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Galleria mellonella as an alternative infection model for *Yersinia pseudotuberculosis*

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We report that larvae of the wax moth (*Galleria mellonella*) are susceptible to infection with the human enteropathogen *Yersinia pseudotuberculosis* at 37 °C. Confocal microscopy demonstrated that in the initial stages of infection the bacteria were taken up into haemocytes. To evaluate the utility of this model for screening *Y. pseudotuberculosis* mutants we constructed and tested a superoxide dismutase C (*sodC*) mutant. This mutant showed increased susceptibility to superoxide, a key mechanism of killing in insect haemocytes and mammalian phagocytes. It showed reduced virulence in the murine yersiniosis infection model and in contrast to the wild-type strain IP32953 was unable to kill *G. mellonella*. The complemented mutant regained all phenotypic properties associated with SodC, confirming the important role of this metalloenzyme in two *Y. pseudotuberculosis* infection models.

INTRODUCTION

The use of non-mammalian models of infection by human pathogens has attracted significant attention over the past few years. These models allow virulence studies to be carried out without the use of mammals and are relatively convenient and easy to use (Garcia-Lara *et al.*, 2005; Mylonakis *et al.*, 2007; Steinert *et al.*, 2003; Tan, 2002). *Caenorhabditis elegans* has been most frequently used as an alternative model of infection, but suffers from the disadvantage that this nematode cannot survive at 37 °C (Mylonakis *et al.*, 2007). Therefore, virulence determinants which are expressed or are optimally active at mammalian body temperatures may not be revealed. In addition, *C. elegans* lacks functional homologues of many components of the mammalian immune system, such as specialized phagocytic cells. More recently, several groups have reported the utility of insects or insect larvae as models of infection (Aperis *et al.*, 2007; Jander *et al.*, 2000; Mylonakis *et al.*, 2007; Schell *et al.*, 2008; Scully & Bidochka, 2006). Insect larvae have the advantage that they can be infected at 37 °C and they possess specialized phagocytic cells, termed haemocytes (Bergin *et al.*, 2005; Mylonakis *et al.*, 2007). The haemocytes perform many of the functions of phagocytic cells in mammals, and are capable of ingesting bacterial pathogens and generating bactericidal compounds such as superoxide via a respiratory burst (Bergin *et al.*, 2005; Lavine & Strand, 2002). Additionally the innate immune systems of insects such as the wax moth *Galleria mellonella* share a high degree of structural and functional homology with the innate immune system of mammals. Defence against bacteria involves both cellular defence (including phagocytosis, nodulization and encapsulation) and humoral defence (including melanization, haemolymph clotting and antimicrobial peptide production). *G. mellonella* larvae are simple to maintain, inexpensive, do not require feeding, and are amenable to biocontainment. The larvae have recently been shown to be susceptible to infection with bacterial pathogens such as *Francisella tularensis* (Aperis *et al.*, 2007), *Burkholderia mallei* (Schell *et al.*, 2008) and *Pseudomonas aeruginosa* (Jander *et al.*, 2000).

The ability of insect haemocytes to generate compounds such as superoxide suggests that virulence mechanisms which allow mammalian pathogens to subvert these killing mechanisms might also play roles in the infection of insects. Superoxide dismutases (SODs) are a group of metalloenzymes identified as playing a key role in subversion of mammalian host cell defences by pathogens. These enzymes catalyse the dismutation of cytotoxic

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Abbreviations: CI, competitive index; i.v., intravenous(ly); SOD, superoxide dismutase.

superoxide anion radicals to molecular oxygen and hydrogen peroxide (McCord & Fridovich, 1969). Killing by this pathway is known to occur in mammalian cells, including macrophages and neutrophils (Djaldetti *et al.*, 2002; Liochev & Fridovich, 1994). Three types of SOD (Fe-SOD, Mn-SOD and Cu-Zn SOD) have been classified according to the metal cofactor present at the active site of the enzyme. Of these, the periplasmic or lipid-anchored SodC (Cu-Zn SOD) is proposed to play a major role in protecting cells from exogenous sources of superoxide. Cytosolic iron-containing SODs (Fe-SOD) and manganese-containing SODs (Mn-SOD) protect cells from intracellular reactive oxygen species, produced when electron-carrying cofactors of redox enzymes are oxidized by molecular oxygen (Hassan & Schrum, 1994; Liochev & Fridovich, 1994). A role for Cu-Zn SOD in protecting cells from extracellular reactive oxygen species, including superoxide anions generated during the macrophage respiratory burst, and in virulence has been demonstrated in several Gram-negative bacteria (Battistoni *et al.*, 2000; De Groote *et al.*, 1997; Gee *et al.*, 2005; Korshunov & Imlay, 2002). The potential role of a SodC orthologue in virulence of *Y. pseudotuberculosis* has yet to be reported.

In previous studies several groups of workers have used *C. elegans* as a model for infection with the human-pathogenic yersiniae (*Yersinia pseudotuberculosis*, *Yersinia pestis* and *Yersinia enterocolitica)*. These studies have been especially valuable for characterizing biofilms produced by these bacteria (Darby *et al.*, 2002; Darby, 2008; Joshua *et al.*, 2003). However, for the reasons outlined above they have been of limited value in identifying virulence determinants important in human infection. Our aims were to investigate whether *G. mellonella* larvae might serve as a model of infection with *Y. pseudotuberculosis*. To investigate whether these larvae are suitable for characterizing mechanisms of virulence which involve the interaction of the pathogen with phagocytic cells, we have constructed a SodC mutant of *Y. pseudotuberculosis* and tested this mutant for virulence in *G. mellonella* larvae.

METHODS

Plasmids, bacterial strains and culture conditions. Plasmids and bacterial strains used in this work are listed in Table 1. Wild-type (WT) *Y. pseudotuberculosis* IP32953 was maintained in Luria–Bertani (LB) broth or on LB agar, incubated at 28 °C. LB broth or agar supplemented with kanamycin at a concentration of 50 μg ml^{-1} was used to culture the *sodC* mutant and complemented strains. *Y. pseudotuberculosis* was cultured at 37 °C on Congo red magnesium oxalate (CRMOX) agar to confirm the presence of virulence plasmid pYV (Bhaduri *et al.*, 1991; Riley & Toma, 1989). *Escherichia coli* was cultured in LB broth or on LB agar at 37 °C. *Y. pseudotuberculosis* expressing green fluorescent protein (GFP) was produced by electroporating IP32953 with plasmid pSB2019, which constitutively expresses GFP. LB supplemented with 50 μg chloramphenicol ml^{-1} was used to select for *Y. pseudotuberculosis* containing pSB2019, and fluorescence was confirmed by microscopy.

Construction of a *Y. pseudotuberculosis* Δ*sodC* mutant. Construction of a *sodC*-deficient mutant was carried out using a previously published method (Derbise *et al.*, 2003). All primers used are listed in Table 2. Briefly, primers were designed for *sodC* to be disrupted that included 20 bp complementary to the 5′ or 3′ kanamycin-resistance gene of the plasmid pK2 followed by 50 bp of upstream or downstream sequence of the *Y. pseudotuberculosis* genome flanking the *sodC* gene. PCR products were generated using plasmid pK2 as a template. Excess template was digested with *Dpn*I and the PCR product was purified using Microcon 100 centrifugal filters (Millipore). PCR products were transformed into *Y. pseudotuberculosis* IP32953/pAJD434 by electroporation. Following overnight incubation at 28 °C in LB supplemented with 0.8 % arabinose, transformants were selected on LB agar supplemented with kanamycin (50 μg ml^{-1}) and trimethoprim (100 μg ml^{-1}) for 48 h at 28 °C. Transformants were screened by PCR using gene-specific and kanamycin-specific primers. Mutant strains were cured of the pAJD434 plasmid by growth at 37 °C in LB medium supplemented with kanamycin (50 μg ml^{-1}). Cured mutant strains were screened for the virulence plasmid pYV by PCR for two genes located on this

Table 1. Bacterial strains and plasmids

Strain or plasmid	Characteristics	Reference
Y. pseudotuberculosis		
IP32953	Wild-type	Chain *et al.* (2004)
IP32953 Δ*sodC*::kan^r	Insertion deletion mutant of YPTB0756 Δ*sodC*	This work
IP32953 Δ*sodC*::kan^r/pOC1	Mutant complemented with pOC1	This work
IP32953 GFP	IP32953/pSB2019; constitutive expression of GFP	Qazi *et al.* (2001)
Plasmids		
pGEM-T-Easy	Cloning vector	Invitrogen
pSB2019	GFP plasmid	Qazi *et al.* (2001)
pAJD434	Encodes λ Red recombinase genes under the control of an arabinose-inducible promoter	Maxson & Darwin (2004)
pAM238	Complementation vector, low-copy-number, spectinomycin-resistant. pGB2 derivative	Churchward *et al.* (1984)
pOC1	*sodC* and upstream native promoter cloned into pAM238 using *Pst*I and *Kpn*I restriction sites.	This work
pK2	pGEM-T-Easy containing kanamycin cassette	Taylor *et al.* (2005)

Table 2. Oligonucleotides

Name	Sequence
YPTB0756H1	TGACAAGGTTAAACCAGACTTAATCAACATATAAGGGATATAACAATATGGATCTGCCACGTTGTGTCTC
YPTB0756H2	CACCAAACTGGCTACGCTCGGGTAGCCAGTTTAACTGTATCAGACGGTTAGCTCTGCCAGTGTTACAACC
YPTB0756for	GCGGGAGTATGAAGCATAAG
YPTB0756rev	CCGATTATCGCAGATTACCC
KanF1	GCCATATTCAACGGGAAACG
KanR1	AAACTCACCGAGGCAGTTCC
LcrVfor	ACAACTGGCTCTGCTAGAAC
LcrVrev	TCACAATACGCCACGCTTAG
VirFfor	TTCCAGAGCGAGGAGTTCAG
VirFrev	ATCCAGCGGCGAAACAATAC

plasmid, *virF* and *yscC*. The presence of the virulence plasmid was also confirmed by culture on CRMOX plates.

Complementation of the Δ*sodC* mutation. The *sodC* gene of strain IP32953 was amplified by PCR, introducing *Kpn*I and *Pst*I restriction sites into the amplicon, which was ligated into the pGEM vector (Invitrogen). The plasmid was digested with *Pst*I and *Kpn*I and the insert was ligated into similarly restricted pAM238 to generate plasmid pOC1. The plasmid was transformed into *E. coli* JM109 (Invitrogen), following the manufacturer's protocol. Transformants were selected on LB agar supplemented with 50 μg spectinomycin ml^{-1} and confirmed by PCR using primers YPTB0756for and YPTB0756rev. Construct pOC1 was subsequently transformed into *Y. pseudotuberculosis* Δ*sodC* by electroporation. Transformants were selected on LB agar supplemented with 50 μg spectinomycin ml^{-1} and confirmed by PCR using primers YPTB0756for and YPTB0756rev.

Superoxide anion assay. The spontaneous oxidation of pyrogallol (1,2,3-trihydroxybenzene) generates O_2^-. To test susceptibility to exogenous superoxide anions, *Y. pseudotuberculosis* IP32953, Δ*sodC* or the complemented *sodC* mutant were grown overnight in LB broth supplemented with 50 μg kanamycin ml^{-1} and diluted to an OD_{590} of 0.1. In three experimental replicates aliquots of 100 μl *Y. pseudotuberculosis* IP32953 were spread on LB agar containing 1000 units catalase ml^{-1}. Aliquots of 100 μl *Y. pseudotuberculosis* Δ*sodC* or the complemented *sodC* mutant were spread onto LB agar supplemented with kanamycin and 1000 units catalase ml^{-1}, in triplicate. Filter paper discs 5 mm in diameter were placed in the centre of the plates, to which was added 5 μl 1 M pyrogallol (Sigma). The plates were incubated overnight at 30 °C and zones of inhibition surrounding the discs were measured.

SOD activity. To measure and compare SOD activity in *Y. pseudotuberculosis* IP32953, Δ*sodC* or the complemented *sodC* mutant, a commercial SOD determination kit (Fluka) was used according to the manufacturer's instructions. Reduction of O_2^- by SOD is measured colorimetrically. Briefly, a water-soluble tetrazolium salt, WST-1 [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] produces a water-soluble formazan dye upon reduction with O_2^-. The rate of the reduction is linearly related to the xanthine oxidase activity and is inhibited by SOD. Therefore, the IC_{50} (50 % inhibition activity) of SOD or SOD-like enzymes can be determined. Overnight cultures were added to the wells of a 96-well microplate in triplicate. Working solutions of kit reagents were prepared and added in quantities directed by the manufacturer's instructions, as were blanks, positive and negative controls. The microtitre plate was incubated at 37 °C for 20 min, after which the absorbance was read at 450 nm in a model 680 microtitre plate reader (Bio-Rad) and the SOD activity calculated.

***Galleria mellonella* infection.** *G. mellonella* larvae were purchased from Livefood UK Ltd (Rooks Bridge, Somerset, UK). Larvae were infected with *Y. pseudotuberculosis* IP32953, Δ*sodC* or the complemented *sodC* mutant. For dosing, the larvae were inverted over a 5 mm diameter plastic tube and 10 μl given by micro-injection (Hamilton) into the right foremost leg. The larvae were incubated at 37 °C and survival and appearance recorded at 24 h intervals. Larvae were scored as dead when they ceased moving, changed from their normal pale cream coloration to brown and failed to respond when gently manipulated with a pipette tip. For confocal microscopy studies, bacteria expressing GFP were used. PBS-injection and no-injection controls were used. Survival 24 h post-infection was recorded. To determine the numbers of bacteria and site of localization in the haemocoel, larvae were chilled on ice for 20 min. The bottom 2 mm of each larva was aseptically removed and haemocoel was drained into a sterile 1.5 ml microcentrifuge tube. For enumeration haemocoel was serially diluted in PBS and the bacterial load per larva was quantified by enumeration of colony-forming units (c.f.u.) on LB agar or LB agar supplemented with kanamycin. PBS-injection and no-injection controls were used. For visualization the haemocoel was drained onto a sterile glass slide. A coverslip was overlaid and haemocoel was visualized using a Zeiss LSM510 META laser confocal microscope. Twenty random fields of view per slide were scored for presence of intracellular and extracellular bacteria.

Murine competitive index assay. *Y. pseudotuberculosis* Δ*sodC* mutant or wild-type strains were grown separately to exponential phase in 20 ml LB with shaking. Broth cultures were then centrifuged (10 min, 4000 ***g***) and the pellet resuspended in 10 ml sterile PBS and centrifuged again (10 min, 4000 ***g***). The bacteria were washed and resuspended in 10 ml PBS and the OD_{600} adjusted to 0.55–0.6 with sterile PBS. Wild-type or mutant bacterial suspensions were then mixed in a 1 : 1 ratio and serially diluted with sterile PBS to give an inoculation concentration of approximately 1×10^3 c.f.u. ml^{-1}, and groups of six female 6-week-old BALB/c mice (Charles River Laboratories) were dosed with 0.1 ml of this suspension by the intravenous (i.v.) route into the tail vein. Retrospective viable counts were determined by plating out dilutions on LB agar and LB kanamycin agar to determine the input ratio. After 5 days, spleens were recovered and passed through sieves (70 μm; Becton Dickinson) to produce a cell suspension in 3 ml sterile PBS. Cell suspensions were serially diluted in sterile PBS and plated onto LB and LB kanamycin agar to determine the output ratio. The competitive index (CI) was calculated as follows: CI=(mutant output/WT output)/(mutant input/WT input) (Freter *et al.*, 1981; Taylor *et al.*, 1987).

Statistic analyses. Graphpad Prism software was used for all statistical analyses. Unpaired *t*-tests using Welch's correction were applied to pooled data from three experimental replicates for

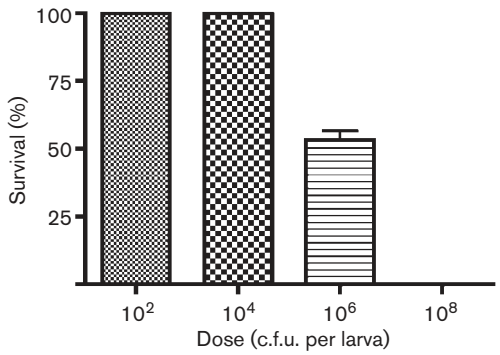

Fig. 1. Survival of *G. mellonella* 24 h after challenge with 10^2–10^8 c.f.u. of *Y. pseudotuberculosis* IP32953. Groups of 10 larvae were challenged and the results shown are the means of three replicates. The error bar indicates standard deviation.

environmental stress, pyrogallol disc assays and *G. mellonella* bacterial load quantification.

RESULTS

G. mellonella are suspectible to a lethal infection with *Y. pseudotuberculosis* IP32953

Initially we challenged groups of 10 *G. mellonella* larvae, by injection into the foreleg, with 10^2, 10^4, 10^6 or 10^8 c.f.u. of *Y. pseudotuberculosis* IP32953 and incubated the larvae at 37 °C. The data from three experiments were pooled and averaged and are shown in Fig. 1. Larvae were scored as dead when they became melanized and ceased moving even when gently disturbed. At 24 h post-infection there was 100 % survival for the 10^2 and 10^4 c.f.u. groups and 53 % survival of the group challenged with 10^6 c.f.u., but none of the larvae challenged with 10^8 c.f.u. survived. At 48 h all of the remaining larvae in the challenge groups had died but we also observed deaths in the control group. Therefore, for subsequent studies results were recorded at 24 h post-challenge.

Fluorescence microscopy was used to establish the cellular location of *Y. pseudotuberculosis* expressing GFP which had been injected into *G. mellonella* larvae. In a repeat experiment larvae challenged with 10^6 c.f.u. of *Y. pseudotuberculosis* IP32953 which were alive at 24 h post-infection were bled and the haemocoel examined using laser confocal microscopy. Bacteria were observed within haemocytes and no extracellular bacteria were recorded (Fig. 2).

Construction of a Y. pseudotuberculosis Δ*sodC* mutant

A mutant of *Y. pseudotuberculosis* IP32953 was constructed in which the *sodC* gene was deleted and replaced with a kanamycin-resistance cassette. This mutant was complemented with the *sodC* gene and 365 bp upstream region containing the cognate promoter carried on plasmid pAM238. To confirm the predicted function of the gene, a commercial SOD activity kit was used to evaluate the levels of SOD produced by the mutant in response to exogenous superoxide anions. A significant reduction in SOD activity (P=0.0132) was observed in the Δ*sodC* mutant strain compared to wild-type *Y. pseudotuberculosis* IP32953. SOD activity levels were restored to wild-type levels in the complemented *sodC* mutant (Fig. 3).

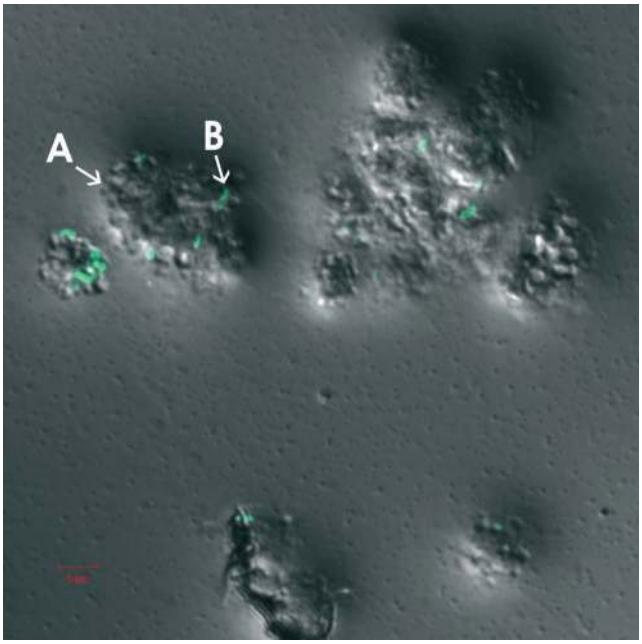

Fig. 2. *Y. pseudotuberculosis* survives within *G. mellonella* haemocytes. *G. mellonella* larvae were infected with 10^6 c.f.u. of *Y. pseudotuberculosis* IP32953 expressing GFP and at 24 h post-infection the haemocoel from surviving larvae was examined using confocal microscopy. A, haemocyte; B, *Y. pseudotuberculosis*. Twenty random fields of view were scored and all bacteria were observed to be surviving within haemocytes with no extracellular bacteria visible. Scale bar, 5 μm.

SodC functions in *Y. pseudotuberculosis* survival during exposure to exogenous superoxide anions

Pyrogallol disc sensitivity assays were used to assess inhibition of growth of wild-type, Δ*sodC* mutant or *sodC* complemented mutant strains by exogenous superoxide anions. A significantly larger zone of inhibition (15 mm) was observed around a lawn of the Δ*sodC* mutant compared to wild-type bacteria (8 mm) (P=0.0318) or the complemented *sodC* mutant (Fig. 4).

Y. pseudotuberculosis Δ*sodC* virulence is attenuated in *G. mellonella*

In initial studies the Δ*sodC* mutant of *Y. pseudotuberculosis* was shown to be attenuated in mice. A CI of 0.16 ± 0.01

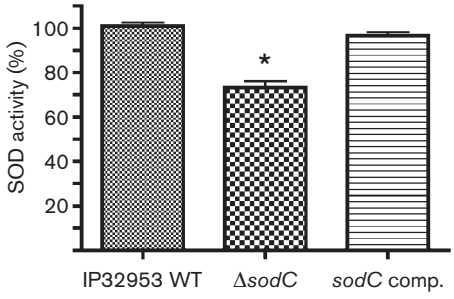

Fig. 3. SOD activity of *Y. pseudotuberculosis* wild-type IP32953, SodC-deficient mutant (Δ*sodC*) and the *sodC* complemented mutant. The results shown are the means of nine replicates. Error bars indicate standard deviation. A significant reduction in SOD activity was measured for the Δ*sodC* mutant ($P=0.0132$).

(mean ± SD) was calculated for the Δ*sod*C mutant when given i.v. with wild-type. To investigate whether the mutant was attenuated in *G. mellonella*, larvae were infected with 10^6 c.f.u. of IP32953, Δ*sodC* or *sodC* complemented strains. Survival 24 h post-infection was observed. Following infection with *Y. pseudotuberculosis* Δ*sodC*, 89 % of infected larvae survived compared to 52 % of larvae infected with wild-type ($P<0.0001$). Virulence was restored in the complemented *sodC* mutant, with no significant difference ($P=0.332$) in survival between wild-type and complemented strains (Fig. 5). These data demonstrate that the presence of *sodC* significantly increases the virulence of *Y. pseudotuberculosis* in *G. mellonella*.

To establish whether killing of *G. mellonella* larvae following infection of IP32953 or the Δ*sodC* mutant correlated with bacterial load, *G. mellonella* larvae were infected with 10^6 c.f.u. wild-type or Δ*sodC* mutant. Bacterial load per larva at 24 h post-infection was measured by enumeration of viable bacteria present in the haemocoel. *Y. pseudotuberculosis* IP32953 infection resulted in a mean bacterial load of 1.2×10^5 c.f.u. per larva, significantly higher than that of larvae infected with the Δ*sodC* mutant, which was 3.7×10^4 c.f.u. per larva ($P=0.0038$) (Fig. 6). These data suggest that *sodC* significantly enhances survival of *Y. pseudotuberculosis* in *G. mellonella*.

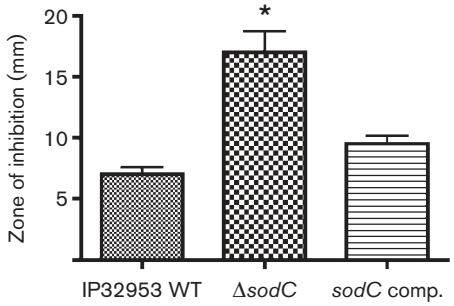

Fig. 4. Role of *sodC* in *Y. pseudotuberculosis* resistance to exogenous superoxide anions. The sensitivity of *Y. pseudotuberculosis* wild-type IP32953, SodC-deficient mutant (Δ*sodC*) or the *sodC* complemented mutant to superoxide was compared using a disc diffusion assay. The results shown are the means of nine or (for the complemented mutant) eight replicates. Error bars indicate standard deviation. A significant increase in the zone of inhibition was observed for the Δ*sodC* mutant ($P=0.0318$). Resistance to superoxide anions, indicated by a reduced zone of inhibition, was restored in the complemented strain.

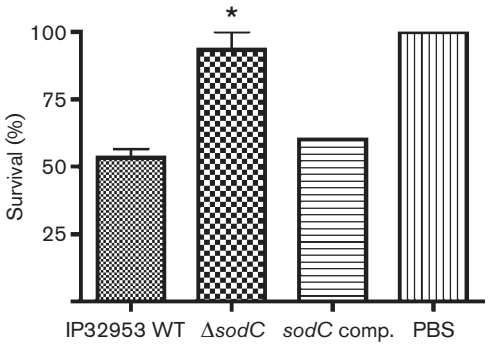

Fig. 5. Survival of *G. mellonella* following infection with 10^6 c.f.u. per larva of *Y. pseudotuberculosis* wild-type IP32953, SodC-deficient mutant (Δ*sodC*) or the *sodC* complemented mutant. The results shown are the means of three replicates, each with 10 larvae. Error bars indicate standard deviation. Larval survival increased significantly following infection with the Δ*sodC* mutant ($P<0.0001$). Larval survival decreased to the level seen with wild-type bacteria following infection with the complemented strain.

DISCUSSION

Recently *G. mellonella* has attracted attention as a model of infection in mammals (Aperis *et al.*, 2007; Schell *et al.*, 2008), and we have shown that these insect larvae are susceptible to a lethal infection with *Y. pseudotuberculosis*. *G. mellonella* (wax moth) larvae are bred commercially as live food for captive reptiles and amphibians. The larvae are the caterpillar stage of the moth and are fully developed as supplied. They are typically 1–2 cm in length, easy to handle and typically survive as larvae for up to 3 weeks before pupating. During this time they do not require feeding and require minimal maintenance. The mechanisms by which the larvae are killed by pathogens are not known and are likely to be pathogen-specific. However, infection of insects is accompanied by the generation of melanin, which is available in the haemocoel and also becomes deposited in tissues (Nappi & Christensen, 2005). As a consequence of melanization, infected *G. melonella* larvae change from their normal cream colour to a pale or dark brown colour. The precise role of melanization in host

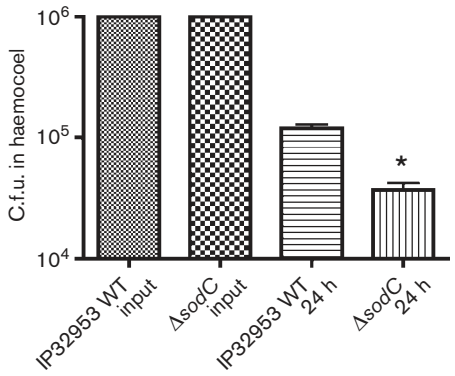

Fig. 6. Bacterial load of *G. mellonella* 24 h post-infection with wild-type IP32953 or SodC-deficient (Δ*sodC*) *Y. pseudotuberculosis*. *G. mellonella* larvae (n=9) were infected with 10^6 c.f.u. per larva of *Y. pseudotuberculosis* wild-type IP32953 or Δ*sodC*. The bacteria in the haemocoel of infected larvae were enumerated 24 h post-infection. The bacterial load of larvae infected with wild-type IP32953 was significantly higher than that of larvae infected with the *sodC* mutant (P=0.0038). Error bars indicate standard deviation.

defence is not known but during this process melanin becomes deposited around pathogens (Nappi & Christensen, 2005).

As an infection model *G. mellonella* has several advantages over invertebrates such as *C. elegans*. Precise doses of the pathogen can be given to *G. mellonella* larvae by injection, enabling an LD_{50} to be determined. In contrast, *C. elegans* is typically infected by allowing the nematodes to graze lawns of bacteria and the dose ingested is difficult both to control and to determine. The low cost and ease of maintenance of the larvae also allow large experimental groups to be used. In this study we routinely used groups of 10 larvae. Overall, the ability to work with large groups of animals and the ability to determine virulence in a quantitative manner makes the *G. mellonella* model of infection ideally suited to comparisons of the virulence of wild-type and mutant bacteria. In this study we have used this model to compare the virulence of WT and *sodC* mutants of *Y. pseudotuberculosis*. The Δ*sodC* mutant of *Y. pseudotuberculosis* was attenuated in *G. mellonella* larvae and showed a reduced ability to colonize the host.

Another major advantage of *G. melonella* larvae compared to many other invertebrate models of infection is the ability to carry out infection studies at the human body temperature of 37 °C. Therefore, genes which are temperature regulated, including many of the virulence genes in *Yersinia*, should be expressed in this infection model. In *G. mellonella*, haemocytes function in the same way as mammalian macrophages and neutrophils in mammals, phagocytosing bacteria and eradicating them through the production of cytotoxic reactive oxygen species including the superoxide anion (Bergin *et al.*, 2005; Mylonakis *et al.*, 2007). After infection of *G. mellonella* with *Y. pseudotuberculosis*, the bacteria accumulated in haemocytes. This finding suggests that *G. mellonella* may be useful for the identification of other genes associated with intracellular survival of *Yersinia*.

In many pathogens SOD plays a key role in protecting the bacterium from antibacterial mechanisms by catalysing the dismutation of cytotoxic superoxide anion radicals to molecular oxygen and hydrogen peroxide (McCord & Fridovich, 1969). The Mn-cofactored SodA has previously been shown to play a role in virulence of *Y. enterocolitica* (Roggenkamp *et al.*, 1997), and a homologue of *sodA* is present in the *Y. pseudotuberculosis* IP32953 genome. In this study we report, apparently for the first time, the construction and characterization of a Δ*sodC* mutant of *Y. pseudotuberculosis* IP32953. The residual SOD activity in the *sodC* mutant is likely to be a consequence of the *Y. pseudotuberculosis* IP32953 SodA and SodB enzymes. As expected, the Δ*sodC* mutant showed an increased susceptibility to exogenous superoxide, but not to other stresses, and the wild-type phenotype could be restored by complementation. In addition the mutant was attenuated in mice by the i.v. route of infection. These studies confirm the important role of SodC in two infection models and validate the use of *G. mellonella* larvae as an alternative infection model for *Y. pseudotuberculosis*.

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