Conservation of the \( \text{opcL} \) gene encoding the peptidoglycan-associated outer-membrane lipoprotein among representatives of the \textit{Burkholderia cepacia} complex

Maria Plesa, Abdelaziz Kholiti, Karen Vermis, Peter Vandamme, Stavroula Panagea, Craig Winstanley and Pierre Cornelis

1Laboratory of Microbial Interactions, Department of Molecular and Cellular Interactions, Flanders Interuniversity Institute of Biotechnology, Vrije Universiteit Brussel, Building E, Room 6.2, Pleinlaan 2, B-1050 Brussels, Belgium
2,3Laboratory of Pharmaceutical Microbiology and Laboratory of Microbiology, Ghent University, Ghent, Belgium
4Department of Medical Microbiology and Genitourinary Medicine, University of Liverpool, Duncan Building, Liverpool L69 3GA, UK

Members of the \textit{Burkholderia cepacia} complex are Gram-negative \( \beta \)-proteobacteria that are classified into nine genomic species or genomovars. Some representatives of this group of bacteria, such as \textit{Burkholderia multivorans} (genomovar II) and \textit{Burkholderia cenocepacia} (genomovar III), are considered to be dangerous pathogens for cystic fibrosis (CF) patients because of their capacity to colonize CF lungs. The \( \text{opcL} \) gene, which encodes the peptidoglycan-associated outer-membrane lipoprotein (PAL), was detected in the genome of \textit{Burkholderia} sp. LB 400 by a similarity search that was based on the sequence of the \textit{Pseudomonas aeruginosa} PAL, OprL. Primers that could amplify part of \( \text{opcL} \) from \textit{B. multivorans} LMG 13010\(^T\) were designed. This PCR fragment was used as a probe for screening of a \textit{B. multivorans} genomic bank, allowing cloning of the complete \( \text{opcL} \) gene. The complete \( \text{opcL} \) gene could be PCR-amplified from DNA from all genomovars. The sequences of these \( \text{opcL} \) genes showed a high degree of conservation (> 95\%) among different species of the \textit{B. cepacia} complex. OpcL protein that was purified from \textit{B. multivorans} LMG 13010\(^T\) was used to generate mouse polyclonal antisera against OpcL. The OpcL protein could be produced in \textit{Escherichia coli} and detected in outer-membrane fractions by Western blot. \textit{Burkholderia} cells were labelled by immunofluorescence staining using antibodies against OpcL, but only after treatment with EDTA and SDS. The \( \text{opcL} \) gene could be amplified directly from the sputa of 15 CF patients who were known to be colonized by \textit{B. cepacia}; sequence data derived from the amplicons identified the colonizing strains as \textit{B. cenocepacia} (genomovar III, \( n = 14 \)) and \textit{B. multivorans} (\( n = 1 \)).

INTRODUCTION

Bacteria that belong to the \textit{Burkholderia cepacia} complex, originally described as plant pathogens by Burkholder (1950), have now been recognized as major opportunistic pathogens of the respiratory tract in cystic fibrosis (CF) patients (Govan & Deretic, 1996; Mahenthiralingam \textit{et al.}, 2002). Recent taxonomic studies have revealed that strains routinely identified as \textit{B. cepacia} represent a complex of nine closely related species, all of which have been isolated from CF patients or the environment (Mahenthiralingam \textit{et al.}, 2002; Vandamme \textit{et al.}, 2002). These genomovars are very similar phenotypically, but show significant differences at the genomic level, allowing them to be considered as separate species. Next to \textit{B. cepacia} (genomovar I), genomovars V and IX were also identified as genuine \textit{Burkholderia} species, i.e. \textit{Burkholderia vietnamiensis} (Gillis \textit{et al.}, 1995) and \textit{Burkholderia pyrocina} (Vandamme \textit{et al.}, 1997), respectively. Following the identification of distinguishing phenotypic biochemical characteristics and the development of novel molecular diagnostic tools, the names \textit{Burkholderia multivorans} (Vandamme \textit{et al.}, 1997), \textit{Burkholderia cenocepacia}

Abbreviations: CF, cystic fibrosis; PAL, peptidoglycan-associated outer-membrane lipoprotein; PNPG, \( p \)-nitrophenyl \( \beta \)-D-glucoside; SS-PCR, species-specific PCR.

The GenBank/EMBL/DDBJ accession numbers for the sequences described in this article are AY278462–AY278482.
(Vandamme et al., 2003), Burkholderia stabilis (Vandamme et al., 2000), Burkholderia ambifaria (Coenye et al., 2001) and Burkholderia anthinz (Vandamme et al., 2002) were proposed for genomovars II, III, IV, VII and VIII, respectively. Genomovar VI has now been formally named 'Burkholderia dolosu' (Vermis et al., 2004). Burkholderia ubonensis, a soil bacterium that was described by Yabuuchi et al. (2000), may represent a tenth B. cepacia genomovar. B. multivorans and B. cenocepacia strains are known to be highly transmissible between CF patients (LiPuma et al., 1998).

It is important to develop sensitive laboratory diagnostic assays to better distinguish strains of the B. cepacia complex, in order to avoid misidentification of closely related bacteria. Several studies have presented different molecular methods for the detection and classification of these organisms, but these are not always sensitive enough to distinguish between the different B. cepacia genomovars. PCR-RFLP based on the 16S rRNA gene is limited in its ability to differentiate organisms into genomovars. Although widely used for detection and classification, recent articles demonstrate that the 16S rRNA gene is limited in its ability to differentiate strains of the B. cepacia complex (LiPuma et al., 1999; Mahenthiralingam et al., 2002b).

One particular class of outer-membrane proteins, the so-called peptidoglycan-associated lipoproteins (PALs), is conserved among many Gram-negative bacteria, such as Haemophilus influenzae (Deich et al., 1988; Nelson et al., 1988), Escherichia coli (Chen & Henning, 1987), Brucella abortus (Tibor et al., 1994) and Pseudomonas aeruginosa, where it is called OprL (Lim et al., 1997). The exact function of PALs is unknown, but they have been shown to be necessary for resistance to detergents and antibiotics (Cascales et al., 2002) and for integrity of the cell envelope (Rodriguez-Herva et al., 1996). Based on two outer-membrane lipoprotein genes, oprL and oprl, De Vos et al. (1997) developed a multiplex PCR assay for the specific detection and identification of P. aeruginosa in clinical specimens. Similarly, a real-time detection PCR, based on amplification of the oprL gene to detect and quantify P. aeruginosa in wound biopsy samples, was developed (Pirnay et al., 1999).

In this article, we describe the cloning and molecular characterization of the oprL gene, which encodes the PAL from B. multivorans, and show that the corresponding gene can be amplified and used to detect and differentiate strains of the B. cepacia complex, including those from clinical specimens.

**METHODS**

**Bacterial strains.** Tables 1 and 2 list the 50 B. cepacia complex and other β- and γ-Proteobacteria reference strains and/or purified DNA, together with E. coli strains and plasmids, that were used in this study.

**DNA preparation.** DNA was prepared as described by Pitcher et al. (1989).

**Identification of the oprL gene of B. multivorans.** A 400 bp fragment that contained the oprL gene of B. multivorans LMG 13010T, but was missing 32 and 89 bp from the 5′ and 3′ ends of the ORF, respectively, was amplified by using primers that were derived from the oprL sequence of Burkholderia sp. LB 400, available from the US Department of Energy web site (www.jgi.doe.gov), with the following sequences: P33, 5′-ATTGATGGTCGGTGCGCT-3′, corresponding to position 33 of the gene, and P421R, 5′-TCACGGCTTCTTTC CATTTCGCG-3′, corresponding to position 421 of the oprL ORF.

**Construction of a genomic library from B. multivorans.** Genomic DNA from B. multivorans LMG 13010T (20 μg) was partially restricted with Sau3A I by using increasing dilutions of the restriction enzyme in a volume of 100 μl. After 15 min, the reaction was stopped by addition of EDTA and 5 μl aliquots from each tube were analysed by agarose gel electrophoresis. Fractions that contained fragments averaging 30 kb were pooled and their DNA was ligated to BamHI-restricted cosmids pRG930 (Van den Eede et al., 1992). Packaging of ligated DNA and transformation of E. coli HB101 cells were done by using a GIGAPACK III kit, following the recommendations of the manufacturer (Stratagene). Colonies were selected on Luria–Bertani (LB) agar plates with streptomycin (25 μg ml⁻¹) and spectinomycin (50 μg ml⁻¹) and transferred with sterile toothpicks to individual wells of 96-well microtitre plates that each contained 100 μl of the same medium. After incubation at 28 °C overnight, colonies were replicated by using an alcohol- and flame-sterilized replicator on agar plates. Plates were incubated overnight and an autoclave-sterilized nitrocellulose membrane (Hybond C, positively charged; Amersham) was laid on top and left for 1 h at room temperature. The membrane was first laid on a filter that was soaked in 1 % SDS to lyse the cells, and then on a filter that was soaked in denaturation solution (1 M NaOH, 1-5 M NaCl) for 15 min After

<table>
<thead>
<tr>
<th>Table 1. E. coli strains and plasmids used in this study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strain/plasmid</strong></td>
</tr>
<tr>
<td>---------------------------</td>
</tr>
<tr>
<td>E. coli HB101</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>E. coli DH5a</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>pRG930</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>pCR2.1</td>
</tr>
</tbody>
</table>
Table 2. *B. cepacia* complex strains and other β- and γ-proteobacteria reference strains that were used in this study

<table>
<thead>
<tr>
<th>Species/genomovar</th>
<th>Strains (LMG accession no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Positive for opcL amplification:</strong></td>
<td></td>
</tr>
<tr>
<td><em>B. cepacia</em> (genomovar I)</td>
<td>LMG 1222(^T), LMG 6963, LMG 6964, LMG 6032, LMG 18821, R-9944</td>
</tr>
<tr>
<td><em>B. multivorans</em> (genomovar II)</td>
<td>LMG 13010(^T), LMG 16668, LMG 16660, R-10041</td>
</tr>
<tr>
<td><em>B. cenocepacia</em> (genomovar III)</td>
<td>LMG 16656(^T), LMG 16671, R-1909, LMG 14276, R-922, R-13524, LMG 16654</td>
</tr>
<tr>
<td><em>B. stabilis</em> (genomovar IV)</td>
<td>LMG 14294(^T), LMG 15951, LMG 14086, LMG 6997, LMG 7000, LMG 18140</td>
</tr>
<tr>
<td><em>B. vietnamiensis</em> (genomovar V)</td>
<td>LMG 10929(^T), LMG 10823, LMG 10824, R-13251, LMG 16230</td>
</tr>
<tr>
<td><em>B. cepacia</em> (genomovar VI)</td>
<td>R-10358, LMG 18946, R-2879, LMG 21443, LMG 21820</td>
</tr>
<tr>
<td><em>B. ambifaria</em> (genomovar VII)</td>
<td>LMG 19182(^T), R-9927, LMG 17828, LMG 17829, R-14255</td>
</tr>
<tr>
<td><em>B. anthina</em> (genomovar VIII)</td>
<td>LMG 20980(^T), R-18629, R-11752, LMG 20981, LMG 20982</td>
</tr>
<tr>
<td><em>B. pyrrocinia</em> (genomovar IX)</td>
<td>LMG 14191(^T), LMG 21822, R-11794, R-12631</td>
</tr>
<tr>
<td><em>B. ubonensis</em></td>
<td>LMG 20358(^T), R-11767, R-5630</td>
</tr>
<tr>
<td><strong>Negative for opcL amplification:</strong></td>
<td></td>
</tr>
<tr>
<td><em>Burkholderia terricola</em></td>
<td>R-11772</td>
</tr>
<tr>
<td><em>Burkholderia graminis</em></td>
<td>R-11762</td>
</tr>
<tr>
<td><em>Burkholderia fungorum</em></td>
<td>R-1469</td>
</tr>
<tr>
<td><em>Burkholderia gladioli</em></td>
<td>LMG 18158, LMG 18160, R-18878</td>
</tr>
<tr>
<td><em>Burkholderia glumae</em></td>
<td>PG1</td>
</tr>
<tr>
<td><em>Ralstonia insidiosa</em></td>
<td>LMG 18101</td>
</tr>
<tr>
<td><em>Ralstonia mannituolica</em></td>
<td>LMG 18097</td>
</tr>
<tr>
<td><em>Ralstonia pacsula</em></td>
<td>LMG 3244(^T)</td>
</tr>
<tr>
<td><em>Pandoraea apista</em></td>
<td>LMG 19513</td>
</tr>
<tr>
<td><em>Pandoraea pmonensu</em></td>
<td>R-17877</td>
</tr>
<tr>
<td><em>Alcaligenes xylosoxidans</em></td>
<td>R-16906, R-16907, R-16909</td>
</tr>
<tr>
<td><em>Bordetella bronchiseptica</em></td>
<td>R-16913</td>
</tr>
<tr>
<td><em>Bordetella hinzii</em></td>
<td>R-16903</td>
</tr>
<tr>
<td><em>Stenotrophomonas maltophilia</em></td>
<td>LMG 10997, LMG 11109, LMG 10887</td>
</tr>
</tbody>
</table>

Cloning of the opcL gene from *B. multivorans* LMG 13010\(^T\) in expression vector pQE60. The *B. multivorans* LMG 13010\(^T\) opcL gene (without the start and stop codons) was PCR-amplified by using primers pONC1 (5′-CATGCGATGTTGCGAAAAAAGTTCGTCG3′), which contained an NcoI restriction site and corresponded to the beginning of the ORF, and pOLBR300 (5′-CGGGATCCCTTTGGTAGCGG3′), which contained a BamHI restriction site and corresponded to the end of the ORF. A fragment of 469 bp that contained the opcL gene was restricted with BamHI and NcoI and cloned into a pQE60 expression vector (Westburg). The resulting plasmid was transformed into *E. coli* M15 cells (which contained the pREP4 repressor plasmid) and colonies were selected on LB agar plates with ampicillin (100 μg ml\(^{-1}\)) and kanamycin (25 μg ml\(^{-1}\)).

Purification of the recombinant OpcL protein. The OpcL lipoprotein was purified with an Ni-NTA spin kit under denaturing conditions, following the recommendations of the manufacturer (Qiagen). Purity of OpcL was checked by SDS-PAGE followed by silver staining.

Obtainment of polyclonal antibodies against OpcL. BALB/c mice between 6 and 10 weeks old were immunized subcutaneously with 3 μg purified OpCL protein that was emulsified in complete Freund’s adjuvant. Second and third immunizations were done after 21 and 42 days, respectively, with the same amount of protein emulsified in incomplete Freund’s adjuvant and without adjuvant, respectively. Blood samples were taken from the eye vein before the first immunization and 20 and 41 days after the second and third immunizations, respectively. Mice were killed after 63 days. Antibody specificity was checked against total membrane fractions of *B. multivorans* LMG 13010\(^T\) and *E. coli* M15 by Western blot.

Total extracts and outer membranes. Total and outer membranes were obtained from the bacterial pellet of a 50 ml culture by the Sarkosyl differential membrane solubilization method (Filip et al., 1973) as described previously (Cornelis et al., 1989; Lim et al., 1997).

Immunofluorescence assays of bacterial cells. Cell pellets from *B. multivorans* LMG 13010\(^T\), *E. coli* M15 and *E. coli* M15–opcL were obtained from 1-5 ml cultures that had been grown until they reached an OD\(_{600}\) of 0.5 and then, when necessary, the cultures were induced for 3 h with IPTG. After washing with PBS, cells were incubated for 1 h with 1% BSA (w/v) in PBS to block unspecific binding sites. Then, 500 μl polyclonal anti-OpcL FITC-conjugate (1:1000) was allowed to react with the cells for 1 h, followed by three washing steps with PBS. After washing, each pellet was pre-equilibrated for 10 min in equilibration buffer from the SlowFade AntiFade kit (Molecular Probes). Following removal of equilibration buffer, 50 μl component A and component B
antifade reagents (1:1) was added for staining for 15 min, after which cells were examined under normal and UV light by using a Leitz Wetzar microscope.

**PCR amplification of opcL.** Each 25 μl reaction mixture contained 1× PCR buffer, 2 mM each dNTP, 50–100 ng template DNA, 0.2 U Taq polymerase enzyme (Invitrogen) and 30 pmol each primer [OLBam60 (‘-CGGGATCTGTAAGTGGGTGAGG-3‘), corresponding to the beginning of the opcL gene (60 bp from the 5‘ end), and OLKR511 (5‘-GGGTACCTTACCGGGTGAGG-3‘), corresponding to the end of the ORF of the opcL gene]. The amplification program comprised a 3 min initial denaturation step at 94 °C, followed by 30 cycles of amplification, each of which comprised a denaturation step at 94 °C for 40 s, annealing at 66 °C for 40 s and extension at 72 °C for 45 s, followed by a final extension of 10 min at 72 °C. Negative controls contained either the PCR mix without template DNA or with genomic DNA from related species, mentioned in Table 2. An aliquot (5 μl) of the PCR mixture was subjected to electrophoresis in 1 % (w/v) agarose gel.

**Amplification of opcL from sputa of CF patients.** Sputum samples were collected, liquefied with mixing at room temperature for 20 min in an equal volume of Sputasol (Oxoid) and stored at −70 °C prior to DNA extraction. Liquefied sputum (300–500 μl) was centrifuged at 13 000 g for 5 min. The supernatant was discarded and the pellet was resuspended in 500 μl PBS. After repetition of the centrifugation step, DNA was isolated from the pellet by using a QIAamp DNA Mini kit (Qiagen), following the manufacturer’s protocol for tissue. A 5 μl aliquot of this DNA in a total reaction volume of 50 μl was used directly in PCR assays, using an annealing temperature of 58 °C. The presence of bacterial DNA that was susceptible to PCR amplification was confirmed for each sample by PCR detection of 16S rRNA gene sequences by using the primer pair PSL and PSR, as described by Campbell et al. (1995). Following purification by using Microspin S-400 HR columns (Amer sham Biosciences), opcL PCR amplicons were sequenced directly (Lark Technologies, Saffron Walden, UK) by using the same primers that were employed in the PCR amplification (OLBam60 and OLKR511). As a further control, amplification of the cable pilin gene (cblA) was carried out, as described by Goldstein et al. (1995).

**DNA sequence and phylogenetic analysis.** Amplified fragments (450 bp) that corresponded to the opcL gene were cloned into vector pCR2.1 (TA Cloning kit; Invitrogen) or into vector pUC19, restricted with Smal. Sequencing was done for at least two clones from each genomovar by Eurogentec (Seraing, Belgium) by using universal primers. Multiple sequence alignments and a phylogenetic tree were constructed by using the BioNumerics 2.1 software package (Applied Maths), based on the neighbour-joining method (Saitou & Nei, 1987).

### RESULTS

**Search for PAL homologues in Burkholderia sp. LB 400**

The amino acid sequence from *P. aeruginosa* OprL was used in a BLAST comparison with translated *Burkholderia* sp. LB 400 genomic sequences; this was the only genome sequence that was available at the time this work was initiated (http://www.jgi.doe.gov). One protein sequence gave 42 % similarity at the amino acid level, with a higher degree of similarity at the C-terminal end. The corresponding gene was named opcL. An amino acid alignment of OpcL from *Burkholderia* sp. LB 400 with other PALs revealed similarity levels that ranged from 36 to 66 % (Fig. 1). Highest similarity (68 %) was to the PAL from *Ralstonia metallidurans* (http://www.jgi.doe.gov).

---

**Fig. 1.** Identity between the translation products of the opcL gene from *B. multivorans* LMG 13010T (B.m), *Burkholderia* sp. LB 400 (B.s), *B. cenocepacia* J2315 (B.c), *R. metallidurans* (R.m), *R. solanacearum* (R.s) and the oprL gene from *P. aeruginosa* (Pa). CONS, Consensus.
Amplification of the opcL gene of B. multivorans

Several primer pairs were designed, based on the opcL gene sequence of Burkholderia sp. LB 400, in order to amplify the opcL gene of B. multivorans LMG 13010T. Of all primers tested, only P33 and P421 gave rise to the amplification of a single fragment with the expected size of 400 bp (data not shown). The identity of this fragment as opcL was confirmed by direct nucleotide sequence analysis of the PCR product. As the opcL fragment amplified from B. multivorans is incomplete (missing the 5’ and 3’ ends), a cosmid genomic library was prepared from B. multivorans in order to clone and sequence the complete opcL gene.

Screening of the B. multivorans genomic bank

Of 3000 clones in the cosmid genomic library from B. multivorans LMG 13010T, three clones that hybridized with the 400 bp B. multivorans opcL fragment were selected. The complete opcL gene was further sequenced and its product was compared with those of other reported PALs by using the BioNumerics software program. These included the PALs from Burkholderia sp. LB 400 (http://www.jgi.doe.gov), P. aeruginosa (Lim et al., 1997), E. coli (Chen & Henning, 1987), H. influenzae (Nelson et al., 1988), Pseudomonas putida (Rodríguez-Herva et al., 1996), R. metallidurans (http://www.jgi.doe.gov), Ralstonia solanacearum (http://www.jgi.doe.gov) and B. cenocepacia J2315 (http://www.sanger.ac.uk). The alignment between OpcL from B. multivorans, Burkholderia sp. LB 400 and B. cenocepacia revealed 82 and 95 % similarity, respectively. OpcL showed only 42 % similarity to OprL from P. aeruginosa (Fig. 1).

Outer-membrane localization of OpcL

Production and outer-membrane localization of B. multivorans LMG 13010T OpcL were demonstrated by SDS-PAGE and Western blot analyses. An 18 kDa protein band that corresponded to OpcL was clearly visible in the outer-membrane preparations from B. multivorans LMG 13010T and E. coli M15 pQE60–opcL, as detected by Western blot using a polyclonal anti-OpcL serum. No protein of the same molecular size could be detected from E. coli M15 on SDS-PAGE, nor on Western blot (Fig. 2a and b).

Immunofluorescence assay

Immunofluorescence experiments were performed by using the mouse polyclonal antibody that was raised against the E. coli-produced and gel-purified OpcL. The polyclonal antibody M15–OpcL gave a positive reaction with E. coli M15 cells that were expressing opcL (Fig. 3b), resulting in the staining of elongated cells that were also clearly observed in visible light (Fig. 3a). No fluorescence was observed with E. coli M15 cells (Fig. 3d); furthermore, these cells had a normal appearance when observed in visible light (Fig. 3c). No fluorescence was observed for B. multivorans LMG 13010T cells without prior treatment with EDTA and SDS (results not shown).

PCR amplification of the opcL gene in B. cepacia complex strains

Based on the B. multivorans opcL sequence, we designed primers OL Bam60 and OLKR511 to amplify the opcL gene (lacking the part that encodes the signal peptide sequence) from 50 different strains that belong to the B. cepacia complex. A fragment of 450 bp was amplified for all strains.
Identity of the 450 bp fragment as opcL was subsequently confirmed by direct nucleotide sequence analysis of PCR products. No amplification product was detected for related species of β-proteobacteria, including Burkholderia species that do not belong to the B. cepacia complex (Table 2).

**Sequence comparison and phylogenetic analysis of the opcL gene**

opcL gene sequences from two to three strains in each genomovar were compared at the nucleotide level for the presence of substitutions, with the B. multivorans LMG 13010T opcL sequence as reference. The 21 opcL sequences (corresponding to different genomovars) were extremely conserved, with 96% nucleotide sequence similarity between B. cepacia complex strains. Construction of an opcL-based phylogenetic tree of the B. cepacia complex was carried out by using the B. multivorans LMG 13010T opcL nucleotide sequence to root the tree. The resulting nucleotide sequence-based phylogenetic tree is shown in Fig. 4.

**Amplification of opcL from sputa of CF patients**

The PCR assay that used the primer set OLBam60/OLKR511 was tested on sputum samples from 18 adult CF patients, all but three of whom were known to be colonized by B. cepacia complex strains. In accordance with colonization status, PCR amplicons were obtained from 15 of 18 samples. Sample PCR assay results are presented in Fig. 5. The 15 amplicons were sequenced and, in each case, yielded unequivocal sequence data without the requirement for cloning the PCR product. Fourteen of the 15 sequences were identical and showed 100% similarity to opcL from B. cenocepacia (results not shown). The same samples were also PCR-positive for the cblA gene, a marker for the B. cenocepacia ET12 lineage (Clode et al., 2000). The other sequence was identified as B. multivorans, also with 100% similarity.

**DISCUSSION**

**Identification of Burkholderia strains by using opcL as a probe**

The main goal of this study was to develop a rapid and sensitive additional method for specific detection and identification of B. cepacia complex strain isolates from natural and hospital environments. Early detection and accurate identification of B. cepacia complex strains are extremely important in the case of CF patients (Mahenthiralingam et al., 2002). Phenotypic identification techniques are often insufficiently reliable for the differentiation of strains within the B. cepacia complex. The API 20NE numerical identification system (bioMérieux) is considered to be the best system for biochemical identification of all B. cepacia complex strains except for B. stabilis and B. cepacia (Henry et al., 2001). Discrimination could be improved if the API 20NE database were to incorporate negative p-nitrophenyl β-D-glucoside (PNPG) results and no growth at 4 °C as diagnostic criteria for B. stabilis (Henry et al., 2001). B. multivorans, B. stabilis, B. vietnamiensis and genomovar VI were distinguished from other members of the B. cepacia complex by tests such as sucrose and adonitol acidification, PNPG utilization and growth at 42 °C (Henry et al., 2001).
Oxidation of sugars and oxidase reaction were also useful to distinguish *Burkholderia gladioli*, *Ralstonia pickettii* and *Pandoraea* spp. (Henry et al., 2001).

Genomic analysis techniques proved to be more reliable tools for identification of *B. gladioli*, as demonstrated by species-specific PCR (SS-PCR) based on the 16S and 23S rRNA genes (Bauernfeind et al., 1998; Whitby et al., 2000). By using PCR-RFLP of the 16S rRNA gene, Segonds et al. (1999) analysed 51 presumed *B. cepacia* clinical isolates, of which only 24 (47 %) were identified as *B. cepacia* and 15 (28 %) could not be identified completely. In the same study, one isolate was identified as *Aeromonas salmonicida* because of negative assimilation tests, and six of the 11 isolates that could not be identified by molecular tests were identified completely by use of the oxidase test.

Several groups have investigated SS-PCR and RFLP analysis as a tool to identify *B. cepacia* complex genomovars (Bauernfeind et al., 1998; LiPuma et al., 1999; Segonds et al., 1999; Whitby et al., 2000). Reliable SS-PCR methods to identify *B. multivorans* and *B. vietnamiensis*, based on the 16S rRNA and 23S rRNA genes, have been described by LiPuma et al. (1999). Sequence similarity of the 16S–23S rRNA genes among genomovars I, II and IV was, however, too high to allow the design of species-specific primers (LiPuma et al., 1999). Another SS-PCR based on recA has also been described (Vandamme et al., 2000; Mahenthiralingam et al., 2002). Specificity and sensitivity of the recA-based PCR assay for 85 clinical and 17 environmental isolates of *B. multivorans* were both 100 % (Vermis et al., 2002b). The same high specificity and sensitivity were obtained for *B. ambifaria* (six clinical and 47 environmental isolates), but the assay lacked sensitivity (72 %) in the case of *B. cepacia* genomovar I (29 clinical and 42 environmental isolates), which cross-reacted with all *B. pyrocinia* isolates examined (Vermis et al., 2002b).

Our analysis of *opcL* genes in strains of the *B. cepacia* complex demonstrated that it is a suitable additional marker for detection and identification of *B. cepacia* complex isolates and differentiation from other *Burkholderia* species that are not part of the complex, such as *Burkholderia fungorum*, *Burkholderia graminis*, *Burkholderia terricola*, *B. gladioli* and *Burkholderia glumae*. No amplification was obtained from other *C226*-proteobacteria, such as *Ralstonia*, *Bordetella*, *Pandoraea* and *Alcaligenes*. Alignment of the *opcL* gene from the *B. cepacia* complex shows a low degree of nucleotide sequence variation (97 % similarity), whereas the gene product (the Opcl lipoprotein) is better conserved.
conserved, showing 98 % similarity. In the phylogenetic tree based on comparison of opcL sequences, *B. multivorans* LMG 13010^T^ clusters together with strains LMG 16668 and LMG 16660, with good bootstrap support between 83 and 100 % within the same genomovar. However, in this phylogenetic tree, not all strains that belong to the same genomovar cluster together.

In the tree based on sequence similarity of the opcL gene, strains of *B. multivorans* (genomovar II), *B. stabilis* (genomovar IV), ‘*B. dolosa*’ (genomovar VI), *B. ambifaria* (genomovar VII) and *B. pyrocinia* (genomovar IX) clustered together, but the most defined clusters were observed for strains that belong to *B. multivorans* and *B. ambifaria*. Such easy differentiation of *B. multivorans* and *B. ambifaria* could be interesting, as Vandamme et al. (1997) showed that *B. multivorans* strains were mainly found in CF patients, whereas most of the highly transmissible strains were reported to be strictly specific to *B. multivorans* or *B. cenocepacia* (Segonds et al., 1999; Vandamme et al., 2003).

### Detection of *Burkholderia* strains from sputa

Although performed on a limited number of samples, we have demonstrated that amplicons can be generated from the sputa of patients who are known to be colonized by *B. cepacia* complex strains. Sequence analysis allowed unambiguous identification of strains and revealed that the majority of patients were colonized by *B. cenocepacia* (genomovar III), confirming the results of other studies (LiPuma et al., 2001; Speert et al., 2002; Cunha et al., 2003).

### Expression of opcL and localization of the OpcL protein

The OpcL protein could be detected in the outer membranes of *E. coli* and *Burkholderia* spp. alike. Expression of the opcL gene in *E. coli* had an effect on the morphology of the cells; they appeared to be more elongated, an observation that is consistent with the results of another study where oprL from *P. aeruginosa* was expressed in *E. coli* (Lim et al., 1997). The polyclonal antiserum that was raised against the denatured OpcL protein recognized epitopes that were exposed at the surface of the *E. coli* host, as shown by immunofluorescence experiments, but this was not the case when examining *Burkholderia* cells. Again, this situation is similar to that observed for oprL expression in *E. coli* and *P. aeruginosa* (Lim et al., 1997).

We are now in the process of purifying OpcL from *E. coli* cells that express opcL, in order to use this antigen to detect the presence of antibodies in sputa of CF patients infected with *Burkholderia* spp.

### Conclusion

This study shows that opcL is an interesting probe for the detection and identification of *B. cepacia* complex strains, either from colonies or in the sputa of CF patients.

### ACKNOWLEDGEMENTS

This research was financed by an FWO grant (krediet aan navorsers 1.5.208.01N); M. P. received a fellowship from an OZR grant from Vrije Universiteit Brussel.

### REFERENCES


Woodcock, D. M., Crowther, P. J., Doherty, J., Jefferson, S., DeCruz, E.,