**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., 2003; 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
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 ΔsodC 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 DpnI 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 plasmid.

**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Y. pseudotuberculosis</strong></td>
<td>Wild-type</td>
<td>Chain et al. (2004)</td>
</tr>
<tr>
<td>IP32953</td>
<td></td>
<td>This work</td>
</tr>
<tr>
<td>IP32953 ΔsodC::kan'</td>
<td>Insertion deletion mutant of YPTB0756 ΔsodC</td>
<td>This work</td>
</tr>
<tr>
<td>IP32953 ΔsodC::kan’pOC1</td>
<td>Mutant complemented with pOC1</td>
<td>Qazi et al. (2001)</td>
</tr>
<tr>
<td>IP32953 GFP</td>
<td>IP32953/pSB2019; constitutive expression of GFP</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-T-Easy</td>
<td>Cloning vector</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pSB2019</td>
<td>GFP plasmid</td>
<td>Qazi et al. (2001)</td>
</tr>
<tr>
<td>pAM238</td>
<td>Complementation vector, low-copy-number, spectinomycin-resistant, pGB2 derivative</td>
<td>Churchward et al. (1984)</td>
</tr>
<tr>
<td>pOC1</td>
<td>sodC and upstream native promoter cloned into pAM238 using PstI and KpnI restriction sites.</td>
<td>This work</td>
</tr>
<tr>
<td>pK2</td>
<td>pGEM-T-Easy containing kanamycin cassette</td>
<td>Taylor et al. (2005)</td>
</tr>
</tbody>
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Complementation of the \(\Delta sodC\) mutation. The \(sodC\) gene of strain IP32953 was amplified by PCR, introducing \(Kpn\) and \(Pst\) restriction sites into the ampiclon, which was ligated into the plasmid vector (Invitrogen). The plasmid was digested with \(Pst\) and \(Kpn\) and the insert was ligated into similarly restricted pAM238 to generate plasmid pOCI. The plasmid was transformed into \(E. coli\) JM109 (Invitrogen), following the manufacturer’s protocol. Transformants were selected on LB agar supplemented with 50 \(\mu\)g spectinomycin ml\(^{-1}\) and confirmed by PCR using primers YPTB0756for and YPTB0756rev. The microtitre plate was incubated at 37 \(^{\circ}\)C and the SOD activity calculated.

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, \(\Delta sodC\) or the complemented \(sodC\) mutant were grown overnight in LB broth supplemented with 50 \(\mu\)g kanamycin ml\(^{-1}\) and confirmed by PCR using primers YPTB0756for and YPTB0756rev. Construct pOCI was subsequently transformed into \(Y. pseudotuberculosis\) \(\Delta sodC\) by electroporation. Transformants were selected on LB agar supplemented with 50 \(\mu\)g spectinomycin ml\(^{-1}\) and confirmed by PCR using primers YPTB0756for and YPTB0756rev.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>YPTB0756H1</td>
<td>TGACAAGGTAAACACGAGCTTAATCAACATATAAGGATATTACAAATATTGCTGCGCAGTTTCTGCTC</td>
</tr>
<tr>
<td>YPTB0756H2</td>
<td>CACCAAACTGGCTACGCTCGGGTAGCCAGITTTAATCTGTATCAGACGGTTAGCTCTGCGGTGTTAACC</td>
</tr>
<tr>
<td>YPTB0756fors</td>
<td>GCCGGGATATGAAAGCATAAG</td>
</tr>
<tr>
<td>YPTB0756rev</td>
<td>CGGATTACCGAGATTACCCC</td>
</tr>
<tr>
<td>KanF1</td>
<td>GCCATATTTAAGGGGAAAAGC</td>
</tr>
<tr>
<td>KanR1</td>
<td>AAACCTAGCCCGAGGTTCCC</td>
</tr>
<tr>
<td>LcrVfor</td>
<td>ACAACTGGCTCCTGCTAGAAC</td>
</tr>
<tr>
<td>LcrVrev</td>
<td>TCACAAATCGCCGAGGTTCAG</td>
</tr>
<tr>
<td>VirFfor</td>
<td>TTCCAGAGCCGAGGTTCAG</td>
</tr>
<tr>
<td>VirFrev</td>
<td>ATCCAGCGGCGAAACAATAC</td>
</tr>
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</table>

Galleria mellonella infection. \(G. mellonella\) larvae were purchased from Livefood UK Ltd (Rooks Bridge, Somerset, UK). Larvae were infected with \(Y. pseudotuberculosis\) IP32953, \(\Delta sodC\) or the complemented \(sodC\) mutant. For dosing, the larvae were inverted over a 5 mm diameter plastic tube and 10 \(\mu\)l given by micro-injection (Hamilton) into the right foremost leg. The larvae were incubated at 37 \(^{\circ}\)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. \(Y. pseudotuberculosis\) \(\Delta sodC\) 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 0.5–0.6 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 \times 10^7 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 \(\mu\)m; Becton Dickinson) to produce a cell suspension in saline. \(G. mellonella\) 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...
environmental stress, pyrogallol disc assays and *G. mellonella* bacterial load quantification.

**RESULTS**

**G. mellonella** are susceptible 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 visible. Scale bar, 5 μm.

![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.](http://mic.sgmjournals.org)

![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.](http://mic.sgmjournals.org)

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 ΔsodC 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).

**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, ΔsodC 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 ΔsodC mutant compared to wild-type bacteria (8 mm) (*P*=0.0318) or the complemented sodC mutant (Fig. 4).

**Y. pseudotuberculosis ΔsodC virulence is attenuated in *G. mellonella***

In initial studies the ΔsodC mutant of *Y. pseudotuberculosis* was shown to be attenuated in mice. A CI of 0.16 ± 0.01
(mean ± SD) was calculated for the ΔsodC mutant when given i.v. with wild-type. To investigate whether the mutant was attenuated in *G. mellonella*, larvae were infected with 10⁶ c.f.u. of IP32953, ΔsodC or sodC complemented strains. Survival 24 h post-infection was observed. Following infection with *Y. pseudotuberculosis* ΔsodC, 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 ΔsodC mutant correlated with bacterial load, *G. mellonella* larvae were infected with 10⁶ c.f.u. wild-type or ΔsodC 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 1.2×10⁵ c.f.u. per larva, significantly higher than that of larvae infected with the ΔsodC mutant, which was 3.7×10⁴ c.f.u. per larva (*P*=0.0038) (Fig. 6). These data suggest that sodC significantly enhances survival of *Y. pseudotuberculosis* in *G. mellonella*.

**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 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. mellonella* larvae change from their normal cream colour to a pale or dark brown colour. The precise role of melanization in host...
pathogen can be given to invertebrates such as C. elegans. Precise doses of the pathogen can be given to G. mellonella larvae by injection, enabling an LD₅₀ 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 ΔsodC mutant of Y. pseudotuberculosis was attenuated in G. mellonella larvae and showed a reduced ability to colonize the host.

Another major advantage of G. mellonella 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 ΔsodC 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 ΔsodC 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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