Burkholderia pseudomallei kills the nematode Caenorhabditis elegans using an endotoxin-mediated paralysis

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Summary
We investigated a non-mammalian host model system for fitness in genetic screening for virulence-attenuating mutations in the potential biowarfare agents Burkholderia pseudomallei and Burkholderia mallei. We determined that B. pseudomallei is able to cause ‘disease-like’ symptoms and kill the nematode Caenorhabditis elegans. Analysis of killing in the surrogate disease model with B. pseudomallei mutants indicated that killing did not require lipopolysaccharide (LPS) O-antigen, aminoglycoside/macrolide efflux pumping, type II pathway-secreted exoenzymes or motility. Burkholderia thailandensis and some strains of Burkholderia cepacia also killed nematodes. Manipulation of the nematode host genotype suggests that the neuromuscular intoxication caused by both B. pseudomallei and B. thailandensis acts in part through a disruption of normal Ca²⁺ signal transduction. Both species produce a UV-sensitive, gamma-irradiation-resistant, limited diffusion, paralytic agent as part of their nematode pathogenic mechanism. The results of this investigation suggest that killing by B. pseudomallei is an active process in C. elegans, and that the C. elegans model might be useful for the identification of vertebrate animal virulence factors in B. pseudomallei.

Introduction
Two closely related bacteria of the genus Burkholderia, Burkholderia mallei, the causative agent of human and equine glanders, and Burkholderia pseudomallei, which causes melioidosis, have been recognized as potential biowarfare agents. Relatively little is known about the molecular events involved in the pathogenesis caused by either of these microorganisms. Burkholderia mallei and B. pseudomallei are related by their nucleotide sequence similarity and by the aetiology of the diseases they cause (Neubauer and Meyer, 1997; Woods et al., 1999). The clinical manifestations of both infections range from pyoderma to fatal septicaemia. Bacteria have been isolated from every organ in severely infected individuals, including the brain (Asche, 1991). Human infections with either organism frequently result in septic-pneumonia, and can be fatal even under aggressive antibiotic therapies (Neubauer and Meyer, 1997; Alibek and Handelman, 1999; Dance, 1999; Woods et al., 1999).

Burkholderia mallei is an obligate pathogen that has been eliminated from North America and some parts of the world by quarantine and slaughter of infected horses (Neubauer and Meyer, 1997). In contrast, Burkholderia pseudomallei is a soil saprophyte endemic in south-east Asia and Australia (for review see (Yabuuchi and Arakawa, 1993; Dance, 1999; Woods et al., 1999). Typically, fatal human infections with B. pseudomallei correlate with host risk factors including chronic alcoholism or diabetes. The relationship between these conditions and the defects in host defence leading to a fatal clinical outcome is unknown. The saprophytic lifestyle and the existence of known risk factors suggest that B. pseudomallei is an opportunistic human pathogen. Despite the association of severe disease with compromised hosts, both bacterial species have shown that they are highly infectious in humans if aerosol exposure occurs (Neubauer and Meyer, 1997; Alibek and Handelman, 1999). Person-to-person spread has so far been rare (McCormick et al., 1975; Kunakorn et al., 1991). Intensive study of the molecular pathogenesis mechanisms used by potential biowarfare agents may lead to more effective countermeasures, including both therapeutic treatments and protective vaccines.

Recently, the human pathogen Pseudomonas aeruginosa was shown to use an overlapping set of virulence factors to cause disease in mice, plants, insects and nematodes (Rahme et al., 1995; Tan et al., 1999a). Use of this discovery has led to the development of non-vertebrate genetic model systems to screen for bacterial virulence factors and defence mechanisms in the animal host (Mahajan-Miklos et al., 1999; Tan et al., 1999a, b).

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Because non-vertebrate models have few ethical or cost constraints as subjects for experimentation and are genetically tractable themselves (Caenorhabditis elegans and Arabidopsis thaliana), extensive in vivo mutant screens have been developed. Many genes that would not have otherwise been identifiable as pathogenesis factors have been discovered using lower order hosts (Mahajan-Miklos et al., 1999; Tan et al., 1999a, b). Importantly, host targets and resistance factors can also be identified and characterized in these systems (Darby et al., 1999). This report documents the development and characterization of a nematode—B. pseudomallei model.

Results

To establish a non-vertebrate host model system for studying pathogenic bacteria, candidate hosts must be screened for their pathogenesis phenotype. Tan et al. (1999) evaluated C. elegans as a potential model host for P. aeruginosa by a simple plate assay. The plate mortality assay used a standard C. elegans solid growth medium that had the usual food source (Escherichia coli) substituted with a potential pathogen. The outcome was determined by identifying the ‘victor’. If the nematodes were unaffected, within a few days, they devoured the lawn of bacteria, and thousands of progeny nematodes were visible on the plate. However, if the bacteria killed the nematodes, few if any of the progenitors transferred to the pathogenic lawn survived. Intermediate pathogenesis contained modest nematode population growth.

Burkholderia species kill nematodes

Four strains of B. pseudomallei, four strains of B. mallei, seven strains of the Burkholderia cepacia complex and a single strain each of Burkholderia thailandensis, Burkholderia cocovenenans, Burkholderia pyrrocina and Ralstonia pickettii were tested for their ability to kill nematodes. Many strains killed the nematodes or only allowed a modest population growth relative to the E. coli OP50 negative control strain (Table 1). Under MYOB medium conditions, B. pseudomallei, B. thailandensis and B. cepacia species were nematocidal, but not the B. mallei isolates. The B. pyrrocina, B. cocovenenans and R. pickettii isolates had a weak effect upon population growth (Table 1).

Interestingly, many but not all the tested strains exhibited enhanced killing relative to MYOB medium under the high-osmolarity conditions of PGM medium (Table 1). Phenazines, superoxide-generating small-molecule toxins, are produced by P. aeruginosa PA14 grown under PGM agar conditions (Mahajan-Miklos et al., 1999), and many of the strains tested may have a similar killing mechanism under PGM agar growth conditions.

Table 1. Burkholderia kill nematodes.

| Strain                  | MYOB | PGM | Reference/source                                   |
|-------------------------|------|-----|---------------------------------------------------|
| E. coli OP50 and DH5a   | ++ ++| ++ ++| A. Golden (NIH Bethesda)                          |
| P. aeruginosa PA14      | ++   | 0   | Tan et al. (1999b); M.W. Tan (MGH)                |
| B. cepacia ATCC 25416   | 0    | 0   | ATCC; D. DeShazer (USAMRIID)                      |
| B. multivorans c568b    | ++ ++| ++  | D. DeShazer (USAMRIID)                            |
| B. cepacia K56-2        | ++   | 0   | D. DeShazer (USAMRIID)                            |
| B. cepacia Fco 362      | 0    | +   | D. DeShazer (USAMRIID)                            |
| B. vietnamiensis Fco 369| ++++| ++  | D. DeShazer (USAMRIID)                            |
| B. cepacia ATCC 27515   | 0    | 0   | ATCC; D. Waag (USAMRIID)                          |
| B. cepacia ATCC 35130   | +    | 0   | ATCC; D. Waag (USAMRIID)                          |
| B. cocovenenans ATCC 33664| ++ ++| 0   | ATCC                                              |
| B. pyrrocina ATCC 15958 | ++ ++| 0   | ATCC                                              |
| R. pickettii ATCC 27512 | ++ ++| ++  | ATCC                                              |
| B. thailandensis E264   | 0    | 0   | Brett et al. (1998); D. DeShazer (USAMRIID)       |
| B. pseudomallei E203    | 0    | 0   | Brett et al. (1997); D. DeShazer (USAMRIID)       |
| B. pseudomallei 316c    | 0    | 0   | Brett et al. (1997); D. DeShazer (USAMRIID)       |
| B. pseudomallei NCTC 4845| + | 0   | D. Waag (USAMRIID)                               |
| B. pseudomallei 1026b   | 0    | 0   | Brett et al. (1997); D. DeShazer (USAMRIID)       |
| DD503 = 1026b (amr-R-opRA: rpsL  | 0    | 0   | Moore et al. (1999); D. DeShazer (USAMRIID)       |
| SRM117 = 1026b(wbl::Tn5-OT182) | 0 | 0   | DeShazer et al., (1998); D. DeShazer (USAMRIID)   |
| DD213 = 1026b(gspP::Tn5-OT182) | 0 | 0   | DeShazer et al., (1999); D. DeShazer (USAMRIID)   |
| MM36 = 1026b(bcr::Tn5-OT182) | 0 | 0   | DeShazer et al., (1997); D. DeShazer (USAMRIID)   |
| B. mallei NCTC 10229    | ++++ | +   | D. Waag (USAMRIID)                               |
| B. mallei NCTC 10260    | ++++ | +   | D. Waag (USAMRIID)                               |
| B. mallei ATCC 23344    | ++++ | +   | Fritz et al. (1999); ATCC; D. Waag (USAMRIID)     |

Virulence was assayed in a plating assay on either MYOB or PGM medium. 0 = no living nematodes. +s reflect the number of surviving nematodes: ++ = 1–100 nematodes; +++ = 101–250; ++++ = 251–500; +++++ = 501–1000; ++++++ = 1000 or more. All counts at 86 h. Plates were inoculated with three L4-stage N2 Bristol nematodes at 0 h. E. coli OP50 and DH5a were used as negative controls. P. aeruginosa PA14 was used as the positive control for PGM-mediated killing. 0s confirmed 2N.
Only the genomovar IV *B. cepacia* Fco 362 did not kill nematodes as well under PGM agar conditions (Table 1).

Some of the pathogenic strains supported small or modest nematode population growth, whereas others allowed no growth (Table 1). The strain-specific differential in population size suggested variable killing. An LT₅₀ assay (Tan et al., 1999b) was used to explore nematode killing further. The LT₅₀ is the calculated time at which 50% of the nematode population was observed to cease movement (see Experimental procedures). Differences in LT₅₀ times represent different rates of killing. The kinetics of killing by *B. pseudomallei* and *B. thailandensis* strains are shown in Fig. 1 and summarized in Table 2. The L4-stage *C. elegans* LT₅₀ for *B. pseudomallei* strains ranged between 16 h and 23 h of feeding. The *B. thailandensis* strain E264 killed most efficiently, with an LT₅₀ time of about 10 h. Because the *Burkholderia viettamensis* strain Fco 369 was found not to kill nematodes (Table 1), it was used as a more suitable 'non-pathogenic' negative control for LT₅₀ analysis.

Characterized *B. pseudomallei* mutations have little effect on nematode killing

*Burkholderia pseudomallei* knock-out mutations affecting aminoglycoside and macrolide efflux pumping, DD503 (DeShazer et al., 1998), lipopolysaccharide (LPS) O-antigen, SRM117 (DeShazer et al., 1998), general protein secretion machinery (gspD), DD213 (Brett et al., 1998), and the flagella (*fliC*), MM36 (Brett et al., 1998), showed little effect upon the ability to kill L4-stage *C. elegans* (Table 1). Detailed LT₅₀ analysis of the mutant strains versus wild-type N2 *C. elegans* confirmed the initial observation (data not shown).
Table 2. N2 versus *Burkholderia* sp. LT50 summary.

| Strain                  | LT50 (h of feeding ± SD) |
|-------------------------|--------------------------|
| *E. coli* OP50          | ND                       |
| *P. aeruginosa* UCBPP-PA14 | > 30 (MYOB)a             |
| Starvation              | > 30                     |
| *B. cepacia* ATCC 25416 | 13.5 ± 3.5               |
| *B. cepacia* K96-2      | ~30                      |
| *B. cepacia* Fco 362    | 19.9 ± 2.7               |
| *B. thailandensis* E264 | 9.5 ± 1.6                |
| *B. pseudomallei* 1026b | 17.7 ± 1.7               |
| *B. pseudomallei* NCTC 4845 | 19.8 ± 0.8               |

Average *C. elegans* N2 Bristol LT50 times (in hours) for various strains. *n =* 3 repetitions of triplicate time point experiments. Values derived by calculation of LT50 (see Experimental procedures). All experiments conducted at 23°C in MYOB agar medium with synchronized L4-stage N2 nematodes.

a. PA14 data not shown; see Tan et al. (1999b) and Mahajan-Miklos et al. (1999).

Nematode death syndrome

Observations of the phenotype of dying nematodes suggested that killing by *B. pseudomallei* and *B. thailandensis* was an active process. Nematode pathogenesis appeared to affect locomotor functions, as evidenced by the rapid onset of lethargy. Locomotion visibly decreased after exposure for as little as 1 h, and the rate of foraging (side-to-side feeding head movement) was similarly affected in the same time frame. Pharyngeal pumping was affected by more prolonged feeding and, after 4 h, ~50% of the nematode population had stopped pumping (Table 3). In addition, nematodes fed on lawns of *B. pseudomallei* and *B. thailandensis* exhibited an egg-laying-deficient (egl-d) phenotype. Nearly all L4-staged nematodes that fed on *B. pseudomallei* (or *B. thailandensis*) became ‘bags-of-worms’ early in adulthood. The ability of the pathogens to inhibit egg-laying was measurable. Both *B. pseudomallei* 1026b and *B. thailandensis* E264 were able to inhibit egg-laying in adults homozygous for a constitutive egg-laying mutation. The egg-laying inhibition conditioned a >40% decrease in accumulated eggs within 4 h. Over the next 20 h of feeding, few additional eggs were sown (Table 3). ‘Bags-of-worms’ are the result of internal hatching of retained embryos (Riddle et al., 1997).

Even after the nematodes were returned to the *E. coli* lawn, killing was observed. L4-stage N2 Bristol animals fed on *B. pseudomallei* 1026b, NCTC 4845 or *B. thailandensis* E264 for >8 h did not recover once transferred back onto *E. coli* (OP50) plates (Table 3). Feeding for shorter intervals allowed a stochastic chance of recovery that appeared to be strain specific (Table 3). Although 50% of the nematodes stopped pumping at 4 h, this phenotype did not correlate with animal survival to reproduction. Approximately 80% of the nematodes had stopped pharyngeal pumping by 8 h (Table 3).

Every life stage of *C. elegans* was susceptible to *B. pseudomallei* and *B. thailandensis*, except unhatched embryos (data not shown). L3-stage and dauer (a developmentally arrested life stage) animals survived longer than L4/adult animals, probably because the less developed (or arrested) L3/dauer animals fed less than the voracious L4/adults (Riddle et al., 1997). Dead nematodes were often found lying coiled. Embryos sown in lawns of E264, NCTC4845 and 1026b did hatch, but the L1 did not survive. Starved *C. elegans* is able to arrest its development at the L1 stage as a response to the lack of available food (Riddle et al., 1997). The E264- and 1026b-fed L1s were not simply starvation arrested, as they also did not recover once they were transferred back to OP50.

Curiously, nematodes killed in the lawn of bacteria took on a ghostly and hollow ‘shell-like’ appearance ~48 h after the L4s were first introduced. The nematode shells induced by *Burkholderia* strains1026b, NCTC 4845 and E264 were termed ‘chalk-mark ghosts’. Chalk-mark ghosts appeared to have no discernible internal cell structures. Often the ghosts eroded to a mere outline of where a nematode died in the bacterial lawn. Although some ghosts were formed by adults that died and subsequently became ‘bags-of-worms’, ghosts were also found from animals too young to have ‘bagged’. Examination of many chalk-mark ghosts found that every

Table 3. Prolonged feeding blocks recovery, pharyngeal pumping and egg-laying.

| Starting food strain | 4 h switch no. of animals (day 4) | 4 h % pumping (day 1) | 4 h mean no. of eggs | 8 h switch no. of animals (day 4) | 8 h % pumping (day 1) | 24 h mean no. of eggs |
|----------------------|-----------------------------------|----------------------|---------------------|----------------------------------|----------------------|---------------------|
| *E. coli* OP50       | > 1000                            | 94 (± 5)             | 75 (± 2)            | > 1000                           | 92 (±330)            |
| *B. thailandensis* E264 | 4 (± 5)                            | 37 (± 8)             | 45 (± 1)            | 0 (± 0)                           | 18 (± 5)             |
| *B. pseudomallei* 1026b | 3 (± 2)                            | 55 (± 5)             | 48 (± 1)            | 2 (± 2)                           | 23 (± 1)             |
| *B. pseudomallei* NCTC 4845 | 73 (± 52)                          | ND                   | ND                  | 35 (± 42)                        | ND                   |
| Starvation           | > 1000                            | 96 (± 6)             | ND                  | ND                               | ND                   |

The experiment began with ~20 L4s/strain (see Experimental procedures). Animals were assessed for pumping and general disposition at the indicated times, at which point three live L4s were switched back (by hand) from the test strain to OP50. Nematode population size counted at day 4. *n =* 3, except for 8 h pumping data (*n =* 1). Values are means ± (SD). ND, not determined.

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life stage became a ghost after dying on the *Burkholderia* lawn. Attempts to isolate the ghosts for more detailed characterization were unsuccessful.

**Nematode killing exploration**

The relatively rapid death rate (10–18 h) and the death syndrome’s phenotypic components suggested that the nematocidal killing observed may be mediated by, or in concert with, intoxication. Bacteria producing a toxin may exhibit cell-free killing. Conditioned culture medium may kill and/or dead bacteria may kill as well as live bacteria. Bacterial cell-free nematode toxicity was not detected (data not shown). Bacterial culture filtrates from *B. pseudomallei* and *B. thailandensis* liquid (LB and MYOB), as well as ‘conditioned’ solid (MYOB) agar, did not kill *C. elegans*. In contrast, when *P. aeruginosa* PA14 was grown on a 0.2 μm filter on PGM agar and the filter was lifted from the PGM agar plate, the ‘conditioned’ agar killed nematodes in a cell-free manner, confirming the results of Mahajan-Miklos et al. (1999).

Although neither the *B. pseudomallei* nor the *B. thailandensis* isolates tested possessed a diffusible nematocidal agent, it was possible that *B. pseudomallei* and *B. thailandensis* exhibited cell-associated toxicity. However, UV-inactivated *B. pseudomallei* and *B. thailandensis* were no longer nematocidal (Table 4). In marked contrast, gamma-irradiated lawns of *B. thailandensis* and *B. pseudomallei* were nematocidal. Lawns irradiated with as much as $9 \times 10^6$ rad retained the ability to kill nematodes and suppress population growth (data not shown), even though no colony-forming units were recoverable from any of the $1.4 \times 10^6$ rad (60 min) or larger irradiation doses. Although irradiated lawns killed the nematodes, killing analysis showed that wild-type N2 Bristol nematodes died two to four times more quickly when fed live bacterial lawns than gamma-irradiation-killed bacterial lawns [Fig. 2; living bacteria are not essential for pathogenesis (killing indicated by line A); however, living/proliferating bacteria make up a large portion, roughly half, of the killing activity (indicated by B)].

These data suggest that the nematode killing process consists of an intoxication mechanism plus additional mechanisms dependent upon living bacteria. The skew towards the slower killing relative to Fig. 1 is a reflection of the mixed multistaged nematode population assayed.

*Caenorhabditis elegans* respond to environmental stimuli. Nematodes exhibit aversion to high-salt environments and a preference for high-sugar environments. In addition, they tend to migrate within a temperature gradient to the temperature at which they were reared (Dusenbery et al., 1975). Further, Jones and Candido (1999) found that *C. elegans* would stop feeding in response to environmental intoxicants, such as ethanol, methanol, heavy metals and the toxic phthalimide fungicide captan (Jones and Candido, 1999). L4-stage N2 Bristol animals exhibited no aversion to eating *B. thailandensis* or *B. pseudomallei* over *E. coli* (Table 5). Behavioural ‘choice’ between 1026b and E264 was monitored similarly; the nematodes fed randomly (i.e. also without aversion; *B. pseudomallei* data not shown). If the E264 or 1026b were intoxicating the animals or were otherwise a generally bad food source, the nematodes did not indicate behaviourally that they were able to sense any toxicity or nutritional deficiency.

**Host genotype modulates survival**

Darby et al. (1999) demonstrated that host mutations in *C. elegans* conferred resistance to a neuromuscular paralysis phenotype conditioned by the PAO isolate of *P. aeruginosa* when grown upon brain–heart infusion (BHI) medium. As the death phenotype conditioned by *B. pseudomallei* and *B. thailandensis* resembled that of *P. aeruginosa*, we determined the affect of *egl-9* mutations upon host survival. Further, as the toxicity of the pathogens appeared to affect the nematodes’ neuromuscular signalling, we screened a variety of characterized *C. elegans* mutations in neural response pathways looking for mutations that would modify positively (or negatively) nematode host survival. Figure 3 depicts the results.

Loss-of-function mutations in the *egl-9* gene, ([n571]...
and (n586]), whose product is of unknown function, enhanced nematode survival but did not provide qualitative resistance to either *B. thailandensis* or *B. pseudomallei* grown upon MYOB agar (Fig. 3A; data not shown). *Pseudomonas aeruginosa* PAO did not kill wild-type *C. elegans* N2 or egl-9 mutants under the MYOB agar conditions assayed. For a direct comparison, BHI medium was used to grow *B. thailandensis* E264, *B. pseudomallei* 1026b and *P. aeruginosa* PAO. Although the egl-9 mutations did confer resistance to PAO paralytic killing under BHI medium, confirming the results of Darby et al. (1999), the mutant animals still succumbed to both the E264 and the 1026b with about the same kinetics as that of MYOB medium-grown strains (data not shown). LT50 analysis showed that egl-9 (n571) homozygotes lived more than twice as long as wild-type N2 under MYOB conditions (Table 6).

Interestingly, mutations that disrupted normal Ca2+ signal transduction also had an affect upon nematode survival (Fig. 3B and Table 6). Loss-of-function mutations affecting L-type voltage-gated Ca2+ channel subunits expressed in the body wall muscle (*unc-36*) and the neuromuscular junction (*egl-19*), encoded by the egl-19(n582) and unc-36(n251) alleles, decreased nematode survival in homozygotes. Conversely, gain-of-function mutations affecting *egl-19* (n2368)sd and (ad695)sd enhanced homozygote survival (Fig. 3B; Table 6; data not shown). Double-mutant analysis suggests that *unc-36* If, egl-19 If double-mutant animals were more sensitive to the pathogenic *Burkholderia* sp. than the single mutants alone, and that the egl-19(qf) alleles could partially mitigate the negative affect of an *unc-36(If)* allele (most sensitive *unc-36(n251)*; egl-19(n582) > unc-36(n251) = egl-19(n582) > unc-36(n251); egl-19(n2368) > wild type = unc-36(n251); egl-19(ad695) > egl-19(ad695) = egl-19(n2368) least sensitive) (Fig. 3B, Table 6; data not shown). Mutations in the *unc-2* gene ([unc-2(e55)],[61] which also encodes a similar L-type voltage-gated Ca2+ channel subunit involved in neurotransmitter adaptation (Schafer and Kenyon, 1995), had little effect upon survival (data not shown).

The *C. elegans* L-type Ca2+ channel encoded by the *egl-19* and *unc-36* genes provide the Ca2+ necessary for signal transduction processes that are mediated in part by the calcium- and calmodulin-dependent protein kinase II (CaMKII) gene, *unc-43* (Rongo and Kaplan, 1999). Because *egl-19* and *unc-43* act in the same neuromuscular signalling pathway, mutations in the *unc-43* gene were also examined. We tested five *unc-43* alleles in a killing assay against *B. thailandensis* E264 and calculated the LT50s for each of the alleles (Fig. 3B, Table 6; data not shown). Homozygotes for the gain-of-function *unc-43* allele (n498) had an LT50 that was almost three times longer than that of wild-type animals (Table 6). Animals homozygous for the null allele, *unc-43*(e408), also exhibited an extended LT50 time relative to wild type, but the extension was less dramatic (Table 6). Homozygotes for hypomorphic *unc-43* alleles ([n498n1179] and [n498n1186]) behaved similar to the null allele homozygotes (Table 6).

Because many of the single- and double-mutant combinations tested are paralysed, it was important to confirm that the accelerated *Burkholderia* sp.-induced paralytic death resulted from the presence of the specified alleles and was not simply a consequence of already being paralysed. To assess the specificity of the interaction, we examined the LT50 of another highly paralysed mutant animal, *unc-22(If)* homozygotes (Moerman and Baillie, 1979). Animals homozygous for two different mutant alleles of the *unc-22* gene ([unc-22(s7) and *unc-22(s177)*] stopped twitching at a rate similar to wild-type N2 Bristol animals (data not shown).

**Discussion**

**Lower order hosts to model pathogenesis**

The objective of this study was to establish a lower order model for the identification of important virulence factors in pathogenic bacteria. The data shown here suggest that the disease phenotype observed in nematode hosts after exposure to *B. pseudomallei* may be valuable for investigating the pathogenesis of these bacteria, especially as few of its virulence determinants are understood. Many members of the *Burkholderia* genus tested were able to kill nematodes (Table 1).
Fig. 3. C. elegans genotype modulates survival.
A. *egl-9* versus E264 and PAO on MYOB. Comparison of the relative pathogenicity of *P. aeruginosa* PAO and *B. thailandensis* E264 and the effect of two alleles of the *egl-9* gene [wild-type (N2) and (n571)]. Values plotted represent averages from three independent analyses. Blue lines represent nematodes fed *E. coli* OP50. Wild-type N2 Bristol animals are depicted by blue diamonds. Green lines are animals fed upon MYOB-grown *P. aeruginosa* PAO, whereas red lines are MYOB-grown *B. thailandensis* E264. Yellow circles represent *C. elegans* *egl-9*(n571) homozygotes. SD is shown as error bars. L4 animals were used for each assay. Although *egl-9*(n586) homozygous animals are not depicted, they performed similar to the *egl-9*(n571) homozygotes, confirming the results of Darby et al. (1999). Notice that PAO has little killing activity under MYOB media relative to E264. Under BHI media, PAO kills wild type, but not *egl-9* mutants; E264 kills both *egl-9* mutants and wild type, although *egl-9* mutations enhance survival.
B. Ca$^{2+}$ signal transduction mutations affect survival versus E264. The differential effect upon survival of the L-type Ca$^{2+}$ channel genotype. Values plotted represent mean values from four triple-point analyses. Green lines indicate genotypes that have enhanced survival relative to wild type (blue lines). Red lines indicate genotypes that have reduced survival relative to wild type. Red dashed lines are further reduced in survival. Diamonds indicate the presence of specific *egl-19* alleles indicated by colours: green fill, *egl-19*(n2368); and red fill, *egl-19*(n582). Open circles represent *unc-36*(n251) homozygotes, whereas wild-type N2 is indicated by blue-filled triangles. *unc-43*(n498) homozygous animals are depicted by yellow squares. Because many of the mutant lines grew more slowly than the rest of the lines analysed, developmental synchronization of all the lines for simultaneous assay was impossible; therefore, 40–100 L4 young adult animals were hand picked for each assay. The LT$\text{$_{50}$}$s were calculated from these data (see Experimental procedures) and are in Table 6.

The finding that *B. mallei* was not pathogenic in this surrogate model system suggests that nematode killing by this genus may not represent general correlates of animal virulence. However, the observed lack of pathogenesis by *B. mallei* in nematodes is not surprising, as this organism has no known environmental reservoir. Although *B. mallei* was not directly amenable to study in the surrogate model, *B. pseudomallei* appeared to be.
All *B. pseudomallei*, *B. thailandensis* and *B. cepacia* species had strains capable of killing nematodes. However, pathogenicity in nematodes may not correlate directly with pathogenicity in mammals, as the most nematocidal strain tested was *B. thailandensis*. *Burkholderia thailandensis*, like *B. pseudomallei*, is a soil saprophyte, often co-isolated from the same environmental sample (Brett et al., 1997; 1998). Classically, arabinose assimilation has been a discriminator of *B. pseudomallei*-like species virulence. Arabinose non-\-assimilators are much more virulent than arabinose assimilators. Recently, Brett et al. (1997; 1998) suggested that arabinose assimilators belong to species other than *B. pseudomallei*; the designation for one of those species was *B. thailandensis*.

*B. thailandensis* strains are markedly reduced in mammal virulence when compared with *B. pseudomallei*. The hamster LD$_{50}$ for *B. pseudomallei* is less than 10 bacteria in 48 h (DeShazer et al., 1997). The hamster LD$_{50}$ of *B. thailandensis* is 10$^5$ times higher than that of *B. pseudomallei* (Brett et al., 1997; 1998); it does, however, still kill hamsters. Although clearly not as adept at animal killing as *B. pseudomallei*, *B. thailandensis* possesses some characteristics that suggest it may represent an intermediate form of pathogen. Harley et al. (1998) showed that *B. thailandensis*, like *B. pseudomallei* and *B. mallei*, is capable of invading and multiplying inside eukaryotic cells. Brett et al. (1997) showed that *B. thailandensis* is the most cytotoxic of the related species. Further, there is at least one report of a human clinical infection with an arabinose-assimilating *B. pseudomallei*-like strain (Lertpatanasuwan et al., 1999). Combining these reports with the results presented here suggests that arabinose-assimilating *B. pseudomallei*-like strains deserve more intensive study.

Nematode killing is an active process

Genetic analysis using available *B. pseudomallei* mutants suggested that killing is an active process. Nematode killing did not depend upon secreted exoenzyme proteases, lipases or other ‘digestive’ enzymes because *B. pseudomallei* harbouring gspD insertion mutations (DD213 and C21) and wild-type 1026b killed equally (Table 1; data not shown). The role of other secretion systems needs to be investigated. Any role for type III and type IV secretion systems in *B. pseudomallei* vertebrate pathogenesis has not been reported. *B. pseudomallei* mutants in an RND class efflux transporter (DD503) killed as well as wild-type bacteria, suggesting that nematode killing is not caused by the export of bacterial products such as aminoglycoside or macrolide antibiotic compounds (Table 1; data not shown). *Streptomyces avermitilis*, another soil saprophyte species, produces potent antihelminthic macrolides called avermectins (Ormond, 1983). Avermectin resistance-conferring mutants have been isolated and characterized in *C. elegans* (avr-14; avr-15) (Dent et al., 2000). Pathogenic *Burkholderia* sp. kill ivermectin-resistant mutant *C. elegans* at the same rate as wild-type animals (A. L. O’Quinn and J. A. Jeddeloh, unpublished data), also positing against the action of known antihelminthic macrolides.

*Burkholderia pseudomallei* harbouring mutations in LPS O-antigen synthesis (SRM117), which are modestly attenuated in Syrian hamsters, guinea pigs and diabetic infant rats (Bryan et al., 1994; DeShazer et al., 1998), induce nearly wild-type pathogenesis in nematodes (A. L. O’Quinn and J. A. Jeddeloh; Table 1; data not shown). Motility has a negligible role in nematode pathogenesis because the *fliC* knock-out strain (MM36) kills as fast as...
the 1026b parent strain (A. L. O’Quinn and J. A. Jeddelloh; Table 1; data not shown).

Theoretically, nematode killing could exist along a continuum of relevance, ranging from an active process initiated by the bacteria to a passive process manifested by starvation. Under some culture conditions, bacteria of the genus *Yersinia* inhibit growth of *C. elegans* in a manner that suggests that the animals are unable to feed normally (C. Darby, personal communication). Starvation caused little observable mortality under the 30 h window during the LT₅₀ assays (Fig. 1). Starvation among nematodes increases foraging and pharyngeal-pumping behaviours (Avery and Horvitz, 1990). The data presented here suggest that both these processes were inhibited by *B. pseudomallei* (Table 3), arguing against a non-specific, starvation-based lethal mechanism and positing some active aspect to the nematode killing observed.

Feeding on *B. pseudomallei* or *B. thailandensis* for long intervals ensured death even when the animals were returned to a non-pathogenic *E. coli* lawn (Table 3). Feeding for 4 h was long enough to compromise the population size attainable at day 4 substantially, as well as to make 50% of the nematodes stop feeding and laying eggs (Table 3). Feeding cessation, however, did not correlate with whether or not the animals survived to breed. Survival was reduced further at longer feeding intervals (Table 3). The loss of survival at the extended feeding interval suggests that either a lethal dose of some type of toxin was delivered and/or that longer feeding ensured that the pathogen multiplicity of infection became high enough to establish an infection with a lethal outcome.

Further evidence of the specific nature of the *B. pseudomallei*–nematode interaction is presented in Table 4. UV-inactivated bacteria were no longer able to kill nematodes, although bacterial lawns inactivated with radioactive cobalt continued to kill *C. elegans*. The photosensitive, radiation-resistant nematode killing activity had no apparent upper ceiling, as lawns inactivated with $9 \times 10^6$ rad continued to kill nematodes (data not shown). The nematode death phenotype conditioned by gamma-killed bacteria was consistent with that of live bacteria. Irradiated bacteria continued to inhibit locomotion, feeding and egg-laying behaviours. However, there was one noticeable difference. The animals killed by irradiated bacteria appeared to be less constipated than those killed by live bacteria (J. A. Jeddelloh, unpublished observation). Bacterial population growth within the *C. elegans* intestinal tract is correlated with pathogenesis in *P. aeruginosa* (Tan et al., 1999b). Analogously, irradiated bacteria killed more slowly than live bacteria (Fig. 3). Combining the results of these observations suggests that nematode pathogenesis by *B. thailandensis* and *B. pseudomallei* involves an intoxication mechanism plus additional factors that depend upon living bacteria for delivery. The simplest interpretation would be that living/proliferating bacteria deliver more of the toxin. Because gamma-irradiation inactivated *B. thailandensis* E264, and *B. pseudomallei* 1026b kills nematodes, the feasibility of using irradiated plate lawns to purify and characterize the toxic moieties is under exploration.

**Nematode killing by intoxication?**

An advantage in using *C. elegans* over cultured cells to model pathogenesis is that, as an animal, it can sense and respond to environmental stimuli with observable changes in behaviour (Dusenbery et al., 1975; Hedgecock and Russell, 1975; Wolinsky and Way, 1990). *C. elegans* ceases feeding in response to environmental intoxicants in a dose-dependent fashion and resumes feeding when the intoxicant is removed (Jones and Candido, 1999).

A similar pattern of feeding cessation behaviour was observed after feeding the nematodes *B. pseudomallei* or *B. thailandensis*. Feeding cessation occurred in $\approx 50\%$ of the nematode population within 4 h (Table 3). Feeding cessation as a behavioural response to feeding on pathogens seems to indicate that the nematodes were able to ‘sense’ the apparently harmful nature of the food source, and subsequently ‘decided’ not to eat more. Because the close relative *B. pyrrhocinia* produces the potent fungicide pyroloxtin (Hammer et al., 1997; 1999), and *B. pyrrhocinia* has little nematocidal activity (Table 1), it is unlikely that the nematode feeding cessation induced by *B. pseudomallei* or *B. thailandensis* is mediated through bacterial production of antifungal/antibiotic products.

Another way to explore the ability of the nematodes to perceive any apparent toxicity of *B. pseudomallei* or *B. thailandensis* was to allow the nematodes to ‘choose’ the food source; aversion to pathogenic bacteria may reflect a perception of toxicity by the nematodes. The food choice experiments indicated that the animals were not averse to feeding on *B. pseudomallei* or *B. thailandensis* (Table 5). A tantalizing alternative hypothesis regarding the mechanism of feeding cessation exists that may unify both observations.

According to this alternative hypothesis, the animals cannot ‘sense’ the pathogens to be a bad food source or intoxicating. In this model, feeding cessation, as well as loss of locomotor activity and egg-laying, are consequences of a bacterial pathogenesis factor that affects either the neurons controlling these actions or the ability of the muscles to respond to normal neuronal stimuli. Under this hypothesis, there is no, or little, cellular perception of acute toxicity.

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C. elegans–Burkholderia species interaction involves paralytic killing

The functional impairment of the locomotor, pharyngeal and vulval muscles in C. elegans suggests that B. pseudomallei and B. thailandensis may use some type of neurotoxin or paralytic agent as part of their nematode pathogenic mechanism. Conflicts exist in the B. pseudomallei literature regarding the presence of toxins, which may result from differences in the assays and cell types used to detect them (Nigg et al., 1958; Brett et al., 1997; Haase et al., 1997; Häussler et al., 1998). Early toxicological investigations of B. pseudomallei suggest that it may possess a neurotoxic/paralytic activity (Nigg et al., 1958). Several examples of neurotoxicity are documented among human and animal melioidosis cases (Nigg et al., 1958; Narita, 1982; Woods et al., 1992; Smith et al., 1997).

Loss of regulated L-type voltage-gated Ca\(^{2+}\) channel activity could result in paralysis by inhibition of signalling both presynaptically and post-synaptically. Voltage-gated Ca\(^{2+}\) channels mediate both signalling processes (Lee et al., 1997). Further, pathogenesis by uropathogenic E. coli has recently been shown to use a pathogenesis mechanism involving host L-type Ca\(^{2+}\) channels (Uhlen et al., 2000), suggesting that attack by prokaryotes upon eukaryotic second messenger signalling pathways may be a general pathogenesis strategy.

Nematode paralysis and death caused by Burkholderia sp. may result from a toxin-mediated inability to restore Ca\(^{2+}\) membrane potentials. Perhaps the action of the gain-of-function alleles in the egl-19 L-type Ca\(^{2+}\) channel subunit and the unc-43 CaMKII partially impedes the establishment and perception of bacterially perturbed changes in Ca\(^{2+}\) signals, resulting in a slowing of killing/paralysis. Whatever the specific mechanism, double-mutant analysis (Fig. 3B and Table 6) suggests that both the body wall muscles (unc-36) and the neuromuscular junction (egl-19) are targeted by the bacteria.

Both the egl-19 gene and the egl-9 genes appear to be expressed in a similar spectrum of nematode cell types (Lee et al., 1997; Darby et al., 1999). egl-9 is a protein of unknown function. Loss-of-function mutations in the egl-9 gene have the same phenotype as gain-of-function mutations in the egl-19 gene and the unc-43 gene with respect to Burkholderia species survival enhancement. Perhaps egl-9 is interacting with egl-19 or unc-43 as a mediator or modulator of their activity. Double mutants would provide a clue and may define another genetic function for egl-9, outside of its effect upon egg-laying (egl = egg-laying deficient); double mutant construction is under way.

Experimental procedures

Strains/maintenance

Bacterial strains were maintained on LB agar. Burkholderia mallei were maintained on LBG (LB + 4% glycerol). Burkholderia pseudomallei mutants were maintained on LB supplemented with streptomycin (100 μg ml\(^{-1}\)) and tetracycline (50 μg ml\(^{-1}\)). Overnight cultures were grown in LB or LBG at 37°C. Bacillus anthracis and Bacillus subtilis were cultured on BHI liquid medium and BHI plates. N2 Bristol C. elegans were maintained by growth on E. coli OP50 spotted onto standard C. elegans growth agar (MYOB). Population growth occurred at 23°C. Standard protocols for growth, growth medium and manipulation of C. elegans have been described by Riddle et al. (1997), DA1032 (avr-14; avr-15) was kindly provided by L. Avery (UTSW, Dallas, TX, USA). The following mutant strains were obtained from CGC (University of Minnesota, St Paul, MN, USA): BC23, BC177, CB55, CB251, CB408, CB904, CB1072, CB1091, CB1111, CB1112, CB1114, CB11152, DA695, DA823, DA939, DA944, DA945, DA1031, DA1034, DA1035, DA1051, DA1055, KP1097, MT1092, MT1201, MT1212, MT2426, MT2598, MT2605, MT3198, MT6129 and PR1158.

Nematocidal activity assays

Bacteria were grown aerobically overnight in 2–3 ml of LB liquid cultures and spotted (10–20 μl) onto the C. elegans growth medium: MYOB, NG, NGM (NG + 0.15 M sorbitol), PG or PGM (PG + 0.15 M sorbitol). Animals were hand transferred onto the spots and monitored according to the procedure of Tan et al. (1999b). Growth of the nematode populations was compared with growth on E. coli OP50. The positive control for nematode pathogenesis was P. aeruginosa PA14 (Mahajan-Miklos et al., 1999; Tan et al., 1999).

Killing kinetic assays and LT\(_{50}\) calculation

Analysis was performed blind to animal genotype using hundreds of hypochlorite-synchronized juvenile (L4-stage) animals in a time course experiment (Tan et al., 1999b). Three 6-well culture plates were prepared by spreading overnight bacterial cultures with a Q-tip into each of the six wells. After overnight growth at 37°C, the uniform lawns of the bacterial strain to be analysed were allowed to cool to 23°C. Synchronized animals were inoculated into each well of the test plates at three intervals; the first plate was started at 09.00 (0 h), the second at 12.00 (3 h) and the last at 15.00 (6 h). Starvation was assessed by inoculating synchronized L4 animals into foodless MYOB plates and observing the ‘ratio still moving’ over the course of the 30 h experiment (number moving/total number). All assays involving B. pseudomallei were performed under BSL-3 conditions with a microscope-equipped camera in a biological safety cabinet and a video monitor for remote viewing. Rather than scoring loss of reflex action as a marker for nematode death, death was confirmed by high-power (120 x) visual inspection. The percentage still moving was determined by averaging the movement ratios and multiplying them by 100 for each time point among at least three experiments. The LT\(_{50}\) was calculated from the mean percentage still moving values using a non-linear
logistic, three-parameter regression of the form \( y = a / \left[ 1 + (x / x_0)^{br} \right] \), where \( a \) and \( b \) are parameters fitted to the curve and \( x_0 \) is the LT_{50}. The calculations were made using SIGMAPLOT (version 4.0, SigmaPlot) and EXCEL (version 5/95, Microsoft) for the PC.

Food source switching

Approximately 20 L4-stage animals (L4s) were transferred to B. pseudomallei 1026b, NCTC 4845, B. thailandensis E264 or E. coli OP50 and allowed to feed for 4 h or 8 h. Individuals were then transferred back onto E. coli, and the resulting population was counted at day 4. L4s were examined at the time of transfer back onto E. coli to note whether they had stopped pharyngeal pumping. ‘Starvation’ was assessed by nematode transfer to empty MYOB for 4 h or 8 h, followed by transfer back to OP50 for further monitoring. To minimize the carry-over of pathogenic bacteria, the experiments were also performed in parallel with plates supplemented with tetracycline at 20 \( \mu \)g ml\(^{-1}\). The bacterial food source for these plates was E. coli DH5\( \alpha \) carrying the plasmid pBR322 (n = 3). The data from these plates were averaged into the data in Table 3. Because the tetracycline kills the Burkholderia sp. outside and inside the nematodes, the tetracycline experiment animal counts reflect the action of the toxin, and the data without tetracycline presumably reflect the action of both bacterial proliferation and intoxication.

Egg-laying inhibition

Egg-laying inhibition was monitored using homozygous egl-19(n2368) host animals. The (n2368) allele expresses an egl-c (constitutive egg-laying) phenotype and, as such, it retains very few eggs in its uterus. Because few eggs are retained by adults, accumulation of the eggs on a plate is easy to monitor. Saturated overnight cultures (100 \( \mu \)l) of E264, OP50 or 1026b were spotted onto MYOB agar and spread out to confluency with a sterile cotton swab. The plates were incubated overnight at 37°C, allowed to cool to room temperature and then seeded with 10 adults plate\(^{-1}\). The number of embryos sown was counted at 4 h and 24 h. Two plates per bacterial species were examined in parallel. The experiment was performed twice. The average number ± SD of embryos per plate is shown in Table 3.

Toxin diffusion

LB overnight cultures (100 \( \mu \)l) of B. pseudomallei 1026b, B. thailandensis E264, E. coli OP50 and P. aeruginosa PA14 were spotted onto either 100 mm MYOB agar plates (100 mm PGM agar plates for PA14) or MYOB(PGM) overlain with a 0.2 \( \mu \)m Nytran (Schleicher and Schuell; through Midwest Scientific) filter disk. After incubating the spotted plates at 37°C overnight, the plates were allowed to cool to room temperature. The plates containing the bacterial lawn supported upon the filters had the filters lifted from the plates. E. coli OP50-grown N2 Bristol C. elegans were washed with 10 ml of sterile M9 + 10 mM MgCl\(_2\) and collected by low-speed centrifugation four times. The animals were counted by measuring the number of animals in 20 \( \mu \)l of the resuspension. Approximately 20 of the washed multi-stage animals were spotted either into the bacterial lawns or onto the plates that had the lawn filter lifted, and mortality was monitored at 6 h and 24 h.

UV/gamma inactivation

Aerobic overnight bacterial cultures were spotted onto 12 MYOB agar plates (PGM for PA14) per species. The inoculated plates were allowed to grow overnight at 37°C. The following day, the plates were inverted on a UV transilluminator (mix 260–280 nm) for 0 s, 6 s, 39 s, 1 min, 10 min and 60 min or exposed to radioactive \(^{60}\)Co (23 333 rad min\(^{-1}\)) for the same time increments. Two plates were exposed in parallel. The irradiated plates were allowed to cool to room temperature, and then a P200 pipette tip was stabbed into the lawn of bacteria. The lawn stab was rinsed into 1 ml of LB liquid medium and serially diluted (logs of 10) to a ratio of 1:1 \( \times 10^{-6}\). Colonies were counted after overnight growth at 37°C. After the plate stab, 3 L4-stage N2 Bristol C. elegans were transferred onto each plate (day = 0). The total population size was counted after 4 days incubation at 23°C (day = 4). Any threshold to the radiation doses was tested by extending the UV exposure to 120 min, and the gamma irradiation exposures were confirmed at 130 min (\( \approx 3 \times 10^{6}\) rad) and 385 min (∼9 \( \times 10^{6}\) rad). All increased exposures resulted in no change to the experimental outcomes, while visibly discoloring the Petri dishes containing the inactivated bacteria. The 1.4 \( \times 10^{6}\) rad plates were tested three times, and the 3 \( \times 10^{6}\) rad and 9 \( \times 10^{6}\) rad plates were checked twice.

Toxin killing kinetic

Samples of 100 \( \mu \)l from saturated aerobic overnight bacterial cultures of B. thailandensis E264 were spotted onto four (60 mm) MYOB agar plates and spread out to confluency with a sterile cotton swab. After overnight growth at 37°C, the plates were allowed to cool to room temperature (∼23°C). Two plates were selected and exposed to radioactive \(^{60}\)Co at a dose of 3 \( \times 10^{6}\) rad. The bacterial lawns were next stabbed with a P200 pipette tip. The lawn stab was rinsed into 1 ml of LB liquid medium and serially diluted (logs of 10) to a ratio of 1:1 \( \times 10^{-6}\). Colonies were counted after overnight growth at 37°C. The next day, after confirmation of the gamma inactivation, 50 multistaged N2 C. elegans were placed into the lawns. The ratio still moving was monitored at 24 h and 48 h after introduction in parallel for both the live and the inactivated lawns. The plates were incubated at room temperature over the course of the experiment. The experiment was performed twice, and the data reported are mean values ± SD.

Food choice

Numerous L4-stage N2 Bristol animals were placed in various positions on a series of plates that contained E. coli and B. thailandensis E264 (or B. pseudomallei 1026b). Three plates were prepared, each containing a thin longitudinal band of bacterial lawn of E. coli and E264 (or 1026b). Three

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zones were demarcated: zone A, bounded by the edge of the plate and the stripe of *E. coli* OP50; zone B, the space between the *E. coli* and the *B. thailandensis* E264 (or 1026b); and zone C, the space between the outer edge of the E264 (1026b) and the edge of the Petri dish. Approximately 20 hypochlorite-synchronized L4 animals were placed into zone A on plate 1, the mid-point of zone B on plate 2 and zone C on plate 3. Six hours later, the location and disposition of the feeding nematodes were determined.

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**Note added in proof**

Since submission and acceptance of this manuscript, recently obtained results support the model of Ca\(^{2+}\) signalling disruption by the pathogenic *Burkholderia* sp. During the course of LT50 studies with the unc-43 (n498) homozygotes the normally over-stimulation induced uncoordinated phenotype of the animals appeared to be alleviated by feeding upon the nematocidal *Burkholderia* sp. Recent investigation, using a nematode track assay, has revealed that the paralytic endotoxin mediated the observed phenotypic suppression (A. L. O’Quin and J. A. Jeddeloh, unpublished data). *E. coli* fed CaMKII constitutive kinase mutant animals were observed to sew few tracks in 24 h. CaMKII gain-of-function mutants fed upon *B. thailandensis*, however, left as many tracks as wild-type N2. The toxin alone probably mediated the observed suppression, because feeding upon gamma irradiation-killed *B. thailandensis* lawns continued to suppress the uncoordinated phenotype (more tracks). UV-killed, *B. thailandensis*-fed animals exhibited the converse phenotype; they resembled those fed upon *E. coli* (fewer tracks). Observation of the interaction of unc-43 (n498) homozygotes with non-nematocidal *Burkholderia* sp. revealed no phenotypic suppression. Further, homozygotes for an unrelated unc mutation (unc-24) also exhibited no observable alleviation of its ‘unc’ phenotype when feeding upon *B. thailandensis*. The observation of specific phenotypic suppression and the reported survival enhancement based upon the Ca\(^{2+}\) signalling genotype suggests that normal Ca\(^{2+}\) signalling may be perturbed by the endotoxin.

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