Chapter 4

Virulence in a *Caenorhabditis elegans* host model Bcc species

As seen in the introduction, the Bcc is a group of closely related bacterial species, (Coenye *et al.*, 2001, Vandamme *et al.*, 1997), that thanks to their unusually large genomes possess unsurpassed metabolic capacities, that allow them to colonize extremely diverse habitats (Chiarini *et al.*, 2006, Vandamme & Dawyndt, 2011).

In natural environment Bcc species can be found in several ecosystems, where they are generally considered highly beneficial : many isolates protect commercially useful crops against bacterial and fungal diseases, can promote plants growth, fix nitrogen and degrade several man-made toxic agents, in particular chlorinated aromatic compounds (Vial et al., 2011, Mahenthiralingam et al., 2005, Chiarini et al., 2006). On the other hand, these bacteria can also cause lethal infections in immuno-compromised individuals like those affected by CF (Saldias & Valvano, 2009, Mahenthiralingam *et al.*, 2008).

The prevalence (2009 and 2010) of chronic infection by Bcc is reported to vary between 0 and 12% of the CF population attending various CF centres (Ciofu *et al.*, 2013), that it is not high compared to other CF pathogens but Bcc infections often correlates with poorer prognosis, longer hospital stays and an increased risk of death (Mahenthiralingam et al., 2005). Among the reasons for this high mortality rate in Bcc infected patients there are the ability of these bacteria to produce a wide variety of potential virulence factor and their high resistance to antibiotics: they are intrinsically resistant to many antibiotics and can develop *in vivo* resistance to essentially all classes of antimicrobial drugs (Mahenthiralingam et al., 2005, Drevinek & Mahenthiralingam, 2010).

Actually the Bcc comprises 18 validly named species (Table 1) (Vandamme & Dawyndt, 2011, Peeters *et al.*, 2013). Among them, *B. cenocepacia* and *B. multivorans* are the two Bcc species that predominate in CF patients. In particular *B. cenocepacia* is considered one of the most serious pathogens and most of the studies on mechanisms of Bcc virulence have been conducted on this lineage (Holden *et al.*, 2009). The other species of the Bcc are much less characterize and

some have only recently been defined (Vandamme & Dawyndt, 2011, Peeters et al., 2013).

A characterization of the 18 type strains of the Bcc species is in progress in our laboratory from various viewpoint. In particular, sequencing of their genomes and their phenotypic characterization by Phenotype Microarray are still in progress and therefore they are not included in this thesis. We also studied the pathogenicity determinants of these strains by using the non-vertebrate host *Caenorhabditis elegans* (Tedesco *et al.,* submitted for publication).

C. elegans has been proven to be a reproducible and genetically tractable model to validate microbial virulence traits that are involved in mammalian infections (Schwager *et al.*, 2013, Uehlinger *et al.*, 2009), and there is an extensive literature for the usefulness of the nematode to model virulence and antimicrobial efficacy in a variety of bacteria (Wu *et al.*, 2013, Bhatt *et al.*, 2011) including the pathogens *B. pseudomallei* (Lee *et al.*, 2011, O'Quinn *et al.*, 2001, Lee *et al.*, 2013). For example, the set up of a *C. elegans* - *P. aeruginosa* pathogenesis model system has facilitated the systematic dissection of both host and pathogen responses (Tan *et al.*, 1999a, Tan *et al.*, 1999b, Feinbaum *et al.*, 2012).

Bcc strains are able to kill *C. elegans* through different mechanisms that include the intestinal accumulation/colonisation and/or production of toxic compounds, the latter causing first paralyses of the worms and then death (Kothe *et al.*, 2003, Huber *et al.*, 2004). The *C. elegans*-Bcc interaction was the subject of intensive investigation for the last decade (Huber et al., 2004, Kothe et al., 2003, Sousa *et al.*, 2011, Tegos *et al.*, 2012, O'Grady *et al.*, 2012, Cooper *et al.*, 2009, Schwager et al., 2013, Cardona *et al.*, 2005, Springman *et al.*, 2009, Sousa *et al.*, 2010, Markey *et al.*, 2006).

In our work (Tedesco et al., submitted for publication) a multistep approach was used. Firstly, two different assays to evaluate the Bcc virulence determinants were set up and used with the type strains of the 18 Bcc species (eight of which had

never been characterized before for their capability of killing *C. elegans*): the Slow Killing Assay (SKA), correlates worm mortality with intestinal bacterial accumulation/colonisation (Huber et al., 2004, Kothe et al., 2003) while the Fast Killing Assay (FKA) allows to demonstrate and evaluate the secretion of bacterial toxins (or alternative virulence factors) capable of paralysing the worms and consequently promoting their death (Huber et al., 2004, Kothe et al., 2003). A pathogenicity scale for Bcc strains under investigation was established ranging from score 0 to score 3. Data obtained revealed that both killing mechanisms, *i.e*. i) bacterial accumulation/colonisation in the worms intestine as it was also visualised by microscope analysis, and ii) production of diffusible toxins-virulence factors are exhibited by most of Bcc strains.

The Bcc strains that displayed high pathogenicity score in the nematode FKA were used for further analysis revealing that toxin/compound production is growth-phase dependent and even though the compound produced by one of strain was not UV and heat labile. This compound should be further biochemically and mechanistically investigated, through its purification and characterisation.

Lastly, we analysed the behaviour of *C. elegans* mutants strains [*mrp-3(ok955)* and *mrp-4(ok1095)*] impaired in ABC CF trans-membrane conductance regulator (CFTR) -like transporters, towards the Bcc selected strains. Our findings suggested that the nematode ABC transporters *mrp-3* and *mrp-4* were also involved in the Bcc infection process and further studies are required to better understand the role of these transporters.

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Novel insights into the interaction of *Burkholderia cepacia* complex strains revealed through the model host system *Caenorhabditis elegans*

(Submitted for publication)

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ABSTRACT

Aim: The main goal of this work was to try to define the interaction between members of the *Burkholderia cepacia* complex and the nematode host *Caenorhabditis elegans*, in order to gain knowledge of pathogenicity traits and identify strains with high virulence. These strains may be for future experimental tests aimed to uncover Bcc virulence genes that can be exploited as novel therapeutic target against Bcc. The nematode *C. elegans* has been recently established as a useful host model for studying virulence factors and for the assessment of novel therapeutic strategies.

Materials & Methods: With this aim, two different toxicity tests were used: a slow killing assay (SKA) and a fast killing assay (FKA). The latter was performed onto two different *C. elegans* larval stages. A pathogenicity score was defined for the evaluation of the Bcc panel members toxicity towards *C. elegans*, based on the percentage of survival worms.

Results & Conclusions: Firstly, our data showed that nematode killing is due to the bacterial accumulation in the whole intestine on SKA, while worm death is mediated by paralyzing toxins on FKA. Secondly, we provided evidence that the toxins produced by some Bcc strains are secreted in the medium and are able to kill the nematodes and are UV and heat stable. Finally, we noticed different pathogenic score of some Bcc strains when infecting the multidrug resistance *C. elegans* mutant strains *mrp-3(ok955)* and *mrp-4(ok1095)* and this evidence suggest the nematode ABC transporters *mrp-3* and *mrp-4* are involved in the Bcc infection process.

Keywords: *Burkholderia cepacia* complex (Bcc), non-vertebrate hosts, *Caenorhabditis elegans* virulence, pathogenicity, multidrug resistance

Introduction

Pathogen resistance to commonly used antimicrobials is a worldwide concern and a major challenge for modern medicine [1]. The major manifestation of resistance entails the appearance of microorganisms able to overcome lethal or inhibitory doses for a variety of antimicrobial compounds. Consequently, these phenomena as a total have lead to the generation of an elite class of multi-drug resistance (MDR) bacteria. These microorganisms possess effective and dynamic virulence and pathogenic capabilities, both of nosocomial and community-acquired origin, and emerged not only in the developing world, but also the developed world.

The *Burkholderia cepacia* complex (Bcc) occupies a critical position among Gram-negative multi-drug resistant (MDR) bacteria. It consists of at least 18 closely related species inhabiting different ecological niches, including plants and animals [2-4].

Many Bcc strains are opportunistic human pathogens and represent a serious concern for Cystic Fibrosis (CF) patients and immune-compromised individuals. The two most clinically relevant species are *B. cenocepacia* and *B. multivorans*, accounting for >85% of all Bcc infections in CF patients. Bcc strains are naturally resistant to many antibiotics such as cephalosporin, β-lactams, polymyxins and aminoglycosides and therefore Bcc infections are very difficult to eradicate [5, 6]. The MDR phenomena related with the Bcc were attributed to: i) the ability of its members to reduce outer membrane permeability; ii) the production of β-lactamase enzymes and the alteration of antibiotic targets [7]; iii) a variety of virulent factors with most notable lipases, proteases and other secreted products including system-associated effectors [8]; iv) the presence of multidrug efflux pumps with emphasis in members of the Resistance-Nodulation cell Division (RNDs) [9, 10].

To fight the infection of these bacteria there is a serious need of development of new strategies, one of which is dissecting the virulence and pathogenicity determinants of these pathogens to identify novel therapeutic targets. This task has been facilitated by using the non-vertebrate host models *Drosophila melanogaster*, *Galleria melonella*, *Caenorhabditis elegans* and *Danio rerio.* These non-vertebrate hosts have been systematically also used to evaluate the efficacy of antimicrobial compounds and treatments [11-16]. The nematode *Caenorhabtidis elegans* is a widespread multicellular organism, a self-fertilizing hermaphrodite with a rapid generation time. Adults can reach the maximum length of 1 mm, and in optimal condition they can produce about 300 genetically identical progeny in 3 days, allowing the cultivation of nematodes and the establishment of homogenous populations.

The free-living nematode *C. elegans* has been proven to be a reproducible and genetically tractable model to validate microbial virulence traits that are involved in mammalian infections [15, 16]. The simulation of a bacterial infection in a nematode is a subject of continuous investigation with emphasis in the methodology surrounding physiological responses between the host and the pathogen.

There is an extensive body of literature for the usefulness of the nematode to model virulence and antimicrobial efficacy in *S. aureus* [17], *E. coli* [18] and a variety of Gram-negative bacteria including the pathogens *Burkholderia pseudomallei* [19-21].

Pseudomonas aeruginosa causes a broad spectrum of opportunistic infections in immunocompromised individuals and in CF patients. The set up of a *C. elegans* - *P. aeruginosa* pathogenesis model system has facilitated the systematic dissection of both host and pathogen responses [22-24]. Bcc strains are able to kill *C. elegans* through different mechanisms that include the intestinal accumulation/colonisation and/or production of toxic compounds, the latter

causing first paralyses of the worms and then death [25, 26]. The *C. elegans*-Bcc interaction was the subject of intensive investigation for the last decade [8, 16, 25-33]. Even though some correlation of bacterial genotype with the physiology of either the bacterial or nematode strains has been traced, those studies are mainly focused on the microbe-nematode interaction. Moreover, the availability of *C. elegans* mutants (*mrp-3(ok955)* and *mrp-4(ok1095))* impaired in ABC CF trans-membrane conductance regulator (CFTR) -like transporters and the fact that CF is a genetic disease due to a loss of function in the CFTR may shed some light on the comprehension of the ability of Bcc strains in infecting CF patients.

In this study we employed a panel of 18 Bcc strains (eight of which were previously analyzed by Cardona et al, 2005 [25]) representing all the 18 different species recognized so far, and with a different origin, in order to shed additional light on the nematode-Bcc pathogenic interaction by: i) correlating nematode death with either an intestinal accumulation/colonisation and/or ii) a presence of a diffusible toxin or compounds; iii) implicating the nematode larval stage on the infection susceptibility iv) checking the response to Bcc strains by two *CFTR*-like multi drug resistant *C. elegans* mutants (*mrp-3(ok955)* and *mrp-4(ok1095)*), which are impaired in ABC membrane transporters.

Materials and Methods

Bacterial strains, nematode strains and growth conditions

C. elegans Bristol N2, RB1028 (*mrp-3(ok955*) X) and VC712 (*mrp-4(ok1095*) X) strains were obtained from the *Caenorhabditis* Genetic Center (CGC). All strains were recovered from frozen stocks, and routinely kept on NGM (Nematode Growth Medium) plates seeded with *E. coli* OP50 as a food source [34]. The panel of Bcc strains used in this work belongs to the Bcc strains collection at the University of

Gent, Belgium, and is listed in Table 1. When available, information on the geographic and biological sources of the isolates was also included in the Table 1. Bcc and *E. coli* OP50 cells were routinely grown in Luria-Bertani broth (LB) (10 g/L Bacto-tryptone, 5 g/L Yeast extract, 10 g/L NaCl) at 37°C.

Nematode Slow Killing Assay (SKA) and Fast Killing Assay (FKA)

Slow-killing assays were performed using the *C. elegans* strains N2, *mrp-3* and *mrp-4* mutants. Plates containing 3 ml of NGM agar (Peptone 2.5 g/L, NaCl 2,9 g/L, Bacto-Agar 17 g/L, $CaCl_2$ 1 mM, Cholesterol 5 µg/mL, KH_2PO_4 25 mM, $MgSO_4$ 1 mM) were seeded with 50 µl of the overnight Bcc cultures, normalized to an OD_{600}, of 1.7 and incubated 24 h at 37 °C to allow the formation of a bacterial lawn, and. *C. elegans* N2 strain, *mrp-3* and *mrp-4* mutants were synchronized by bleaching treatment [35], and twenty to forty L4 larvae were transferred to each plate and incubated at 20 °C for three days. The plates were scored for living worms every 24 h.

Fast-killing assays were carried out in 2.5-cm-diameter agar plates, containing about 3 ml of PGS medium [25] (Peptone 12 g/L, Glucose 12 g/L, Sorbitol 27.25 g/L, NaCl 12 g/L, Bacto-Agar 17 g/L, $CaCl_2$ 1 mM, Cholesterol 5 µg/mL, KH_2PO_4 25 mM, $MgSO_4$ 1 mM). Plates were seeded with fifty µl of the overnight Bcc cultures, normalized to an OD_{600} of 1.7 and incubated 24 h at 37 °C to allow the formation of a bacterial lawn. Then, L1 and L4 *C. elegans* larvae from N2 strain and *mrp-3* and *mrp-4* mutants were collected from NGM plates, washed with M9 medium ($Na_2HPO_4{\cdot}7H_2O$ 12.8 g/L, Na_2HPO_4 (anhydrous) 6 g/L, KH_2PO_4 3 g/L, NaCl 0.5 g/L, NH_4Cl 1 g/L) and 30-40 live animals were spotted onto the bacterial lawn. The plates were then incubated at 20 °C and scored for living worms every 24 h. In both assays, *E. coli* OP50 was used as a negative control. A worm was considered dead when it no

longer responded to touch. For statistical purposes, 5 replicates per trial were carried out. The same pathogenic scale was used in both assays and the incubation time was defined in 3 days for L1 larvae, and in 5 days for L4 larvae at 20 °C.

Microscopy analysis

C. elegans N2 Bristol L4 larvae were grown on NGM plates seeded with 50 µl of the overnight Bcc culture normalized to an OD_{600} of 1.7 and incubated 24 h at 37 °C to allow the formation of a bacterial lawn. NGM plates seeded with *E. coli* OP50 and with the same number of worms were used as control. Plates were incubated at 20 °C, and after two different time-points (4 and 24 h) the nematodes were inspected with a Zeiss Axioskop microscope equipped with differential interference contrast (DIC) employing 10x, 20x, 40x, 63x and 100x objectives and 10X eyepiece. Images were collected with a Zeiss Axiocam MR digital camera.

Toxin Diffusion assay

LB overnight cultures (100 µl) of Bcc strains and *E. coli* OP50 as control, were spread on sterile 0.22 µm Millipore Nitrocellulose (Darmstadt, Germany) filter disk located onto 2.5 cm PGS plates [25]. After an incubation at 37 °C overnight, the filter together with the bacterial lawn was removed and the plates were allowed to cool to room temperature. 30-40 hypochlorite-synchronised *C. elegans* L4 larvae worms were spotted onto the conditioned agar. Paralysation and mortality of the worms were detected at 4 and 24 h. The experiments were performed twice, and data reported are mean values ± SD.

Toxin Diffusion assay at late growth phase

100 µL of Bcc overnight cultures, in LB of and *E. coli* OP50 as control for all the Table 1 strains were spread on sterile 0.22 µm Millipore Nitrocellulose (Darmstadt, Germany) filter disk located onto 2.5 cm PGS plates.

Different sets of plates for all strains tested were prepared, and each set was incubated at 37 °C for 24 h, 48 h and 72 h. After the incubation the filter with the bacterial lawn was removed and the plates were allowed to cool to room temperature. 30-40 hypochlorite-synchronised *C. elegans* L4 larvae were spotted onto the conditioned agar. Paralysis and mortality of the worms were detected at 4 and 24 h. The experiments were performed in triplicate, and the data reported are mean values ± SD.

Bacterial cell-free culture filtrate assay

One colony of an individual Bcc isolate was inoculated into 1 mL LB broth and incubated overnight at 37 °C. The bacterial culture was centrifuged at 4000 x *g* for 15 minutes at 4 °C and the supernatant collected and filtered through a 0.22 µm pore size syringe membrane filter unit (Millipore, Billerica, MA). The supernatant (100 µl) was used to spread onto PGS plates. Supernatant of *E. coli* OP50 growth represented the control.

The toxicity of some heat-killed Bcc strains plated onto PGS plates was also tested; for this purpose 1 ml of overnight culture of Bcc strains was incubated at 65 °C for 1 h, and the cells were spread onto PGS plates for the FKA. Twenty to forty L4- and L1-larvae of *C. elegans* N2 were spread onto these plates and incubated at 20 °C; the toxicity, by counting the surviving worms, was evaluated at 4 and 24 h. Mortality of the worms was monitored at 4-hour intervals. Heat-killed *E. coli* OP50 was used in place of Bcc strains as the negative control. Three independent experiments were performed for each isolate.

UV and Heat treatment

UV treatment: fifty µL of overnight cultures of *Burkholderia cepacia* LMG 1222 adjusted to and *E. coli* OP50 as control were spotted onto PGS agar plates. Plates were incubated overnight at 37°C and then exposed to UV (254 nm, 1.5 J/cm^2) for 10, 20 and 30 min. Four plates were exposed in parallel for each bacterium. The irradiated plates were allowed to cool to room temperatures and 20 to 30 N2 L4 synchronized larvae were spotted on the agar on 3 plates for each condition. As control, a fourth plate was instead washed with 200 µL of sterile water and the collected bacteria were transferred on a new PGS plate and incubated at 37 °C. Survival worms were checked at 24 h.

Heat treatment: fifty µL of overnight cultures of *B. cepacia* LMG 1222 and *E. coli* OP50 as control were spotted onto PGS agar plates. Plates were incubated overnight at 37 °C and then incubated at 65 °C for 1 h and 2 h. Four plates were exposed in parallel for each bacterium. The heated plates were allowed to cool to room temperatures and 20 to 30 N2 L4 synchronized larvae were spotted on the agar on 3 plates for each condition. As control, another plate was washed with 200 µL of sterile water and the bacteria were transferred on a new PGS plate and incubated at 37 °C. Survival worms were checked at 24 h.

Statistical analysis

All the Kaplan-Meier survival curves were analyzed by using the Graph-pad Prism 5 software. All p-value were calculated with "Log-rank (Mantel-Cox) Test". For Toxin diffusion assay and Heat-UV experiments, data reported considered by mean values of triplicate ± SD. p-value between sample and referring controls were calculated with “Z test” with the Primer software.

Results and Discussion

Overall experimental strategy

The main goal of this work was to try to define the pathogenicity interaction attributed to members of the Bcc complex employing the nematode host *C. elegans*. To this purpose, a multistep approach was used. Firstly, a panel of taxonomically, environmentally and clinically diverse Bcc strains was used (**Table 1**) to infect the worms. The 18 strains were obtained from a variety of habitats, clinical settings and conditions spanning from cystic fibrosis patients (9 isolates), animal infection (1 isolate), onion (1 isolate), and soil rizhosphere (7 isolates). Apart from the wide geographic and environmental distribution of the selected isolates, the panel comprehensively covers all the known identified species of the Bcc complex. It should be noted that *B. latens* LMG 24064, *B. diffusa* LMG 24065, *B. arboris* LMG 24066, *B. seminalis* LMG 24067, *B. metallica* LMG 24068, *B. pseudomultivorans* LMG 26883, *B. lata* 22485, *B. contaminans* LMG 23361 were recently added as novel Bcc species and have not been previously characterized for their pathogenicity towards *C. elegans* [4, 36, 37]. There is a well-demonstrated variety and diversity in the infections assays used for Bcc and a justified need to identify and validate consistent pathogenicity models. This fact prompted the set up of *C. elegans* assays tailored to evaluate Bcc virulence determinants and properties: i) the Slow Killing Assay (SKA), performed on a low osmolarity medium (NGM, Nematode Growth Medium), was set up and assigned to correlate worm mortality with intestinal bacterial accumulation/colonisation [25, 26]; ii) the Fast Killing Assay (FKA) was carried out on a high osmolarity medium (PGS medium) to demonstrate and evaluate the secretion of bacterial toxins (or alternative virulence factors) capable of paralysing the worms and consequently promoting their death [25, 26]. Mostly important, by comparisons of the "infectivity" of nematodes

between *E. coli* OP50 and Bcc isolates, a pathogenicity scale for Bcc strains under investigation was established ranging from score 0 to score 3 (see Fig. 1). The parameters for scoring were the following: i) a strain (isolate) was considered to be non-pathogenic when no symptom of disease was observed during the course of nematode infection, which was fixed to be 3 days and a percentage of still alive worms ranged from 85 to 100% (pathogenic score 0); ii) the pathogenic score 1 corresponded to a percentage of alive worms, ranging between 84 to 45%, iii) when a range from 44 to 10% of worms were still alive after 3 days of incubation the pathogenic score was 2; iv) lastly, when the percentage of alive worms ranged between 9 and 0% the score was 3.

It should be noted that 9 out of the 18 isolates-strains were not previously analysed for their ability to infect and kill *C. elegans*, and in the remaining cases the mortality assays were carried out (only) on slow killing medium [30, 38].

The Bcc strains that displayed high pathogenicity score in the nematode FKA were used for further analysis to correlate score with actual virulence factors responsible for the nematocide activity. In order to perform a preliminary analysis of the toxins, we also tested whether these compounds were UV and/or heat labile.

Lastly, we analysed the behaviour of *C. elegans* mutants towards the Bcc selected strains.

Nematode Slow-Killing Assays (SKA)

The pathogenicity of the 18 Bcc strains was investigated using SKA [25], which was carried out cultivating N2 Bristol wild type (w.t.) *C. elegans* L4 synchronised larvae on a bacterial lawn grown on NGM. Data obtained are summarised in **Table 1,** whose analysis revealed a variety of "virulent phenotypes" among the 18 Bcc representatives:

i) Only 3 out of 18 Bcc strains (*B. diffusa* LMG 24065, *B. metallica* LMG 24068, *B. stabilis* LMG 14294) displayed high nematocide activity (Pathogenic score 3) towards L4 N2 Bristol *C. elegans* larvae. Viable nematodes were not detectable in the plates after 3 days of incubation at 20°C.

ii) Six out of 18 Bcc strains (*B. ambifaria* LMG 19182, *B. dolosa* LMG 18943, *B. pseudomultivorans* LMG 26883, *B. pyrrocinia* LMG 14191, *B. lata* LMG 22485 and *B. multivorans* LMG 13010) were unable to kill worms, i.e. the whole population was viable (Pathogenic Score 0).

iii) A quite interesting behaviour was observed for *B. ubonensis* LMG 20358, *B. diffusa* LMG 24065, *B. cenocepacia* LMG 16656 strains; after 48 h of incubation on these strains, most of dead worms displayed a "bags of worms" phenotype, with the presence of many L1 worms within the adult ones. It should be noted that the "bags of worms" was the result of internal hatching embryos.

Intriguingly, nematodes killed in the lawn of bacteria took on a ghostly and hollow "shell-like" appearance about 48 h after the L4 were firstly introduced, and their shells induced by *B. ubonensis* LMG 20358, *B. metallica* LMG 24068 and *B. stabilis* LMG 14294 were defined as "chalk-mark ghosts". This shape is characteristic for not having a discernible internal cell structures. Often the ghosts eroded to a mere outline.

Bacterial intestinal accumulation/colonisation

The Bcc strains subset with paramount ability to infect and kill nematodes in the SKA assay (score 3), that is *B. stabilis* LMG 14294, *B. metallica* LMG 24068 and *B. diffusa* LMG 24065 was assessed for their ability to colonize the *C. elegans* intestine. Thus, *C. elegans* N2 Bristol L4 larvae were grown on NGM plates seeded with Bcc strains, using NGM plates seeded with *E. coli* OP50 and with the same number of worms as control. The nematodes were inspected at different

incubation time with a Zeiss Axioskop microscope for the visualization and evaluation of the bacterial accumulation/colonisation in the intestinal lumen.

Nematodes exposed for 24-h to *B. metallica* LMG 24068 resulted to have the intestinal lumen packed with bacteria, whereas worms fed with *E. coli* OP50 showed a thin intestinal lumen, as expected (**Fig. 2 A-B**). These data suggested that *B. metallica* LMG 24068 cells were able to proliferate within the entire nematode intestine and therefore slow-killing resembled with an infection-like process (**Fig. 2 E-F**). The same experiment, performed with strain *B. pseudomultivorans* LMG 26883, exhibiting a low pathogenicity *vs* N2 Bristol *C. elegans* (0 score on SKA) revealed that after 24-h of infection (**Fig. 2 C-D**) accumulation of LMG 26883 cells was observed only in the first part of the intestine, but not in the whole. This finding might indicate that even non-pathogenic Bcc strains were able to pass intact through the pharynx and proliferate in the intestine. Moreover, this observation might suggest that the accumulation/colonization of the Bcc in the whole nematode gut, especially in the last part of the intestine, might be responsible for worm's death [26].

Nematode Fast Killing Assays (FKA)

The pathogenicity of the 18 Bcc strains was also investigated for the ability of producing toxins on high-osmolarity media, by cultivating N2 Bristol wild type *C. elegans* L4 synchronised larvae on a bacterial lawn grown on PGS. Data obtained are summarised in **Table 1**, and revealed a wide array of "virulent phenotypes" among the 18 Bcc members also on FKA [25].

The phenotypic observation for dying worms on FKA suggested that nematodes infection to be an rapid process. Nematodes appeared to rapidly loose locomotor functions, as shown by the rapid onset of lethargy. Locomotion visibly decreased after exposure for 4 h, and the rate of foraging was similarly affected in the same

time frame. On FKA five strains (*B. ambifaria* LMG 19182, *B. cepacia* LMG 1222, *B. contaminans* LMG 23361, *B. metallica* LMG 24068, *B. stabilis* LMG 14294) were highly active (score 3) against L4 N2 larvae and only 2 strains (*B. multivorans* LMG 13010 and *B. vietnamiensis* LMG 10929) were completely ineffective in killing worms. For the highly active strains almost 100 % of mortality occurs in 24 h (**Fig 3 A-B-C)** and this rapid killing kinetics suggested a potential role for diffusible toxins as part or as a totally separate infectious process component.

We further performed the FKA on L1 worms. This relies on the fact that the majority of studies performed to assess pathogenicity of Bcc strains on *C. elegans* was carried out on L4 larval stage; however, it has been previously reported that the age of adult animals and the composition of a population may impact the infection assay readout [26, 30]. The safe assumption was made that animals in the early larval stages were more susceptible and sensitive to infection. This concept was consecutively tested by using synchronized L1 and L4 larval populations in infection with panel members from the Bcc strains (as described in Materials and Methods). Data obtained are shown in **Table 1**. A different response of L1 and L4 larvae to the presence of Bcc cell populations was revealed; as expected, the 18 Bcc strains have a stronger nematocide activity against L1 larvae than on L4 larvae.

Interestingly, five strains (*B. ambifaria* LMG 19182, *B. cepacia* LMG 1222, *B. contaminans* LMG 23361, *B. metallica* LMG 24068, *B. stabilis* LMG 14294) demonstrated to be highly active (score 3) towards both L1 and L4 larvae and the majority of worms were found dead within 24 h. On the contrary, three strains (*B. arboris* LMG 24066, *B. latens* LMG 24064, and *B. seminalis* LMG 24067) appeared to have a *C. elegans* larval stage dependent effect; they were highly active on L1 larvae, whereas the L4 larvae seemed to be less susceptible by these Bcc isolates (**Table 1**).

Also, strain *B. vietnamiensis* LMG 10929 was completely ineffective towards *C. elegans* at any larval stage tested.

The comparison of data obtained in the FKA and SKA carried out on L4 N2 Bristol *C. elegans* larvae revealed that three *Bcc* strains (*B. ambifaria* LMG 19182, *B. cepacia* LMG 1222 and *B. stabilis* LMG 14191) possess high nematocide activity in the FKA, whereas the same strains were unable to kill the worms in the SKA (score 0 and 1). It should be noted that these strains have been isolated from soil; this finding may suggest that Bcc pathogenesis might be related to the ecological niche.
The *B. metallica* LMG 24068 and *B. stabilis* LMG 14294 deserve a further and more comprehensive observation and analysis, since these two strains were very active in both *C. elegans* SKA and FKA.

Toxin Diffusion assay

To evaluate whether the partition of diffusible secreted factors (toxins or other virulence chemical signals-lead molecules) contribute to Bcc-mediated killing of *C. elegans*, we assayed the survival of nematodes exposed to culture filtrates alone and then it was checked by the toxin diffusion filter assays. The experiments were carried out on a reduced panel of Bcc strains, *i.e.* those possessing high nematocide activity *vs* N2 Bristol *C. elegans* in the L4 larvae on FKA (*B. diffusa* LMG 24065, *B. contaminans* LMG 23361, *B. cepacia* LMG 1222, *B. ambifaria* LMG 19182, *B. metallica* LMG 24068, *B. stabilis* LMG 14294 and *B. anthina* LMG 20980). Hence, each of these Bcc strains was plated onto a small filter, placed on a PGS-plate and incubated overnight at 37 °C. Afterwards, the filter was removed from the plate and synchronised L4 larvae N2 Bristol were placed onto the conditioned agar. Data obtained are shown in **Fig. 4** and revealed that, depending on the plated Bcc strain, a different percentage of worms appeared to be paralyzed after 4 h even if they were not in contact with the bacteria. In particular, about 30% of the worms placed on the Bcc *B. ambifaria* LMG 19182 strain were still mobile and active, whereas the remaining nematode population appeared motionless and paralysed. Among the

tested Bcc strains, *B. ambifaria* LMG 19182 was the most active nematode killer in terms of a diffusible toxin/chemical. Indeed, after 24 h of incubation only a 20% of the total number of nematodes was still moving on LMG 19182 plates (**Fig. 4)**. A diverse nematode behaviour was also observed on conditioned agar by the other *Bcc* strains. The worms' survival in the presence of *B. anthina* LMG 20980 cells was accountable for optimal nematode survival when compared with the other Bcc members. In fact, after 24 h of incubation about 75% of the worms were still alive, under the same assay conditions. In some cases (*B. diffusa* LMG 24065, *B. stabilis* LMG 14294, *B. anthina* LMG 20980) we observed that paralyzed worms were able to move again and survive. One plausible explanation for the diverse nematode behavioural response might be related to the short half-life of the diffusible toxin-virulence determinants produced by those strains that requires continuous production.

On the contrary, when *E. coli* OP50 was used as control after 4 h of incubation no mortality has been observed. Interestingly, when the toxin filter assay was performed on NGM medium, no paralysis or mortality was detected. This evidence is in full agreement with the finding that on NGM media worms death might be due to bacterial colonization, while FKA in mediated by secreted toxins.

Toxin Diffusion assay at late growth phase

Several studies have correlated virulence factors activation to quorum sensing mechanisms and cascades [25, 38, 39]. This implies that key pathogenic determinants, including toxin production, may be expressed in steady-state [14]. The high activity towards N2 Bristol *C. elegans* in the L4 larvae e on toxin diffusion assay prompted the investigation for the thorough answer to whether toxin production was dependent on the bacterial growth phase. Therefore, a further analysis was carried out on three strains, i.e. *B. cepacia* LMG 1222, *B. stabilis* LMG

14294 and *B. metallica* LMG 24068, whose cells at different growth stages were used for the toxin diffusion assay.

We have chosen these three strains because they were responsible for high degree of *C. elegans* mortality, which occurred in about 36 hours on FKA (Figure 3). Different sets of plates with filters plated with each of the three strains incubated at 37 °C for 24h, 48h, and 72h. At each incubation time-point, filters with the bacterial lawn were removed and the plates were allowed to cool to room temperature; 30-40 -synchronised *C. elegans* L4 larvae were then spotted onto the conditioned agar and observed after 4 and 24 h. The whole body of data obtained is reported in **Fig 5**. As a general trend, a remarkable nematode mortality was observed in the plates that were incubated for 72 h. This time-comprehensive assay revealed that, regarding *B. metallica* LMG 24068 and *B. cepacia* LMG 1222 strains, 4h of incubation led to lack of mobile worms. The fraction of nematode populations that sustained some movement at 4h and 24h significantly decreased and it was dependent on the incubation time of Bcc strains. Only for *B. cepacia* LMG 1222, we observed that a little percentage of worms paralyzed after 4h, moved again at 24h. Again, the possible explanation for this behaviour might rely on the short half-life of the diffusible toxins/compounds produced by the *B. cepacia* LMG 1222 strain.

In an attempt to correlate the bacterial cell-free culture filtrates with the diffusible toxins, assays were performed exposing worms to crude extracts of the Bcc strains grown for 24 h at 37 °C. However, no killing of worms was observed (data not shown). It is likely that extensive extract (bioassay driven) purification will be necessary to identify and concentrate toxins and the additional virulence determinants, which mediate the fast killing. The same data set demonstrated that, for all the isolates tested, the secreted molecules under investigation were incapable to mediate the complete nematode killing. This might be due either to the binding of the toxin to the filters or the instability of toxin in the supernatant.

UV and Heat Treatment of *B. cepacia* LMG 1222 toxins

As it was challenging to dissect the chemical features of the toxin-signalling molecule(s) a reasonable follow up was to assess its/their stability, with emphasis in standard physic-chemical properties such as the resistance to UV or heat exposure. We choose as a model system the *B. cepacia* LMG 1222 strain, since it displayed a pathogenic score 3 and 1 on FKA and SKA, respectively. Thus, the virulence of this strain might be related to the way of toxins production, rather than the colonization; therefore, this strain might represent a good candidate for performing toxins characterization. Accordingly, PGS plates were seeded with *B. cepacia* LMG 1222 cells (as described in Materials and Methods) and irradiated with 1.5 J/cm^2 for 10, 20 and 30 min; afterwards L4 larvae N2 Bristol were placed onto the conditioned agar. Plates were washed and the collected bacteria were seeded again onto a novel plate that was incubated at 37 °C to assess the percentage of surviving bacteria as a control. Colony Forming Units (CFUs) were observed only for plates irradiated for 10 min. Data obtained are reported in **Fig. 6A**. A remarkable nematode mortality was detected on the *B. cepacia* LMG 1222-plated and subsequently irradiated plates, in comparison with *E. coli* OP50-plated control. Indeed, about the 50% of the worm population remained alive after 24 h of incubation on plates irradiated for 30 min and 1 h. However, the percentage of worms' mortality was comparable to that obtained when L4 larvae were spotted on PGS plates seeded with living bacteria (**Figure 4**). On the contrary, on *B. cepacia* LMG 1222 plates irradiated for 10 min the nematode survival detected was lower, probably due to insufficient bacterial cell killing at short time of irradiation. These data suggested that the toxin/compound produced by *B. cepacia* LMG 1222 was not UV-labile under the irradiation conditions reported above.

The heat resistance of the putative toxins-virulence factor(s) attributed to *B. cepacia* LMG 1222 was also investigated. The experimental design included incubation (exposure) of PGS plates seeded with *B. cepacia* LMG 1222 cells at 65 °C for 1 and 2 h. Then, L4 larvae N2 Bristol were placed onto the conditioned agar. As a control, a plate was washed with 200 µL of sterile water and the collected bacteria were incubated at 37 °C to assess the effect of heat challenge to the Bcc cell population. For all the experiments, we observe 0 CFUs. Data obtained are shown in **Fig 6B**. The nematode mortality rate detected on the *B. cepacia* LMG 1222 heated plates was remarkably higher than the control on *E. coli* OP50. Surprisingly, high worm mortality was observed in both conditions tested. In fact, after 24 h of incubation only 17% and 7% of worms were still alive on the plates heated for 1 and 2 h respectively. These data suggested that the compound(s)/toxin(s) produced by Bcc *B. cepacia* LMG 1222 cells was/were not heat-labile.

Killing of *mrp-3* and *mrp-4 C. elegans* mutants by Bcc strains

ATP binding cassette (ABC) systems are responsible for the import and export of a wide variety of molecules across cell envelopes and comprise one of largest protein superfamilies found in Bacteria, Archaea and Eucarya [40]. ABC systems play important roles in the several *C. elegans* behaviour and development. It has been demonstrated that ABC transporter genes encoded in the *C. elegans* genome are required for robust RNAi [41]. In host-pathogen interactions it has been demonstrated that ABC systems play important roles in bacterial lifestyle, virulence and survival for both the involved parts [24, 42, 43]. Recent data highlighted a significant role of ABC transporters in virulence and pathogenicity for bacteria belonging to the genus *Burkholderia* [24, 44]. This analysis prompted a comprehensive profile of the Bcc strains against two *CFTR*-like multi drug resistant

nematode mutants, such as *mrp-3(ok955)* and *mrp-4(ok1095)*, which are impaired in ABC membrane transporters, in order to shed some light on the role that these transporters might play during Bcc infection, although they are phenotypically comparable to the wild type. Both the SKA and FKA assays were performed using all the 18 Bcc strains. Data reported in Table 1 and concerning the SKA N2 Bristol, *mrp-3* and *mrp-4* datasets, revealed a differential effect of the Bcc strains on the nematode strains. In particular: i) *B. contaminans* LMG 23361 was very active towards L4 *mrp-3* larvae, whereas no effect was detected in the wild-type and *mrp-4* nematode mutants, ii) *mrp-3* and N2 Bristol were severely impaired by the presence of *B. cenocepacia* LMG 16656 and *B. seminalis* LMG 24067, while no apparent pathogenic effects were observed in mutant *mrp-4*.

The FKA performed on the L1 *C. elegans mrp-3* and *mrp-4* mutants revealed a different behaviour of the mutants in comparison with the *C. elegans* w.t. for 10 out of 18 Bcc strains used (*B. arboris* LMG 24066, *B. cenocepacia* LMG 16656, *B. dolosa* LMG 18943, *B. pseudomultivorans* LMG 26883, *B. pyrrocinia* LMG 14191, *B. seminalis* LMG 24067, *B. ubonensis* LMG 20358, *B. vietnamiensis* LMG 10929, *B. lata* LMG 22485, *B. multivorans* LMG 13010).

Interestingly, both the *C. elegans* transporter mutants (*mrp-3* and *mrp-4*) appeared to be very sensitive to *B. pseudomultivorans* LMG 26883 and *B. lata* LMG 22485 compared with the w.t. N2 Bristol nematodes.

The FKA was also performed on the L4 *C. elegans mrp-3* and *mrp-4* larvae mutants, and it revealed a different response of the nematode transporter mutants when compared with the w.t. *C. elegans* strain for the following strains: *B. anthina* LMG 20980, *B. cenocepacia* LMG 16656, *B. dolosa* LMG 18943, *B. ubonensis* LMG 20358, *B. multivorans* LMG 13010, *B. pyrrocinia* LMG 14191, *B. contaminans* LMG 23361 and *B. lata* LMG 22485 (**Table 1**). In particular, LMG 18943 strain was found to be very toxic for *C. elegans mrp-3* mutant (pathogenic score 3), whereas it was not highly virulent for the wild type and *mrp-4* mutant (pathogenic score 1). Also, *B.*

multivorans LMG 13010 seemed to be less toxic in the wild type nematodes whereas it appeared strongly pathogenic towards the *mrp-3* and *mrp-4 C. elegans* strains.

These data suggest a significant role of *mrp-3* and *mrp-4* in Bcc infection process but the multitude of the data set pinpointed the needs for a comprehensive experimental design and investigation to demonstrate the specific roles for the *C. elegans mrp-3* and *mrp-4* genes during the infection.

Conclusions

The aim of this work was to analyze the nematocide activity of strains spanning the entire set of known Bcc species, some of which recognized as crucial invasive clinical opportunistic pathogens in lung infections with an arsenal of virulence factors yet to be elucidated [45]. The use of non-vertebrate hosts to profile Bcc virulence and pathogenicity has been a subject of intense investigation and the first set of results are promising but circumstantial [27, 29, 30]. In this paper we tested the interaction between *C. elegans* and Bcc by using an approach, which differs from that previously used [30] and which relies on the use of i) a set of isolates without characterized pathogenicity properties, ii) two different killing assays, iii) two different larval stages, and iv) two knock out mutants impaired in ABC transporter. Data obtained revealed that both killing mechanisms, *i.e*. i) bacterial accumulation/colonisation in the worms intestine as it was also visualised by microscope analysis, and ii) production of diffusible toxins-virulence factors, are exhibited by most of Bcc pathogens. Each Bcc strain was tested for the ability to accumulate in the last part of the intestine and for the production of paralyzing toxins. Recent studies demonstrated that several Bcc strains produce a haemolytic toxin required for full virulence [46]. This toxin is synthesized by non-ribosomal peptide synthase pathway, typical of complex secondary metabolite. These

compounds, were former known as occidiofungins or burkholdines, and have been shown to have antifungal activity [47].

Here we showed that the toxin/compound production is growth-phase dependent and even though the compound produced by strain LMG 1222 was not UV and heat labile. This compound should be further biochemically and mechanistically investigated, through its purification and characterisation. It was also defined the pathogenicity of 8 *Burkholderia* species, which were recently added to Bcc (*B. latens* LMG 24064, *B. diffusa* LMG 24065, *B. arboris* LMG 24066, *B. seminalis* LMG 24067, *B. metallica* LMG 24068, *B. pseudomultivorans* LMG 26883, *B. lata* 22485, *B. contaminans* LMG 23361) and they were never characterized for the capability of killing *C. elegans*. Among these strains, *B. metallica* LMG 24068 and *B. diffusa* LMG 24065 (isolated from CF patients) revealed to be the most virulent ones in both SKA that FKA.

B. metallica LMG 24068 and *B. stabilis* LMG 14294 were a notable exception since they exhibited high killing capability in both the SKA and FKA.

Nine out of 18 strains were already characterized [30, 38], but only SKA was carried out. The results obtained for these strains were in agreement with the previous studies for 5 strains (*B. anthina* LMG 20980, *B. ubonensis* LMG 20358, *B. vietnamiensis* LMG 10929, *B. cenocepacia* LMG 16656, *B. dolosa* LMG 18943), while we revealed a different pathogenicity score on *C. elegans* when incubated in presence of *B. ambifaria* LMG 19182, *B. cepacia* 1222, *B. pyrrocinia* 14191, *B. stabilis* LMG 14294. These variations may be due to the differences in establishing virulence scores between these studies.

We also reported that strains isolated from CF patients were more virulent on SKA than strains isolated from environments. This finding might suggest that toxins production may be a common mechanism of virulence for the majority of Bcc strains, while Bcc isolated from CF patients have acquired different pathogenic traits that allow them to infect and colonize hosts.

ABC systems play important roles in the several *C. elegans* lifestyle. Multidrug resistance-associated protein (MRP), which belongs to the ABC transporter superfamily transporters in *C. elegans* are involved in the protection of organisms against exogenous toxins or heavy metals by cellular detoxification processes [48, 49]. These transporters have been also found to be involved in other roles such as differentiation [50]. Our findings suggested that the nematode ABC transporters *mrp-3* and *mrp-4* were also involved in the Bcc infection process and further studies are required to better understand the role of these transporters.

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Executive summary

- The *Burkholderia cepacia* complex (Bcc) consists of at least 18 closely related species inhabiting different ecological niches, including plants and animals.
- Many Bcc strains are opportunistic human pathogens and represent a serious concern for Cystic Fibrosis (CF) patients and immune-compromised individuals.
- Dissecting the pathogenicity determinants of these pathogens been facilitated by using the non-vertebrate host *Caenorhabditis elegans*

Aims of the study

- The main goal of this work was to define the pathogenicity interaction attributed to members of the Bcc complex employing the nematode host *C. elegans.*
- We aimed to identify virulence strains to use for future experimental tests to uncover Bcc virulence genes, that may be potential therapeutic targets

Conclusion

- The majority of Bcc strains tested are able to kill *C. elegans* by both intestinal colonization, both toxins production.
- L1 larvae are more sensitive than L4 larvae to Bcc infection on FKA.
- Most the Bcc strains tested are able to produce and secrete a paralyzing toxin that is not UV/Heat labile.
- The multidrug resistance *C. elegans* transporters *mrp-3(ok955)* and *mrp-4(ok1095)* are involved in pathogenicity.

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Legend to the figures:

Fig.1: Kaplan-Meier survival plots for L4 N2 larvae fed with exemplifying Bcc strains for different pathogenic score grown on PGS medium. Worms fed on: LMG 24068 (Score 3; solid line; n = 113; 0% survival at day 3); LMG 24067 (Score 2; dashed line; n=150; 21% survival at day 3); LMG 18943 (Score 1; dotted line; n=198; 70% survival at day 3); LMG 13010 (Score 0; dot-dash line; n=120; 91% survival worms). n: Number of worms at day 0. All p-value, comparing each survival curve between them, resulted to be < 0.0001, calculated with "Log-rank (Mantel-Cox) Test" with the Graph-pad Prism 5 software.

Fig. 2: The ability of *Bcc* strains to accumulate in *C. elegans* intestinal lumen was evaluated with microscopic analysis. Red arrows indicate the nematodes intestine. **A)** Intestinal lumen of one L4 N2 larvae after 4 h of incubation on NGM plate spotted with OP50 *E. coli*, and **B)** after 24 h of incubation on the same plate. **C)** Intestinal lumen of one L4 N2 larvae after 4 h of incubation on NGM plate spotted with *B. pseudomultivorans* LMG 26883 (Pathogenicity score 0 on Slow Killing assay, and **D)** after 24 h of incubation on the same plate. **E)** Intestinal lumen of one L4 N2 larvae after 4 h of incubation on NGM plate spotted with LMG 24068 (Pathogenicity score 3 on Slow Killing assay, and **F)** after 24 h of incubation on the same plate.

Fig. 3: Kaplan-Meier survival plots for L4 N2 larvae fed with: *E. coli* OP50 (solid lines), *Bcc* strains on NGM (dashed lines), *Bcc* strains on PGS (dotted lines). n: Number of worms at day 0.
A) The killing ability of *Bcc* strain LMG 24068 on slow killing assay medium (n = 80) was compared with the ability on fast killing assay medium (n = 113).

B) The killing capacity of *Bcc* strain LMG 1222 on slow killing assay medium (n = 93) was compared with the ability on fast killing assay medium (n = 184).
C) The killing ability of *Bcc* strain LMG 14294 on slow killing medium (n = 87) was compared with the ability on fast killing medium (n = 161).
p-values were calculated between survival curves on FKA and SKA of each bacteria, and resulted to be < 0.0001 calculated with "Log-rank (Mantel-Cox) Test" with the Graph-pad Prism 5 software.

Fig. 4: Secreted compounds or toxins mediate fast killing. Bcc strains or control *E. coli* OP50 were spread on sterile filter disk located onto 2.5 cm PGS medium plates. After an incubation at 37 °C overnight, the filter together with the bacterial lawn were removed and 30-40 hypochlorite-synchronised *C. elegans* L4 larvae were spotted onto the conditioned agar. Paralysation and mortality of the worms were detected at 4 and 24 h. Data represent mean values of three independent experiment and SD values are reported. p-value were calculated with “Z test” with the Primer software. p-values were calculated between sample (Bcc) and control (OP 50) at the corresponding time, and resulted to be < 0.0001, otherwise is indicated with: *= p-value < 0.01; **= non significant p-value.

Fig. 5: Toxin production depending on the bacterial growth phase. Bcc strains or control *E. coli* OP50, were spread on sterile filter disk located onto 2.5 cm PGS medium plates. Different sets of plates for all strains tested, were incubated at 37°C for different length of times (24 h, 48 h, 72 h). After the incubation the filter together with the bacterial lawn were removed and 30-40 hypochlorite-synchronized *C. elegans* L4 larvae were spotted onto the conditioned agar. Paralysis and mortality of the worms were detected at 4 and 24 h. The experiments were performed in triplicate, and the data reported are mean values ± SD. p-value

were calculated with “Z test” with the Primer software. p-values were calculated between sample (Bcc) and control (OP 50) at the corresponding time, and resulted to be < 0.0001 otherwise is indicated with: *= p-value < 0.005; **= -value < 0.05. Control samples were omitted in the graphic for clarity.

Fig. 6: Effect of UV irradiation and heat on the compounds secreted by *Burkholderia cepacia* LMG 1222. **A) UV treatment**. PGS medium plates with LMG 1222 or control *E. coli* OP50 were incubated overnight at 37 °C and then were allowed to stay in an UV oven for 10, 20, 30 min. After the incubation, 30-40 hypochlorite-synchronized *C. elegans* L4 larvae were spotted onto the irradiated agar. Worms’ survival was detected at 24 h. The experiments were performed in triplicate, and the data reported are mean values ± SD. p-value were calculated with “Z test” with the Primer software. p-values were calculated between sample (Bcc) and control (OP 50) at the corresponding time, resulted to be < 0,0001 otherwise is indicated with: *= p-value < 0.005.

B) Heat treatment. PGS medium plates with LMG 1222 or control *E. coli* OP50 were incubated overnight at 37°C and then were allowed to stay at 65°C for 1h and 2h. Then, 30-40 hypochlorite-synchronized *C. elegans* L4 larvae were spotted onto the plates. Worms’ mortality was detected at 24 h. The experiments were performed in triplicate, and the data reported are mean values ± SD. p-value between calculated with “Z test” with the Primer software. p-values were calculated between sample (Bcc) and control (OP 50), at the corresponding time p-value resulted to be < 0.0001.

Figure 1

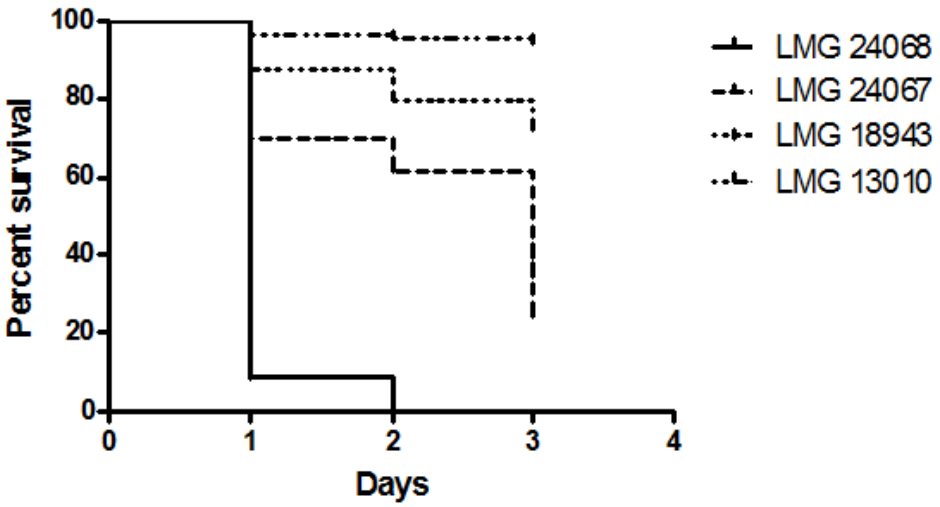
Percent survival
100
80
60
40
20
0
0
1
2
3
4
Days
LMG 24068
LMG 24067
LMG 18943
LMG 13010

Figure 2

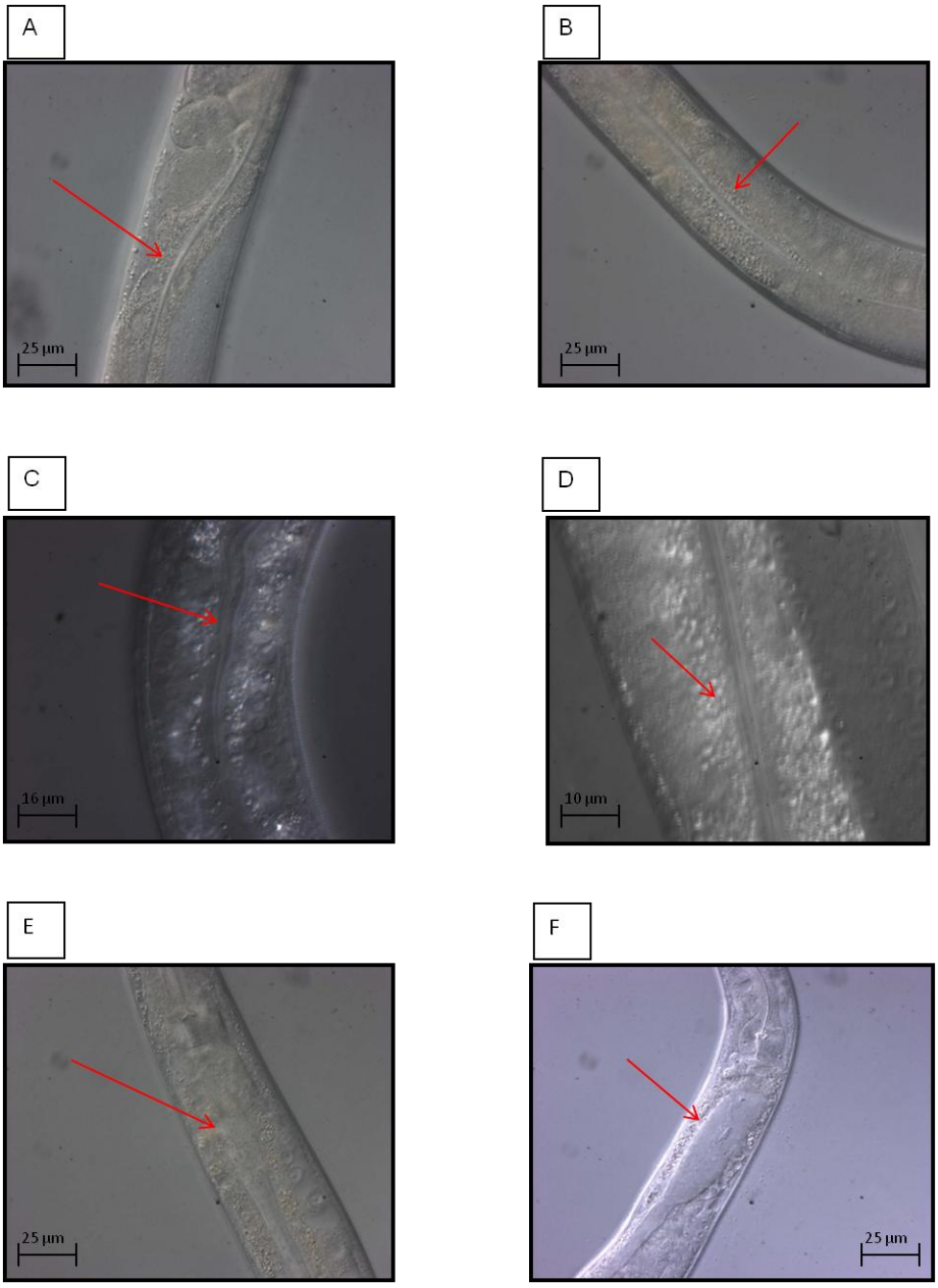

Figure 3

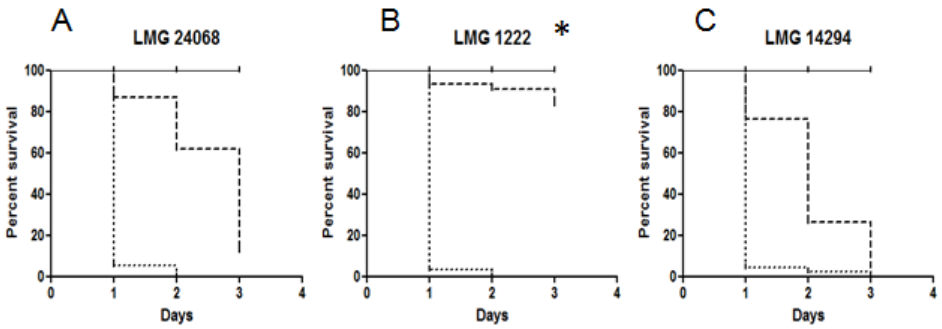
A
LMG 24068
B
LMG 1222
*
C
LMG 14294
Percent survival
Days
100
80
60
40
20
0
0
1
2
3
4

Figure 4

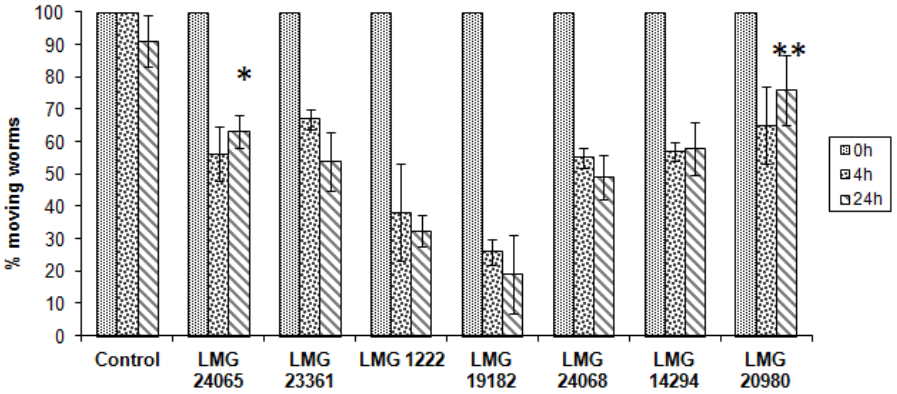
% moving worms
100
90
80
70
60
50
40
30
20
10
0
*
**
Control
LMG 24065
LMG 23361
LMG 1222
LMG 19182
LMG 24068
LMG 14294
LMG 20980
0h
4h
24h

Figure 5

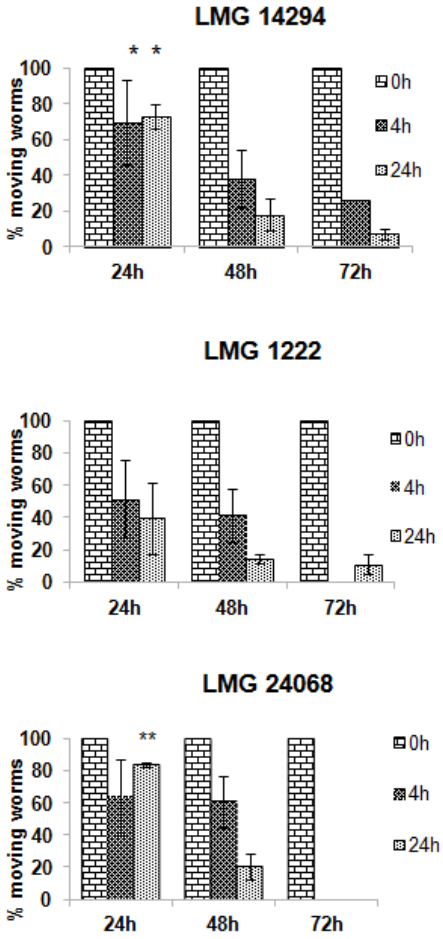
LMG 14294
* *
% moving worms
100
80
60
40
20
0
0h
4h
24h
24h
48h
72h
LMG 1222
% moving worms
100
80
60
40
20
0
0h
4h
24h
24h
48h
72h
LMG 24068
**
% moving worms
100
80
60
40
20
0
0h
4h
24h
24h
48h
72h

Figure 6

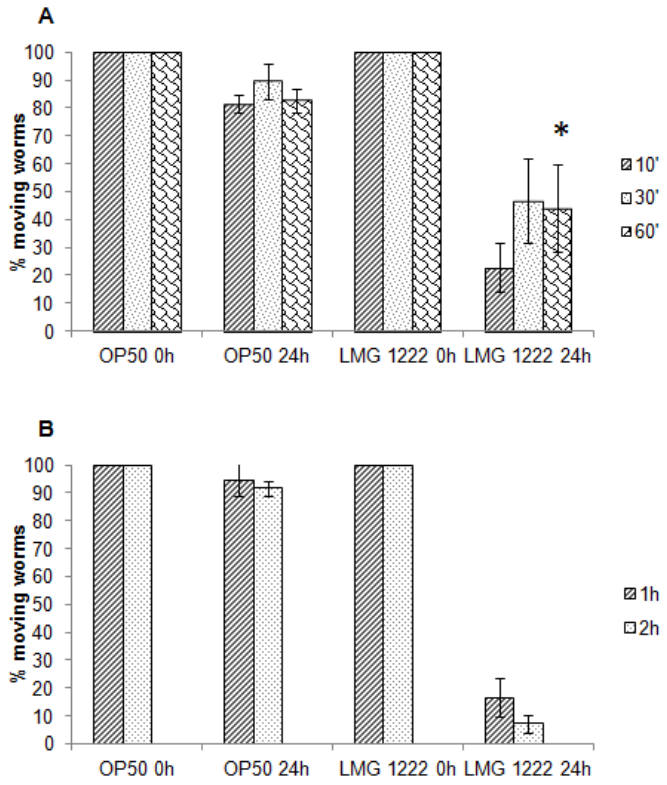
A
% moving worms
100
90
80
70
60
50
40
30
20
10
0
*
10'
30'
60'
OP50 0h
OP50 24h
LMG 1222 0h
LMG 1222 24h
B
% moving worms
1h
2h
OP50 0h
OP50 24h
LMG 1222 0h
LMG 1222 24h

Table 1. *Burkholderia cepacia* complex used in this work and pathogenicity score relative to the different killing assays and the *C. elegans* strains used.

Abbreviations: Soil = Soil rhizosphere, AI = Animal Infections, CF = Cystic Fibrosis patients

Pathogenic scores: 0 = 100 % > Survival worms > 85%; 1 = 84 % > Survival worms > 45%; 2 = 44% > Survival worms > 10%; 3 = 9 % > Survival worms > 0.

Species	Strain	Source	Slow killing assay			Fast killing assay					
			L4			L4			L1		
			N2	mrp-3	mrp-4	N2	mrp-3	mrp-4	N2	mrp-3	mrp-4
Burkholderia cepacia	LMG1222	Allium cepa	1	1	0	3	3	3	3	3	3
Burkholderia multivorans	LMG13010	CF	0	1	0	0	2	1	2	2	3
Burkholderia cenocepacia	LMG16656	CF	2	2	0	2	2	0	3	2	2
Burkholderia stabilis	LMG14294	CF	3	3	3	3	3	3	3	3	3
Burkholderia vietnamiensis	LMG10929	Soil	1	0	0	0	0	0	2	2	3
Burkholderia dolosa	LMG18943	CF	0	0	1	1	3	1	2	3	3
Burkholderia ambifaria	LMG19182	Soil	0	1	1	3	3	3	3	3	3
Burkholderia anthina	LMG20980	Soil	2	2	2	2	2	1	3	3	3
Burkholderia pyrrocinia	LMG14191	Soil	0	0	0	2	1	1	3	2	1
Burkholderia ubonensis	LMG20358	Soil	2	2	1	2	2	1	2	2	2
Burkholderia latens	LMG24064	CF	1	0	0	1	1	1	3	3	2
Burkholderia diffusa	LMG24065	CF	3	2	2	2	2	2	2	2	2
Burkholderia arboris	LMG24066	Soil	1	1	1	1	1	1	3	1	3
Burkholderia seminalis	LMG24067	CF	2	1	0	2	1	1	3	3	2
Burkholderia metallica	LMG24068	CF	3	3	3	3	3	3	3	3	3
Burkholderia lata	LMG22485	Soil	0	0	1	1	1	2	1	2	2
Burkholderia contaminans	LMG23361	AI	1	3	1	3	2	2	3	3	3
Burkholderia pseudomultivorans	LMG26883	CF	0	0	0	1	1	1	1	3	3