Caenorhabditis elegans killing assay as an infection model to study the role of type III secretion in *Burkholderia cenocepacia*

The *Burkholderia cepacia* complex (BCC) consists of a group of related genospecies that include important opportunistic pathogens, especially in relation to cystic fibrosis (CF) (Mahenthiralingam *et al.*, 2005). The most common and pathogenically important member of the BCC is *Burkholderia cenocepacia*, which along with *Burkholderia multivorans* accounts for the vast majority of BCC isolates from CF patients.

Type III secretion (TTS) systems are implicated in the pathogenicity of a number of Gram-negative bacterial pathogens (Winstanley & Hart, 2001). Previously we cloned and sequenced the TTS gene cluster from *B. cenocepacia* ET12 lineage strain J2315 (GenBank AY028431). Furthermore we identified and sequenced TTS gene regions for strains representing genomovar I (*B. cepacia*) and *B. multivorans* (strain E243). Whilst genomovar I lacks TTS structural genes, it contains flanking genes, suggesting that a deletion has occurred. *B. multivorans* contains a gene cluster equivalent to that of *B. cenocepacia* J2315 (Glendinning *et al.*, 2004). It has been demonstrated that a TTS mutant of *B. cenocepacia* J2315 is attenuated for virulence in a murine model of infection (Tomich *et al.*, 2003), suggesting a role for TTS in pathogenicity. However, the precise role of TTS remains unclear, and the secreted effectors themselves have yet to be identified.

The *Caenorhabditis elegans* infection model has been used extensively to study bacterial virulence and host-pathogen interactions (Sifri *et al.*, 2005; Alegado *et al.*, 2003). The model has been tested with various members of the BCC (Cardona *et al.*, 2005), but has been used most effectively with *B. cenocepacia* strain H111 to demonstrate the importance of quorum-sensing (QS) regulation in virulence (Huber *et al.*, 2004). As an alternative to mammalian animal models, we investigated the potential use of a *C. elegans* killing assay to study the role

of TTS in *B. cenocepacia*. Using the pKNOCK vector system (Alexeyev, 1999), TTS knock-out mutants were constructed in two different strains of *B. cenocepacia*. A *bcsN* mutant of *B. cenocepacia* H111, analogous to the mutant used in a previous study (Tomich *et al.*, 2003), was constructed by cloning an internal region of the *bcsN* gene (encoding ATPase) into pKNOCK-Km. The region was PCR-amplified from *B. cenocepacia* using the oligonucleotide primers 5'-ACCTCTAGAAAGATGATCGACACGCCG-3' (forward primer containing an *Xba*I restriction site) and 5'-ATCCTGCAGCTGGTACTTCGCGATCAAC-3' (reverse primer containing a *Pst*I restriction site), and cloned into pKNOCK-Km plasmid digested with *Xba*I and *Pst*I. In addition, a *bcsQR-virB1* knock-out mutant (*bcsQ* mutant) of *B. cenocepacia* C5424 (ET12 lineage) was constructed using the pKNOCK-Cm vector (Alexeyev, 1999). A 2.5 kb *Sac*II-*Xho*I fragment of the *B. cenocepacia* J2315 genome, comprising part of *bcsQ*, all of *bcsR* and part of *virB1* (Glendinning *et al.*, 2004), was cloned into pKNOCK-Cm. The pKNOCK-Km-*bcsN* and pKNOCK-Cm-*bcsQ* constructs were subsequently introduced into *B. cenocepacia* H111 and *B. cenocepacia* C5424, respectively, by electroporation. Cells from 5 ml overnight broth cultures were harvested, washed twice in 10% (v/v) glycerol and subsequently resuspended in 0.5 ml of the same solution. Bacterial suspension (100 µl) was mixed with DNA, transferred to 0.2 cm electrocuvettes, and subjected to a pulse of 2.5 kV (25 µF capacitance and 400 Ω resistance) using a GenePulser II apparatus (Bio-Rad). After electroporation and the addition of 0.9 ml L broth, cells were incubated at 37 °C for 2 h prior to plating onto media containing kanamycin (50 µg ml⁻¹; for pKNOCK-Km-*bcsN*) or chloramphenicol (50 µg ml⁻¹; for pKNOCK-Cm-*bcsQ*). Genomic DNA was isolated from putative mutants using a Wizard Genomic DNA

Purification kit (Promega). The position of the insertion was determined by cloning out the pKNOCK-Km/Cm regions into the *Kpn*I site of pUC19 and sequencing using vector and internal primers. Sequencing confirmed that the pKNOCK construct had inserted into *bcsN* and *bcsQ* respectively. TTS mutant and wild-type strains were phenotypically similar with respect to growth rate in Luria broth and a series of 20 biochemical tests using the API ZYM kit (bioMérieux).

B. cenocepacia wild-type and two TTS mutant strains were tested in small-scale agar-plate assays for their ability to kill *C. elegans*, essentially using the method described by Köthe *et al.* (2003). Bacterial lawns of the test strains and an *E. coli* control strain were prepared on nematode growth (NG) media to test for infective (slow-killing) effects. Small-scale assays were used in multi-segmented Petri dishes, in which each test was performed in an area of 1.5 cm². After bacterial growth for 24 h at 37 °C, L4 larvae or adult worms (~50 and ~10 for the *bcsQ* and *bcsN* mutants, respectively) were placed on the bacterial lawn. The plates were incubated at 23 °C and scored for live worms at intervals using a microscope set at ×50 magnification. Results were obtained from three independent experiments (or two for the *bcsN* mutant), each experiment testing the ability of the wild-type strain, mutant strain and *E. coli* OP50 to kill *C. elegans*.

A comparison of the *bcsN* TTS mutant with *B. cenocepacia* H111 wild-type in the *C. elegans* slow-killing model of infection at time intervals of 24, 48 and 72 h post-infection indicated a significant difference ($P < 0.05$) at the 48 and 72 h time-points (Fig. 1a). When the *bcsQ* mutant was compared with wild-type *B. cenocepacia* C5424, at time intervals of 6, 18, 24 and 42 h, a significant difference ($P < 0.05$) was only observed at the 18 h time-point (Fig. 1b). At the 24 and 72 h time-points, all worms had been killed by

Fig. 1. *C. elegans* killing assays. (a) *C. elegans* percentage viability measured at three time-points post-infection; black bars, *E. coli* OP50; white bars, *B. cenocepacia* H111; grey bars, *B. cenocepacia* H111-*bcscN*. (b) *C. elegans* percentage viability measured at the 18 h time-point.

both wild-type and mutant strains, whereas at the 6 h time-point, less than 10 % of worms had been killed by either wild-type or mutant. Our findings demonstrate that TTS mutants kill *C. elegans* more slowly than wild-type strains, but that the effect can only be observed at particular time-points that can vary between *B. cenocepacia* strains. Cardona *et al.* (2005) have reported significant variation between the *C. elegans*-killing activities of members of the BCC. Interestingly, strain C5424 was included in the study, but scored as non-pathogenic to *C. elegans*. Both in our study and the study of Cardona *et al.* (2005), *B. cenocepacia* C5424 was obtained

as part of an experimental strain panel deemed representative of the BCC (Mahenthiralingam *et al.*, 2000). In our hands, *B. cenocepacia* C5424 was effective at killing *C. elegans*. The discrepancy may indicate that one or other version of strain C5424 has mutated.

Slow killing occurs over 2–3 days and involves the accumulation of bacteria in the intestinal lumen (Köthe *et al.*, 2003). Clearly, since mutation did not prevent host infection and killing, factors independent of the TTS system contribute to nematode killing by the BCC. However, our observations suggest that, given the

right choice of strain and assay conditions, the *C. elegans* killing assays may provide a more accessible model for the study of defined mutations in the BCC TTS system and should facilitate efforts to resolve the role of this secretion system. *C. elegans* is well characterized genetically and many mutants are available for study (<http://biosci.umn.edu/CGC/CGChomepage.htm>). It has been proposed as a useful model for studying host–pathogen interactions, and especially innate immune responses to infection (Gravato-Nobre & Hodgkin, 2005). As yet, there are no known conditions *in vitro* that will trigger the expression of the BCC TTS system genes (Glendinning *et al.*, 2004; Tomich *et al.*, 2003). Studies addressing the interaction between BCC and *C. elegans* may well provide us with a clearer idea of the role of TTS in the pathogenicity of these organisms.

We would like to acknowledge funding from the UK Cystic Fibrosis Trust. In addition, we are grateful to Dr Mikhail Alexeyev (University of South Alabama) for providing the pKNOCK vectors.

Kevin M. Markey, Kerry J. Glendinning, J. Alun W. Morgan, C. Anthony Hart and Craig Winstanley

Division of Medical Microbiology and Genitourinary Medicine, University of Liverpool, Daulby Street, Liverpool L69 3GA, UK

Correspondence: Craig Winstanley (C.Winstanley@liv.ac.uk)

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