Depletion of host cell riboflavin reduces *Wolbachia* levels in cultured mosquito cells

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Abstract

Wolbachia is an obligate intracellular alphaproteobacterium that occurs in arthropod and nematode hosts. *Wolbachia* presumably provides a fitness benefit to its hosts, but the basis for its retention and spread in host populations remains unclear. *Wolbachia* genomes retain biosynthetic pathways for some vitamins, and the possibility that these vitamins benefit host cells provides a potential means of selecting for *Wolbachia*-infected cell lines. To explore whether riboflavin produced by *Wolbachia* is available to its host cell, we established that growth of uninfected C7–10 mosquito cells decreases in riboflavin-depleted culture medium. A well studied inhibitor of riboflavin uptake, lumiflavin, further inhibits growth of uninfected C7–10 cells with an LC_{50} of approximately 12 µg/ml. Growth of C/wStr1 mosquito cells, infected with *Wolbachia* from the planthopper, *Laodelphax striatellus*, was enhanced in medium containing low levels of lumiflavin, but *Wolbachia* levels decreased. Lumiflavin-enhanced growth thus resembled the improved growth that accompanies treatment with antibiotics that deplete *Wolbachia*, rather than a metabolic advantage provided by the *Wolbachia* infection. We used the polymerase chain reaction to validate the decrease in *Wolbachia* abundance and evaluated our results in the context of a proteomic analysis in which we detected nearly 800 *w*Str proteins. Our data indicate that *Wolbachia* converts riboflavin to FMN and FAD for its own metabolic needs, and does not provide a source of riboflavin for its host cell.

Keywords

Wolbachia; Mosquito cell line; Selection; Riboflavin; Lumiflavin; Proteomics

Introduction

The obligate intracellular bacterium, *Wolbachia pipientis* occurs in a wide range of arthropods, including some species of mosquitoes, and in filarial worms vectored by mosquitoes and other biting flies. In insects, *Wolbachia* is typically considered a reproductive parasite, while in filarial worms, *Wolbachia* is an obligate mutualist thought to provide nutritional supplements such as riboflavin, which cannot be produced by the filarial host (Foster *et al.* 2005). In mosquitoes, *Wolbachia* is vertically transmitted in the egg

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cytoplasm, but its presence in developing testes causes reproductive distortions such as cytoplasmic incompatibility (Beckman and Fallon 2013), which favor *Wolbachia* invasion of uninfected host populations. Although fitness effects associated with *Wolbachia* have been difficult to identify in mosquitoes, in which antibiotic-cured populations differ little from infected cohorts (Islam and Dobson 2006), **Wolbachia's** ability to invade uninfected populations provides a potential tool for controlling insect pests, including vectors of arthropod-borne disease (Sinkins and Gould 2006).

Implementation of *Wolbachia*-based control strategies will be facilitated by genetic manipulation and transformation of *Wolbachia* with genes encoding fluorescent proteins and/or other desirable markers, as has already been achieved with closely related intracellular microbes in the order Rickettsiales (reviewed by Beare *et al.* 2011). Several strains of *Wolbachia* can be propagated in insect cell lines, and we have been interested in identifying metabolic interactions that will support recovery of mosquito cells that harbor normal, and eventually transformed, *Wolbachia*. Here, we explore whether riboflavin (vitamin B2) biosynthesis by *Wolbachia* provides a selective advantage to infected mosquito cells using the C/*w*Str1 cell line (Fallon *et al.* 2013a), which maintains a robust, persistent infection of *Wolbachia* from the planthopper, *Laodelphax striatellus* (Noda *et al.* 2002). Riboflavin is an essential nutrient for mosquito host cells, and despite genomic reduction typical of obligate intracellular bacteria, *Wolbachia* retains the ability to produce riboflavin (Wu *et al.* 2004; Dunning Hotopp *et al.* 2006).

Although provisioning of B vitamins has been implicated in a few insects that have evolved a mutualistic relationship with *Wolbachia* (Hosokawa *et al.* 2010), our results indicate that reducing availability of exogenous riboflavin to mosquito host cells compromises survival of *Wolbachia*. The robust *Wolbachia* infection in C/*w*Str1 cells requires adequate host cell access to exogenous riboflavin, which cannot be supplemented by *Wolbachia's* biosynthetic activity. In this cell culture system, *Wolbachia's* retention of a pathway for riboflavin biosynthesis fails to provide a fitness benefit to its host cell.

Materials and Methods

Cells and culture conditions

We derived C/*w*Str1 cells (Fallon *et al.* 2013a) by infecting *Aedes albopictus* C7–10 cells with *w*Str from an uncloned mosquito cell line (Noda *et al.* 2002). C7–10 control cells and C/*w*Str1-infected cells were maintained in Eagle's medium containing 5% heat-inactivated fetal bovine serum (E-5 medium; Shih *et al.* 1998). C7–10 cells were subcultured weekly, at a 10-fold dilution into fresh medium. C/*w*Str1 cells were subcultured at 2-wk interval by adding 2 ml of cells, resuspended from a 12-ml culture to 5 ml of fresh E-5 medium, in 25-cm^2 flasks. On alternate weeks, flasks (containing 7 ml of cells plus medium) were supplemented with an additional 5 ml of fresh E-5 medium to establish a 12 ml culture. Passage number and other details relevant to individual experiments are described in the figure legends. All experiments were replicated with two or more independent populations of cells, and within an experiment, variation between data points from replicate samples was typically less than 5%. Figures show representative experiments.

Analytical methods

Cells were counted with a Coulter electronic cell counter, and metabolic activity was assayed using methylthiazole tetrazolium (MTT; Fallon *et al.* 2013b). Infection status was monitored by fluorescence microscopy and by evaluating *Wolbachia* DNA content using the polymerase chain reaction (PCR) with *Wolbachia*-specific primers S12F (5′-GCACTAAGGTGTATACTACAACTCC) and S7R (5′-GCCTTATTAGCTTCAGCCAT) as described previously (Fallon *et al.* 2013b). The concentration of riboflavin in E-5 medium is 0.2 μg/ml. Riboflavin-free medium was prepared by replacing the commercially available 100X vitamin stock (GIBCO, Cat No. 11120) with individually dissolved vitamins (Sigma-Aldrich Cat No. B4639, biotin; P5155, pantothenate; C7527, choline chloride; F8758, folic acid; I7508, myoinositol; N3014, β-NAD; P6155, pyridoxal HCl; R9504, riboflavin; T1270, thiamine HCl) lacking riboflavin. When the riboflavin-free medium was supplemented with riboflavin, growth of cells was identical to that in standard medium with commercial components. Stock concentrations (1.0 mg/ml) of lumiflavin (Sigma-Aldrich, L4879) were prepared in 20-mM NaOH and diluted in culture medium to the final concentrations described in the figure legends.

Mass spectrometry

As detailed in proteomics-based analyses of *Wolbachia* from filarial nematodes (Darby *et al.* 2012), we estimated relative abundance levels (RAL) for individual proteins based on counts of MS-matched unique peptide spectra in a series of four MS data sets from a large scale analysis of the *w*Str proteome. Among a total of 797 proteins, RAL values ranged from 0.2 to 58.2 (Baldridge GD, Witthuhn BA, Higgins LA, Markowski TW, Fallon AM. Proteomic analysis of an insect *Wolbachia* in mosquito cells reveals robust expression of a constitutive stress response, a complete type IV secretion system and potential supplementation of bacterial amino acid pools by proline, glutamate, glycine and cysteine degradation, unpublished). The subset of these proteins involved in riboflavin metabolism is discussed here.

Results

We began this study by evaluating sensitivity of wild type C7–10 cells to culture medium lacking riboflavin, normally present at a final concentration of 0.2 μg/ml (see Shih *et al.* 1998). Vitamin-free medium was supplemented with fetal bovine serum, at a final concentration of 5% (E-5 medium), which added riboflavin to a concentration of about 8 ng/ml (see Said and Ma 1994). C7–10 cells were plated at 1×10^5 and 1×10^4 cells/ml in 100 mm dishes (12 ml total volume) in complete medium, or in riboflavin-depleted medium, and counted after 7 d (Fig. 1). Cell counts in the absence of riboflavin (−RF) were reduced to 59 and 83% of controls (+RF) for initial cell densities of 10^4 and 10^5 cells/ml, respectively.

In mammalian cells, riboflavin is acquired from the medium by the transporter proteins, RFT1 and RFT2, recently characterized in rats and humans (Yonezawa *et al.* 2008; Yamamoto *et al.* 2009). Homologous hypothetical proteins occur in various mosquito species, with *E* values ranging from 5e-87 to 2e-71 (Accession Nos. GI : 157104132; GI :

347963328; GI : 170040965; GI : 568255901; GI : 170038973, www.ncbi.nlm.nih.gov/), suggesting that mosquito cells use a similar transport system. Mosquito cells were sensitive to lumiflavin, a strong inhibitor of rat RFT2 (Said and Ma 1994; Yamamoto *et al.* 2009), with an LC_{50} of approximately 12 μg/ml (Fig. 2). Consistent with its inhibition of riboflavin uptake, lumiflavin reduced cell growth in complete (RB+) medium to 29% of control levels (Fig. 3, compare columns *1* and *2*). In riboflavin-depleted (RB−) medium (Fig. 3, columns *3* and *4*), lumiflavin further reduced cell numbers to 19% of controls in complete medium (Fig. 3, compare columns *1* and *4*).

We previously showed that growth of C/*w*Str1 host cells measured by cell number and metabolic activity measured by reduction of MTT are enhanced by treatment with the antibiotics rifampicin and tetracycline, which are used routinely to eliminate *Wolbachia* from insects or cell lines. This difference in growth of C/*w*Str1 cells with and without antibiotics presumably reflects, at least in part, diversion of host resources to support *Wolbachia* replication, which is reduced, but not completely eliminated, by short-term exposure to antibiotics. In contrast to antibiotics, a test compound that would provide a selective advantage to *Wolbachia*-infected cells would be expected to increase growth of infected cells, without an accompanying decrease in *Wolbachia* levels. In a previous study, we noted that improved C/*w*Str1 cell growth in low concentrations of the oxidizing agent paraquat did not support in vitro selection of *Wolbachia*-infected cells based on increased expression of host antioxidant activity because *Wolbachia* itself is more sensitive to paraquat than are the host mosquito cells (Fallon *et al.* 2013b).

The effect of lumiflavin resembles that of paraquat (Fig. 4*A*). In the absence of lumiflavin, growth of C/*w*Str1 cells, or metabolic activity reflected by MTT values, reached about 60% of control levels with tetracycline and rifampicin. At concentrations ranging from 2.5 to 10 μg/ml, lumiflavin increased cell numbers and MTT values to about 80% of values in the presence of rifampicin and tetracycline before dropping back to initial levels for cell counts on day 4, or dropping below initial MTT levels on day 6. Microscopic (data not shown) and biochemical comparisons of samples from the same experiment showed that lumiflavin reduced *Wolbachia* abundance relative to control cells (Fig. 4*B*; compare *lanes 3* and *4* to *lanes 5* to *12*), although the effect was weaker than that with antibiotics (Fig. 4*B*; compare *lanes 3* and *4* to *lanes 1* and *2*). In addition, flow cytometric data (to be described in detail elsewhere) with two additional, independent populations of cells were consistent with the increased cell numbers and reduced *Wolbachia* numbers in lumiflavin-treated samples depicted in Fig. 4. These results suggest that C/*w*Str1 host cells do not benefit from riboflavin produced by *Wolbachia*. Conversely, a BLAST search indicates that the bacterial *imp*X gene from *Fusobacterium nucleatum*, which encodes a putative riboflavin transporter used as a query in the rat RFT2 study (Yamamoto *et al.* 2009), has no homologs in *Wolbachia* or other members of the Rickettsiales, suggesting that *Wolbachia* is unlikely to have access to metabolic pools of riboflavin acquired from the medium by host cells.

As a final consideration, we compare these experimental observations with a recently completed analysis of the *w*Str proteome in C/*w*Str1 cells (Baldridge GD, Witthuhn BA, Higgins LA, Markowski TW, Fallon AM. Proteomic analysis of an insect *Wolbachia* in mosquito cells reveals robust expression of a constitutive stress response, a complete type

IV secretion system and potential supplementation of bacterial amino acid pools by proline, glutamate, glycine and cysteine degradation, unpublished). The present data suggest that *Wolbachia* and their host cells are unlikely to share riboflavin pools. Figure 5 summarizes the riboflavin biosynthetic pathway in *Wolbachia*, with accession numbers for gene homologs in *w*Pip, the annotated *Wolbachia* genome that most closely matches the majority of *w*Str-encoded proteins. For those proteins detected in our proteomic analysis, relative abundance levels (RAL) are given at right (nd, not detected in the proteomic analysis), and in the schematic at left, more intense shading designates the more abundant enzymes. Riboflavin is synthesized from one molecule of GTP, a product of the purine synthesis pathways, condensed with 3,4-dihydroxy-2-butanone 4-phosphate derived from two molecules of ribulose 5-phosphate provided by the pentose phosphate pathway (Bacher *et al.* 2000). Of the enzymes that participate in riboflavin biosynthesis, RibB (3,4-dihydroxy-2-butanone 4-phosphate synthase), was the most abundant in our proteomic analysis of *w*Str. The RAL score for RibF, which acts downstream of riboflavin biosynthesis, converting riboflavin to FAD and FMN cofactors, approaches that for RibB, whose high RAL score potentially reflects functions outside the riboflavin pathway (Jin *et al.* 2003; Knegt *et al.* 2008; McDonagh *et al.* 2011). The high RAL score for RibF, relative to RibA, RibD, RibH, and RibC, is consistent with *Wolbachia's* production of riboflavin solely for its own metabolic needs, rather than to provision its host cell.

Discussion

Despite considerable research, it has been difficult to identify metabolic processes that underlie *Wolbachia's* interactions with its diverse hosts and presumably contribute a fitness advantage that accompanies its invasion of host populations (see Harcombe and Hoffmann 2004; Islam and Dobson 2006). In insects, *Wolbachia* is typically a reproductive parasite, but it may be symbiotic in the wasp, *Asobara tabida*. In *A. tabida, Drosophila simulans,* and *Aedes aegypti* RML12 cell line, *Wolbachia* has been implicated in iron homeostasis (Kremer *et al.* 2009), but the dynamics of iron partitioning between insect hosts and *Wolbachia* have been difficult to quantify by manipulation of dietary iron (Brownlie *et al.* 2009). *Wolbachia* that infect filarial worms are obligate symbionts that have coevolved with their nematode hosts. Annotation of the genome of *w*Bm, a symbiont of *Brugia malayi*, initially suggested that *Wolbachia* might provide heme, nucleotides, riboflavin, and FAD to its nematode host (Foster *et al.* 2005). Moreover, Li and Carlow (2012) reported that in RPMI medium, doxycycline-induced inhibition of worm motility and reproduction were partially restored by supplementing the medium with riboflavin. Aside from these data, however, Darby et al. (2012) recently reported that *Wolbachia* strain *w*Oo, which infects *Onchocerca ochengi*, a filarial parasite of cattle, lacks pathways for both riboflavin and heme provisioning, and suggest that in this system, *Wolbachia* may generate ATP for its host. Moreover, secondary loss of *Wolbachia* in members of the Onchocercidae (McNulty *et al.* 2010) implies that the symbiotic relationship is not universal in filarial worms.

In insects, obligate endosymbionts such as *Buchnera aphidicola* in aphids and Wigglesworthia glossinida in tsetse flies occur in distinct symbiotic organs known as bacteriomes; in contrast, *Wolbachia* typically occurs in non-specialized cells and tissues and is generally considered facultative or parasitic. An exception is the bedbug, *Cimex*

lectularius, wherein *Wolbachia* occurs in bacteriocytes and functions as an obligate nutritional mutualist (Hosokawa *et al.* 2010). Disruption of bedbug development by rifampicin treatment was ameliorated when the blood meal was supplemented with a mixture of B vitamins, including the four vitamins (riboflavin, folic acid, pantothenate, and pyridoxine) for which *Wolbachia* retains complete biosynthetic pathways (Dunning Hotopp *et al.* 2006), but investigation of individual vitamins was not reported.

The possibility that *Wolbachia* provisions mosquito cells with one or more vitamins is consistent with early nutritional studies indicating that mosquitoes require riboflavin and pantothenic acid for larval growth, and pyridoxine and folic acid for pupation (Lichtenstein 1948; Singh and Brown 1957). If riboflavin produced by *Wolbachia* was available to its host cell, it might provide a convenient method to select for infected cells, for example, after *Wolbachia* transformation. Riboflavin provides a particularly attractive candidate because mosquito genomes encode a homolog of rat and human riboflavin transporter proteins, which can be inhibited by lumiflavin, a water-soluble compound produced by photolysis of riboflavin. Although the present study demonstrates that uninfected mosquito cells respond to riboflavin depletion and to inhibition of riboflavin transport, we find no evidence for a bacterial contribution to the host riboflavin pool in *Wolbachia*-infected cells. Moreover, *Wolbachia* growth is diminished when availability of exogenous riboflavin to the host cell is reduced, and proteomics data suggest that *Wolbachia* requires the riboflavin it produces for synthesis of FAD and NAD cofactors. Nevertheless, we cannot eliminate the possibility that the metabolic costs of the *Wolbachia* infection obscure any small benefit that might be derived from riboflavin production. Once transformation of *Wolbachia* is achieved, direct manipulation of the *Wolbachia* genome may allow us to exploit the many biotechnological implications that have emerged from investigation of riboflavin biosynthesis and transport (Long *et al.* 2010; Abbas and Sibirny 2011). For example, Coquard *et al.* (1997) described a mutation in the *Rib*C gene in *Bacillus subtilis* that causes riboflavin overproduction.

Insects provide complex and diverse relationships with *Wolbachia*, and our own observations indicate that strains of *Wolbachia* from various sources differ in their adaptability to clonal *A. albopictus* cell lines (Fallon 2008; Fallon and Witthuhn 2009). Host cell factors that regulate infection and replication of *Wolbachia* are largely unexplored, and many strains of *Wolbachia* have not yet been established in cell lines. Using the *w*Str system, we anticipate that further exploration of *Wolbachia* metabolism in the context of proteomics data will facilitate manipulation of *Wolbachia*-infected cells, provide a framework for *Wolbachia* transformation and genetic manipulation, and improve establishment of recalcitrant *Wolbachia* strains in insect cell lines.

Acknowledgments

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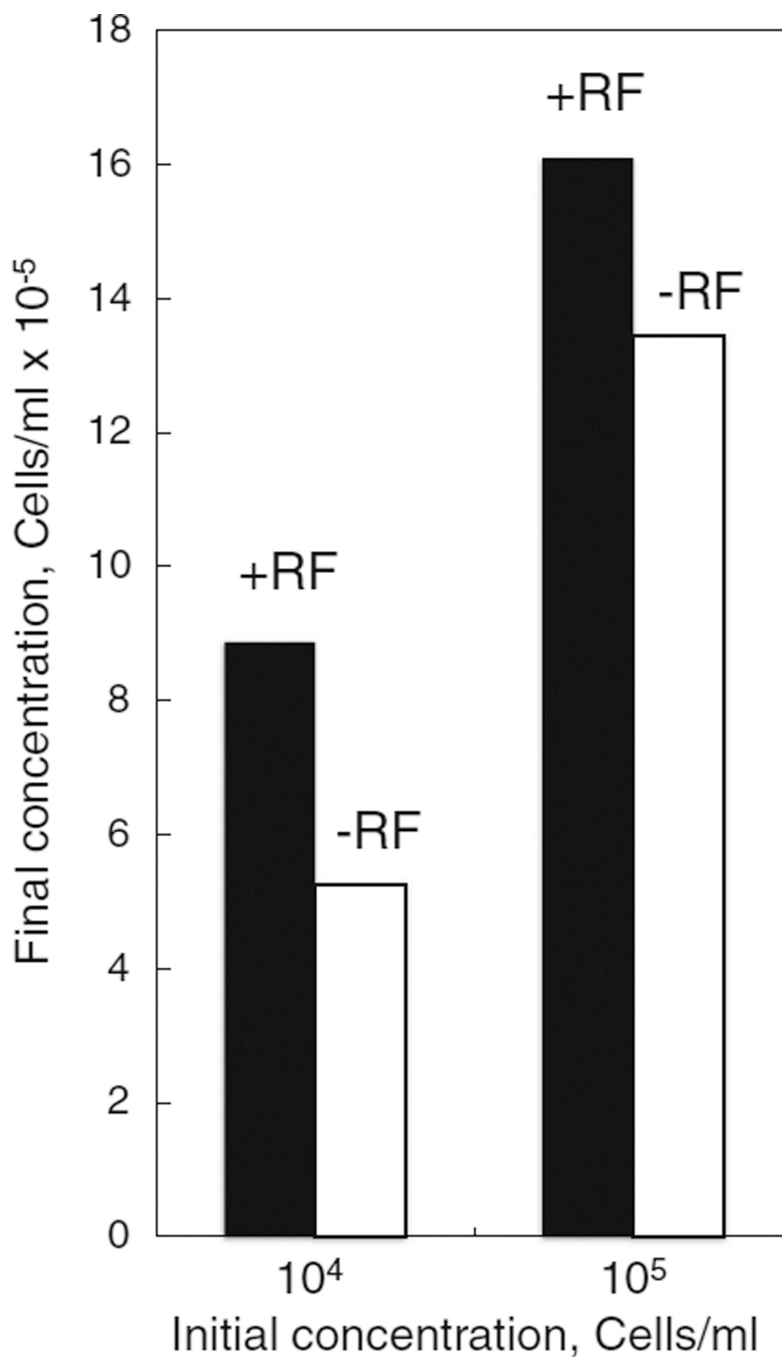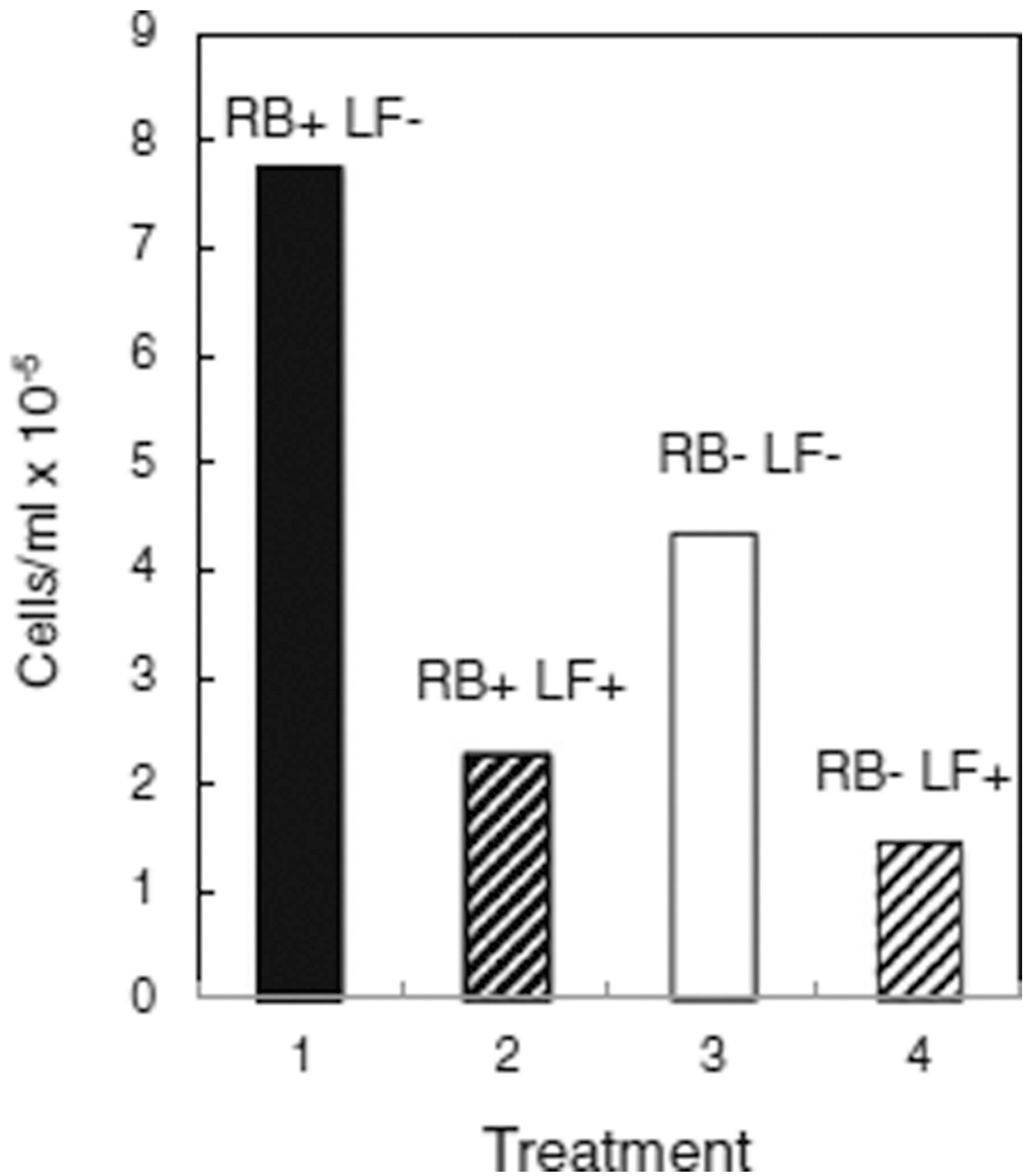
Figure 1.
Growth of C7–10 cells is sensitive to riboflavin depletion. Cells were plated at 1×10^5 and 1×10^4 cells/ml in 100-mm plates, in 12 ml of complete medium (*solid bars*) or riboflavin-deficient medium (*open bars*). After 7 d, cells were resuspended and counted with a Coulter electronic cell counter. *Values* are averages from duplicate plates; variation between values was typically less than 5%.
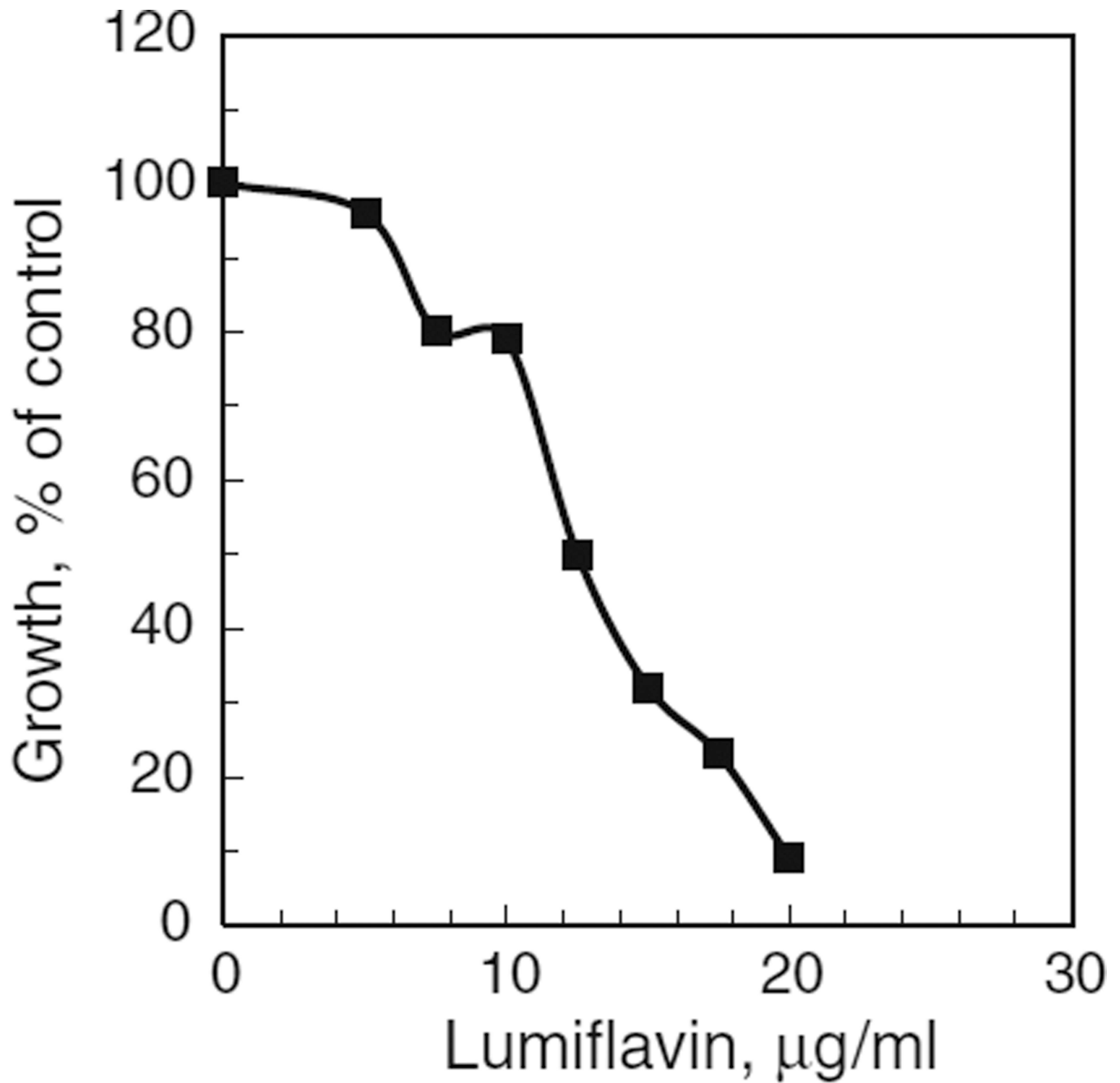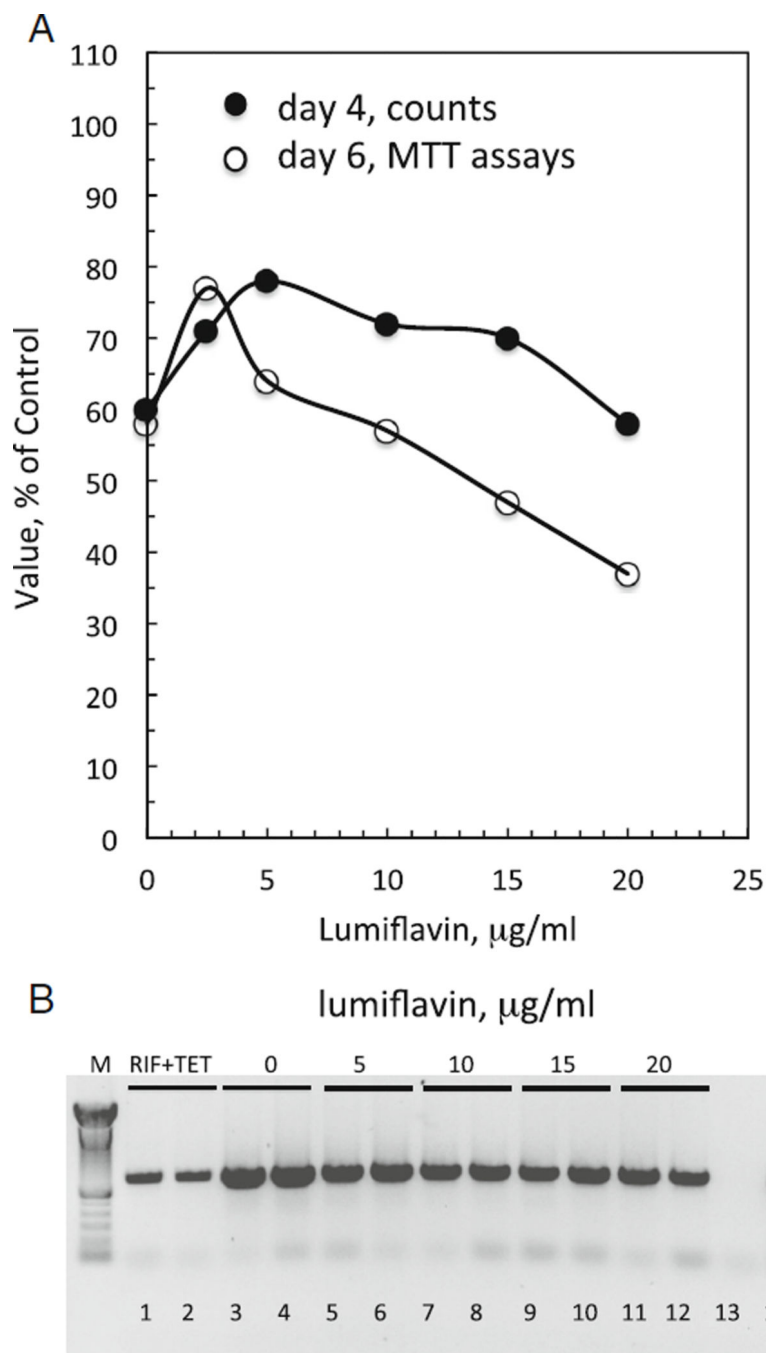
Figure 2.
Effect of lumiflavin on growth of uninfected cells. Cells were plated at 1×10^5 cells/ml, 2 ml/35 mm plate, in E-5 medium containing the indicated concentrations of lumiflavin. After 5 d, cells were counted with a Coulter electronic cell counter. Values are plotted as percentages of controls without lumiflavin (4.8×10^6 cells/plate).

Figure 3.
Lumiflavin reduces cell growth in the presence or absence of riboflavin. C7–10 cells were plated at a density of 10^4/ml in complete medium (*solid bar*) and in riboflavin-free medium (*open bar*) and counted after 10 d. *Hatched bars* (lanes 2 and 4) represent samples that were supplemented with lumiflavin at a final concentration of 5 μg/ml.
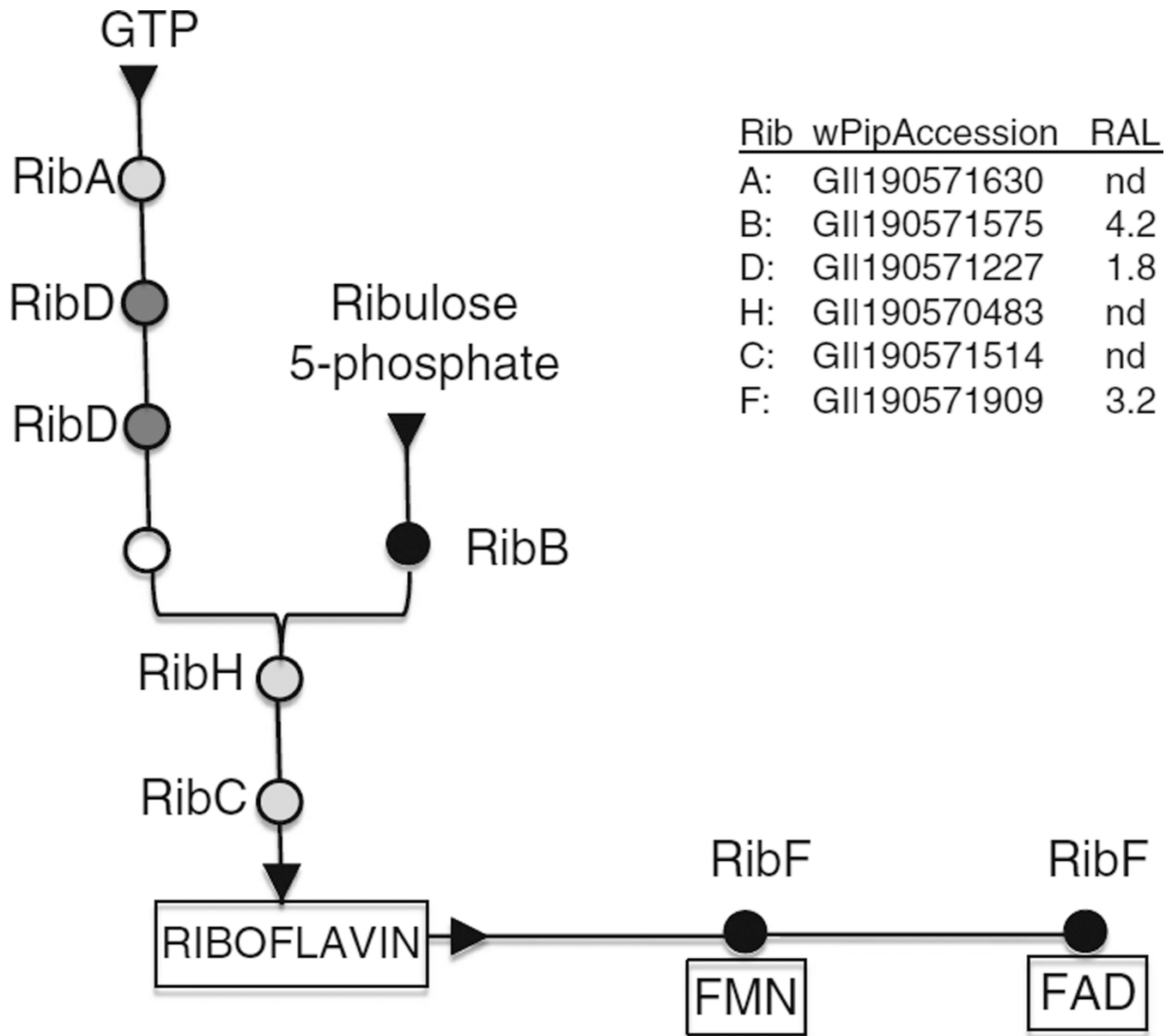
Figure 4.
Low levels of lumiflavin enhance growth of C/*w*Str1 cells, and decrease *Wolbachia* levels. (*A*) C/*w*Str1 cells at passage 60 were seeded in 35-mm plates at a density of 1×10^5 cells/ml (2 ml/plate) in the absence or presence of lumiflavin at the indicated concentrations. A second set of control plates without lumiflavin was supplemented with rifampicin and tetracycline to suppress *Wolbachia* growth. Values from these plates were established as 100%. In the absence of antibiotics and lumiflavin, values for C/*w*Str1 cells reach about 60% of levels with antibiotics, as indicated in the Figure. After 4 d, duplicate plates were

resuspended and counted with a Coulter electronic cell counter (*closed circles*). Values are expressed as percentages of counts in the presence of rifampicin and tetracycline (100% =15.3 × 10^5 cells/plate). After 6 d, duplicate plates were assayed for metabolic activity by MTT (*open circles*; 100%=2.32 OD_{570}), and duplicate samples were harvested for isolation of DNA. (*B*) Relative amounts of *Wolbachia* determined by the polymerase chain reaction (PCR). PCR reactions were carried out as described previously (Fallon *et al.* 2013b). *Lanes* show products from two independent DNA extractions under each experimental condition. *Lanes 1 and 2* show suppression of *Wolbachia* grown in the presence of rifampicin and tetracycline for 6 d after plating. *Lanes 3* and *4* show control C/*w*Str1 cells; *lanes 5–12* show cells with lumiflavin at the indicated concentrations. *Lane 13* shows no DNA control; *lane 14* shows positive control DNA.

Figure 5.

Pathways for riboflavin biosynthesis in *Wolbachia. Circles* indicate enzymes that participate in riboflavin biosynthesis from two substrates, GTP derived from purine biosynthesis, and ribulose 5-phosphate derived from the pentose phosphate pathway. *RibA* GTP cyclohydrolase II; *RibD* bifunctional deaminase and reductase; *open circle* unidentified enzyme (*see* Bacher *et al.* 2000; Abbas and Sibirny 2011); *RibB* 3,4-dihydroxy-2-butanone 4-phosphate synthase; *RibH* lumazine synthase; *RibC* riboflavin synthase; *RibF* riboflavin kinase and FAD synthase. At the left, *shading* represents relative expression level for these pathways, based on data in a related proteomics study (Baldridge GD, Witthuhn BA, Higgins LA, Markowski TW, Fallon AM. Proteomic analysis of an insect *Wolbachia* in mosquito cells reveals robust expression of a constitutive stress response, a complete type IV secretion system and potential supplementation of bacterial amino acid pools by proline,

glutamate, glycine and cysteine degradation, unpublished; *see the legend* at the right). *Black* represents highly expressed enzyme; *dark gray* represents moderately expressed; *light gray* represents not detected (nd) in the proteomics study. Accession numbers are from the annotated *w*Pip (Pel) genome.