Identification of *Burkholderia cepacia* complex Pathogens by Rapid-Cycle PCR with Fluorescent Hybridization Probes

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Running Title: FRET probes for *B. cepacia* complex differentiation

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Summary

Members of the *B. cepacia* complex are important bacterial pathogens in cystic fibrosis patients (CF). The *B. cepacia* complex currently consists of nine genetic subgroups (genomovars) with different epidemiological relevance and possibly different pathogenic potential in humans. In this study we developed a new approach for the rapid identification of *B. cepacia* genomovar I, *B. multivorans* (genomovar II) *B. cenocepacia* (lineage III-A and III-B), *B. stabilis* (genomovar IV) and *B. vietnamiensis* (genomovar V) causing the large majority of infections in CF patients. The method is based on the detection of differences in the *recA* gene sequence by using rapid-cycle PCR and genomovar-specific fluorescence resonance energy transfer (FRET) probes. The genomovar status of all 39 *B. cepacia* complex strains tested (genomovars I to V) was identified by melting curve analysis. Each FRET probe produced a specific fluorescence signal only with the respective genomovar but not with other *B. cepacia* complex strains and *Burkholderia spp*. Our identification system is easy to handle and reveals *B. cepacia* complex genomovar status I to V from culture isolates within about one hour.

Introduction

Burkholderia cepacia first described by Burkholder as the cause of soft rot in onions is commonly found throughout the environment and causes opportunistic infections in humans. During the last few years it has been recognized that organisms previously identified as *B. cepacia* appear to be very heterogeneous and constitute a complex of phenotypically similar species among which at least nine *Burkholderia* species can be differentiated by molecular methods. In patients with cystic fibrosis (CF) *B. cepacia* complex strains are associated with the "cepacia syndrome", a necrotizing pneumonia with bacteraemia, which is often fatal (Isles et al., 1984). The use of *B. cepacia* complex strains as a fertiliser and pesticide may serve as a source of infections for patients although there has been no proof so far that agriculturally used strains led to infections of CF patients (Balandreau et al., 2001; LiPuma et al., 2002). Person-to-person transmission of *B. cepacia* complex strains through social contact has been shown (Agodi et al., 2002; Govan et al., 1993) leading to attempts to reduce this risk by segregation of patients (Thomassen et al., 1985; Muhdi et al., 1996). Due to its poor prognosis after allogenic lung transplantation (Snell et al., 1993; Chaparro et al., 2001) some transplant centers excluded patients colonized with *B. cepacia* complex strains from lung transplantation programs (Aris et al., 2001).

So far *B. cepacia* genomovar I, *B. multivorans* (genomovar II) (Vandamme et al., 1997), *B. cenocepacia* (genomovar III) (Vandamme et al., 2003), *B. stabilis* (genomovar IV) (Vandamme et al., 2000), *B. vietnamiensis* (genomovar V) (Gillis et al., 1995), *B. dolosa* (genomovar VI) (Vermis et al., 2004; Coenye et al., 2001), *B. ambifaria* (genomovar VII) (Coenye et al., 2001), *B. anthina* (genomovar VIII) and *B. pyrrocinia* (genomovar IX) (Coenye et al., 2001) have been identified within the *B. cepacia* complex. Epidemiological studies indicated differences in transmission frequence and pathogenicity within the CF population depending upon genomovar status (Biddick et al., 2003). Especially *B.*

cenocepacia seems to be associated with high transmission frequence and poor prognosis (Woods et al., 2004).

Several methods have been used in order to identify genomovar status of members of the *B. cepacia* complex. Phenotypic tests separated reliably only *B. multivorans* and *B. stabilis* (Henry et al., 2001). 23S rDNA- and 16S rDNA-based PCR assays allowed differentiation of *B. multivorans* and *B. vietnamiensis*, but not *B. cepacia*, *B. cenocepacia* or *B. stabilis* (LiPuma et al., 1999; Bauernfeind et al., 1999). Whitby et al. (Whitby et al., 2000) developed a PCR algorithm of the 16S-23S rDNA spacer region being able to separate *B. cenocepacia* and *B. stabilis* but not *B. cepacia*. Conventionally amplified fragment length polymorphism analysis (AFLP) can be used for *B. cepacia* complex differentiation but is technically demanding while automated AFLP still requires visual examination for correct identification of all isolates (Coenye et al., 1999). Restriction fragment length polymorphism (RFLP) of 16S ribosomal DNA led to the identification of groups but was unable to distinguish many strains of *B. cepacia*, *B. cenocepacia*, *B. stabilis* and *B. pyrrocinia* (Segonds et al., 1999; Vermis et al., 2002). Discrimination of *B. cepacia* genomovar I, *B. multivorans*, *B. cepacia* genomovar III, *B. stabilis*, *B. vietnamiensis* and *B. ambifaria* is possible by RFLP analysis of the PCR amplified *recA* gene using *B. cepacia* complex-specific *recA* primers (Mahenthiralingam et al., 2000; Coenye et al., 2001). This approach was also used by McDowell et al. (McDowell et al., 2001) for direct detection of *B. cepacia* complex strains in sputum of CF patients. Most *B. cepacia* complex genomovars can also be identified by using *recA*-based genomovar-specific primers (Mahenthiralingam et al., 2000). Based on this approach nested PCR assays have been described that improve the sensitivity of *B. cepacia* complex detection from sputum specimens (Moore et al., 2002; Drevinek et al., 2002). A recent evaluation of genomovar-specific *recA*-based PCR tests by Vermis et al. (Vermis et al., 2002) revealed that PCR primers designed to be specific for *B. cepacia* genomovar I cross-reacted with *B. pyrrocinia* and failed to detect some genomovar I isolates. Since the rapid and

reliable detection of *B. cepacia* complex organisms is important for infection control measures as well as for the clinical management of patients, a rapid method for genomovar determination suitable for daily use in clinical laboratory is desirable.

The aim of this study was to develop a rapid test based on LightCycler technology for the differentiation of the most common *B. cepacia* complex culture isolates based on the known *recA* gene polymorphism (Mahenthiralingam et al., 2000). The LightCycler technique is based on the principle of conventional PCR but reduces DNA amplification time by increasing temperature transition rate using air for heating and cooling (Cockerill et al., 2002). Mutations within genes can be detected by sequence-specific fluorescence resonance energy transfer (FRET) probes labeled with two different fluorescence dyes that generate a fluorescence signal only when both probes bind to the target sequence. After the amplification process, a melting curve analysis is performed: the fluorescence is monitored with increasing temperature. A fluorescence decrease is obtained when probes melt off. Mismatches between probe sequence and target sequence lead to a lowered melting temperature.

B. cepacia, *B. multivorans*, *B. cenocepacia*, *B. stabilis* and *B. vietnamiensis* usually comprise more than 95% of *B. cepacia* complex isolates in CF patients (Speert et al., 2002; Agodi et al., 2001; LiPuma et al., 2001). We therefore developed an identification protocol based on *B. cepacia* complex-specific LightCycler amplification of the *recA* locus and newly designed genomovar-specific FRET probes for the rapid detection of genomovars I to V.

Methods

Bacterial strains and template preparation. Strains of *B. cepacia* genomovars I, *B. multivorans* (II), *B. cenocepacia* lineages III-A and III-B, *B. stabilis* (IV) and *B. vietnamiensis* (V) were recieved from the BCCM/LMG Bacteria Collection or were clinical isolates of Hannover Medical School which were tentatively identified as *Burkholderia cepacia* after

growth on OFBL agar (Henry et al., 1997) and determination of biochemical profiles using API 20NE. Those strains were further identified by SDS-PAGE of whole-cell proteins and, if necessary, by *recA* RFLP as described (Coenye et al., 2001). All strains including non-*B. cepacia* complex organisms used in this study are listed in TABLE 1. Bacteria were grown over night on Columbia blood agar plates at 37°C. 10 colonies were then mixed with 1 µL DNA free water. 2 µL of this suspension served as template. No further DNA extraction was performed. Besides bacterial suspensions other than *B. cepacia* complex DNA free water also served as negative control.

LightCycler protocol. A LightCycler system (Roche Molecular Biochemicals, Mannheim, Germany) and FRET probes (TIB MOLBIOL, Berlin, Germany) labelled with different LC-Red dyes (640 nm and 705 nm) were used. FRET probes were designed to show a perfect match only to the corresponding genomovar. When FRET probes bind to their target sequence a fluorescence signal is detected by the system. If denaturation occurs a loss of fluorescence is observed. Therefore, if the target sequence shows a mismatch the melting temperature of FRET probes is lowered. Visualization of melting temperature when probes denature from the amplicon and by that loss of fluorencence occurs was done by plotting the negative derivative of fluorescence over temperature versus temperature.

Amplification of the *B. cepacia* complex *recA* gene was performed using a total volume of 18 µL of the ready-to-use reaction mixture “Fast Start DNA Master Hybridisation Probe Kit“ (Roche Diagnostics, Mannheim, Germany) supplemented with 4 mM $MgCl_2$, 10 pM of each primer BCR1 and BCR2 (MWG-Biotech, Ebersberg, Germany) specific for *B. cepacia* complex *recA* gene as described (Mahenthiralingam et al., 2000) (500 nM final concentration) and 2 pM of two probe pairs for genomovar determination (100 nM final concentration). 2 µL of template as described above were added for a total sample volume of 20 µL in each glass capillary.

After an initial denaturation step (95°C, 10 min) 45 cycles consisting of denaturation (95°C, 10 s each), annealing (reached with a touch-down from 66°C to 60°C at 1°C/cycle, 10 s each) and elongation (72°C, 20 s each) were run for *recA* gene amplification. After a final denaturation (95°C, 30 s) melting curve analysis from 40°C up to 75°C was done measuring fluorescence at 640 nm and 705 nm continously. Temperature transition rate was 20°C/s for all changes in temperature except melting curve analysis when rate was lowered to 0.1°C/s.

The sequences of FRET probes used for genomovar determination are listed in TABLE 2. Probe pairs for detection of genomovar I, II and III-A were labeled with LightCycler Red 640 and of genomovar III-B, IV and V were labeled with LightCycler Red 705, respectively. A software colour compensation file was used when FRET probes with labels of different wave lengths were tested simultaneously. Sequences of hybridization probes were based on the alignment of published *recA* sequences of defined *B. cepacia* complex genomovars I to IX (GenBank accession no. AF143774 to AF143795, AF143798 to AF143799, AF323985, AF456003, AF456005 to AF 456007, AF456012 to AF456014, AF456021, AF456027 to AF456033, AF456045, AF456055, AF456058, AF456060, AF456070, AF456076 to AF456077, AF456093, AF456097, AF456102, AF456104, AF456106 to AF456108 and AJ544692) using DNA Star Windows 32 MegAlign 4.00 Sofware.

To determine sensitivity of our LightCycler protocol template suspension was prepared as described above. Serial dilution of template suspension using DNA free water was performed. Diluted probes then were used for LightCycler detection as well as for determination of bacterial CFU after growth over night on Columbia blood agar plates at 37°C.

Results and Discussion

We first tested amplification of the *recA* gene with primers BCR1 and BCR2 (Mahenthiralingam et al., 2000; McDowell et al., 2001) in strains belonging to *B. cepacia* complex genomovar I, II, III-A, III-B, IV and V using LightCycler technology. Under those experimental conditions primers BCR1 and BCR2 also proved to be specific for these genomovars as described by conventional PCR (Mahenthiralingam et al., 2000) and led to a PCR product of 1,043 bp visualized by conventional gel electrophoresis, whereas non-*B. cepacia* complex members did not show an amplification product of this size (not shown).

After alignment of *recA* sequences from genomovar I to IX genomovar-specific areas were used to design FRET probes. Sequences are shown in TABLE 2. Using these probes the genomovar status of all 39 *B. cepacia* complex strains belonging to genomovar I, II, III-A, III-B, IV, and V was correctly identified by melting curve analysis. FIGURE 1 shows the specific peak loss of fluorescence of the detection probes of four representative strains of either *B. multivorans* (B), *B. cenocepacia* III-A (C) or *B. cenocepacia* III-B (D) and of five strains of either *B. cepacia* (A), *B. stabilis* (E) or *B. vietnamensis* (F). Each probe pair was tested against members of all other genomovars and lineages as well as against the non-*B. cepacia* complex strains listed above in order to exclude possible interference. For all other but the corresponding genomovar only unspecific melting points at significantly lower temperatures or no peaks at all were observed including lineages III-C and III-D (data not shown).

The observed melting point differences between strains within one genomovar (FIGURE 1) are most likely due to point mutations in the *recA* sequence part used for hybridization. For example differences of peak melting point between the *B. cepacia* strains LMG 1222^{T} (GenBank accession number AF143786) and strains LMG 18821 and LMG 2161 (GenBank accession numbers AF143787 and AF143788) as shown in FIGURE 1A are due to a single nucleotide mismatch in position 339. We conclude a similar phenomenon for *B.*

cenocepacia III-B (FIGURE 1D) and *B. vietnamiensis* (FIGURE 1F). However, the differences in melting curve analysis are still sufficiently small to distinguish these genomovars from all other genomovars. Our LightCycler method could detect as few as five CFU of all genomovar I to V strains tested. FIGURE 2 shows a representative assay for *B. cepacia* lineage III-B LMG 16659.

In conclusion we were able to develop a rapid-cycle PCR system that allows determination of *B. cepacia* complex genomovars I to V from culture isolates within about one hour using genomovar-specific FRET probes. This approach could also discriminate genomovar I from *B. pyrrocinia* (genomovar IX) since there was no melting point signal with a genomovar I–specific FRET probe with *B. pyrrocinia* as shown in FIGURE 1A. This discrimination has not been possible by conventional genomovar-specific PCR (Vermis et al., 2002). Our method is based on the use of commercially available components under standardized conditions. It should therefore enable any clinical laboratory with access to LightCycler technology to distinguish between these genomovars exhibiting different transmission frequences and probably different pathogenic potential (Agodi et al., 2001; Speert et al., 2002; LiPuma et al., 2001). Rapid-cycle real-time PCR has not only been used for the differentiation of a variety of pathogens from cultures but also for direct detection from clinical specimens. In first experiments we have been able to detect directly genomovars I to V in CF sputum specimens seeded with the respective organisms using this LightCycler technology (data not shown). Future studies will have to evaluate the sensitivity and specifity of this method for clinical specimens.

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1 TABLE 1: Bacterial strains used in this study

***B. cepacia* complex**

B. cepacia genomovar I
- LMG 1222 T
- LMG 2161
- LMG 6864
- LMG 6889
- LMG 17997
- LMG 18821

B. multivorans
- LMG 13010 T
- LMG16660
- LMG 17588
- IMM 107
- IMM 208
- IMM 293
- IMM 302
- IMM 361
- IMM 364
- IMM 365
- IMM 5441

B. cenocepacia - lineage III-A
- LMG 16656 T
- LMG 6981
- LMG 6986
- IMM 314

B. cenocepacia - lineage III-B
- LMG 16659
- LMG 18829
- LMG 18830
- IMM 376

B. cenocepacia - lineage III-C
- LMG 19230
- LMG 19240

B. cenocepacia - lineage III-D
- LMG 21461
- LMG 21462

B. stabilis
- LMG 14294 T
- LMG 14086
- LMG 18870
- IMM 134
- IMM 196

B. vietnamiensis
- LMG 10929 T
- LMG 16232
- LMG 18835
- IMM 253
- IMM 5223

B. dolosa
LMG 18941
LMG 21443
LMG 21820
B. ambifaria
LMG 17828
LMG 19182
LMG 19467
B. anthina
LMG 16670
LMG 20980
LMG 21821
B. pyrrocinia
LMG 14191
LMG 21822
LMG 21823
LMG 21824

non *B. cepacia* complex:
B. gladioli
IMM 60732
B. pseudomallei
IMM E8
B. thailandensis
IMM E27
ATCC 700388
S. aureus
ATCC 25923
P. aeruginosa
ATCC 27853
E. faecalis
ATCC 29212
E. coli
ATCC 25922

ATCC = American Type Culture Collection

IMM = Institute for Medical Microbiology and Hospital Epidemiology, Medical School Hannover

LMG = Laboratorium voor Mikrobiologie, Universiteit Gent

TABLE 2: Sequences of genomovar-specific FRET probes

probe	sequence (5´- 3´)	orientation	position (bp)
I – anchor	LightCycler Red640-CCG AGC TTC GAC GCG TAC TGG A	antisense	305 – 272
I – sensor	GCA GTT CCG GCA CAT TCA C-fluorescein	antisense	329 – 346
II – anchor	LightCycler Red640-GTC CAC GGG CTC GCT CGG	sense	120 – 138
II – sensor	GCC GAG GAC ATC CAG GTC G- fluorescein	sense	100 – 118
III-A – anchor	LightCycler Red640-CGT GAA CGT GCC GGA GCT GC	sense	327 – 346
III-A – sensor	CGT TCA ATA TGC CGC GAA GCT C- fluorescein	sense	303 – 324
III-B – anchor	LightCycler Red705-AAG ATC GGC GGC ACG GCA G	sense	257 – 274
III-B – sensor	CTG CAA GTC ATC GCT GAA CTG C- fluorescein	sense	232 – 253
IV – anchor	TGA TCT CGC AGC CGG ACA CCG- fluorescein	sense	347 – 367
IV – sensor	LightCycler Red705-CGA GCA GGC GCT TGA AAT CAC	sense	369 – 389
V – anchor	LightCycler Red705-AGG TCG TCT CCA CGG GCT CGC T	sense	114 – 134
V – sensor	GGC GAC GTG AAG GAA GAC ATC- fluorescein	sense	91 – 111

LEGEND FIGURE 1:

B. cepacia complex genomovar identification using genomovar-specific FRET probes is shown as screen captures of melting peak analysis. The melting curve analysis is displayed as the first negative derivative of the fluorescence (-dF/dT) versus temperature. F2 refers to channel 2, which is used to measure signals from LightCycler-Red 640 at 640 nm (A to C). F3 refers to channel 3, which measures signals from LightCycler-Red 705 at 705 nm (D to F). The identification of representative strains from with the specific FRET probes for *B. cepacia* (five strains) (A), *B. multivorans* (four strains) (B), *B. cenocepacia* lineage III-A (four strains) (C), *B. cenocepacia* lineage III-B (four strains) (D), *B. stabilis* (five strains) (E), and *B. vietnamiensis* (five strains) (F) is shown. *B. dolosa (*genomovar VI) (brown), *B. ambifaria* (genomovar VII) (purple), *B. anthina* (genomovar VIII) (grey), and *B. pyrrocinia* (genomovar IX) (pink) and DNA-free water (black) served as controls. Detection probes show the highest melting temperature with representative strains of the corresponding genomovar while other genomovars showed lower unspecific melting peaks or no peaks at all.

LEGEND FIGURE 2:

Sensitivity of *B. cepacia* complex genomovar identification is shown as screen capture of melting peak analysis and gel electrophoresis of amplicons of a representative strain (*B. cenocepacia* lineage III-B LMG 16659). Five CFU of *B. cepacia* complex strains were reproducibly detected in genomovars I to V.

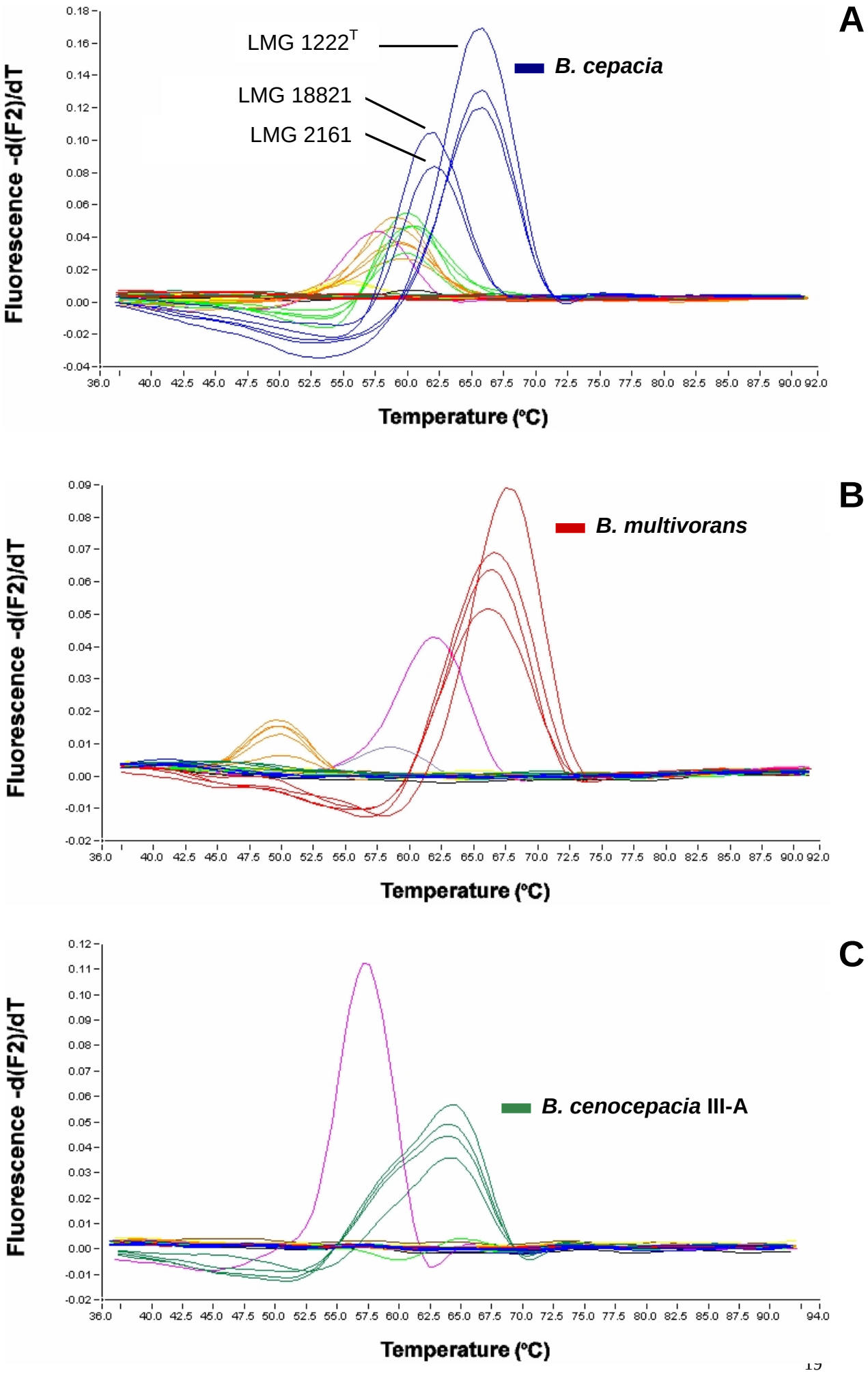
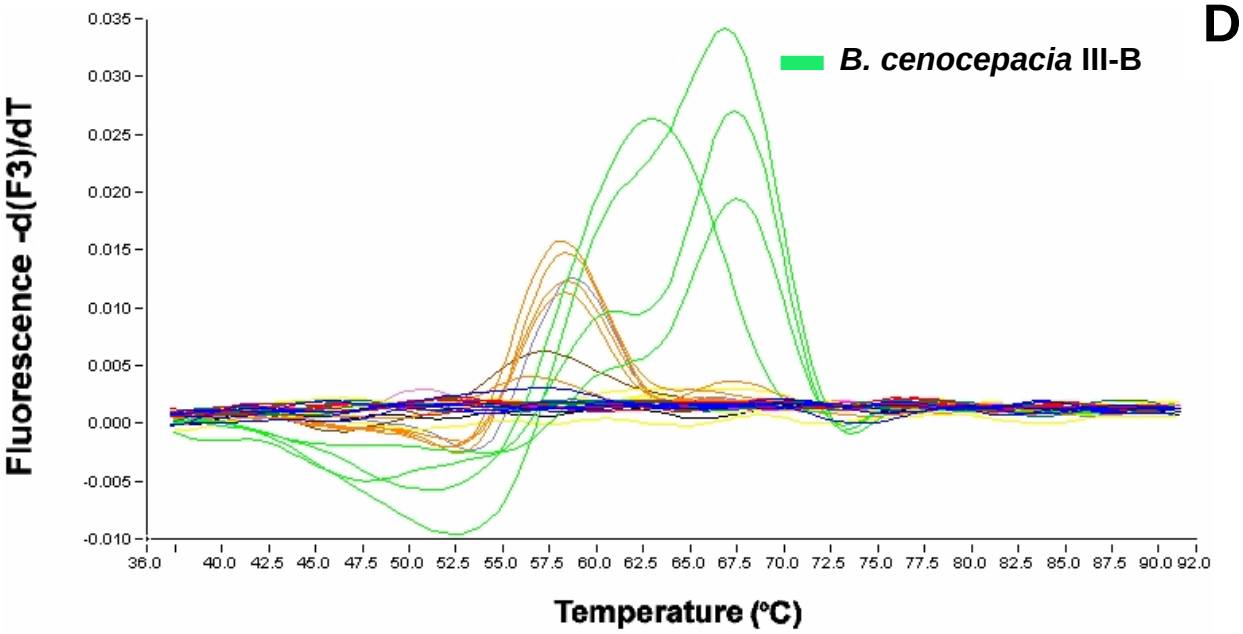
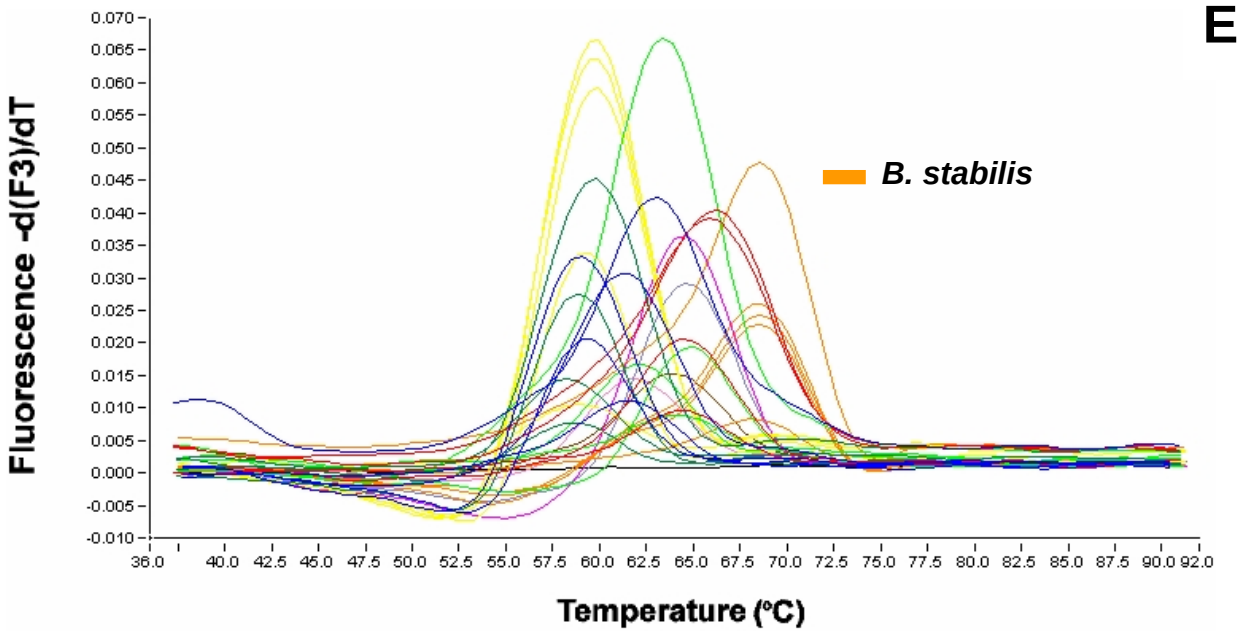

A
LMG 1222T
LMG 18821
LMG 2161
B. cepacia
Fluorescence -d(F2)/dT
Temperature (°C)
B
B. multivorans
Fluorescence -d(F2)/dT
Temperature (°C)
C
B. cenocepacia III-A
Fluorescence -d(F2)/dT
Temperature (°C)

FIGURE 1





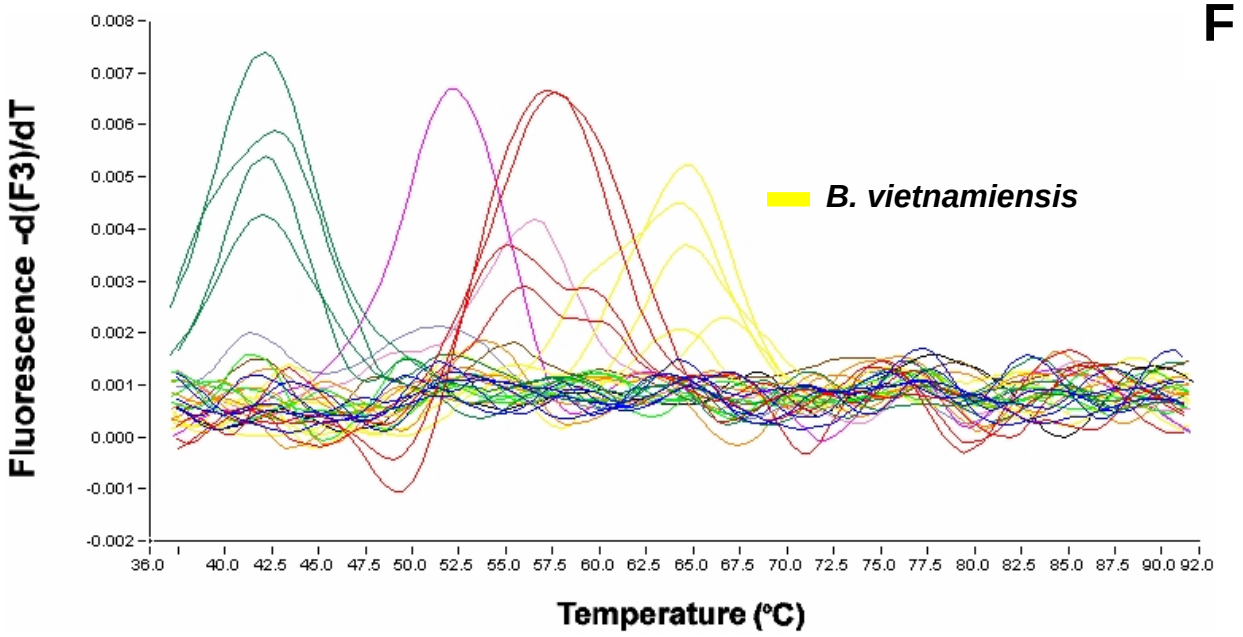


FIGURE 1 continued

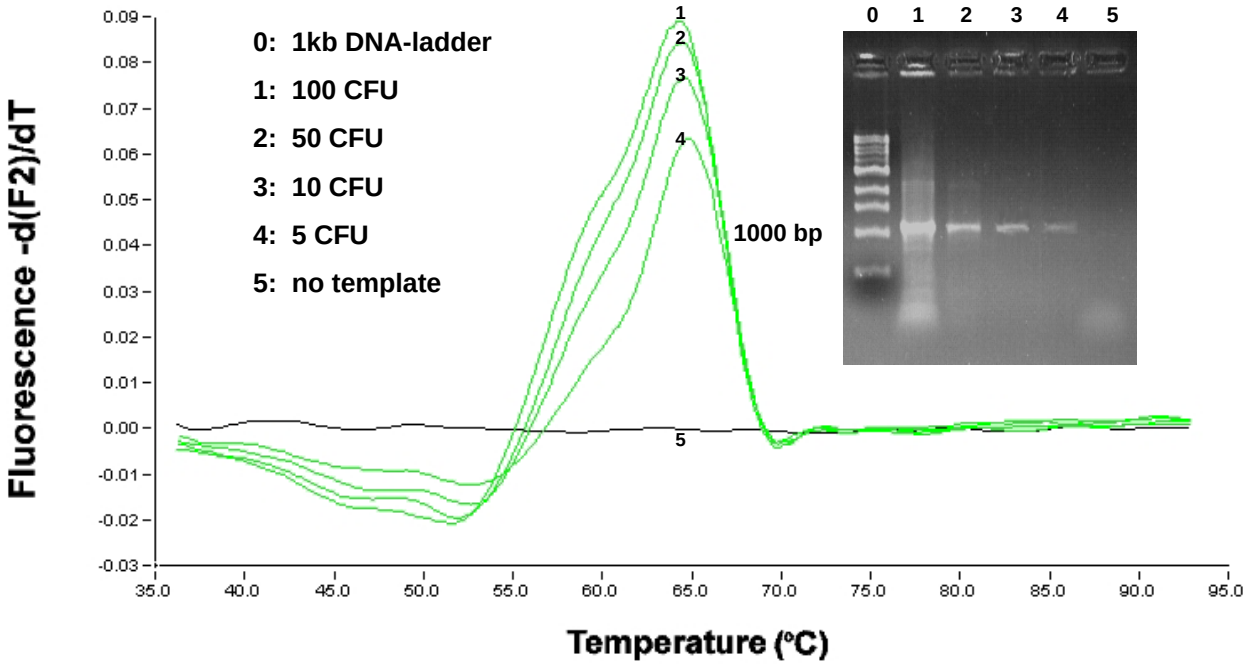

Fluorescence -d(F2)/dT
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
-0.01
-0.02
-0.03
35.0
40.0
45.0
50.0
55.0
60.0
65.0
70.0
75.0
80.0
85.0
90.0
95.0
Temperature (°C)
0: 1kb DNA-ladder
1: 100 CFU
2: 50 CFU
3: 10 CFU
4: 5 CFU
5: no template
1
2
3
4
5
1000 bp
0 1 2 3 4 5

FIGURE 2