Published in final edited form as:

In Vitro Cell Dev Biol Anim. 2013 August ; 49(7): 501–507. doi:10.1007/s11626-013-9634-0.

The oxidizing agent, paraquat, is more toxic to *Wolbachia* than to mosquito host cells

Ann M. Fallon, Cassandra M. Kurtz, and Elissa M. Carroll

Department of Entomology, University of Minnesota, 1980 Folwell Ave, St. Paul, MN 55108, USA

Ann M. Fallon: fallo002@umn.edu

Abstract

Cultured cells provide an important in vitro system for examining metabolic interactions between the intracellular bacterium, *Wolbachia pipientis*, and its insect hosts. To test whether *Wolbachia*-associated changes in antioxidant activities could provide a tool to select for infected cells, we tested the effects of paraquat (PQ) on *Aedes albopictus* mosquito cells. Like mammalian cells, mosquito cells tolerate PQ over a wide range of concentrations, and for considerable lengths of time, depending on cell density at the time of treatment. When mosquito cells were plated at low density and allowed to grow in the presence of PQ, we measured an LC₅₀ of approximately 1–2 μ M. Unexpectedly, cells persistently infected with *Wolbachia* strain wStr, from the planthopper *Laodelphax striatellus*, grew to higher densities in the presence of 1.5 μ M PQ than in its absence. This effect of PQ was similar to the improved growth of host cells that occurs in the presence of antibiotics that suppress the *Wolbachia* infection. A more detailed examination of growth and metabolic sensitivity indicated that wStr is about 10-fold more sensitive to PQ than the mosquito host cells. Microscopic examination confirmed that *Wolbachia* levels were reduced in PQ-treated cells, and DNA estimates based on the polymerase chain reaction (PCR) indicated that *Wolbachia* abundance decreased by approximately 100-fold over a 10-d period. Although *Wolbachia* genomes encode superoxide dismutase, inspection of annotated genomes indicates that they lack several genes encoding products that ameliorate oxidative damage, including catalase, which converts the PQ byproduct, hydrogen peroxide, to molecular oxygen and water. We suggest that loss of multiple genes that participate in repair of oxidative damage accounts for increased sensitivity of *Wolbachia* to PQ, relative to its host cells.

Keywords

Wolbachia; Mosquito cell line; *Aedes albopictus*; Paraquat; Oxidizing agent; Superoxide dismutase

Introduction

Manipulation of interactions between the intracellular alphabacterium, *Wolbachia pipientis*, and mosquito host cells will facilitate use of genetically modified *Wolbachia* strains to

control vector populations. Towards this end, we seek to identify in vitro conditions that favor growth of *Wolbachia*-infected cells, relative to uninfected cells in a mixed population. Brennan et al. (2008) recently described increased abundance of host antioxidant proteins in *Wolbachia*-infected Aa23 *Aedes albopictus* mosquito cells, and suggested that host cell adaptation to *Wolbachia* infection involves induction of reactive oxygen species, coupled with increased expression of proteins with antioxidant activity. To determine whether up-regulated host antioxidant proteins, potentially coupled with additional antioxidant activities expressed from the *Wolbachia* genome itself, might provide an approach to select for *Wolbachia*-infected cells, we compared effects of paraquat (PQ) on infected and uninfected *A. albopictus* mosquito cells.

Paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) is a common, broad-spectrum herbicide with strong oxidizing activity that disrupts photosynthesis in green plants. In animals, PQ cytotoxicity has been associated with a number of mechanisms (Fukushima et al. 2002), but its main target is the mitochondrial matrix (Cocheme and Murphy 2008). Recent evidence shows that the PQ di-cation is transported across the mitochondrial membrane by an unknown carrier protein. Within the mitochondrion, the di-cation PQ^{2+} is reduced to the reactive cation radical, $PQ^{+•}$. The $PQ^{+•}$ radical causes oxidative stress by reacting with molecular oxygen to generate superoxide. In mitochondria, superoxide is converted to hydrogen peroxide by manganese superoxide dismutase (MnSOD; Zheng et al. 2007). Hydrogen peroxide is further reduced to molecular oxygen and water by catalases and peroxidases (reviewed by Fridovich 1997).

Using an *A. albopictus* mosquito cell line persistently infected with *Wolbachia* from the planthopper, *Laodelphax striatellus* (C/wStr1 cells; Fallon et al. 2013), we found that concentrations of PQ that are tolerated by uninfected host cells suppress growth of *Wolbachia*. Thus, rather than providing a means of selecting for *Wolbachia*-infected cells, PQ provides a potential tool to eliminate *Wolbachia* infection. Inspection of available genomes indicates that *Wolbachia* encode a functional homolog of *Escherichia coli* SOD, in which critical residues important to enzyme function are conserved. In contrast, *Wolbachia* genomes lack several genes encoding products that ameliorate oxidative damage, including catalase, which efficiently breaks down hydrogen peroxide. We hypothesize that reduced ability to repair oxidative damage accounts for *Wolbachia*'s high sensitivity to PQ, relative to mosquito host cells.

Materials and Methods

Cells and culture conditions

A. albopictus mosquito cell lines were maintained in Eagle's medium supplemented with nonessential amino acids, vitamins, glutamine, penicillin and streptomycin, glucose, and 5% heat-inactivated fetal bovine serum (E-5 medium) as detailed by Shih et al. (1998). Uninfected cells used in this study included thymidine-kinase deficient TK-6 cells (Mazzacano and Fallon 1992) that served as hosts for infections with *Wolbachia* strain wAlbB (Fallon and Witthuhn 2009), and C7-10 cells (Fallon and Kurti 2005), which were used more recently to establish a particularly robust infection with *Wolbachia* from the

planthopper, *L. striatellus* (Noda et al. 2002). Infected cells, designated C/wStr1, were recovered after prolonged (10 mo) storage as described previously (Fallon et al. 2013).

Paraquat treatment

Paraquat [methyl viologen dichloride hydrate (Sigma/Aldrich, St. Louis MO, Cat # 856177)] was dissolved in distilled water and sterilized by filtration. For long-term toxicity tests, cells were plated at 2×10^5 cells per 35 mm tissue culture dish, in 2 ml of E-5 medium. After 1–2 d (to allow attachment, in the case of *Wolbachia*-infected cells), PQ was added, and cells were allowed to grow until untreated control plates reached confluency (5 d; $\sim 3 \times 10^6$ cells/plate). Cells from duplicate plates were resuspended, diluted in phosphate-buffered saline (Dulbecco and Vogt 1954) and counted with a Coulter electronic cell counter. Cells from a second set of duplicate plates were evaluated for metabolic activity by conversion of methylthiazole tetrazolium (MTT) to a colored formazan product, as described previously (Fallon and Hellestad 2008). With infected cells, additional plates were used for examination of *Wolbachia* by fluorescence microscopy and DNA quantitation by the polymerase chain reaction (PCR), as described below. For short-term toxicity tests, uninfected cells were grown to near confluency in 35 mm culture dishes, refed with PQ in fresh E-5 medium, and MTT assays were performed after 24, 48, and 72 h. Details relevant to particular experiments are provided in the figure legends.

Effects of PQ on *Wolbachia*-infected cells

C/wStr1 cells were pooled from two flasks, counted, and divided into four 60 ml portions at a density of 1×10^5 cells per ml. One portion of cells was used as an untreated control. A second portion was supplemented with PQ at a final concentration of 1.5 μ M; the third portion was supplemented with the antibiotics rifampicin (Rif) and tetracycline (Tet), which suppress *Wolbachia* growth at final concentrations of 0.4 and 5 μ g/ml, respectively; the final portion contained 1.5 μ M PQ and antibiotics. Cells were plated in 35 mm dishes, 2 ml per plate; at 24 h intervals, cells from duplicate plates were resuspended and cells were counted using a Coulter electronic cell counter.

To estimate PQ sensitivity of *Wolbachia*, PQ concentrations ranging from 0.015 to 2 μ M were added to C/wStr1 cells at an initial density of 3.4×10^5 cells per 35 mm plate, with an estimated infection level of 100% based on fluorescence microscopy. After 10 d, cells in duplicate plates were counted, an additional set of duplicate plates was assayed by MTT, a replicate plate was examined by fluorescence microscopy (not shown) and three 100 μ l samples of resuspended cells and medium were assayed for *Wolbachia* by PCR. For microscopy, the medium was aspirated, and an approximately 6 mm diameter circle was drawn on the cell monolayer with a wax pencil to create a shallow well. A mixture of 50 μ M Syto 13 (Invitrogen, Carlsbad, CA) and 50 μ M propidium iodide (5 μ L) was added to the circled area, and cells were covered with a glass coverslip and viewed with an Olympus IX70 fluorescent microscope.

DNA extraction and PCR analysis

For polymerase chain reactions, cells were uniformly resuspended in the growth medium, and DNA was extracted from 100 μ l samples of cells in culture medium (which contained

released *Wolbachia*) essentially as described previously (Fallon 2008). DNA was recovered by ethanol precipitation, washed in 70% ethanol, dried, and resuspended in 100 μ l of 1 mM NaCl. Samples were diluted 1000-fold, and amplified with *Wolbachia*-specific primers S12F (5'-GCACTAAGGTGTATACTACAACCTCC) and S7R (5'-GCCTTATTAGCTTCAGCCAT), which amplify a portion of the prokaryotic ribosomal protein operon encoding ribosomal proteins S12 and S7 (Fallon 2008).

Results

Effects of PQ on mosquito cells

To evaluate the effects of PQ on mosquito cells, we began by exposing near-confluent TK-6 cells to PQ for intervals ranging from 24 to 72 h (Fig. 1). After cells were exposed to 1 to 4 mM PQ for 24 h, MTT values were similar to those of control values at the time of treatment (Fig. 1, compare hatched “time 0” bar at 0 mM PQ to dark gray bars at 1–20 mM PQ). MTT values dropped below 50% of 24 h untreated controls (compare dark gray columns) only at relatively high PQ concentrations of 8 to 20 mM. By 48 h after treatment with 1 mM PQ, MTT values were reduced to 50% of the starting levels (compare light gray bars to the hatched bar), and metabolic activity was reduced by more than 90% (compare open columns) after 72 h exposure to 1 mM PQ.

To establish a more rigorous evaluation of PQ toxicity, we plated mosquito cells at a low density (2×10^5 cells per 35 mm plate) and allowed them to grow in medium containing PQ for approximately 5 d, until untreated control cells reached confluency. Cell numbers were measured using a Coulter electronic cell counter, and metabolic activity was based on MTT assays (Fig. 2). Although residual cell numbers at the highest concentrations of PQ were about 20% in the data set based on cell counts (open circles), LC_{50} values were in the range of 1–2 μ M PQ based on either metabolic activity (closed circles) or cell number (Fig. 2). These initial results with TK-6 cells were further validated with C7–10 cells, which, with *Wolbachia* strain *w*Str, maintain a more robust *Wolbachia* infection (Fallon et al. 2013) than that described earlier with TK-6 cells and *Wolbachia* strain *w*AlbB (Fallon and Witthuhn 2009).

Effects of PQ on *Wolbachia*-infected cells

To test whether host cell antioxidant activities (Brennan et al. 2008) attributed to *Wolbachia* in the Aa23 cell line infected with *w*AlbB (O'Neill et al. 1997) might provide a means to enrich for *Wolbachia*-infected cells, we evaluated effects of PQ on C/*w*Str1 cells, in which the *Wolbachia* infection is sufficiently robust for detection of *Wolbachia* proteins against a background of host cell proteins by gel electrophoresis (Fallon et al. 2013). With C/*w*Str1 cells, suppression of *Wolbachia* by addition of Rif and Tet to the culture medium allows host cells to attain higher densities than untreated, infected C/*w*Str1 cells (Fig. 3, compare open and closed circles), suggesting that *Wolbachia* imposes a metabolic cost on its host cells. When C/*w*Str1 cells were treated with 1.5 μ M PQ, either with (Fig. 3, open squares) or without Rif and Tet (Fig. 3, closed squares), cells grew to intermediate densities. Because PQ treatment alone allowed *Wolbachia*-infected cells (Fig. 3, solid squares) to attain higher densities than *Wolbachia*-infected controls without PQ (solid circles), we suspected that low

levels of PQ reduced levels of *Wolbachia*, albeit to a lesser extent than the combined effects of Rif and Tet. We noted that by day 8, the effect of PQ was slightly enhanced in the presence of antibiotics, but we have not explored whether this modest effect is statistically significant. Visual inspection of cells stained with Syto 13 and propidium iodide by fluorescent microscopy confirmed that *Wolbachia* levels were reduced in PQ-treated cells, relative to untreated control cells.

To further evaluate *Wolbachia* sensitivity to PQ, we added concentrations ranging from 0.015 to 2 μ M PQ to C/wStr1 cells at an initial density of 3.4×10^5 cells per 35 mm plate, with an estimated infection level of 100% based on fluorescence microscopy. After 10 d, cells in duplicate plates were counted (Fig. 4A, open circles), and an additional set of duplicate plates was assayed by MTT (Fig. 4A, closed circles), a replicate plate was examined by fluorescence microscopy (not shown) and three 100- μ l samples of resuspended cells and medium were assayed for *Wolbachia* DNA by PCR (Fig. 4B). On day 10, cell counts ranged from 6.8×10^6 cells per plate in the absence of PQ, to 8.8×10^6 cells per plate at 2 μ M PQ. This modest, 1.3-fold increase in cell numbers was accompanied by more substantial changes in metabolic activity. MTT values varied from 0.45 in the absence of PQ, to a maximum of 2.5 at 0.25 μ M PQ, and declined as PQ concentrations approached the LC₅₀ for the host cells. Based on the transition from lower to higher cell number and MTT values, we estimate that *Wolbachia* is sensitive to PQ at concentrations as low as 0.08 μ M, and thus is approximately 10-fold more sensitive to PQ than uninfected host cells (Fig. 2). To validate microscopic evidence for reduction in *Wolbachia* abundance, we quantified *Wolbachia* DNA amounts by PCR. Even at the lowest levels of PQ, we detected a decrease in *Wolbachia* DNA (Fig. 4B), which was enhanced with increasing PQ concentrations. Using additional serial dilutions (not shown), we estimated an approximately 100-fold decrease in *Wolbachia* DNA at 2 μ M PQ, relative to untreated controls.

In silico analysis of *Wolbachia* antioxidant genes

To understand *Wolbachia*'s response to PQ in the context of proteins known to play key roles in ameliorating oxidative damage, we used BlastP in the NCBI database (<http://www.ncbi.nlm.nih.gov/>; searched as of Dec., 2012) to examine annotated genomes in the *Rickettsiales* (taxid 766), which include *Wolbachia* and related genera of obligate intracellular alphaproteobacteria, including *Anaplasma*, *Ehrlichia*, and *Rickettsia*. Among the superoxide dismutases, catalases, and peroxidases, *Wolbachia* strains wAlbB (ZP_09542721) and wPip (YP_001975359) encode a single SOD gene, which had slightly greater homology to the constitutive, FeSOD (YP_489920), relative to the inducible MnSOD (YP_491542) enzyme in *E. coli* (Fig. 5). The *E. coli* enzymes and their *Wolbachia* homologs shared identities at the active sites identified in the crystal structure of *E. coli* MnSOD (Edwards et al. 1998; Borgshahl et al. 2000), including H26, H30, Y34, H81, D167, E, 170, H171, Y174 (boxed in Fig. 5). However, the *E. coli* enzymes varied at Q146, and the alanine in the *Wolbachia* homologs matched *E. coli* FeSOD, rather than *E. coli* MnSOD (shaded gray in Fig. 5). A similar pattern (alignment not shown) of conservation extended to representative SODs from *Wolbachia*-related alphaproteobacteria in the genera *Rickettsia* (YP_001494799), *Anaplasma* (YP_153998), and *Ehrlichia* (YP_197429). Dismutation of superoxide by MnSOD generates hydrogen peroxide, which itself is a

reactive oxygen species. With the possible exception of *Wolbachia* from the filarial nematode, *Onchocerca volvulus* (Henkle-Durhsen et al. 1998) *Wolbachia* genomes do not encode catalase, which would be predicted to minimize toxicity of hydrogen peroxide.

Discussion

Superoxide dismutases, catalases, and glutathione peroxidases protect cells against oxidative damage caused by low levels of reactive oxygen species (ROS) released as a normal byproduct of aerobic respiration (Fridovich 1997). The observation that *Wolbachia* infection is associated with higher levels of oxidative enzymes (Brennan et al. 2008) led us to explore whether infected cells would have a selective advantage over uninfected cells when treated with PQ, which induces oxidative stress in a wide range of species (Cocheme and Murphy 2008). Sensitivity of animal cells to PQ varies considerably. For example, with Chinese hamster ovary cells assayed by colony formation, Starr et al. (1986) reported an LC₅₀ of 50 μ M, and selected resistant lines by exposing cells to PQ concentrations ranging as high as 0.5 mM. After short-term exposure, Salmon et al. (2005) reported an LC₅₀ of 3 to 4 mM PQ for primary mouse fibroblasts derived from skin biopsies. Like mammalian cells, mosquito cells tolerated PQ over a wide range of concentrations, and for considerable lengths of time, depending on cell density at the time of treatment (compare Figs. 1 and 2). Contrary to our expectations, however, PQ was more toxic to intracellular *Wolbachia* than to its host cell, and caused an approximately 100-fold reduction in *Wolbachia* levels during a 10-d growth cycle.

Better-studied human and *E. coli* models (Zheng et al. 2007; Cocheme and Murphy 2008) have shown that in eukaryotic cells, the PQ di-cation is transported to the mitochondria, where it is converted to an active radical that generates superoxide, which in turn is reduced by MnSOD. Evidence for MnSOD as the primary target for PQ includes protection from PQ toxicity by overexpression of SOD and hypersensitivity to PQ when SOD activity is reduced (reviewed by Cocheme and Murphy 2008). Likewise, RNAi-induced silencing of *Drosophila* mitochondrial SOD increases sensitivity to PQ (Kirby et al. 2002). Reduction of superoxide by MnSOD generates hydrogen peroxide (Zheng et al. 2007).

Like superoxide, hydrogen peroxide is also a reactive oxygen species. Although the details remain under investigation, kinetic studies suggest that hydrogen peroxide interacts with MnSOD to form a product-inhibited complex (Zheng et al. 2007). In most cells, however, hydrogen peroxide is reduced to water and molecular oxygen by catalase, which has one of the highest efficiencies of known enzymes. In the mosquito cell, catalase would be expected to act in concert with MnSOD to relieve PQ-induced oxidative damage.

In contrast to *E. coli*, which expresses three SODs, *Wolbachia* genomes contain a single SOD gene, encoding proteins with 37–43% identity with *E. coli* MnSOD. *Wolbachia* SODs have high conservation of residues that constitute the active site in the *E. coli* enzyme, with the exception of the Q/A substitution at residue 146, which also occurs in *E. coli* FeSOD (see open and shaded boxes in Fig. 5). We note that the active site residues characteristic of *E. coli* MnSOD are likewise conserved in human and mosquito MnSODs (not shown).

In *E. coli*, superoxide acts through the SoxR and SoxS transcription factors (Gu and Imlay 2011), which activate expression of co-regulated genes to restore cellular homeostasis (Pomposiello et al. 2001). Of approximately 100 genes encoding proteins that reduce oxidative stress through the SoxRS response, MnSOD (*sodA*) is most prominent (see Table 1 in Pomposiello et al. 2001). In this context, we note that genomes of *Wolbachia* and other members of the *Rickettsiales* do not encode homologs of the transcription factors SoxR and SoxS, which have presumably been lost with genome streamlining that has occurred in these intracellular microorganisms (Wu et al. 2004). Thus, *Wolbachia* and its close relatives appear to lack an important system that might otherwise enhance protection from oxidative stress. Moreover, with the possible exception of *Candidatus Odysella thessalonicensis*, members of the *Rickettsiales* also lack homologs of *E. coli* endonuclease IV, an inducible DNA repair enzyme activated by PQ (Chan and Weiss 1987).

In the context of oxidative damage repair, we further note that members of the *Rickettsiales* lack homologs of *E. coli* glutathione peroxidase (YP_489972), but do include members of the periredoxin family of enzymes, which feature a conserved cysteine at the active site (in wAlbB, these are ZP_09542550, ZP_09542239, and ZP_09542235; Mavingui et al. 2012). Finally, with the possible exception of *Wolbachia* from the filarial nematode, *O. volvulus* (Henkle-Duhrson et al. 1998), *Wolbachia* genomes do not encode catalase, whose high efficiency could be particularly important in removing hydrogen peroxide resulting from PQ treatment.

In conclusion, our studies indicate that because *Wolbachia* is more sensitive to oxidative damage than its host cell, agents that elicit oxidative stress cannot provide a means of selecting *Wolbachia*-infected cells in vitro. We envision that in mosquito host cells the combined activities of MnSOD and catalase likely contribute to PQ tolerance. In support of this scenario, Mockett et al. (2003) showed that in *Drosophila*, overexpression of catalase in mitochondria, but not in the cytosol, protects against PQ. Although it remains possible that hypothetical proteins encoded by *Wolbachia* have additional, as yet unexplored activities that ameliorate oxidative damage, *Wolbachia*'s higher sensitivity to PQ is supported by in silico analyses, which show that *Wolbachia* and its close relatives lack genes encoding catalase, as well as other regulatory proteins that mediate protection against ROS. Our observations suggest that *Wolbachia*-induced increases in intracellular levels of host antioxidant proteins (Brennan et al. 2008) are not sufficient to protect *Wolbachia* from exogenous inducers of ROS such as PQ. We envision that future improvements in the manipulation of *Wolbachia* in mosquito cell lines will eventually facilitate assays in which *Wolbachia*-encoded activities can be measured independently of host cell activities.

Acknowledgements

This work was supported by NIH grant AI081322 and by the University of Minnesota Agricultural Experiment Station, St. Paul, MN. We thank Gerald D. Baldrige, John F. Beckmann and Grace Li for helpful discussion.

References

- Borgshahl GEO, Pokross M, Chehab R, Sekher A, Snell EH. Cryo-trapping of the six-coordinate, distorted-octahedral active site of manganese superoxide dismutase. *J. Mol. Biol.* 2000; 296:951–959. [PubMed: 10686094]
- Brennan LJ, Keddie BA, Braig HR, Harris HL. The endosymbiont *Wolbachia pipientis* induces the expression of host antioxidant proteins in an *Aedes albopictus* cell line. *PLoS One.* 2008; 3(5):e2083. [PubMed: 18461124]
- Chan E, Weiss B. Endonuclease IV of *Escherichia coli* is induced by paraquat. *Proc. Natl. Acad. Sci. USA.* 1987; 84:3189–3193. [PubMed: 2437576]
- Cocheme HM, Murphy MP. Complex I is the major site of mitochondrial superoxide production by paraquat. *J. Biol. Chem.* 2008; 283:1786–1798. [PubMed: 18039652]
- Dulbecco R, Vogt M. Plaque formation and isolation of pure lines with poliomyelitis virus. *J. Exp. Med.* 1954; 99:167–182. [PubMed: 13130792]
- Edwards RA, Baker HM, Whittaker MM, Whittaker JW, Jameson GB, Baker EN. Crystal structure of *Escherichia coli* manganese superoxide dismutase at 2.1 Å resolution. *J. Biol. Inorg. Chem.* 1998; 3:161–171.
- Fallon AM. Cytological properties of an *Aedes albopictus* mosquito cell line infected with *Wolbachia* strain wAlbB. *In Vitro Cell Dev. Biol.-Animal.* 2008; 44:154–161.
- Fallon AM, Hellestad VJ. Standardization of a colorimetric method to quantify growth and metabolic activity of *Wolbachia*-infected mosquito cells. *In Vitro Cell Dev. Biol.-Animal.* 2008; 44:351–356.
- Fallon AM, Kurti TJ. Cultured cells as a tool for analysis of gene expression. In: Marquardt, WC., editor. *Biology of disease vectors*. 2nd ed.. New York: Elsevier; 2005. p. 539-549.
- Fallon AM, Witthuhn BA. Proteasome activity in a naive mosquito cell line infected with *Wolbachia pipientis* wAlbB. *In Vitro Cell Dev. Biol.-Animal.* 2009; 45:460–466.
- Fallon AM, Baldrige GD, Higgins LA, Witthuhn BA. *Wolbachia* from the planthopper *Laodelphax striatellus* establishes a robust, persistent, streptomycin-resistant infection in clonal mosquito cells. *In vitro Cell Dev. Biol.—Animal.* 2013; 49:66–73.
- Fridovich I. Superoxide anion radical ($O_2^{\cdot -}$), superoxide dismutases, and related matters. *J. Biol. Chem.* 1997; 272:18515–18517. [PubMed: 9228011]
- Fukushima T, Tanaka K, Lim H, Moriyama M. Mechanism of cytotoxicity of paraquat. *Environ. Health Prevent. Med.* 2002; 7:89–94.
- Gu M, Imlay JA. The SoxRS response of *Escherichia coli* is directly activated by redox cycling drugs rather than by superoxide. *Molec. Microbiol.* 2011; 79:1136–1150. [PubMed: 21226770]
- Henkle-Duhrsen K, Eckelt VHO, Wildenberg G, Blaxter M, Walter RD. Gene structure, activity and localization of a catalase from intracellular bacteria in *Onchocerca volvulus*. *Mol. Biochem. Parasitol.* 1998; 96:69–81. [PubMed: 9851608]
- Kirby K, Hu J, Hilliker AJ, Phillips JP. RNA interference-mediated silencing of *Sod2* in *Drosophila* leads to early adult-onset mortality and elevated endogenous oxidative stress. *Proc. Natl. Acad. Sci. USA.* 2002; 99:16162–16167. [PubMed: 12456885]
- Mavingui P, Valiente Moro C, Tran-Van V, Wisniewski-Dyé F, Raquin V, Minard G, Tran FH, Voronin D, Rouy Z, Bustos P, Lozano L, Barbe V, González V. Whole-genome sequence of *Wolbachia* strain wAlbB, an endosymbiont of tiger mosquito vector. *Aedes albopictus*. *J. Bacteriol.* 2012; 194:1840.
- Mazzacano CM, Fallon AM. Thymidine kinase-deficient mutants of *Aedes albopictus* mosquito cells. *In Vitro Cell Dev. Biol.* 1992; 28A:455–458. [PubMed: 1634444]
- Mockett RJ, Bayne AC, Kwong LK, Orr WC, Sohal RS. Ectopic expression of catalase in *Drosophila* mitochondria increases stress resistance but not longevity. *Free Radic. Biol. Med.* 2003; 34:207–217. [PubMed: 12521602]
- Noda H, Miyoshi T, Koizumi Y. In vitro cultivation of *Wolbachia* in insect and mammalian cell lines. *In Vitro Cell Dev. Biol.-Animal.* 2002; 38:423–427.

- O'Neill SL, Pettigrew MM, Sinkins SP, Braig HR, Andreadis TG, Tesh RB. In vitro cultivation of *Wolbachia pipientis* in an *Aedes albopictus* cell line. *Insect. Mol. Biol.* 1997; 6:33–39. [PubMed: 9013253]
- Pomposiello PJ, Bennik MHJ, Demple B. Genome-wide transcriptional profiling of the *Escherichia coli* responses to superoxide stress and sodium salicylate. *J. Bacteriol.* 2001; 183:3890–3902. [PubMed: 11395452]
- Salmon AB, Murakami S, Bartke A, Kopchick J, Yasumura K, Miller RA. Fibroblast cell lines from young adult mice of long-lived mutant strains are resistant to multiple forms of stress. *Am. J. Physiol. Endocrinol. Metab.* 2005; 289:E23–E29. [PubMed: 15701676]
- Shih KM, Gerenday A, Fallon AM. Culture of mosquito cells in Eagle's medium. *In Vitro Cell Dev. Biol.-Animal.* 1998; 34:629–630.
- Starr J, Sela S, Distech CM, Rabinovitch PS, Ogburn CE, Smith AC, Martin GM. Resistance to paraquat in a mammalian cell line. *Somatic Cell Mol. Genet.* 1986; 12:141–152.
- Wu M, Sun LV, Vamathevan J, Riegler M, Deboy R, Brownlie JC, McGraw EA, Martin W, Esser C, Ahmadinejad N, Wiegand C, Madupu R, Beanan MJ, Brinkac LM, Daugherty SC, Durkin AS, Kolonay JF, Nelson WC, Mohamoud Y, Lee P, Berry K, Young MB, Utterback T, Weidman J, Nierman WC, Paulsen IT, Nelson KE, Tettelin H, O'Neill SL, Eisen JA. Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. *PLoS Biol.* 2004
- Zheng J, Domsic JF, Cabelli D, McKenna R, Silverman DN. Structural and kinetic study of differences between human and *Escherichia coli* manganese superoxide dismutases. *Biochemistry.* 2007; 46:14830–14837. [PubMed: 18044968]

Figure 1.

Effect of PQ on near-confluent cells. TK-6 cells were grown in E-5 medium to a density of 1×10^6 per 35 mm plate, and had an MTT value of 2.0 (hatched bar). Medium was aspirated and replaced with fresh E-5 medium containing the indicated concentrations of PQ. At 24 h (dark gray), 48 h (light gray), and 72 h (open bars), MTT values were determined on a set of duplicate plates. At 24 h, cell counts had increased to 2×10^6 in control plates without PQ.

Figure 2.

Effect of PQ on long-term growth of mosquito cells. TK-6 cells were seeded at 2×10^5 per 35 mm plate. PQ (0.1 mM stock in distilled water) was added at the indicated final concentrations 24 h after plating. Cells were allowed to grow until control cells in the absence of PQ reached confluency (3.1×10^6 cells/plate; 5 d after plating). Cells from duplicate plates were counted with a Coulter electronic cell counter (*open circles*) and metabolic activity in duplicate plates was evaluated by an MTT assay (*closed circles*). Values were expressed as % of untreated controls. Similar LC_{50} values (1–2 μM PQ) were obtained in five independent biological experiments using either TK-6 or C7–10 cells.

Figure 3.

PQ treatment increases viability of *Wolbachia*-infected cells. C/wStr1 cells (passage 37) at a density of 1×10^5 cells per ml were treated with PQ (1.5 μ M, closed squares); with antibiotics Rif and Tet (which suppress *Wolbachia* growth) at final concentrations of 0.4 and 5 μ g/ml, respectively (open circles); or both 1.5 μ M PQ and antibiotics (open squares). Control, *Wolbachia*-infected cells (closed circles) were incubated without Rif, Tet, and PQ. Cells were plated in 35 mm dishes, 2 ml per plate. At 24 h intervals, cells from duplicate

plates were resuspended and counted using a Coulter electronic cell counter. Comparable effects of PQ were obtained with a biological replicate with infected cells at passage 38.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

A **B** **Figure 4.**

Wolbachia sensitivity to PQ. (A) PQ (0.015 to 2 μM) was added to C/wStr1 cells (passage 42) at an initial density of 3.4×10^5 cells per 35 mm plate, and an estimated *Wolbachia* infection level of 100%. After 10 d, cells in duplicate plates were counted (*open circles*); an additional set of duplicate plates was assayed by MTT (*closed circles*). (B) At each PQ concentration, 100 μl samples of resuspended cells and medium were assayed for *Wolbachia* abundance by PCR amplification of a portion of a ribosomal protein operon encoding rpS12 and rpS7. PCR products were electrophoresed on 2% agarose gels. *M*, at right, indicates

DNA size markers. A representative gel is shown; three independent DNA samples were assayed. Similar results were obtained in a biological replicate with C/wStr1 cells at passage 38.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

E. coli Fe	1	MSFELPALPYAKDALAPHISAETIEVHYGKHQTYVTNLNNLIKGT-AFEGKSLEETIRSSEG-----GVFNNAQVW	72
E. coli Mn	1	..YT..S....Y...E...FDKQ.M.I..HT.....N.A.AALESLEPE.ANLPV..L.TKLDQLPADKKTVLR...GGHA	80
wAlbB	1	...T...E...D.A..E.Y...N.LNF...D...KG.LNK..E.VEN.-DYQHMEI..LVTKVH.--NNNTVPIF...A...	77
wPip	1	...T...E...D.A..E.Y...N.LNF...D...KG.LNK..E.VEN.-DYQHMEI..LVTKVH.--NNNTVPIF...vA...	77
E. coli Fe	73	NHTFYWNCLAPNAGGEPT--GKVAEIAAASFGSFADFKAQFTDAAIKNFGSGWTWLVKNSDGKLAIVSTSNAGTPLTTDA	150
E. coli Mn	81	..SLFWKG.KK--.TTLQ--.DLKA..ERD...VDN...E.EK..ASR.....A...LK-GD...V...A.QDS..MGEA	155
wAlbB	78	SMKK.G..K.qDD.LL.KK.QDDI.G.DK.CEE.SNYGVSQ.....V...LE-KG..KITK.P.ADL..IYGQ	156
wPip	78	SMKK.G..K.kDD.LL.KK.QDDI.G.DK.CEE.SNYGVSQ.....V...LE-KG..KITK.P.ADL..IYGQ	156
E. coli Fe	151	T-----PLLTVDVWEHAYYIDYRNARPGYLEHFW-ALVNWEFVAKNLAA--	193
E. coli Mn	156	ISGASGFPIIMGI.....LKFQ.R..D.IKE..-NV...DEA.ARF..KK	206
wAlbB	157	V-----M.....L.CQ.R.ID.IKV.LDH.I..D..EE..KKNI	202
wPip	157	V-----M.....L.CQ.R.ID.IKV.LDH.I..D..EE..KKNI	202

Figure 5.

Alignment of *E. coli* and *Wolbachia* SODs. *E. coli* FeSOD (*sodB*; YP_489920.1) and *E. coli* MnSOD (*sodA*; YP_491542.1) are aligned with homologs in wAlbB (ZP_09542721) and wPip (YP001975359). Dots indicate amino acid identities. For the *Wolbachia* genes, *E* values were 2e-48 (wAlbB) and 2e-47 (wPip) for the comparison with *E. coli* MnSOD, and 2e-65 (wAlbB) and 2e-64 (wPip) for the comparison with *E. coli* FeSOD. Boxed residues are involved in enzyme function. The shaded column at Q146 in *E. coli* MnSOD is replaced by an A in *E. coli* FeSOD and in the *Wolbachia* SOD proteins. Two amino acid differences between SOD proteins in wAlbB and wPip are indicated by lower case letters and underlined.