Published in final edited form as:

In Vitro Cell Dev Biol Anim. 2013 January ; 49(1): 66–73. doi:10.1007/s11626-012-9571-3.

***Wolbachia* from the planthopper *Laodelphax striatellus* establishes a robust, persistent, streptomycin-resistant infection in clonal mosquito cells**

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Abstract

The obligate intracellular bacterium, *Wolbachia pipientis* (Rickettsiales: Anaplasmataceae), distorts reproduction of its arthropod hosts to facilitate invasion of naïve populations. This property makes *Wolbachia* an attractive “gene drive” agent with potential applications in the control of insect vector populations. Genetic manipulation of *Wolbachia* will require in vitro systems for its propagation, genetic modification, amplification, and introduction into target insects. Here we show that *Wolbachia* from the planthopper, *Laodelphax striatellus*, establishes a robust infection in clonal C7-10 *Aedes albopictus* mosquito cells. Infected cells, designated C/ϖStr, expressed radiolabeled proteins that were enriched in cells grown in the absence of antibiotics that inhibit *Wolbachia*, relative to cultures grown in medium containing tetracycline and rifampicin. Using mass spectrometry, we verified that tryptic peptides from an upregulated 24 kDa band predominantly represented proteins encoded by the *Wolbachia* genome, including the outer surface protein, Wsp. We further showed that resistance of *Wolbachia* to streptomycin is associated with a K42R mutation in *Wolbachia* ribosomal protein S12, and that the pattern of amino acid substitutions in ribosomal protein S12 shows distinct differences in the closely related genera, *Wolbachia* and *Rickettsia*.

Keywords

Wolbachia; *Laodelphax striatellus*; Mosquito cell lines; Streptomycin resistance; Ribosomal protein S12

Introduction

Wolbachia is an obligate intracellular bacterium first described in reproductive tissues of *Culex pipiens* mosquitoes as Giemsa-stained pleomorphic rickettsia-like microbes (Hertig 1936). Now known to be widespread in insects and other arthropods (Hilgenboecker et al.

2008), *Wolbachia* typically manipulates host reproduction to favor its own transmission (Stouthamer et al. 1999). In mosquitoes, *Wolbachia* causes the reproductive distortion known as cytoplasmic incompatibility, in which eggs from an uninfected female fail to hatch when fertilized by sperm from an infected male (Yen and Barr 1971). Because infected individuals become predominant in a population, *Wolbachia* can potentially be harnessed as a gene drive agent for the control of vector populations (Sinkins and Gould 2006).

Because *Wolbachia* is an obligate intracellular microbe, its manipulation for insect control will be facilitated by developing cell lines to produce infectious *Wolbachia* on a scale suitable for eventual genetic manipulation, transformation, reintroduction into cultured cells, amplification, and, finally, transfer to insect hosts. *Aedes albopictus* Aa23 cells provide the only example of a *Wolbachia*-infected mosquito cell line derived directly from eggs of mosquitoes doubly infected with two strains of *Wolbachia*, *wAlbA* and *wAlbB* (O'Neill et al. 1997). Aa23 cells are an uncloned cell population that maintains a stable, persistent infection with *Wolbachia* strain *wAlbB*, but not *wAlbA*. The tendency of Aa23 cells to grow as clusters of tightly adherent cells, coupled with their long doubling time (Fallon 2008), led us to explore other cell systems that might support a *Wolbachia*-host cell interaction more suitable for biochemical analysis of the *Wolbachia* infection.

When we introduced *wAlbB* into faster growing, clonally derived, thymidine kinase-deficient TK-6 mosquito cells, establishment of the infection was accompanied by upregulation of host protein degradative machinery (Fallon and Witthuhn 2009), but we were unable to detect proteins expressed from the *wAlbB* genome using the criterion that such proteins should preferentially incorporate radiolabeled precursors in the absence of antibiotics that suppress *Wolbachia* growth. Aside from *A. albopictus* Aa23 cells, the NIAS-AeAl-2 cell line (RIKEN Cell Bank, Tsukuba, Japan) has been used to propagate *Wolbachia* from the small brown planthopper, *Laodelphax striatellus* (Noda et al. 2002). *Wolbachia striatellus* (*wStr*) could also be propagated in lepidopteran and mouse L929 cells, suggesting that *wStr* could adapt relatively easily to a wide range of intracellular environments.

Here, we report the establishment of a persistent infection of the clonally derived *A. albopictus* C7-10 cell line with *wStr* from AeAl-2 cells. In C/*wStr* cells, we detected an increased expression of radiolabeled proteins whose synthesis was suppressed by rifampicin and tetracycline and used mass spectrometry to validate the presence of *Wolbachia*-derived peptides in an approximately 24-kDa band with the expected mass of the *Wolbachia* outer surface protein, Wsp. Like *wAlbB* (O'Neill et al. 1997; Fallon and Witthuhn 2009), *wStr* was resistant to streptomycin. To understand the basis for this resistance, we sequenced the region of the *wStr* gene encoding ribosomal protein S12 (rpS12), in which mutations that confer streptomycin resistance have been described in other bacteria. When we aligned rpS12 protein sequences from members of the order Rickettsiales, we found well-conserved substitutions at key rpS12 residues that interact with bacterial 16S rRNA and distinguish the family Anaplasmataceae, which includes *Wolbachia*, from its sister family, Rickettsiaceae.

Materials and Methods

Cells and culture conditions

The NIAS-AeAl-2 cell line (RIKEN Cell Bank, Tsukuba, Japan) infected with *Wolbachia* from the small brown planthopper, *L. striatellus* (Noda et al. 2002) was a generous gift from Dr. T. J. Kurti, University of Minnesota. Because AeAl-2 cells grew poorly in Eagle's medium, and required 20% serum, we directly added a suspension of infected AeAl-2 cells (1 ml) to a confluent monolayer of C7-10 cells (Fallon and Kurti 2005) in 5 ml of antibiotic-free E-5 medium (Shih et al. 1998; Fallon 2008). The resulting C/*wStr* cultures were subcloned at a 1:5 dilution in medium containing 5% serum (E-5 medium) and

monitored by fluorescence microscopy. Infection was not affected by penicillin and streptomycin, which were included at later subcultures. The C/*w*Str infection has remained stable for over 36 mo and maintains the diploid karyotype ($2n=6$) of C7-10 cells, whereas most cells in an AeAl-2 population had ploidy levels of $4n$ or greater (our unpublished observations). The most robust infections were obtained when flasks containing diluted cells were allowed to establish near-confluent monolayers, supplemented with 5 ml of E-5 medium, and harvested approximately 10 d later, when loosely attached cells began to lift off the plastic in small clumps. Cells were dissociated by pipetting, diluted with phosphate-buffered saline (Dulbecco and Vogt 1954), and counted using a Coulter electronic cell counter.

Microscopy

To evaluate infection status, a 5- μ l “scrape” from the surface of the culture flask was obtained with a sterile 3.5-in. pipet tip (MBP XLP pipet tips, catalog # 3542; Molecular BioProducts Inc., San Diego, CA) and resuspended into 5 μ l of the cell-permeable nucleic acid stain Syto11 (5 μ M; Invitrogen, Carlsbad, CA) containing propidium iodide (5 μ M). The staining mix was made in 0.2 ml portions and stored in glass vials at room temperature in the dark. In our hands, diluted Syto11 adsorbed to plastic, even when stored at -20°C .

Biochemical analyses

Methylthiazole tetrazolium (MTT) assays were based on conversion of MTT to a colored formazan product as described previously (Fallon and Hellestad 2008). Cells were typically labeled with ^{35}S [methionine/cysteine], 50 $\mu\text{Ci/ml}$ (Tran [^{35}S] Label; $\sim 1,100$ Ci/ mmol; MP Biomedicals, Solon, OH). To suppress *Wolbachia* growth, rifampicin and tetracycline were added at final concentrations of 0.4 and 5 $\mu\text{g/ml}$, respectively, at the time of plating. At appropriate time points, cells were resuspended in the culture medium, washed in phosphate-buffered saline, harvested by centrifugation, and frozen at -20°C as pellets. For protein analysis, pellets were sonicated in 0.1% sodium dodecyl sulfate (SDS), and radioactivity was measured on a portion of the sample in a liquid scintillation counter. Proteins were separated by electrophoresis on SDS-polyacrylamide gels (Laemmli 1970) using equal amounts of radioactivity per lane. Nonradioactive bands of the 24-kDa protein band were excised from polyacrylamide gels, digested with trypsin, analyzed by mass spectrometry, and matched to peptide and protein candidates essentially as described previously (Fallon and Witthuhn 2009). Variations in database search details were as follows: Cysteine carbamidomethylation was set as a fixed modification; two missed trypsin cleavage sites were specified; Sequest fragment ion tolerance was set to 0.8 Da; Scaffold version 3 (www.proteomesoftware.com) was used for post-processing of Sequest results; and X!Tandem was not used. Scaffold post-processing algorithms included the Peptide Prophet (Keller et al. 2002) and the Protein Prophet (Nesvizhskii et al. 2003). Tryptic peptides were analyzed as detailed in the legend of Table 1.

Polymerase chain reaction

The portion of the *w*Str gene encoding amino acid alterations in ribosomal protein S12 (rpS12) associated with streptomycin resistance was amplified using primers S12F (5'-GCA CTA AGG TGT ATA CTA CAA CTC C) and S7R (5'-GCC TTA TTA GCT TCA GCC AT) with 1 cycle at 95°C for 2 min, 35 cycles at 95°C for 1 min, 56°C for 1 min, 72°C for 1 min, followed by 1 cycle at 72°C for 3 min. Samples were electrophoresed on 2% agarose gels, and the PCR reaction products were sequenced at the University of Minnesota BioMedical Genomics Center. Sequences were verified using a minimum of three reactions with independent DNA template preparations.

Results

Properties of C7-10 cells infected with wStr

C7-10 cells infected with wStr, designated C/wStr, have been maintained since 2009 without loss of infection. Unlike infected AeAl-2 cells (Noda et al. 2002), cultures of C/wStr did not require supplementation with uninfected cells, and during exponential growth, they had a doubling time of approximately 24 h, compared to 15 h for uninfected C7-10 cells (Gerenday and Fallon 1996) and 4–5 d for Aa23 cells (Fallon 2008). Manipulation of C/wStr cells was substantially easier than manipulation of Aa23 cells. Unlike Aa23 cells, C/wStr cells grew in medium containing only 5% serum, formed more uniform monolayers, and did not require treatment with trypsin. However, relative to uninfected C7-10 cells, C/wStr cells had a stronger tendency to aggregate, particularly at high densities; nevertheless, aggregates were relatively small compared to those in Aa23 cultures, and unlike Aa23 cells, aggregates were more easily disbursed by gentle pipetting.

TK-6 cells, which are deficient in thymidine kinase activity and support the growth of wAlbB (Fallon and Witthuhn 2009), also became infected with wStr, but even when supplemented with uninfected cells as described by Noda et al. (2002), infected TK-6 cells grew too slowly to establish populations suitable for routine analysis. By microscopy, infection of TK-6 cells with wStr was substantially more robust than what we have seen with wAlbB (Fallon 2008), again suggesting that wStr was more virulent than wAlbB. Infection in the TK-6 cells was not investigated further.

As has been described for Aa23 cells (O'Neill et al. 1997), *Wolbachia* abundance in C/wStr cells varied considerably among individual cells in a population, especially early after dilution and plating. Efforts to reduce variability by manipulating serum concentrations were unsuccessful. As C/wStr cells approached confluence, typically 100% of the cells were infected, and some cells began to lyse, releasing infectious *Wolbachia* into the culture medium. Nuclei of lysing cells typically stained red with propidium iodide, while nuclei of intact cells, and *Wolbachia*, stained green with Syto11. When diluted and plated at low density (2×10^5 cells/35 mm plate), heavily infected cells often entered a lag period lasting several d before exponential growth commenced. Occasionally, when levels of Syto-11 stained, intracellular particles observed by fluorescence microscopy became low, robust infections could be recovered by subculturing the cells in serum-free medium, followed by restoration of serum after intervals of 2–3 wk. We further noted that when the isolated clones that occasionally appeared in spare flasks were subcultured, they invariably reestablished persistently infected populations. Most flasks yielded low numbers of infected subclones for up to 7 to 10 wk of storage without subculture, at 28–30°C in a 5% CO₂ atmosphere, in medium containing 5% heat-inactivated fetal bovine serum. One such clone was recovered after storage for 10 mo.

In addition to serum, C/wStr cells were maintained in medium containing both penicillin and streptomycin (Shih et al. 1998; Fallon 2008; see also *below*). As described by others (Hermans et al. 2001), the antibiotics rifampicin and tetracycline, either alone or in combination, suppressed *Wolbachia* abundance by 100- to 1,000-fold during a single passage and completely eliminated the *Wolbachia* infection after several consecutive passages (see Fallon 2008). In the presence of tetracycline and rifampicin, C/wStr cells grew to a maximum density of approximately 2×10^6 cells/ml, while untreated cells became stationary at a density of approximately 1×10^6 cells/ml (Fig. 1A). We used the MTT assay to assess relative metabolic activity in the presence and absence of these antibiotics (Fig. 1B). In the presence of rifampicin and tetracycline, C/wStr cells maintained high levels of metabolic activity for 9 d, while metabolic activity in infected cells without rifampicin and tetracycline declined after 4 d of active growth, presumably reflecting diversion of host cell

metabolites to support *Wolbachia* growth. This pattern differs from what we have seen in Aa23 cells, in which antibiotic treatment caused little difference in growth of the host cells (Fallon and Hellestad 2008) and was consistent with our microscopic assessment of a more robust *Wolbachia* infection in C/*w*Str cells, relative to Aa23 cells.

Differential expression of radiolabeled bands in the absence/presence of rifampicin and tetracycline

When labeled with [³⁵S]methionine/cysteine, either for 24-h intervals or continuously for several d, C/*w*Str cells consistently expressed a similar pattern of three strongly induced bands, with masses of approximately 60, 24, and 8 kDa (Fig. 2, see *arrows* at right). The three bands showed higher incorporation of radioactivity in the absence of antibiotics, relative to cultures grown with the *Wolbachia*-inhibitory antibiotics, tetracycline and rifampicin, suggesting that they contained proteins expressed from the *w*Str genome. We repeated this experiment with C/*w*Str cells at various passage levels; in some cases, we saw additional bands that presumably corresponded to *w*Str proteins.

The induced 24-kDa band was a consistently reproducible feature of *w*Str infection that could be observed on Coomassie Blue-stained SDS-PAGE gels, and its mass corresponded to that of a known outer surface protein expected to be abundant in *Wolbachia*. To test whether the 24-kDa band contained *Wolbachia* proteins, the band was excised, trypsin digested, and analyzed by mass spectrometry.

A total of seven proteins were represented by a minimum of three unique spectra that matched peptides from sequenced *Wolbachia* or *Aedes* genomes (Table 1). A total of five proteins matched accessions from the *w*Pip genome, and one matched a protein from the *w*Mel genome. These *Wolbachia* strains are found in the mosquito, *C. pipiens*, and in the fruit fly, *Drosophila melanogaster*, respectively. Because the *w*Str genome has not been sequenced, additional peptides that have diverged from available sequence entries would have been missed in this analysis. A single host-derived protein matching a 25-kDa *Aedes* Hsp70 co-chaperone (GrpE) was also represented by three unique tryptic peptides in the 24-kDa band.

We note that in terms of total peptide spectra, the *w*Pip-like 24-kDa surface antigen, Wsp, was the most highly represented (0.70% of total peptide spectra; eight peptides; 15% coverage) of the six *Wolbachia* proteins detected in the 24-kDa band (Table 1). The second most abundant protein, at 0.37% of total spectra, was represented by probable cleavage fragments of a 69-kDa chaperone protein Dnak (hsp70) from *Wolbachia*, which likely originated from a ~75-kDa *w*Str band (data not shown). In terms of total spectra, *Wolbachia* ribosomal protein S4 (rpS4), with a total of nine peptides and 37% amino acid coverage, was the third best-represented protein in the 24-kDa band, while *Wolbachia* rpL4, adenylate kinase, and RibA (an enzyme involved in riboflavin synthesis), were represented at two- to threefold lower proportion of total spectra. In aggregate, the predominance of *Wolbachia* proteins represented by peptides contained in the rifampicin- and tetracycline-sensitive 24-kDa band supported subsequent full-scale analysis of the *Wolbachia* infection in C/*w*Str cells using proteomic approaches (Baldridge et al. to be described elsewhere).

Streptomycin resistance

When they established the *A. albopictus* Aa23 cell line from the Houston strain of infected mosquitoes, O'Neill et al. (1997) noted that medium containing penicillin and streptomycin did not inhibit the *Wolbachia* infection. Because the streamlined *Wolbachia* genome is deficient in genes involved in cell envelope biogenesis (Wu et al. 2004), resistance of *w*AlbB to penicillin was not surprising. However, streptomycin targets rpS12, which has

been studied in detail because this protein interacts directly with the 16S ribosomal RNA. Because amino acid substitutions at residues K42, R85, and P90 and adjacent positions are associated with streptomycin resistance in *Escherichia coli* and other bacteria (Gregory et al. 2001; Sharma et al. 2007), we used PCR primers that produced a product spanning known sites involved in streptomycin resistance (boxed in Fig. 3A) to examine whether rpS12 genes in *wAlbB* and *wStr* encoded amino acid changes known to be associated with streptomycin resistance. With the exception of a single conservative valine/ methionine change near the C-terminal end of rpS12, deduced amino acid sequences downstream of the forward primer were identical in *Wolbachia* strains *wAlbB* and *wStr* (GenBank accession # JX944712). More recently, the methionine residue in the *wAlbB* rpS12 (ZP_09542662) has been independently confirmed in the context of the *wAlbB* genome project (Mavingui et al. 2012). Relative to its *E. coli* homolog, rpS12 from both *wAlbB* and *wStr* encoded the single K42R substitution (designated by a closed circle in Fig. 3A) most frequently associated with streptomycin resistance in *E. coli*. The K42 residue interacts directly with streptomycin, and recent alanine scanning mutagenesis suggests that the site may be further associated with streptomycin pseudodependence—improved growth in the presence of streptomycin (Sharma et al. 2007).

These observations prompted us to compare all of the rpS12 genes available for members of the order Rickettsiales (Fig. 3B). We note that among the Rickettsiales, the genera *Wolbachia*, *Anaplasma*, *Ehrlichia*, and *Neorickettsia* (family Anaplasmataceae) are more closely related to each other than they are to the genus *Rickettsia* (family Rickettsiaceae; Williams et al. 2007). Overall, the pattern of amino acid residues in the boxed regions reflected these established phylogenetic relationships. First, all *Wolbachia* sequences, both from insects and nematodes, encoded the K42R substitution and were otherwise identical in the boxed residues that interact with 16S rRNA (Fig. 3B). Curiously, the Alphaproteobacterium *Orientia tsutsugamushi*, which causes scrub typhus and is probably vectored by trombiculid mites, also has the K42R substitution with no other changes in the boxed, streptomycin-relevant residues. *O. tsutsugamushi* is the only member of its genus and is not included in the Williams et al. (2007) phylogenetic analysis.

Among the non-*Wolbachia* Anaplasmataceae, rpS12 sequences were more variable. *Ehrlichia ruminantium* was exceptional in that it has the K42R mutation; all other *Ehrlichia*, *Anaplasma*, and *Neorickettsia* had K42, and, unlike the *Rickettsia*, lacked possible compensatory changes in the boxed downstream residues associated with streptomycin resistance. In contrast, all 14 of the *Rickettsia* sequences available in the databases as of July 2012 encoded lysine at position 42. Moreover, in contrast to the Anaplasmataceae, rpS12 sequences in the Rickettsiaceae shared other changes at sites that may affect streptomycin sensitivity, including R53T, R85Q, K87P, and R93K.

Discussion

In a single attempt, *wStr* from the planthopper, *L. striatellus*, readily transferred from AeA1-2 cells to the cloned *A. albopictus* C7-10 cell line. The infected cells (named C/*wStr*) maintained *wStr* as a persistently infected population that did not require supplementation with uninfected host cells. At low densities, infected cells grew at comparable rates and had comparable levels of metabolic activity regardless of the presence of antibiotics that suppress *Wolbachia* growth. As cell densities increased, the presence of *Wolbachia* had an overall negative effect on growth and metabolic activity, consistent with a parasitic, rather than symbiotic, relation with the mosquito host cell. In previous studies with Aa23 cells infected with *wAlbB*, tetracycline treatment failed to elicit differential levels of MTT staining over a period of 10 d (Fallon and Hellestad 2008). However, Aa23 cells grow considerably more slowly than the C/*wStr* cells described here, and microscopic

comparisons with Syto11 stain suggest that the *wAlbB* infection in Aa23 cells is less robust than the *wStr* infection in C/*wStr* cells.

Consistent with microscopic assessment, analysis of radiolabeled proteins produced by C/*wStr* cells allowed detection of *Wolbachia*-encoded proteins. In several independent experiments, a set of three bands showed increased incorporation of radioactivity when cells were labeled in the absence of antibiotics that inhibit growth of *Wolbachia*. Analysis of tryptic peptides from the Coomassie Blue-stained, 24-kDa band revealed peptides from six *Wolbachia* proteins, most abundantly the outer surface protein Wsp, which was expected to be particularly abundant in cells supporting a robust *Wolbachia* infection. The 24-kDa band contained only a single *Aedes* host protein represented by three or more peptides, indicating the suitability of C/*wStr* cells for a more comprehensive proteomic analysis of both host and *Wolbachia* proteins.

Because *Wolbachia* grows only within eukaryotic host cells, which are generally thought to exclude aminoglycoside antibiotics such as streptomycin, it was somewhat surprising to note that both *wStr* and *wAlbB* shared resistance to streptomycin. Moreover, rpS12 in all of the *Wolbachia* strains for which sequence is available contains an amino acid substitution that in better-studied bacteria confers resistance to streptomycin (Sharma et al. 2007). Although its intracellular environment might be expected to protect *Wolbachia* from exposure to other microbes, *Streptomyces* is a genus of more than 500 species of aerobic, Gram-positive bacteria common in soil and decaying vegetation, and the extent to which insects and their associated microbes are exposed to aminoglycosides in their natural environments is unknown. To the extent that eukaryotic cells vary in their ability to take up streptomycin (Oxman and Bonventre 1967), *Wolbachia*'s resistance to this antibiotic may confer an advantage to establishment of an infection. For example, in the absence of streptomycin, *E. coli* with the K42R mutation grows more slowly than wild-type *E. coli* (Gregory et al. 2001), and a reduced growth rate is conceivably adaptive for an intracellular life style. In this context, it will be of interest to examine *Wolbachia* proteins for conserved substitutions in other components of the protein synthetic machinery that affect growth and/or metabolic rates. Finally, if streptomycin resistance proves to be universal among the *Wolbachia*, it provides a phenotype useful for adapting *Wolbachia* isolated from insect tissues to cell culture, as most contaminating microbes would be expected to be streptomycin sensitive. Finally, the conserved rpS12 substitutions noted in the substantially larger group of available *Rickettsia*, which have not to our knowledge been tested experimentally, merit further investigation.

The availability of *Wolbachia* genome sequences from diverse strains (Wu et al. 2004), the successful transfer of *Wolbachia* to mosquito vectors that do not harbor *Wolbachia* in nature (Xi et al. 2005), and the apparent reduction in pathogen density associated with *Wolbachia* infection (Moreira et al. 2009) have stimulated interest in developing *Wolbachia* as a gene drive agent to reduce disease transmission. Because *Wolbachia* replicates only within host cells, we envision that manipulation of this microorganism for insect control will be facilitated by in vitro approaches. Further identification and characterization of host cell lines that produce infectious *Wolbachia* on a scale suitable for transformation and genetic manipulation will facilitate these efforts. Likewise, it will be desirable to develop host cell lines highly susceptible to new infections that can be experimentally established from extracellular (e.g., transformed) *Wolbachia*. Finally, once amplified and tested in vitro, transformed *Wolbachia* will require reisolation under conditions that facilitate reintroduction into target insect hosts. As a first step toward identifying processes that might be genetically manipulated in host cells to support direct manipulation of *Wolbachia* itself, we have begun a large-scale proteomic analysis to investigate host–*Wolbachia* interactions in C/*wStr* cells.

Acknowledgments

This work was supported by NIH grant AI081322 and by the University of Minnesota Agricultural Experiment Station, St. Paul, MN. Mass spectrometry analysis was performed at the University of Minnesota Center for Mass Spectrometry and Proteomics (supporting agencies are listed at <http://www.cbs.umn.edu/msp/about>). We thank John Beckmann and Grace Li for helpful discussion.

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Fig. 1.

C/wStr cells (2×10^5 in 2 ml of E-5 medium) at passage 32 were plated in 35 mm dishes in the absence (*filled circles*, *Wolbachia* present) or presence (*open circles*; *Wolbachia* suppressed) of rifampicin and tetracycline. At daily intervals, cells were counted with a coulter electronic cell counter (A) or incubated for 1 h with MTT (B) and processed as described previously (Fallon and Hellestad, 2008). These data are representative of biological replicates with C/wStr cells at passages 15, 26, and 30.

Fig. 2.

Differential expression of proteins by C/wStr cells. Infected cells from passage 32 (see the legend to Fig. 1) were plated in 35 mm plates in 2 ml of E-5 medium with (+) and without (–) rifampicin and tetracycline. Cells were labeled with [35 S]methionine/cysteine on d 5, 6, 9, and 12, beginning when MTT values of cells in the presence of rifampicin and tetracycline began to diverge (see Fig. 1). Values at *left* indicate molecular mass markers. *Arrows* at right indicate a set of three bands that were more prominent in the absence of antibiotics, i.e., in the presence of “normal” levels of *Wolbachia*. Samples were adjusted to contain equal levels of radioactivity (1.5×10^6 cpm/lane). A similar pattern of bands was obtained when samples were labeled on the indicated d and harvested on day 12 or labeled on the indicated d and harvested 24 h later. Biological replicates of these results were obtained with C/wStr cells at passages 15 and 30.

Fig. 3.

Amino acid alignment of residues associated with streptomycin resistance. (A) Deduced amino acid sequences of rpS12 from wStr and wAlbB aligned with the streptomycin-sensitive homolog from *E. coli*. Boxes designate amino acid regions that interact with 16S rRNA and in which mutations have been described that confer streptomycin resistance (Sharma et al., 2007). The N-terminal residues of wAlbB (shown as dashes for wStr) are upstream of our forward primer and are from ZP_09542662.1. Our experimentally determined downstream sequence of wAlbB shown here was identical to that of ZP_09542662.1. (B) Alignment of available (July 2012) rpS12 sequences from members of the Rickettsiales. Accession numbers are indicated on the left; genera are as follows: *W*, *Wolbachia*; *E*, *Ehrlichia*; *N*, *Neorickettsia*; *A*, *Anaplasma*; *Od*, *Odyssella*; *Or*, *Orientia*; *R*, *Rickettsia*. Genus and family groupings are shown at the extreme right. Boxed regions are as in (A), and key residue positions (42, 53, 85, and 93) are noted at the top of the boxes. Historically, these positions were assigned from the chemically determined amino acid sequence of *E. coli* rpS12, which lacks the N-terminal methionine (see Funatsu et al. 1977) shown in (A). Thus, residue positions in (B) are displaced by one amino acid to be consistent with the literature.

Summary of MS/MS data from the ~24-kDa band from C/ wStr cells grown in the absence of rifampicin and tetracycline. Samples were unlabeled material corresponding to the ~24-kDa proteins indicated by the arrow in Fig. 2, *lane 8*. Accession numbers for *Wolbachia* entries wPip and wMel refer to *Wolbachia* strains from *Culex pipiens* and *Drosophila melanogaster*, respectively. Note that the genome of wStr has not been sequenced. *Aedes* refers to *Aedes aegypti*. The *Aedes albopictus* genome has not been sequenced. Accessions listed here (protein mass is indicated in kilodalton) were represented in MS/MS data by a minimum of three peptides (# *pep*); coverage (% *Cov*) refers to the percent of the total protein sequence represented by detected peptides; and % *spectra* indicates the percentage of total spectra represented by the aggregate number of peptides from each accession

Table 1

Protein	Accession	Species	kDa	#Pep	%Cov	%Spectra	Functional category
<i>Wolbachia</i>							
Surface antigen Wsp	gi 190571332	wPip	24	8	15	0.70	Cell envelope biogenesis/outer membrane
Chaperone protein Dnak (hsp70)	gi 190570602	wPip	69	3	6	0.37	Protein modification/degradation/chaperones
Ribosomal protein S4 3,4-Dihydroxy-2-butanone	gi 190570680	wPip	24	9	37	0.06	Ribosome structure/biogenesis/translation
4-Phosphate synthase (aka RibA)	gi 42520502	wMel	24	3	16	0.03	Coenzyme metabolism/riboflavin biosynthesis
Adenylate kinase	gi 190571566	wPip	24	5	27	0.03	Nucleotide metabolism/transport
Ribosomal protein L4	gi 190571547	wPip	23	3	18	0.02	Ribosome structure/biogenesis/translation
<i>Aedes</i>							
Hypothetical protein AaeL_AAEL004438	gi 157106034	<i>Aedes</i>	25	3	15	0.015	Protein modification/degradation/chaperones