ORIGINAL ARTICLE

Integrated transcriptomic and proteomic analysis of the global response of *Wolbachia* to doxycycline-induced stress

Alistair C Darby^{1,3}, A Christina Gill^{2,3}, Stuart D Armstrong², Catherine S Hartley², Dong Xia², Jonathan M Wastling² and Benjamin L Makepeace²

¹Institute of Integrative Biology and the Centre for Genomic Research, University of Liverpool, Liverpool, Merseyside, UK and ²Institute of Infection & Global Health, Liverpool Science Park IC2, University of Liverpool, Liverpool, Merseyside, UK

The bacterium *Wolbachia* (order *Rickettsiales*), representing perhaps the most abundant vertically transmitted microbe worldwide, infects arthropods and filarial nematodes. In arthropods, *Wolbachia* can induce reproductive alterations and interfere with the transmission of several arthropod-borne pathogens. In addition, *Wolbachia* is an obligate mutualist of the filarial parasites that cause lymphatic filariasis and onchocerciasis in the tropics. Targeting *Wolbachia* with tetracycline antibiotics leads to sterilisation and ultimately death of adult filariae. However, several weeks of treatment are required, restricting the implementation of this control strategy. To date, the response of *Wolbachia* to stress has not been investigated, and almost nothing is known about global regulation of gene expression in this organism. We exposed an arthropod *Wolbachia* strain to doxycycline *in vitro*, and analysed differential expression by directional RNA-seq and label-free, quantitative proteomics. We found that *Wolbachia* responded not only by modulating expression of the translation machinery, but also by upregulating nucleotide synthesis and energy metabolism, while downregulating outer membrane proteins. Moreover, *Wolbachia* increased the expression of a key component of the twin-arginine translocase (*tatA*) and a phosphate ABC transporter ATPase (*PstB*); the latter is associated with decreased susceptibility to antimicrobials in free-living bacteria. Finally, the downregulation of 6S RNA during translational inhibition suggests that this small RNA is involved in growth rate control. Despite its highly reduced genome, *Wolbachia* shows a surprising ability to regulate gene expression during exposure to a potent stressor. Our findings have general relevance for the chemotherapy of obligate intracellular bacteria and the mechanistic basis of persistence in the *Rickettsiales*.

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Introduction

Wolbachia is an obligate intracellular α -proteobacterium (family *Anaplasmataceae*, order *Rickettsiales*) that infects an estimated 40% of terrestrial arthropods, suggesting that it is the most prevalent vertically transmitted symbiont worldwide (Zug and Hammerstein, 2012). In contrast with its pandemic distribution in the Arthropoda, *Wolbachia* has a limited range of infection in the Nematoda, being restricted to ~40% of species in the superfamily

Filarioidea (arthropod-transmitted parasites of vertebrates; the filariae) (Ferri *et al.*, 2011) and a single genus of phytoparasitic Pratylenchidae (Haegeman *et al.*, 2009). The nature of the symbiosis between *Wolbachia* and its host varies considerably both within and between arthropods and nematodes, although some general trends are apparent. First, arthropod *Wolbachia* tend to have relatively large genomes (1.3–1.7 Mb; Wu *et al.*, 2004; Geniez *et al.*, 2012) that frequently contain prophage regions (Kent and Bordenstein, 2010), insertion sequences (Cerveau *et al.*, 2011) and large expansions of protein-coding genes containing ankyrin-like domains (ANKs) (Siozios *et al.*, 2013), whereas their counterparts in the filariae have smaller genomes (0.9–1.1 Mb), in which these features are absent or greatly reduced (Foster *et al.*, 2005; Godel *et al.*, 2012). Second, in many instances of

Correspondence: BL Makepeace, Institute of Infection & Global Health, Liverpool Science Park IC2, 146 Brownlow Hill, University of Liverpool, Liverpool, Merseyside L3 5RF, UK.
E-mail: blm1@liverpool.ac.uk

³These authors contributed equally to this work.

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filaria–*Wolbachia* symbiosis, there is considerable evidence for host–symbiont coevolution over millions of years (Bandi *et al.*, 1998), coupled with an obligate dependency of the nematode on *Wolbachia* for normal embryogenesis and viability (Hoerauf *et al.*, 1999; Langworthy *et al.*, 2000). In contrast, essential mutualisms between arthropods and *Wolbachia* appear to be rare (Dedeine *et al.*, 2001; Hosokawa *et al.*, 2010; Miller *et al.*, 2010). Third, *Wolbachia* can induce a range of reproductive manipulations in arthropods that facilitate vertical transmission (Werren *et al.*, 2008), but to date reproductive phenotypes have not been reported in nematode hosts.

Over the past two decades, *Wolbachia* has aroused intense interest not only because of its fascinating and complex biology but also because of its potential to contribute to disease control. Certain arthropod strains of *Wolbachia* have been demonstrated to reduce vector competence by truncating host life span (McMeniman *et al.*, 2009) and/or by inhibiting the development or proliferation of various pathogens (Kambris *et al.*, 2009; Moreira *et al.*, 2009). In addition, the obligate dependency of important filarial parasites such as *Wuchereria bancrofti* (a cause of lymphatic filariasis) and *Onchocerca volvulus* (the aetiological agent of onchocerciasis) on their *Wolbachia* symbionts renders them susceptible to chemotherapy with tetracycline and rifamycin antibiotics (Hoerauf *et al.*, 2000; Taylor *et al.*, 2005; Specht *et al.*, 2008), which deplete the bacteria from nematode tissues. Unfortunately, this promising approach to the control of two major neglected tropical diseases has been hampered by the treatment regimen required to irreversibly suppress *Wolbachia* in filariae. This comprises 4–6 weeks of daily therapy, which is considered too prolonged by the World Health Organisation for a mass drug administration programme akin to those currently based on conventional anthelmintics (Hoerauf *et al.*, 2011). Nevertheless, antibiotics such as doxycycline remain the only safe drugs that exhibit potent activity against the long-lived adult filariae.

Here, we apply RNA-seq and label-free quantitative proteomics to dissect the phenotypic response of *Wolbachia* to short-term doxycycline exposure. As filarial *Wolbachia* cannot be cultured *in vitro*, we have utilised a mosquito cell line containing a *Wolbachia* strain (wMelPop-CLA) originally derived from *Drosophila melanogaster* (McMeniman *et al.*, 2008), as the drug susceptibility of arthropod- and nematode-derived *Wolbachia* appears to be equivalent (Schiefer *et al.*, 2012, 2013). Our data represent the first global gene expression study of an arthropod *Wolbachia* strain and reveal an unexpected capacity to regulate metabolic pathways and small RNAs during exposure to a potent stressor. Moreover, we identify shifts in the expression of outer membrane proteins and inner membrane transporters that may contribute to antibiotic

tolerance in *Wolbachia*, including a phosphate ABC transporter ATPase associated with reduced susceptibility to antimicrobials in free-living bacteria.

Materials and methods

Cell culture, drug treatment and enrichment of Wolbachia

The *Aedes albopictus* cell line RML-12, stably transfected with *Wolbachia* strain wMelPop-CLA (McMeniman *et al.*, 2008), was kindly provided by Scott O'Neill (Monash University, Victoria, Australia). The cells were maintained as previously described for the *Wolbachia*-infected mosquito cell line Aa23 (Makepeace *et al.*, 2006). For antibiotic treatment, doxycycline hyclate (Fluka, Buchs, Switzerland) was added 4 days after subculture at a final concentration of 0.25 µg ml⁻¹. Treated and control cells were harvested 3 days later. *Wolbachia* were enriched from the host cells using glass bead lysis and filtration, as previously described (Rasgon *et al.*, 2006). The pellet containing the bacteria was stored in RNAlater (Sigma-Aldrich, Gillingham, UK) at 4 °C.

RNA extraction and RNA-seq

Total RNA was extracted from the pellet using TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions, and quantified by RiboGreen fluorimetry (Invitrogen, Paisley, UK) on an Infinite F200 PRO multimode reader (Tecan, Männedorf, Switzerland). To deplete processed transcripts containing a 5'-monophosphate, a fraction of each RNA sample (2.5 µg) was incubated with 1 U of Terminator 5'-phosphate-dependent exonuclease (Epicentre, Madison, WI, USA) in Terminator reaction buffer B according to the manufacturer's instructions. The RNA was purified by phenol–chloroform extraction, quantified as before and stored at –80 °C.

Contaminating genomic DNA was digested using the TURBO DNA-free Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions, and sequencing libraries were prepared using the directional ScriptSeq v2 RNA-seq Library Preparation Kit (Epicentre) as directed by the manufacturer. Twelve libraries, comprising matched pre- and post-exonuclease-treated aliquots from three control and three doxycycline-exposed samples, were multiplexed and sequenced as 100-bp paired-ends on a HiSeq 2000 platform (Illumina, San Diego, CA, USA). Confirmatory runs were also performed using a similar protocol on a MiSeq personal sequencer (Illumina). The resulting reads were mapped to the wMel genome (NCBI RefSeq: NC_002978.6) in Burrows-Wheeler Aligner (Li and Durbin, 2009), and counts per gene were calculated by htseq-count. Differential expression (DE) analysis was performed in edgeR (Bioconductor), using a binomial

distribution model (Robinson and Oshlack, 2010; Robinson *et al.*, 2010). Genes were considered to exhibit DE where the fold change (FC) was ≥ 2 and the *P*-value was < 0.01 . Data were deposited at the NCBI Sequence Read Archive under project ID SRA091852.

Protein extraction and nanoflow liquid chromatography electrospray ionisation tandem mass spectrometry

Before protein extraction, we further purified the *Wolbachia*-enriched material using additional filtration and a Percoll (Sigma-Aldrich) gradient, as used for sequencing of the wBol1 genome (Duplouy *et al.*, 2013). *Wolbachia* pellets were washed twice in Hank's balanced salt solution and stored at -80°C . To solubilise protein, the pellets were sonicated in 25 mM ammonium bicarbonate (Sigma-Aldrich) and 0.1% (w/v) Rapigest (Waters, Elstree, UK) on ice. Proteomic-grade trypsin (Sigma-Aldrich) was added at a protein:trypsin ratio of 50:1, and samples were incubated at 37°C overnight before removal of Rapigest by trifluoroacetic acid precipitation.

Peptide mixtures (2 μl) were analysed by online nanoflow liquid chromatography using the nano ACQUITY-nLC system (Waters) coupled to an LTQ-Orbitrap Velos (ThermoFisher Scientific, Bremen, Germany) mass spectrometer (MS) as previously described (Darby *et al.*, 2012). Thermo RAW files were imported into Progenesis LC-MS (version 4.1, Nonlinear Dynamics, Newcastle, UK), and runs were time-aligned using default settings. Peptide intensities were normalised (using wMel features only) against an auto-selected reference run, and differences in protein expression and associated analysis of variance (ANOVA) *P*-values between seven control and nine doxycycline-treated biological replicates were calculated by Progenesis LC-MS. Spectral data were transformed to MGF files with Progenesis LC-MS, exported using the Mascot search engine (version 2.3.02, Matrix Science, London, UK) and searched against all protein sequences expected to be present in the sample (see Supplementary Materials and Methods). Search parameters were as reported previously (Darby *et al.*, 2012), and the results from Mascot were further processed using the machine-learning algorithm Percolator. The false discovery rate was $< 1\%$, and individual ion scores > 13 were considered to indicate identity or extensive homology ($P < 0.05$). For a protein to be classified as undergoing DE, an FC of ≥ 1.5 and a *P*-value of < 0.01 were required, supported by ≥ 2 unique peptides. Deeper proteome coverage was obtained by fractionation of a single bulk sample using strong anion exchange (Wiśniewski *et al.*, 2009), and the resultant peptide data were used for linear regression analysis against the transcriptomic data set (see Supplementary Materials and Methods). The mass spectrometric data were deposited to the ProteomeXchange Consortium

(<http://proteomecentral.proteomexchange.org>) via the PRIDE partner repository (Vizcaino *et al.*, 2013) with the data set identifier PXD000345 and DOI 10.6019/PXD000345.

Results and Discussion

Features of the wMelPop-CLA transcriptome and proteome

We applied 5'-phosphate-dependent exonuclease treatment to total RNA extracted from *Wolbachia*-enriched cell culture material to enhance representation of unprocessed messenger RNA (mRNA) transcripts containing a 5' triphosphate (Supplementary Figure S1). To maximise our ability to detect gene products from the wMel reference genome, we combined RNA-seq reads with quantitative peptide data obtained from a purified, fractionated isolate of wMelPop-CLA. We found transcript evidence (≥ 2 reads in any one of three untreated biological replicates) and/or peptide evidence (≥ 2 unique peptides from a single-bulk sample) for 798 (66.8%) of 1,195 protein-coding genes predicted from the wMel genome (Wu *et al.*, 2004; Figure 1a). Notably, both the RNA-seq and proteomic data corresponded in detecting zones of limited or absent gene expression within regions of the genome identified as prophages (Wu *et al.*, 2004) or a chromosomal inversion containing several transposase genes (Riegler *et al.*, 2005; Figure 2, Supplementary Table S1). However, we did detect a small number of highly restricted peaks of gene expression in both the sense and antisense orientation within the prophages (Supplementary Figures S2 and S3, Supplementary Table S2).

The degree of global correlation between raw transcript reads and raw protein abundance scores was remarkably low (Figure 1b), as has been noted in other studies on bacteria (Waldbauer *et al.*, 2012). However, within certain Clusters of Orthologous Groups (COGs), the degree of correlation was much higher, with R^2 exceeding 0.8 for 'cell wall, membrane, envelope biogenesis' (Figure 1c). When expression was visualised across all COGs, the largest fold-differences between normalised means for RNA and protein data were observed for the categories 'general function prediction only', 'coenzyme transport and metabolism' and 'carbohydrate transport and metabolism' (where RNA dominated relative to protein in all cases) (Figure 3). Closer inspection of the data revealed that two proteins were vastly overrepresented by ion intensity score (Supplementary Table S3), molecular chaperone GroEL and *Wolbachia* surface protein, which are also highly abundant in the proteomes of the filarial *Wolbachia* strains wBm (Bennuru *et al.*, 2011) and wOo (Darby *et al.*, 2012). In addition, transcripts encoding these two proteins were among the most dominant in the wMelPop-CLA transcriptome (Supplementary Table S4).

Figure 1 Correlations between protein and mRNA expression. (a) Venn diagram of the number of proteins and transcripts quantified using quantitative proteomics and RNA-Seq, respectively. (b) Scatterplot of the relationship between genes quantified in both data sets. (c) Scatterplots for protein and transcript gene expression classified by six key Clusters of Orthologous Genes (COG) categories. In (b) and (c), mean non-normalised RNA reads ($n = 3$) were $\log_{10}$ -transformed and non-normalised protein abundances ($n = 1$) were subjected to a $\log_{10}(10^{-3})$ transformation. Scatterplots display the rectilinear equation and coefficient of determination (R^2).

Identification of differentially expressed genes after doxycycline treatment

Wolbachia strain wMelPop-CLA was grown in mosquito cells with and without doxycycline to identify changes in RNA expression and protein abundance. For the analysis of transcriptional DE, 549 genes passed our filter (see Supplementary Materials and Methods), of which 36 were classified as upregulated and 32 as downregulated following doxycycline treatment (Supplementary Table S5, Figure 2a). To determine parallel changes in the proteome, 434 wMel proteins were identified by the presence of ≥ 2 unique peptides. Of these, 10 proteins were found to be upregulated and 28 were downregulated (Supplementary Table S6, Figure 2b). The wMel genome contains an abundance of hypothetical proteins, and, accordingly,

37% of genes exhibiting DE fell into this category (Supplementary Tables S7 and S8). Putative functions could be assigned to several of these features, including an upregulated *relE*-like addiction module toxin (WD0124) and a downregulated protein with similarity to stringent starvation protein B (WD0128). These may have roles in mRNA degradation (Maisonneuve *et al.*, 2011) and the regulation of proteolysis following ribosome stalling (Lessner *et al.*, 2007), respectively. Importantly, despite this high proportion of uncharacterised genes among the regulated data set, 81.6% of all genes showing DE at the RNA and/or protein level had orthologues in at least one filarial *Wolbachia* genome, or represented conserved non-coding RNAs (Supplementary Tables S5–S8). In contrast, 61.6% of 258 orthologues found only in arthropod *Wolbachia*

Figure 2 Differential expression of transcripts and peptides across the *Wolbachia* strain wMel chromosome. The wMel genome was used as a reference for strain wMelPop-CLA. Circles in both panels are numbered sequentially from the perimeter to the centre. Circles 1 and 2 represent protein-coding genes (blue) on the positive and negative strands, respectively, and the third circle shows structural RNA genes (rRNA (purple), sRNA (green) and tRNA (pink)), prophage regions (yellow) and the site of a chromosomal inversion (red). (a) Circles 4–9 are heat maps of transcript counts from control (4–6) and doxycycline-treated replicates (7–9), with high expression coloured red and low expression coloured blue; circle 10 is a plot of differential expression (fold-change) between control (outer profile) and treatment (inner profile). Note that expression from tRNA and rRNA genes was forced to baseline. (b) Circles 4–19 are heat maps of protein abundance (summed peptide ion intensity scores) from control (4–10) and doxycycline-treated replicates (11–19), with high abundance coloured red and low abundance coloured blue; circle 20 is a plot of differential abundance (fold-change) between control (outer profile) and treatment (inner profile).

Figure 3 Mean abundance of transcripts and peptides across COG groups for *Wolbachia* strain wMel. Pie charts representing (a) the mean number of unadjusted transcript reads and (b) the mean protein abundance (summed peptide ion intensity score) for untreated *Wolbachia* cells, normalised to the number of genes per Cluster of Orthologous Groups (COG). The COGs are labelled clockwise from 12 o'clock and n per COG is shown in parentheses.

strains displayed insufficient expression to be included in the analysis of DE for both RNA and protein (data not shown).

Effects on nucleotide synthesis, energy metabolism and membrane composition

Analysis of DE identified changes in genes involved in a variety of cellular processes (Figure 4), although three broad categories contained the majority (~60%) of the regulated genes with an annotation: translation and ribosome assembly; nucleotide, cofactor and energy metabolism; and DNA replication and transcription. Tetracyclines bind with high affinity to prokaryotic ribosomes, preventing docking of aminoacyl-tRNA and inhibiting translation (Griffin *et al.*, 2010). Thus, the upregulation of ribosomal protein genes that we observed (Supplementary Figure S4) was fully anticipated, and has been reported previously from studies on free-living bacteria exposed to translational inhibitors (VanBogelen and Neidhardt, 1990; Evers *et al.*, 2001; Ng *et al.*, 2003). However, we also noted a surprisingly broad impact of doxycycline on interconnected metabolic pathways involved in energy generation and *de novo* nucleotide synthesis (Figure 5). The *Wolbachia* genome encodes several glycolytic enzymes, and three of these were upregulated after doxycycline treatment (Figure 5).

As *Wolbachia* lack enzymes that catalyse the irreversible reactions of glycolysis, those remaining in the genome function predominantly to supply precursors for nucleotide and phospholipid synthesis *via* the gluconeogenesis pathway (Foster *et al.*, 2005). Accordingly, three enzymes that participate in *de novo* nucleotide generation were also upregulated at the mRNA level, as was an enzyme involved in phospholipid synthesis (Figure 5). Thus, upregulation of glycolytic enzymes could reflect a regulatory mechanism to increase the size of the substrate pool for these pathways. Moreover, the upregulation of enzymes involved in phospholipid synthesis is compatible with a possible role for membrane remodelling in reducing doxycycline uptake. In support of this hypothesis, we observed concurrent downregulation of two *Wolbachia* surface protein paralogues and a further five predicted membrane proteins (Supplementary Tables S5–S7). A significant reduction in outer membrane protein abundance has also been demonstrated both in tetracycline-resistant strains of free-living bacteria, such as *E. coli* (Lin *et al.*, 2010) and *Acinetobacter baumannii* (Yun *et al.*, 2008), and in doxycycline-insensitive isolates of *Orientia tsutsugamushi* (Chao *et al.*, 2009), another member of the *Rickettsiales*.

Three inner membrane transporters were upregulated following exposure to doxycycline, including

Figure 4 Annotated *wMel* genes differentially expressed following doxycycline treatment. Bar chart of fold changes for individual genes (proteins, red; RNA, blue; locus tags, right) exhibiting statistically significant ($P < 0.01$) differential expression after doxycycline treatment, grouped into broad functional categories. Within each group, genes are ranked in ascending order by P -value. For those genes where a significant change was apparent at both the protein and mRNA level, the smaller P -value was used.

Figure 5 Metabolic pathways and membrane components affected by doxycycline stress in *Wolbachia* strain wMel. Summary of the major metabolic pathways, membrane transporters and respiratory chain components that are affected by doxycycline exposure. Differentially expressed gene transcripts are coloured blue and differentially expressed proteins are coloured red. Arrows indicate the direction of change (up represents a significant increase under doxycycline stress). Numbers in parentheses denote the fold change between doxycycline-treated and control samples.

the ATPase subunit (PstB) of the high-affinity phosphate uptake system (Supplementary Tables S5 and S6; Figure 5). Mutations in the *pstB* gene lead to increased susceptibility to aminoglycosides in *Pseudomonas aeruginosa* (Krahn *et al.*, 2012), whereas overexpression of this gene is associated with ciprofloxacin resistance in *Mycobacterium smegmatis* (Bhatt *et al.*, 2000). Moreover, the antimicrobial preservative sodium benzoate has been shown to increase transcription of *pst* genes in *E. coli* O157:H7 (Critz *et al.*, 2010). Upregulation of PstB by doxycycline in *Wolbachia* could reflect an increased demand for nucleotide or phospholipid synthesis, or may provide a means of drug efflux. In addition, a sodium-alanine symporter (the most highly regulated annotated transcript in our study) and a *tatA* homologue exhibited significant upregulation (Supplementary Table S5). In *E. coli*, the twin-arginine translocation (Tat) system consists of three proteins (TatA, TatB and TatC) and is responsible for the export of fully folded proteins, including redox enzymes requiring cofactor insertion before translocation (Harrison *et al.*, 2005; Lee *et al.*, 2006). One predicted substrate of the Tat translocase in the *Anaplasmataceae* (Nuñez *et al.*, 2012) is the fatty acid biosynthesis enzyme 3-oxoacyl-ACP reductase (*fabG*), which also

displayed elevated gene expression after doxycycline exposure (Supplementary Table S5). Interestingly, an upregulated hypothetical protein (WD0222; Supplementary Table S8) showed some amino-acid sequence similarity with TatB from *Anaplasma marginale* (65% query coverage, 29% similarity), and the latter provides partial restoration of function to *E. coli* Δ *tatB* mutants (Nuñez *et al.*, 2012).

The wMel genome encodes enzymes for the synthesis of riboflavin and flavin adenine dinucleotide, which are essential cofactors that are provisioned by strain wBm in its mutualistic relationship with *Brugia malayi*, a human filarial pathogen (Li and Carlow, 2012). Two of the enzymes in this pathway showed DE at the protein level in different directions (Figure 5). Although the net effect of these changes would require experimental validation, comparisons with other bacteria suggest that guanosine triphosphate (GTP) cyclohydrolase II constitutes the rate-limiting enzyme (Hümbelin *et al.*, 1999), and thus doxycycline probably suppresses riboflavin synthesis. Indeed, it has been known since the early phase of the antibiotic era that tetracyclines inhibit riboflavin metabolism; conversely, riboflavin is a competitive inhibitor of tetracycline-induced bacteriostasis (Foster and

Pittillo, 1953). Moreover, this interaction is potentially deleterious to bacterial cells, as the vitamin acts as a photosensitizer of the drug, producing reactive oxygen species in the presence of visible light (Castillo *et al.*, 2007).

Effects on host–symbiont interactions and global regulators

As ANKs represent a protein domain that is widespread in eukaryotes but relatively scarce in prokaryotes (Sedgwick and Smerdon, 1999), the high numbers of ANK-encoding genes in arthropod-associated *Wolbachia* have led to the speculation that they may be important in symbiont–host interactions (Siozios *et al.*, 2013). Despite this, substantive functional data for the role of ANKs in *Wolbachia* have yet to be obtained. In arthropod *Wolbachia*, ANK-encoding genes are often found in close proximity to phage clusters, and there is evidence for lateral gene transfer *via* bacteriophages both within and between *Wolbachia* supergroups (Ellegaard *et al.*, 2013; Siozios *et al.*, 2013). In our study, four ANKs, including three located in prophage regions, were downregulated after doxycycline treatment (Figure 4, Supplementary Tables S5 and S6). This suggests that doxycycline could have an impact on interactions between *Wolbachia* and host cells during a relatively short treatment period, even if symbiont densities are not substantially affected. Indeed, it is possible that this contributes to the mechanism of action of doxycycline against filarial *Wolbachia* such as wBm, in which an orthologue of ANK WD0754 is conserved (Foster *et al.*, 2005; Supplementary Table S6). In addition, our data strongly suggest that doxycycline does not induce the lytic cycle of bacteriophage WO in wMelPop-CLA, despite the well-known ability of the tetracyclines to enhance phage production in free-living bacteria (Kaur *et al.*, 2012).

The characterisation of global regulation in obligate intracellular bacteria is constrained by the paucity of available genetic tools, although gene expression studies in which external stressors are applied can provide valuable insights into potential regulatory networks. In this context, we observed upregulation of two transcripts involved in cell division in our study: the bacterial ‘tubulin’ *ftsZ*, and a gene encoding a Fic family protein (Supplementary Table S5). In *E. coli*, the adenylylase Fic is essential for cell division (Komano *et al.*, 1991) and may have a role in intracellular signalling, although in some pathogenic bacteria it is secreted as a virulence factor that AMPylates host cell proteins (Roy and Mukherjee, 2009). In *Wolbachia*, it is probable that upregulation of these transcripts forms part of a regulatory effort to overcome cellular stasis following inhibition of translation. Two other transcripts that have global regulatory roles in other bacteria were downregulated following drug exposure: *ctrA* and *ssrS* (6S RNA)

(Supplementary Table S5). The response regulator CtrA has been demonstrated in *Ehrlichia chaffeensis* (family *Anaplasmataceae*) to be upregulated during the differentiation of dense-cored cells and is associated with stress resistance (Cheng *et al.*, 2011); hence, it is surprising that *ctrA* transcripts decreased during doxycycline treatment of *Wolbachia*. Furthermore, we failed to detect any significant changes in other components of the *E. chaffeensis* CtrA regulon (BolA, SurE and peptidoglycan-associated lipoprotein), despite their conservation in wMel. It is possible that although CtrA is present in all members of the *Anaplasmataceae* sequenced to date, its role has diverged between genera; indeed, significant differences exist in its C-terminal amino-acid residues between *Anaplasma*, *Ehrlichia*, *Neorickettsia* and *Wolbachia* (Cheng *et al.*, 2011).

The non-coding RNA *ssrS* is an important factor controlling the rate of intracellular replication in *Legionella pneumophila*, as deletion of this gene reduces the ability of the pathogen to multiply in eukaryotic cells (Faucher *et al.*, 2010). In a transcriptomic study of *Wolbachia* strain wOo from the filarial nematode *Onchocerca ochengi*, the expression of *ssrS* was significantly higher in symbionts located in the female worm gonad, where bacterial replication is relatively rapid than in somatic tissues in which *Wolbachia* divides slowly (Darby *et al.*, 2012). Thus, *ssrS* is a key contender as a global regulator of growth rate in *Wolbachia*, and its reduced expression following exposure to a bacteriostat supports such a role.

Susceptibility to tetracyclines in the Rickettsiales

Although the tetracycline derivatives have been in clinical use since the late 1940s, they remain the treatment of choice for infections caused by obligate intracellular bacteria (McOrist, 2000). In marked contrast to recent global trends for free-living bacterial pathogens, reports of antibiotic resistance in obligate intracellular bacteria are very rare, and geographical spread of such isolates has not become clinically significant to date. For instance, a 2- to 5-day course of doxycycline is still effective for the treatment of spotted fever group rickettsiae (Botelho-Nevers *et al.*, 2012), and although isolates of the scrub typhus agent *O. tsutsugamushi* with reduced doxycycline susceptibility were reported from northern Thailand in the mid-1990s (Watt *et al.*, 1996) this drug has remained effective in other endemic areas (Rajapakse *et al.*, 2011). However, despite this apparent lack of heritable resistance, many members of the *Rickettsiales* can persist following tetracycline treatment and may recrudescence months or even years after the cessation of therapy (Andrew and Norval, 1989; Stuenkel and Bergstrom, 2001; McClure *et al.*, 2010). This is also the case for *Wolbachia*, as a 2-week course of oxytetracycline only depleted the bacteria transiently from

O. ochengi worms, with full recovery of both the endosymbionts and their host 6 months later (Gilbert *et al.*, 2005). Failure of tetracyclines to penetrate into worms can effectively be ruled out as an explanation for the necessity of prolonged treatment, as tetracycline accumulation in filariae has been visualised during human infection by autofluorescence (Tobie and Beye, 1960).

These reports suggest that *Wolbachia* and its relatives are capable of producing subpopulations that are phenotypically tolerant to tetracyclines. Indeed, as antibiotic tolerance or 'metabolic resistance' is a universal property of bacteria and yeasts (Lewis, 2012), its occurrence in the *Rickettsiales* is inevitable. However, the potential significance of the phenomenon apparently has not been recognised, perhaps because anti-*Wolbachia* therapy of filariae is a relatively recent development that accentuates this feature of the *Rickettsiales*. Two characteristics of filarial *Wolbachia* appear to facilitate tetracycline tolerance: the low level of bacterial growth in the adult worm soma (McGarry *et al.*, 2004) and their location beneath a physical barrier (the nematode cuticle) that is normally impervious to the mammalian immune system (Hansen *et al.*, 2011). These properties may promote tolerance because the proportion of 'persister' bacteria is 10^{-5} during exponential growth, but increases to 10^{-2} in stationary phase (Lewis, 2007).

Mechanisms of antibiotic tolerance

To date, most studies of antibiotic tolerance have focused on free-living bacteria and facultative intracellular pathogens exposed to bactericidal antibiotics. Functional analyses of the persister phenotype in tractable organisms have identified global regulators, toxin-antitoxin modules and antioxidant enzymes as having critical roles in maintaining antibiotic tolerance in slow-growing subpopulations (Kint *et al.*, 2012). However, many of these candidate molecules and their associated pathways are either absent in *Wolbachia* genomes or have become severely limited. For instance, sigma factors are important for persistence in mycobacteria (Michele *et al.*, 1999), but *Wolbachia* has only two of these regulators and the expression of neither was changed in our study. Similarly, decades of research in *E. coli* and more recently in *M. tuberculosis* have highlighted the central role of toxin-antitoxin modules in antibiotic tolerance (Gerdes and Maisonneuve, 2012). Although we did note the upregulation of a *relE* toxin-like transcript (Supplementary Table S8), this gene is not conserved among other *Wolbachia* genomes (including those from nematode strains) and levels of the Lon protease that mediates its effects (Maisonneuve *et al.*, 2011) were unaffected in our study. Thus, it is unlikely that toxin-antitoxin modules are of general importance in the response of *Wolbachia* to doxycycline. Conversely, some persister gene

candidates did exhibit parallels between studies in *E. coli* and our own experiment. Specifically, knockout of the *dnaK* gene (Supplementary Table S5) reduced the rate of persister formation in *E. coli* by 22-fold in one study, and deletion of a gene (*ygfA*) involved in the stabilisation of *ssrS* (although not knockout of *ssrS* itself) also had a significant, four-fold effect (Hansen *et al.*, 2008). However, we could not identify a homologue of *ygfA* in any *Wolbachia* strain by BLAST analysis. Finally, in the context of the reduced levels of oxidative stress associated with antibiotic tolerance in *P. aeruginosa* (Nguyen *et al.*, 2011), the upregulation of *ppnK* (Supplementary Table S5) in our study suggests that *Wolbachia* also induces an antioxidant response following antibiotic exposure. Nevertheless, the underlying mechanism must be quite different from that for *P. aeruginosa*, as *Wolbachia* apparently lacks the stringent response pathway.

Potential impacts on the symbiotic relationship

The global regulation of gene expression in *Wolbachia* during antibiotic stress is further complicated by the potential demands of the host cell in the symbiotic partnership. Clearly, in this context, caution is required when extrapolating data obtained from an arthropod symbiont cultured *in vitro* to congeneric endobacteria in filarial nematodes. For instance, uncharacterised phage-associated protein genes (including those with ANK domains) are almost entirely absent in filarial *Wolbachia*, and the putative deletion in the wMel-Pop-CLA chromosome (Supplementary Table S1) suggests a degree of genomic plasticity that is probably unlikely in filarial *Wolbachia*. However, it is noteworthy that the pathways containing the largest number of genes undergoing DE were involved in highly conserved aspects of core metabolism (e.g., *de novo* nucleotide synthesis and energy generation). In a previous gene expression study of *Wolbachia* (strain wOo), ATP provisioning was identified as the primary contender for the contribution of the symbiont to the mutualistic relationship (Darby *et al.*, 2012). The observed upregulation of PstB may represent an additional compensatory process to maintain ATP production, although in this case coupling of phosphate uptake to drug efflux (Bhatt *et al.*, 2000) could be critical to the survival of the microbial partner.

As natural and anthropogenic sources of antibiotics are widely dispersed in the environment, significant exposure of *Wolbachia* to these compounds in arthropod hosts is probably commonplace. Indeed, recent studies have examined the impact of antimicrobial compounds on arthropod-associated microbiota in both terrestrial (Adams *et al.*, 2011) and aquatic (Edlund *et al.*, 2012) habitats, including in a collembolan species (*Folsomia candida*) that has an obligate dependency on *Wolbachia* for egg hatching (Timmermans and

Ellers, 2009; Giordano *et al.*, 2010). Interestingly, oral dosing of *F. candida* with oxytetracycline failed to eliminate the *Wolbachia* infection, and the authors speculated that this was due to detoxification of the antibiotic by the host, its intestinal flora, or 'resistant' *Wolbachia* (Giordano *et al.*, 2010). In addition, the potential complexity of interactions between symbionts and environmental pollutants was revealed by an expression microarray analysis in *F. candida* exposed to cadmium, which demonstrated an increase in gene expression across the penicillin and cephalosporin biosynthesis pathway (Nota *et al.*, 2008). Although *Wolbachia* is not susceptible to β -lactams and the impact of cadmium on the collembolan microbiome was not quantified, this study suggests that the population dynamics of arthropod symbionts could be disrupted not only by antibiotics but also by indirect effects of abiotic stressors.

Conclusions

Our integrated transcriptomic and proteomic study has revealed that a 70-year-old drug with a well-defined target displays a plethora of effects on gene expression, even in a bacterium with a highly reduced genome. Thus, our data support the emerging paradigm of antibiotics as agents that act by exploiting and disrupting genome-wide bacterial regulatory networks (Kohanski *et al.*, 2010), which, in the context of a mutualistic symbiosis, may be equally or more important than inhibiting bacterial growth. A fuller understanding of the ability of obligate intracellular bacteria to compensate for tetracycline-mediated inhibition of translation may allow the development of new strategies to overcome persistence in these remarkably resilient organisms.

Conflict of Interest

The authors declare no conflict of interest.

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