Identification of species of the *Burkholderia cepacia* complex by sequence analysis of the *hisA* gene

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Bacteria of the *Burkholderia cepacia* complex (Bcc) are opportunistic human pathogens that can cause serious infections in the lungs of cystic fibrosis patients. The Bcc is a complex taxonomic group and comprises 17 closely related species of both biotechnological and clinical importance that have been discriminated by a polyphasic taxonomic approach. In this study we focused on the *hisA* gene, which encodes a 1-(5-phosphoribosyl)-5-[5-(5-phosphoribosylamino)methylideneamino]imidazole-4-carboxamide isomerase involved in histidine biosynthesis, as a new target gene to discriminate among the Bcc species. PCR primers were designed to amplify a *hisA* DNA fragment of 442 bp from 78 strains representative of all the 17 Bcc species known at the time of writing. The nucleotide sequences of the amplicons were determined and aligned with the 54 Bcc sequences available in databases. Then a phylogenetic tree was constructed on the basis of this alignment and this revealed that this *hisA* region allows discrimination of all Bcc species, suggesting that this gene fragment can be used for the identification of Bcc strains. In addition, an 11 nucleotide letter code for the rapid discrimination of Bcc species was identified.

INTRODUCTION

Cystic fibrosis (CF) is the most common critical genetic disease in white populations and leads to the production of an altered CFTR protein, a transmembrane conductance regulator (Tsui *et al.*, 1985), whose dysfunction induces an anomaly in various exocrine gland secretions that as a result are dehydrated, resulting in the production of dense and viscous mucus. This results in multiple organ system impairment, especially in the respiratory tract where the sticky mucus promotes a (persistent) colonization by one or more bacterial (opportunistic) pathogens. These chronic infections represent the major cause of morbidity and mortality in CF patients. Even though *Pseudomonas aeruginosa* has been recognized as the most common pathogen in the lower airways of CF patients during the last three decades, new emergent pathogens such as *Burkholderia cepacia* complex (Bcc), *Stenotrophomonas maltophilia* and *Achromobacter xylosoxidans* have been detected (Dakin *et al.*, 2002; Saiman & Siegel, 2004). In particular, Bcc are intrinsically resistant to multiple antibiotics and pose the risk of spread within the CF community (Govan *et al.*, 1993) with hospitalization being a risk factor for acquisition (Holmes *et al.*, 1999; Smith *et al.*, 1993; Whiteford *et al.*, 1995). Moreover, Bcc strains can enter the blood circulation and lead to fulminating septicemia with acute respiratory failure called ‘cepacia syndrome’ (Mahenthiralingam *et al.*, 2005). In contrast to these pathogenic properties, Bcc bacteria, which are also found in natural habitats, have a considerable ecological and biotechnological importance; in fact, they can degrade many chemical compounds (Fries *et al.*, 1997) and produce a number of substances that antagonize soilborne plant pathogens (Bevivino *et al.*, 2000; Heungens & Parke, 2000). Therefore, the Bcc represents an interesting and heterogeneous taxonomical entity, containing strains from different environments and with different metabolic abilities. Mechanisms responsible for the colonization of natural habitats, especially the rhizosphere, and antagonistic activity against plant pathogens appear to be similar to those responsible for the colonization of human organs and tissue, and pathogenicity, as
suggested by Holden et al. (2009), who stated that ‘80 % of the (B. cenocepacia) J2315 virulence functions have orthologous matches to other B. cenocepacia strains, 74 % have matches to B. contaminans strain 383, and 68 % have matches to B. pseudomallei. Many of these functions therefore represent Burkholderia-wide functions, which may promote survival in challenging and complex environments such as the soil and rhizosphere but may also have utility in the CF lung’. On this basis, it has been supposed that the natural environment serves as a ’reservoir’ for pathogenic strains (Berg et al., 2005).

Extensive taxonomic studies carried out in the last decade (Mahenthiralingam et al., 2000; Vandamme et al., 1997; Vermis et al., 2002), revealed that Bcc consists of (at least) 17 closely related species (Vanlaere et al., 2008, 2009). Although a polyphasic approach was used to delineate these species, species level identification is most efficiently achieved through recA analysis (Mahenthiralingam et al., 2000) or, for some problematic strains (Vanlaere et al., 2009), multilocus sequence analysis (Baldwin et al., 2005). We recently reported the genetic and evolutionary analysis of a 4800 bp region containing the histidine biosynthetic ‘core’ genes (hisBHAF) from a panel of 44 Bcc strains (Papaleo et al., 2009). In that work a phylogenetic analysis of the four genes was also carried out. Interestingly, the analysis of the phylogenetic tree, constructed using a 756 bp hisA nucleotide sequence from the 44 Bcc strains, revealed that it was possible to group strains from the same Bcc species in coherent clusters and to differentiate Bcc species. In addition, the hisA gene sequence also allowed discrimination between the four Burkholderia cenocepacia lineages referred to as B. cenocepacia IIIA, IIIB, IIIC and IID (Vandamme et al., 2003). The aim of the present study was to evaluate the discriminatory power of the hisA sequence within the Bcc using a larger panel of strains, representing all 17 current species, from both environmental and clinical sources.

**METHODS**

**Bacterial strains and growth conditions.** All strains were previously identified using a polyphasic-taxonomic approach and/or recA or multilocus sequence analyses (Mahenthiralingam et al., 2000; Vandamme et al., 1997). The experimental panel used in this work consisted of 132 Bcc strains of different origin (Supplementary Table S1 available with the online journal); from 78 of these the nucleotide sequence of the hisA gene was determined in this work. Strains were grown aerobically for 48 h at 37 °C in Luria broth (Sambrook et al., 1989) or in Luria agar.

**PCR amplification of a 442 bp hisA gene region from Bcc strains.** The DNA or the cell lysate of 16 FCF (Florence cystic fibrosis) bacterial isolates were a gift from the Anna Meyer Children’s Hospital (Department of Paediatrics, Division of Paediatrics, Infectious Diseases, Cystic Fibrosis, University of Florence; Viale Pieraccini 24, I-50139 Florence, Italy). Cell lysates were prepared by lysing of 2–3 colonies grown overnight in Luria broth, according to a previously described method (Vandamme et al., 2002). PCR amplification of the hisA gene region was performed in a 50 μl reaction mixture containing 2 μl cell lysate, 2 U Taq DNA polymerase (Bioline), 250 μM each dNTP, 0.6 μM each primer, 1.5 mM MgCl2 and 1× PCR buffer. A primary denaturation treatment of 2 min at 95 °C was performed and amplification of the hisA gene was carried out for 30 cycles consisting of 30 s at 95 °C, 45 s at 67 °C and 1 min at 72 °C, with a final extension of 10 min at 72 °C. Thermal cycling was performed with a gene Amp PCR system 9700 instrument (Applied Biosystems). The primers designed and used in this study were A-442_for (AGGACCCGGCGGCGAT) and A-442_rev (TGCA-GCATCCCCGTCCG) whose 5’ ends anneal at positions 89 and 530, respectively.

**DNA sequencing.** For sequencing, the PCR products were purified using the MinElute gel extraction purification kit (Qiagen) according to the manufacturer’s instructions. The nucleotide sequence of a 442 bp hisA gene region was determined on both strands according to the method of Sanger et al. (1977), using an Applied Biosystems BigDye terminator cycle sequencing kit, version 3.1, according to the manufacturer’s instructions. Thermal cycling was performed with a gene Amp PCR system 9700 instrument (Applied Biosystems). Each of the 78 sequences determined was submitted to GenBank and was assigned the accession number shown in Supplementary Table S1 (available with the online journal).

**DNA sequence analysis.** The hisA sequences available in the National Center for Biotechnology Information (NCBI) database were retrieved using the BLASTn option of the BLAST program (Altschul et al., 1997), using default parameters. All the 132 hisA sequences were aligned using the CLUSTAL W program (Thompson et al., 1994) in the BioEdit package (Hall, 1999). Multialignments were then used to construct phylogenetic trees using the distance-based neighbour-joining algorithm implemented in the MEGA4 software (Tamura et al., 2007), setting the pairwise deletion or the complete deletion option and performing 1000 bootstraps replicates to infer statistical support. The phylogenetic trees were also constructed including or eliminating the shortest sequences in order to check whether the branching order and strain clustering might change. The data obtained (data not reported) revealed that no significant difference was found in the diverse phylogenetic trees.

**RESULTS AND DISCUSSION**

**Identification of the shortest hisA region able to discriminate among Bcc species**

In order to try to find the shortest region of the hisA gene that was easily PCR-amplifiable and that was able to discriminate between Bcc species, the multialignment of the entire hisA gene from 44 sequences from a previous study (Papaleo et al., 2009) was analysed. Two regions, each surrounded by highly conserved sequences that could represent targets for primers in PCRs were selected. They spanned from nt 149 to nt 417 (the 269 bp hisA gene region) and from nt 89 to nt 530 (the 442 bp hisA gene region) (Supplementary Fig. S1 available with the online journal). The multialignments of each of the two regions were used for the construction of the phylogenetic trees shown in Fig. 1, whose analysis revealed that the 442 bp hisA region allowed discrimination of all Bcc species and the four lineages within B. cenocepacia, even though the topology of this tree was partly different from that of the entire hisA gene tree. The tree based on the 269 bp gene region did not allow discrimination of all Bcc species. Thus, the 442 bp hisA region was chosen and used for further experiments.
Fig. 1. Phylogenetic trees constructed using the nucleotide sequence of the entire \textit{hisA} gene (a) and two regions thereof (b, c) from 44 Bcc strains. The orthologous sequences from \textit{B. xenovorans} LB400 and \textit{R. eutropha} H16 were used as outgroups.
PCR amplification of the hisA DNA region from a panel of 78 Bcc strains

A set of primers (A-442_for and A-442_rev) was designed for the PCR amplification of the 442 bp hisA fragment as described above. Primers and amplification program were first tested on the DNA of 12 strains representative of different Bcc species. Different annealing temperatures and amplification conditions were tested. Data obtained (not shown) revealed that 67°C was the optimal annealing temperature, because lower temperatures gave unspecific PCR products. An amplicon of the expected size was then obtained from each of the 12 strains, suggesting that these primers might be successfully used to amplify the 442 bp hisA region from every Bcc strain. Therefore, the two primers were employed in PCRs on a panel of 78 Bcc strains (Supplementary Table S1 available with the online journal). In this way, a DNA fragment of the expected size was obtained from each of the 78 strains (data not shown). Each amplicon was then purified from agarose gel using the MinElute gel extraction purification kit (Qiagen) according to the manufacturer's instructions.

Sequencing and phylogenetic analysis of hisA gene region

The nucleotide sequence of each of the 78 purified amplicons was determined using primers A-442_for and A-442_rev. Each nucleotide sequence was used as a query in a BLAST probing (Altschul et al., 1997) of the NCBI non-redundant nucleotide database. Moreover, the CLUSTAL W (Thompson et al., 1994) multialignment of the 78 Bcc sequences and other orthologous sequences revealed the absence of insertions, deletions or stop codons in the sequences obtained in this work (data not shown).

A multialignment including the 78 Bcc new sequences, 40 Bcc sequences previously analysed (Papaleo et al., 2009), 16 Bcc sequences of the hisA gene retrieved from the NCBI database (Supplementary Fig. S2 available with the online journal) and the orthologous sequences from Burkholderia xenovorans LB400 (locus tag: Bxe_A0403) and Ralstonia eutropha H16 (locus tag: H16_A341) was obtained using the CLUSTAL W program (Thompson et al., 1994). The entire multialignment is reported as Supplementary Fig. S2 (available with the online journal). The multialignment was used to construct a phylogenetic tree (Fig. 2).

Fig. 2. Phylogenetic tree constructed using the nucleotide sequence of the 442 bp hisA region from 132 Bcc strains. The orthologous sequences from B. xenovorans LB400 and R. eutropha H16 were used as outgroups. The tree accounts only for the overall topology, whereas the real phylogenetic distances between species can be inferred from the tree reported in Supplementary Fig. S3 (available with the online journal).
used to construct the phylogenetic trees shown in Fig. 2 and in Supplementary Fig. S3 (available with the online journal) using the neighbour-joining method (Saitou & Nei, 1987). Interestingly, the analysis revealed that, with few exceptions, strains of the same species shared a high degree of sequence similarity and were clustered together in the tree. At the same time, each species is clearly separated from each other. The only exceptions are represented by Burkholderia lata strains LMG 14095 and R-18628, and Burkholderia diffusa strain LMG 24268. Vanlaere et al. (2009) already reported that the two B. lata strains were aberrant; the reason why the B. diffusa strain clusters aberrantly is unclear. Finally, as in the recA-based and multilocus-based phylogenetic trees, sequences representing the IIIA, IIIB, IIIC and IID lineages within B. cenocepacia (Mahenthiralingam et al., 2000; Vandamme et al., 2003) were clearly separated from each other and formed four different clusters.

The topology of the hisA tree was different from that obtained with recA sequences (Vanlaere et al., 2008, 2009) in that the branching order of some species was different. However, most nodes in the hisA trees were supported by bootstrap values higher than 75%. These results further indicate that like the multilocus-sequence-based approach (Baldwin et al., 2005), the phylogenetic tree based on 442 bp hisA sequences, separated all 17 Bcc species in different clusters (with B. cenocepacia sequences also being divided in 4 lineages) with high bootstrap values. Hence, these data indicate that this gene provides another locus that is useful in discriminating most Bcc strains into the proper species.

### An 11 letter code useful for the identification of Bcc bacteria

The data reported above showed that the phylogenetic analysis of the 442 bp hisA gene fragment can represent an additional powerful tool in discriminating most Bcc strains. However, in order to allow the identification of bacteria belonging to Bcc but avoiding the need to construct a phylogenetic tree, we selected a reduced set of polymorphic and informative sites [single nucleotide polymorphisms (SNPs)] whose combination could permit the identification of Bcc strains by ‘simply’ determining the nucleotide present at each of these sites and comparing the obtained results with a consensus code for each species/subspecies. To this purpose the entire hisA multialignment (reported in Supplementary Fig. S2 available with the online journal) was re-examined and a consensus sequence was then obtained for each species and for each of the four B. cenocepacia lineages (Supplementary Fig. S4 available with the online journal). The 20 consensus sequences were then compared using the CLUSTAL W program (Thompson et al., 1994) (Supplementary Fig. S4 available with the online journal). In this way 93 polymorphic sites were detected. All the discriminatory sites were analysed and 11 of them, contained within

### Table 1. Eleven letter code for the Bcc hisA gene that can be used for the identification of bacteria belonging to the Bcc

<table>
<thead>
<tr>
<th>Bcc species</th>
<th>SNP position: entire hisA gene/442 bp hisA region*</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. ambifaria</td>
<td>C A Y S G C G C C T A</td>
</tr>
<tr>
<td>B. anthina</td>
<td>C G C C G G G B C G</td>
</tr>
<tr>
<td>B. arboris</td>
<td>Y A C G G C C C C A</td>
</tr>
<tr>
<td>B. cenocepacia IIIA</td>
<td>T C C G A C C G T A</td>
</tr>
<tr>
<td>B. cenocepacia IIIB</td>
<td>T C C S A C C G C G</td>
</tr>
<tr>
<td>B. cenocepacia IIIC</td>
<td>T A C C C A C T C G</td>
</tr>
<tr>
<td>B. cenocepacia IIID</td>
<td>T G C C A C C G G C</td>
</tr>
<tr>
<td>B. cepacia</td>
<td>C A C C G G C G C</td>
</tr>
<tr>
<td>B. contaminans</td>
<td>C A C G A C C C G</td>
</tr>
<tr>
<td>B. diffusa</td>
<td>C A C T A C C G</td>
</tr>
<tr>
<td>B. dolosa</td>
<td>C G T C G G C C</td>
</tr>
<tr>
<td>B. dolosa</td>
<td>Y A C G G C C G</td>
</tr>
<tr>
<td>B. latens</td>
<td>C G C C A C C G</td>
</tr>
<tr>
<td>B. latens</td>
<td>T C T C A C C G</td>
</tr>
<tr>
<td>B. metallica</td>
<td>C G C G G T G C</td>
</tr>
<tr>
<td>B. multivorans</td>
<td>C G C C G G C C</td>
</tr>
<tr>
<td>B. pyrocinia</td>
<td>C A C G G C C G</td>
</tr>
<tr>
<td>B. seminalis</td>
<td>C C S A C C Y C</td>
</tr>
<tr>
<td>B. stabilis</td>
<td>C G C G G C G</td>
</tr>
<tr>
<td>B. ubonensis</td>
<td>T G C C C G C C</td>
</tr>
<tr>
<td>B. vietnamiensis</td>
<td>C G C C C G C</td>
</tr>
</tbody>
</table>

B, C or G or T; R, A or G; Y, C or T; S, G or C.

*SNP position refers to the nucleotide sequence of the entire hisA gene and of the 442 bp hisA region.
| Bcc species | B. ambifaria | B. anthina | B. arboris | B. cenocepacia IIIA | B. cenocepacia IIIB | B. cenocepacia IIIC | B. cenocepacia IID | B. contaminans | B. diffusa | B. dolosa | B. lata | B. latens | B. metallica | B. multivorans | B. pyrrocincia | B. seminalis | B. stabilis | B. ubonensis | B. vietnamiensis | Total mismatches | Mean value* |
|-------------|--------------|-------------|-------------|---------------------|---------------------|---------------------|---------------------|----------------|------------|-----------|---------|----------|-------------|--------------|--------------|-------------|-----------|-----------|-----------|-----------|
| B. ambifaria | –            | 2           | 2           | 7                   | 6                   | 6                   | 5                   | 2               | 3         | 5         | 2        | 3        | 5           | 7            | 3            | 4           | 4         | 3         | 4         | 3         | 4.0       |
| B. anthina   | –            | 4           | 7           | 5                   | 6                   | 3                   | 2                   | 4               | 4         | 2         | 4        | 2        | 7           | 5            | 3            | 5         | 4         | 2         | 4         | 3.6       |
| B. arboris   | –            | 4           | 3           | 4                   | 5                   | 3                   | 2                   | 4               | 4         | 4         | 1        | 5        | 6           | 3            | 2            | 2         | 3         | 4         | 1         | 6.7       |
| B. cenocepacia IIIA | – | 1         | 4           | 4                   | 6                   | 5                   | 5                   | 8               | 3         | 5         | 4        | 7        | 5           | 2            | 5            | 5         | 6         | 6         | 4         | 9.4       |
| B. cenocepacia IIIB | – | 2         | 2           | 4                   | 4                   | 4                   | 6                   | 2               | 3         | 2         | 6        | 4         | 1           | 4            | 5            | 4         | 4         | 5         | 4         | 6.8       |
| B. cenocepacia IIIC | – | 3         | 4           | 5                   | 4                   | 7                   | 3                   | 4               | 4         | 4         | 6        | 5         | 4           | 6            | 6            | 4         | 4         | 6         | 4         | 5         | 8.8       |
| B. cenocepacia IID | – | 3         | 5           | 5                   | 4                   | 4                   | 3                   | 4               | 5         | 6         | 4        | 3         | 3           | 2            | 2            | 4         | 4         | 3         | 2         | 7.3       |
| B. cepacia   | –            | 5           | 4           | 3                   | 4                   | 6                   | 2                   | 4               | 3         | 4         | 2        | 4        | 4           | 6            | 4            | 2         | 4         | 4         | 3         | 6.9       |
| B. contaminans | –          | 2           | 6           | 4                   | 3                   | 7                   | 5                   | 4               | 2         | 5         | 7        | 4         | 8           | 4            | 2            | 5         | 9         | 4         | 3         | 8.2       |
| B. diffusa   | –            | 1           | 2           | 5                   | 7                   | 3                   | 4                   | 5               | 8        | 4         | 5         | 8        | 4           | 6            | 3            | 4         | 4         | 3         | 4         | 8.6       |
| B. dolosa    | –            | 5           | 5           | 6                   | 3                   | 6                   | 4                   | 3               | 3        | 4         | 3        | 4         | 3           | 2            | 4            | 5         | 6         | 2         | 3         | 6.2       |
| B. lata      | –            | 4           | 4           | 4                   | 1                   | 3                   | 2                   | 4               | 5         | 2         | 4        | 5         | 9           | 4            | 3            | 6         | 2         | 4         | 3         | 6.9       |
| B. latens    | –            | 5           | 6           | 5                   | 3                   | 4                   | 4               | 6             | 1        | 7         | 3         | 5         | 9           | 4            | 3            | 6         | 2         | 4         | 3         | 7.5       |
| B. metallica | –            | 9           | 5           | 4                   | 7                   | 6                   | 4               | 3             | 2         | 4         | 5        | 2         | 4           | 5            | 6            | 4         | 5         | 9         | 1         | 10.4      |
| B. multivorans | –           | 5           | 4           | 2                   | 4                   | 5                   | 9               | 1             | 4         | 7         | 2        | 4         | 4           | 5            | 4            | 6         | 8         | 2         | 4         | 9.1       |
| B. pyrrocincia | –           | 4           | 3           | 6                   | 6                   | 8               | 4             | 3            | 6         | 6         | 8        | 2         | 4           | 4            | 3            | 6         | 8         | 2         | 4         | 8.4       |
| B. seminalis | –            | 4           | 5           | 4                   | 4                   | 3               | 2             | 4            | 3         | 6         | 6        | 8         | 4           | 4            | 3            | 6         | 8         | 2         | 4         | 8.4       |
| B. stabilis  | –            | 4           | 3           | 7                   | 0                   | 3               | 2             | 4            | 3         | 6         | 4        | 2         | 4           | 3            | 6            | 8         | 2         | 4         | 3         | 7.0       |
| B. ubonensis | –            | 4           | 9           | 3                   | 7                   | 4               | 2             | 4            | 3         | 6         | 4        | 2         | 4           | 3            | 6            | 8         | 2         | 4         | 3         | 9.3       |
| B. vietnamiensis | –       | 77          | 4           | 0                   | 0                   | 0               | 0             | 0            | 0         | 0         | 0        | 0         | 0           | 0            | 0            | 0         | 0         | 0         | 0         | 7.7       |

Total 1597 4.38

*Number of mismatches between a given species and the other ones.
in a 259 bp region of the hisA fragment (located at position 231, 237, 294, 318, 331, 342, 369, 387, 441, 464 and 489), were finally selected. As shown in Table 1, the combination of the nucleotides located at these sites allowed design of an 11 letter code that allowed discrimination all the 17 Bcc species and B. cenocepacia lineages. The possibility that in the future new Bcc strains belonging to different species might share the same discriminating nucleotide in 1 (or more) of the 11 positions cannot be a priori excluded. However, data reported in Table 2 revealed that the total number of mismatches between the 20 species/subspecies is 1597 and the mean value of mismatches between each species was 4.38 (ranging between 3.6 and 8.3); thus, suggesting that the occurrence of such events, even though possible, is of very low probability.

Conclusions

In this work we have reported the use of the hisA gene for the discrimination of all 17 Bcc species. The structure of this gene resembles that of 16S rRNA genes. The HisA protein belongs to the histidine biosynthetic ‘core’, consisting of four genes (hisBHAF) (Fani et al., 2007; Papaleo et al., 2009), whose products have been hypothesized to form a metabolon, that is, a multicomplex enzyme constituted by transiently interacting proteins. Thus, these proteins exhibit strong functional and structural constraints, which limits the number of mutations that can occur in the genes encoding them, since they might interfere with the correct assembly of the metabolon. Accordingly, we reported earlier that hisB also allows discrimination among Bcc species, although to a lower extent than hisA (Fani et al., 2007; Papaleo et al., 2009). The data obtained revealed that the hisA segment of about 400 bp is easily amplifiable from Bcc strains and that the phylogenetic analysis of this region allows the assignment of most Bcc strains to a given Bcc species. The multialignment of Bcc hisA sequences revealed the presence of conserved regions intermixed with variable ones.

The analysis of the entire multialignment of the hisA region consensus sequences enabled selection of 11 SNPs, whose combination allows discrimination of the Bcc species. This, in turn, permits the identification of a Bcc strain without performing a phylogenetic analysis. In conclusion, in our opinion the hisA gene represents a new promising tool for the identification of Bcc bacteria.

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