complex have been identified in CF sputum cultures, most clinical isolates belong to B. cepacia genomovar III [4, 5]. Furthermore, at a subspecies level, some specific strains are more commonly found in CF than others. Most of these so-called “epidemic” strains are B. cepacia genomovar III. These include the transatlantic ET12 clone, which is responsible for infecting many patients with CF in Canada and the United Kingdom [6], the PHDC clone, which infects nearly all B. cepacia complex–infected patients receiving care in large CF treatment centers in 2 mid-Atlantic US cities [7], and a clone, which is responsible for numerous infections in the midwestern region of the United States [8] (here designated the Midwest clone). Recent studies [9, 10] have shown that B. cepacia genomovar III is also present in various environmental niches, including the rhizosphere of several widely cultured plants, such as maize, wheat, and lupin. The observation that infection-control measures have reduced, but not eliminated, new infections has raised speculation that the environment serves as the reservoir for acquisition of novel B. cepacia genomovar III strains; analysis of a large number of environmental and clinical isolates recovered from the same geographic area will be required to answer this question.

Over the past 2 decades, the relationship between B. cepacia complex isolates has been assessed by various methods, including ribotyping [8, 11], multilocus enzyme electrophoresis (MLEE) [12, 13], randomly amplified polymorphic DNA (RAPD) typing [14, 15], pulsed-field gel electrophoresis (PFGE) [15–17], and flagellin typing [18, 19]. Most of these methods are primarily suitable for comparison of sets of isolates obtained during outbreak investigations; their utility for answering questions with regard to long-term epidemiology is limited [20, 21]. To date, the tool most used in studies addressing questions regarding global epidemiology has been MLEE. In this method, allelic variation in sets of randomly selected housekeeping genes is indexed through the electrophoretic mobility of the corresponding enzymes [22]. Multilocus sequence typing (MLST), in which allelic variation of several loci is examined by direct nucleotide sequencing, has emerged recently as a powerful and portable replacement for MLEE [20]. However, MLST requires high sequencing throughput and is, compared with other methods, relatively expensive [23, 24].

An alternative to MLST is multilocus restriction typing (MLRT) [25], in which variation at several loci is indexed by restriction analysis of polymerase chain reaction (PCR)–amplified genes. Data generated in this way are amenable to analysis by a variety
of methods that allow an assessment of relatedness between distinct clonal lineages, including split decomposition analysis, a recently developed technique used to analyze the structure of dissimilarity matrices [26]. Unlike many other algorithms used to construct trees, split decomposition does not force data into a bifurcating tree and allows the detection of conflicting data, suggestive of recombination. This method has been applied recently in studies of viral evolution [27, 28] and in the analysis of bacterial population structure [29–31].

The aim of this study was to evaluate the use of MLRT for typing of B. cepacia genomovar III isolates collected worldwide over an extended period. As an initial step in the assessment of MLRT, we compared data obtained by MLRT and PFGE for a large number of B. cepacia genomovar III isolates, including isolates belonging to major epidemic lineages and environmental isolates, that were collected in the United States, Canada, the United Kingdom, Belgium, and Australia. The data generated by MLRT were also analyzed by use of split decomposition analysis.

**Materials and Methods**

**Bacterial strains and growth conditions.** Of 73 B. cepacia genomovar III isolates included in this study, 54 were recovered from sputum cultures of patients with CF in the United States between 1986 and 2000. Isolates were grown aerobically on Mueller-Hinton broth (Becton Dickinson), supplemented with 1.8% (wt/vol) agar, and incubated overnight at 32°C. All isolates were identified by use of a polyphasic approach employing 16S rDNA- and recA-based PCR assays, as described elsewhere [1, 32].

**PFGE genotyping.** Preparation of agarose-embedded DNA, macrorestriction digests with SpeI, and PFGE were performed as described elsewhere [7]. B. cepacia genomovar III AU2725 was included multiple times on each gel to allow intra- and inter-gel normalization. Densitometric analysis, normalization and interpolation of PFGE patterns, and numerical analysis using the Pearson’s product-moment correlation coefficient were performed, using the Molecular Analyst Fingerprinting Plus software (BioRad).

**MLRT.** DNA from each isolate was prepared by heating 1 or 2 colonies at 95°C for 15 min in 20 µL of lysis buffer containing 0.25% (wt/vol) SDS and 0.05 M NaOH. After lysis, 180 µL of distilled water was added to the solutions, which were then stored at −20°C. Fragments from each of 5 chromosomal loci were obtained by PCR. These included nearly complete open-reading frames of recA, gyrB, flIC, cepIR, and dsbA. The RecA protein is essential for DNA repair and recombination [32], whereas DNA gyrase is a topoisomerase type II that regulates supercoiling of double-stranded DNA [33]. The flIC gene encodes the flagellin protein and has been used as a marker in epidemiologic studies [18, 19]. The cepIR locus codes for the genes involved in quorum-sensing and includes cepI, which directs the synthesis of signaling molecules (N-acyl-homoserine lactones), and cepR, which encodes the cognate transcriptional regulator protein [34, 35]. The dsbA gene encodes a periplasmic disulfide bond oxidoreductase, which is likely involved in the formation of metal efflux and multidrug resistance systems [36].

All PCRs were done in 25-µL reaction mixtures containing 2 µL of DNA solution, 1 U Taq polymerase (Gibco BRL), 250 mM each dNTP (Gibco BRL), 5 µL of 5M betaine (Sigma) (except for the amplification of the gyrB locus), 1X PCR buffer (Qiagen), and 20 pmol of each oligonucleotide primer (table 1). Amplification was carried out using a PTC-100 programmable thermal cycler (MJ Research). After initial denaturation for 2 min at 94°C, 30 amplification cycles were completed, each consisting of 1 min at 94°C, 45 s at the appropriate annealing temperature (table 1), and 1 min at 72°C. A final extension of 10 min at 72°C was applied. Ten microliters of PCR-amplified product was digested, using 5 U of restriction enzyme and the appropriate buffer for 3 h at 37°C. The following restriction enzymes were used: HaeIII (Gibco BRL; recA), MspI (Promega; gyrB, flIC, and cepIR), and DdeI (Gibco BRL; dsbA).

The resulting restriction fragments were separated by electrophoresis in 3% agarose gels (BioRad) in 0.5X Tris-borate–EDTA buffer at 6.5 V/cm for 70 min and visualized by staining with ethidium bromide. A 100-bp ladder (Promega) was used as standard. Gel images were digitized, using a GelDoc 2000 (BioRad), and stored as TIF image files. Digitized images were converted and normalized with Molecular Analyst Fingerprinting Plus software (BioRad). After normalization, we defined a set of bands to each normalized densitometric curve. Similarity between the patterns was calculated, using the Dice coefficient as implemented in the Molecular Analyst Fingerprinting Plus software (both tolerance and optimization parameters were set to 1%). Restriction patterns for each locus were considered to be the same if they had the same number of bands and if they demonstrated ≥92% similarity following cluster analysis, using the above-

### Table 1. Polymerase chain reaction primers used for multilocus restriction typing of Burkholderia cepacia genomovar III.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Target</th>
<th>Sequence, 5′→3′</th>
<th>Annealing temperature, °C</th>
<th>Amplicon size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>BCR1</td>
<td>recA</td>
<td>TGACCGGCGGAAAAGGCAA</td>
<td>58</td>
<td>1043</td>
</tr>
<tr>
<td>BCR2</td>
<td>recA</td>
<td>CTTCTTCTGGCATCAGCACTGTCCTC</td>
<td>65</td>
<td>1260</td>
</tr>
<tr>
<td>UP-1</td>
<td>gyrB</td>
<td>GAGTCTACATGGACCTGCTTGCCATGCGNGNGGNAARTTYGCA</td>
<td>62</td>
<td>1000</td>
</tr>
<tr>
<td>UP-2r</td>
<td>gyrB</td>
<td>AGCGAGTAGATCGGACGCGCCTCAGCAGACCTGTCAT</td>
<td>57</td>
<td>1800</td>
</tr>
<tr>
<td>BC4</td>
<td>flIC</td>
<td>CTGGACTCAGAAGCAGGTGACACCTGGAAC</td>
<td>58</td>
<td>1043</td>
</tr>
<tr>
<td>BCR12</td>
<td>flIC</td>
<td>ACAGTTTGCCGGTTTCTGTC</td>
<td>65</td>
<td>1260</td>
</tr>
<tr>
<td>cepIR-Ba1V</td>
<td>cepIR</td>
<td>GGAAACGGCGGTTCCGGCCTAGCAGCAGCAGCAGCAG</td>
<td>57</td>
<td>1000</td>
</tr>
<tr>
<td>cepIR-Ba2R</td>
<td>cepIR</td>
<td>CGTGGAGCTGGACGCGGCGGAAAGGACGCGGAC</td>
<td>62</td>
<td>2160</td>
</tr>
<tr>
<td>dshAB</td>
<td>dshA</td>
<td>CCGCCGTTCGGCGAAGGCGGCTCG</td>
<td>62</td>
<td>1800</td>
</tr>
<tr>
<td>dshAR</td>
<td>dshA</td>
<td>TACGGGTTCGGCGGCGGCT</td>
<td>58</td>
<td>1043</td>
</tr>
</tbody>
</table>
defined parameters. Different restriction patterns for each locus were considered to represent different alleles, and each allele was assigned an arbitrary number.

The allelic profile (consisting of the allele number for each locus) for each isolate was entered into a database (Excel; Microsoft). The allelic profiles were used to construct a tree based on the unweighted-pair group method with average (UPGMA) linkage of distance, using the START (Sequence Type Analysis and Recombinational Tests) software package (http://outbreak.ceid.ox.ac.uk/software.shtml). START was also used to convert the data set to a distance matrix in NEXUS format. These matrices were then used for split decomposition analysis [26], using SplitsTree 2.0 [37] (http://bibiserv.techfak .uni-bielefeld.de/splits).

Results

**PFGE.** Reproducibility of PFGE profiles was determined by analyzing multiple independently prepared agarose-embedded DNA samples of isolate AU2725. The correlation level between patterns obtained was > 90% (data not shown). PFGE profiles were considered to belong to the same cluster if they shared ≥ 65% similarity following numerical analysis (corresponding to a difference of ≤ 6 bands [data not shown]). By using this cutoff value, 10 clusters could be delineated, whereas several strains occupied separate positions in the cluster analysis (figure 1). Cluster P1 was composed of all 12 isolates belonging to the ET12 clone. Clusters P2 and P3 each contained 3 isolates, while clusters P4, P6, P8, and P9 each contained 2 isolates. Cluster P5 was composed of 5 isolates. Clusters P7 and P10 comprised 12 isolates belonging to the PHDC clone and 15 isolates belonging to the Midwest clone, respectively. Fourteen isolates occupied separate positions in the dendrogram. Despite multiple attempts, we were not able to obtain a PFGE pattern of isolate AU3010.

**MLRT.** Reproducibility of MLRT was determined by generating the 5 restriction fragment–length polymorphism (RFLP) patterns of 6 isolates in duplicate. After numerical analysis, the same allelic profiles were obtained, indicating a reproducibility of 100% (data not shown). Among the 73 isolates studied, 39 different allelic profiles could be recognized (data not shown). The number of different alleles present per locus was as follows: 5 (recA), 10 (gyrB), 14 (fliC), 13 (cepIR), and 26 (dsbA). The frequency of each allele is shown in figure 2.

The UPGMA tree shown in figure 3 was constructed on the basis of the allelic profiles of all isolates studied. Three major subgroups (designated A, B, and C) could be recognized; within these subgroups, several clusters could be delineated at a linkage distance of 0.38 (resulting in clusters containing isolates that differed in not more than 2 loci), whereas multiple isolates occupied separate positions. The major cluster (A4) in subgroup A comprised all representatives of the ET12 epidemic lineage plus 2 additional isolates. Several smaller clusters within subgroup A (designated A1, A2, A3, A5, and A6) could be delineated as well, and these contained 5, 2, 2, 3, and 2 isolates, respectively. Two major clusters could be delineated within subgroup B. The first (cluster B1) contained all isolates belonging to the PHDC lineage, whereas the second (cluster B3) contained all 14 isolates belonging to the Midwest clone. Subgroup B also contained a smaller cluster (B2) containing 3 isolates. Two clusters can be seen in subgroup C (C1 and C2), both containing 2 isolates. All isolates in subgroup C are environmental isolates from Australia.

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**Figure 1.** Dendrogram derived from the unweighted-pair group method, with average linkage of correlation coefficients between pulsed-field gel electrophoresis patterns. Arrow, 65% similarity cutoff.
Comparison of typing results obtained by PFGE and MLRT. Our data demonstrate an excellent correlation between PFGE and MLRT. Most clusters delineated in the dendrogram based on MLRT allelic profiles have corresponding clusters in the dendrogram based on PFGE patterns (figures 1 and 3); overall, 92% of all isolates clustered in the same way by either method. However, a few discrepancies between these methods were noted. Isolates AU0604 and AU0824 have identical allelic profiles, and they both cluster with the ET12 isolates by MLRT analysis; however, they appeared to be distinct by PFGE analysis. Of interest, both isolates were recovered from patients with CF attending the same treatment center. AU2107 and AU2105 similarly appear to be only distantly related on the basis of PFGE, but they group in the same cluster (A3) by MLRT. Again, both isolates were recovered from patients with CF attending the same treatment center. AU2107 and AU2105 similarly appear to be only distantly related on the basis of PFGE, but they group in the same cluster (A3) by MLRT. AU1202 groups closely with AU0903 and AU3014 (both isolated from patients with CF attending the same treatment center) in MLRT cluster A5, it occupies a separate position in the PFGE dendrogram. The converse is true for AU2622: this isolate groups in PFGE cluster P3 but occupies a separate position in the MLRT dendrogram. On the basis of PFGE, AU0918 clusters with the Midwest clone (cluster P10). MLRT analysis indicates that AU0918 could be related to the Midwest clone, grouping with cluster B3 at a linkage distance of 0.5; however, it was isolated from a CF patient receiving care on the US West Coast. Thus, the data deduced from MLRT are in agreement with the available epidemiologic data for most (5/7) of these isolates for which MLRT and PFGE results are discordant.

Split decomposition analysis. The relationships between all isolates within each MLRT subgroup were further analyzed by using split decomposition. The split graph, including all members of subgroup A, shows an almost bushlike or starlike structure (figure 4A) and has a low fit parameter (49.1%), indicating that not all information couldfaithfully be represented in a tree. Gradual pruning of the outer branches to completely resolve the relationships between isolates belonging to clusters A2, A3, and A4 resulted in the graph shown in figure 4B. The fit parameter improved to 100% when comparing only cluster A2, A3, and A4 isolates, and the split graph showed a combination of tree- and networklike features. Split decomposition analysis of isolates belonging to MLRT subgroup B (figure 4C) resulted in a largely resolved (fit parameter is 81.0%) split graph with limited networking. Analysis of all subgroup C isolates (figure 4D) resulted in a completely resolved (fit parameter is 100%) split graph with no networking.

Discussion

Originally known as plant pathogens, B. cepacia complex bacteria have been recognized as important opportunistic path-
Infections with *B. cepacia* complex in CF are associated with increased morbidity and mortality [2, 3]. Most infected patients with CF harbor *B. cepacia* genomovar III, but differences in outcome among patients infected with this species suggest important differences in the epidemiology and pathogenic potential among different strains [4, 5]. Most methods used for bacterial strain typing are designed to rapidly distinguish outbreak-related epidemic strains from nonrelated strains, and they are often not capable of answering questions addressing more global epidemiologic issues [22, 38]. PFGE is considered to be the standard method for epidemiologic typing, and guidelines for interpreting DNA restriction patterns generated by PFGE have been published [39]. However, few studies employing PFGE have been conducted using strains collected from diverse locations over longer periods of time; indeed, published interpretive guidelines are not appropriate for studies of large populations of organisms collected over extended periods [39].

Indexing of variation in sets of housekeeping genes provides a good basis for estimating overall levels of genotypic variation in populations of microorganisms [22], and methods based on this principle (including MLEE, MLST, and MLRT) should provide better insights into the global epidemiology of bacterial pathogens. A better understanding of the relationships among distinct *B. cepacia* genomovar III strains is necessary to optimize infection-control strategies, better define the risks associated with specific strains, and assess the potential threat posed to patients with CF by environmental isolates.

An excellent correlation between the clustering results obtained with PFGE and MLRT was found when similarity coefficient cutoff values of 65% and 0.38 were used, respectively. The cutoff used for PFGE in this study is lower than cutoff values reported elsewhere for other organisms [40, 41]. Factors affecting cutoff values are the number of bands in the pattern, the coefficient used for numerical analysis, and the time and space scales considered in the study. The 65% cutoff used in this study corresponds to a difference of ≤6 bands among macrorestriction profiles, and the grouping obtained is largely in agreement with the available epidemiologic data. Similarly, a cutoff of 0.38 for MLRT also resulted in a grouping largely in agreement with epidemiologic data. Most of the MLRT clusters delineated contained isolates with allelic profiles differing in only one locus, and no clusters contained isolates that differed in >2 loci.

Isolates belonging to the 3 major epidemic lineages investigated (ET12, PHDC, and the Midwest clone) grouped in MLRT clusters A4, B1, and B3, respectively. All ET12 isolates had identical MLRT allelic profiles, although their PFGE patterns were rather diverse. It was shown elsewhere that ET12 isolates were also indistinguishable by MLEE [12], although some variation could be seen using RAPD [14] and PFGE [42]. Among the PHDC isolates investigated, 3 different MLRT allelic profiles could be found. These allelic profiles were different only in the *dsbA* locus. As is obvious from figure 2, this locus is the most diverse of the loci used in our scheme. It is therefore not surprising that isolates collected over a period of 14 years differ in this locus. Isolates belonging to the Midwest clone had either of 2 MLRT allelic profiles, also being different only in the *dsbA* locus. Isolates belonging either to the PHDC or the Midwest clone were distinctly different from other isolates investigated, confirming our PFGE results and previously published data based on RAPD, BOX-PCR typing, PFGE, and ribotyping [7, 8].

A key advantage of MLRT is that meaningful relationships between distant clusters can be established. The 3 major subgroups...
(A, B, and C) apparent by using MLRT were not distinctly delineated in the PFGE data set; in general, no meaningful relationships between clusters or groups of clusters could be established on the basis of the PFGE data. The most obvious examples of this are the 5 MLRT subgroup C isolates, all of which were collected from soil and plants in Australia. Although individual isolates of this subgroup were clustered similarly by both methods, the overall relationships between these 5 isolates were not revealed by PFGE. Thus, MLRT correctly reflects the epidemiologic relationships between these isolates, whereas PFGE does not. Findings such as this suggest that MLRT may be the method of choice in assessment of isolates belonging to widespread epidemic lineages.

The relationships between isolates in each major MLRT subgroup were further analyzed by use of split decomposition analysis. With this method, distance matrices are decomposed in a

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**Figure 4.** Split graphs showing the interrelationships of members of multilocus restriction typing (MLRT) subgroups A (A), B (C), and C (D).
number of binary splits, and these splits can then be represented as a network in which the nodes and tips of the branches correspond to individual entries [26]. Split decomposition allows for the identification of related groups in data sets that contain conflicting signals due to the occurrence of genetic recombination and, thus, are not always clearly supported by a unique tree [26, 37]. Analysis of the complete MLRT subgroups B and C resulted in split graphs with high fit parameters (figure 4C and 4D), indicating that most or all information present in the data set could accurately be presented in the graph and that genetic recombination in these subgroups is not very frequent. Split decomposition analysis of all subgroup A isolates resulted in a split graph with a bush- or starlike appearance and low fit parameter, indicating the presence of conflicting data. Pruning of this graph and split decomposition analysis of the data from isolates from clusters A2, A3, and A4 resulted in a split graph with a high fit parameter that confirmed the relationships obvious from the UPGMA tree.

From these analyses, it is clear that clusters A2, A3, and A4 are more closely related to each other than to other subgroup A isolates; the exact relationship of clusters of these isolates toward other subgroup A isolates has however been obscured by the presence of conflicting data, probably caused by genetic recombination. This suggests that there may be significant differences in the rate of recombination (and thus in population structure) between different B. cepacia genovar III subgroups. A more comprehensive analysis of a larger number of isolates will be required to resolve these issues; however, our data show that MLRT and data analysis using split decomposition will be valuable tools in addressing these questions.

During the last decade, PFGE has increasingly been used for the epidemiologic investigation of outbreaks of bacterial infections [23, 39, 43]. Guidelines and interpretative criteria for the analysis of discrete sets of isolates obtained during outbreaks have been proposed, but these are not appropriate for the analysis of large populations of organisms collected over extended periods [39]. Several multicenter studies have shown that the reproducibility of PFGE patterns obtained in different laboratories using different methodologies is low and that high standardization is mandatory for obtaining reproducible results [44, 45]. Variation within PFGE patterns has been noted in isolates cultured repeatedly, and insertion of transposons in the genome can have a significant impact on the banding pattern [46]. Most important, PFGE is too discriminatory for studies assessing long-term epidemiology, because factors that may dramatically alter macrorestriction banding profiles (such as insertions, deletions, and inversions [39]) may obscure similarity among epidemiologically related isolates.

Methods that index neutral variation that accumulates slowly in the genome are better suited for answering global epidemiologic questions [20]. It is in this regard that MLST has been found valuable for the study of the global epidemiology of infectious agents [20, 21]. This method was validated in studies on Neisseria meningitidis [20], and more recently MLST schemes have been developed for Streptococcus pneumoniae [47], Staphylococcus aureus [48], Streptococcus pyogenes [49], and Campylobacter jejuni [30]. The main advantages of MLST include its relative simplicity and its portability; the main drawback of the method is that it relies entirely on DNA sequencing, which is expensive. Therefore, it is anticipated that studies employing MLST will be limited to a small number of reference laboratories or large clinical laboratories [24]. MLRT should provide a valid alternative to MLST in that it can provide robust data via analysis of multiple chromosomal loci without a requirement for high sequencing throughput, making it considerably less expensive. The method has recently been explored in studies of S. pneumoniae [25] and Helicobacter pylori [50]. Data obtained by RFLP analyses can be converted into band tables (containing the molecular weights of all bands in a pattern) that can easily be shared among laboratories, thus providing excellent portability.

Our data indicate that MLRT can be used to compare B. cepacia genovar III isolates collected in larger-scale (both temporal and spatial) studies. Insights into the structure of bacterial populations are essential to enhance our understanding of the molecular basis of variation in disease frequency and severity, host adaptation of clonal lineages, and the relationship between disease-causing isolates and naturally occurring bacterial clones [21, 22]. The development of an MLRT scheme and the use of advanced methods for data analysis will allow us to further investigate the global epidemiology of B. cepacia genomovar III and will aid in answering outstanding questions regarding the source of transmissible B. cepacia genomovar III strains and the potential of environmental isolates to cause infection in patients with CF.

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References


