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Strategies of the home-team: symbioses exploited for vector-borne disease control

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Symbioses between eukaryotes and unicellular organisms are quite common, with examples copiously disseminated throughout the earth's biota. Arthropods, in particular, owe much of their ecological success to their microbial flora, which often provide supplements either lacking in the limited host diet or which the hosts are unable to synthesize. In addition to harboring beneficial microbes, many arthropods (vectors) also transmit pathogens to the animals and plants upon which they prey. Vector-borne diseases exact a high public health burden and additionally have a devastating impact on livestock and agriculture. Recent scientific discoveries have resulted in the development of powerful technologies for studying the vector's biology, to discover the weak links in disease transmission. One of the more challenging applications of these developments is transgenesis, which allows for insertion of foreign DNA into the insect's genome to modify its phenotype. In this review, we discuss an approach in which the naturally occurring commensal flora of insects are manipulated to express products that render their host environment inhospitable for pathogen transmission. Replacing susceptible insect genotypes with modified counterparts with reduced pathogen transmission ability, might provide a new set of armaments in the battle for vector-borne disease reduction.

Symbioses vary in their evolutionary history, with some being recently established and others being ancient. The impact of a particular symbiosis on host physiology can also range from parasitic to mutualistic, and its association can span from facultative to obligate (Figure 1). Typically, the presence of a highly specialized obligate mutualist is not only beneficial but an absolute necessity to both partners, with an existence short of the other nonviable because of a shared reliance for one another as a result of a long co-evolutionary period. The facultative parasite, at the opposite end of the symbiosis continuum, establishes an association only if the opportunity presents itself and imposes a net negative effect on host fitness for its own selfish evolutionary success. The commensal, a term arising from the Latin 'commensalis' defined as 'at table together', lies at the center of the symbiosis spectrum. The commensalist receives benefit(s) while simultaneously presenting no detrimental cost or advantages to its host. Recent advances in diagnostics and systematics tools have greatly advanced the field of symbiosis and have revealed the presence of a consortium of microbes that co-exist and intimately influence the biology of multicellular animals and plants. In fact, it is thought that the phenotypes of eukaryotes result from the combined actions of products synthesized by heterogeneous and often co-evolved multiple genomes. Moreover, symbionts can be manipulated to reduce or eliminate their hosts' ability to transmit diseases. In this review, we focus on the midgut symbiotic flora of insects [1–4].

Interactions between symbionts and hosts

Eukaryotic hosts can acquire their symbionts by maternal inheritance (transovarial or acquisition *in utero*) or environmental acquisition (via the surrounding habitat, with a new infection established at each generation) (Table 1) [5]. While residing in the bodies of their multicellular hosts, most symbiotic microbes encounter an elaborate series of obstacles. Similar to pathogens, these beneficial bacteria first have to adhere to and enter their host cells (often specialized tissues and cell types) while avoiding the host's defense system, then multiply, and finally exit host cells to colonize new cells or hosts. Recent studies into beneficial symbiont genomes reveal that loci typically attributed to contributing towards

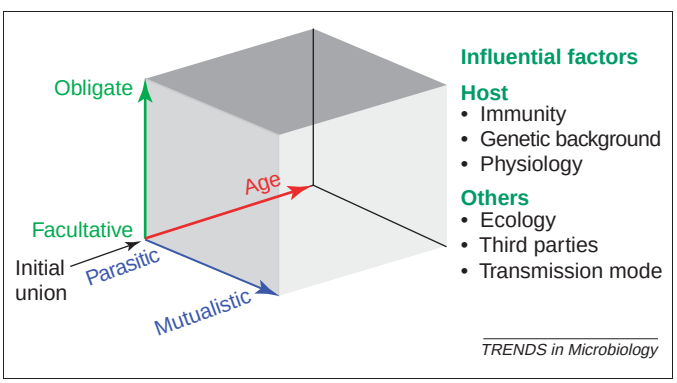

Figure 1. Symbiotic associations can be described according to: their impact on host biology spanning from parasitic to mutualistic (X); the nature of the association varying from facultative to obligate (Y); and the age of the symbiosis between partners (Z). Host and environmental factors influence the dynamics of the relations through time.

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virulence in a pathogen, such as a functional type III secretion system [6,7] and a urease gene cluster [8], have been retained. It is possible that pathogenesis, through an attenuated adaptive process, might have given rise to beneficial relationships which now fulfill important host needs [9,10].

The majority of insects are involved in some form of symbiosis and offer exceptional model systems to study various aspects of these interactions [11]. Insects whose diets consist of a single food source, such as vertebrate blood or plant phloem, harbor symbionts (P for primary symbionts) that provide nutritional supplementation, and without which loss of fertility and a reduction in longevity occurs. These obligates typically reside within specialized epithelial cells (bacteriocytes) that form an organ (bacteriome) which houses these intracellular 'captive genomes'. Based on recent phylogenetic reconstruction studies, the obligates from aphids, tsetse flies, whiteflies and carpenter ants form closely related distinct lineages within the γ-subdivision of Proteobacteria (Figure 2) [12,13]. In each insect system, the relationships have been maternally transmitted for millions of years and display concordance with the host species. As a result of successive bottlenecks because of their strict maternal mode of transmission and small effective population size, genetic drift is exacerbated and these genomes now display increased mutational rates [14]. Genome sequencing projects completed for *Wigglesworthia glossinidia* from tsetse flies [15], several *Buchnera* species from aphids [16–18] and *Blochmannia floridanus* from carpenter ants [8] reveal traits that are similar to those observed in obligate intracellular pathogens, such as drastically reduced genome sizes and extreme adenine–thymine (A + T) bias [19].

Symbionts, which are obligate to specific aspects of the host biology, can also be horizontally acquired following host embryogenesis. The juvenile Hawaiian sepiolid squid

Table 1. Examples of different insect orders harboring bacterial partners and their relevance towards host biology[a]

Insect order	Bacterial partner	Distribution/putative function/transmission
Blattaria		
Cockroach	Blattobacterium (Flavobacteria)	[c]Fat body/urate recycling/V
Isoptera		
Termite	Blattobacterium (Flavobacteria)	[c]Fat body/nitrogen fixation/V
	Anaerobes, methanogens	Gut/urate recycling/?
Phthiraptera		
[b]Sucking lice	Many bacterial types	[c]Body/nutrition/V
Hemiptera		
Bedbug	γ-Proteobacteria	[c]Abdomen/nutritional/V
[b]Triatomine bug	Actinomycetes, Corynebacterium	Extracellular (gut lumen)/vitamins/coprophagy
	Arsenophonus triatominarum (γ-Proteobacteria)	Intracellular (broad)/?/V
[b]Stinkbug	γ-Proteobacteria	Midgut crypts/nutritional/capsule probing
Homoptera		
[b]Aphid		
	P – *Buchnera aphidicola* (γ-proteobacteria)	[c]Body/amino acids/V
	S – PASS, PAUS, PABS (γ-proteobacteria)	Broad/defense, temperature tolerance/V
	PAR (α-proteobacteria)	Hemolymph/?/V
	Spiroplasma (Mollicutes)	Hemolymph/?/V
[b]Whitefly		
	P – γ-Proteobacteria	[c]Body/nutritional/V
	S – γ-Proteobacteria	[c]Body/nutritional/V
[b]Mealybug		
	P – *Tremblaya princeps* (β-proteobacteria)	[c]Body/nutritional/V
	S – γ-Proteobacteria	[c]Within P/slowing P degradation/V
[b]Sharpshooter		
	Baumannia cicadellinicola (γ-Proteobacteria)	[c]Anterior abdomen/nutritional/V
[b]Psyllid		
	P – *Carsonella ruddii* (γ-proteobacteria)	[c]Body/nutritional/V
	S – γ-Proteobacteria	[c]Synctial region/?/V
Diptera		
[b]Tsetse fly		
	P – *Wigglesworthia glossinidia* (γ-Proteobacteria)	[c]Anterior midgut/vitamins/V
	S – *Sodalis glossinidius* (γ-proteobacteria)	Extra- and intracellular/?/V
Sheep ked	Unknown	[c]Midgut/nutrition/V
Bat fly	Unknown	[c]Abdomen/nutrition/V
Hymenoptera		
Carpenter ant	*Blochmannia camponotii* (γ-proteobacteria)	[c]Midgut/colony-founding, growth/V
Coleoptera		
Cereal weevils	SOPE, SZPE (γ-proteobacteria)	[c]intestine/nutritional/V

[a]Abbreviations: P, primary symbiont; PABS, pea aphid *Bemisia*-type symbiont (also described as T-type); PAR, pea aphid *Rickettsia;* PASS, pea aphid secondary symbiont (also described as R-type); PAUS, pea aphid U-type symbiont (also described as U-type); S, secondary symbiont; SOPE, *Sitophilus oryzae* primary endosymbiont; SZPE, *Sitophilus zeamais* primary endosymbiont; V, vertical transmission mode.
[b]Vectors of important animal and plant diseases: sucking lice (typhus, relapsing fever, trench fever); triatomine bugs (Chagas disease); stinkbugs (various plant diseases); aphids, whiteflies, mealybugs, and psyllids (various plant viruses); sharpshooters (Pierce's disease); and tsetse flies (African trypanosomiasis).
[c]Bacteriocyte.

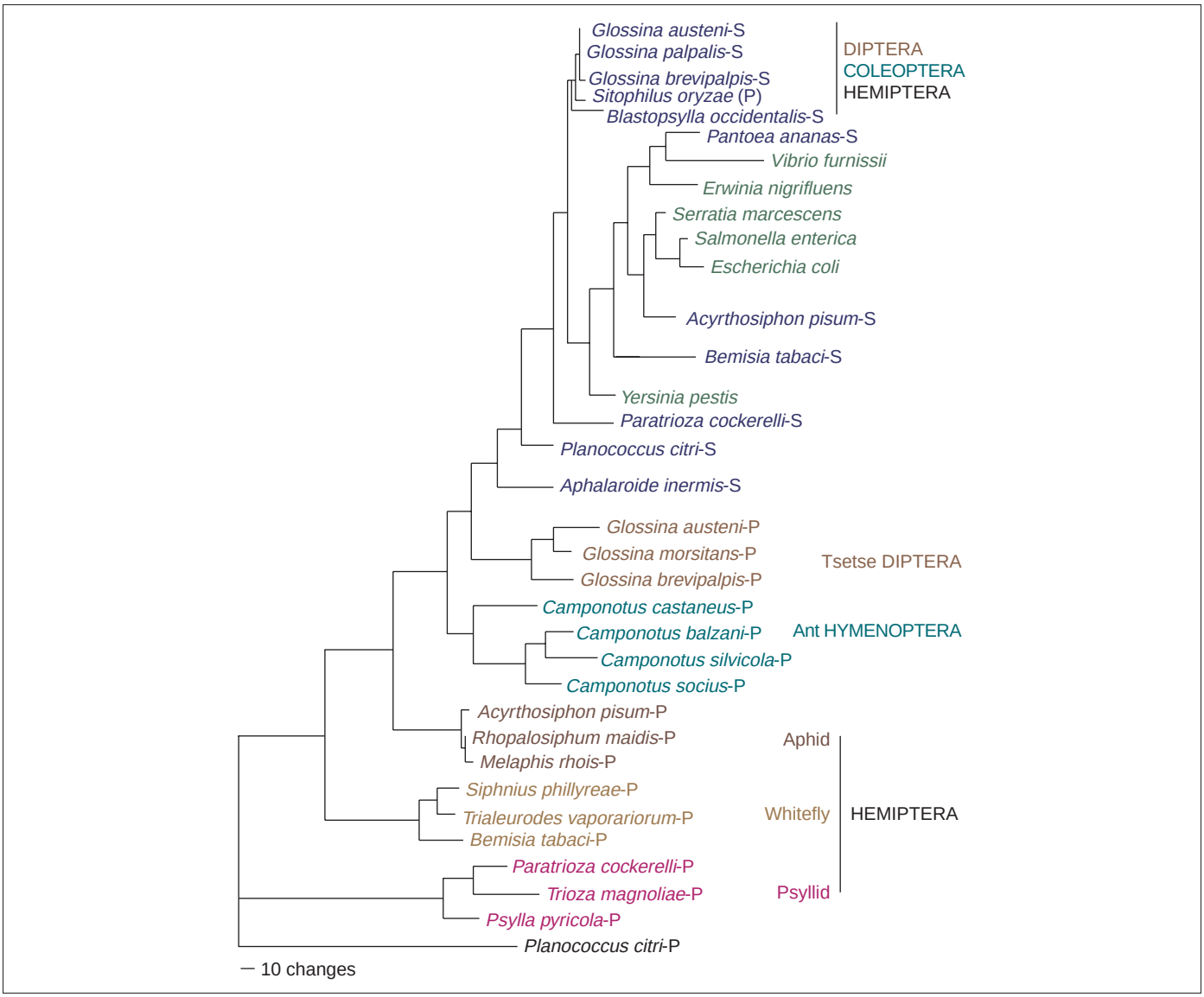

Figure 2. Phylogenetic tree showing the taxonomic position of primary (P) and secondary (S) symbionts characterized from insect species in distant orders determined by 16S rDNA sequence. Species names in green indicate the closely related free-living bacteria within γ-proteobacteria. The nodes shown in the figure had bootstrap values above 50 using the neighbor-joining method with 500 replications. The P-symbiont of the mealybug in the β-subdivision of Proteobacteria was used as an outgroup. The GenBank accession numbers for the 16S rDNA sequences are as follows: *Glossina austeni*-S (U64869), *Glossina palpalis*-S (U64867), *Glossina brevipalpis*-S (U64870), *Sitophilus oryzae* (AF005235), *Blastopsylla occidentalis*-S (AF077608), *Pantoea ananas*-S (Z96081), *Vibrio furnissii* (X76336), *Erwinia nigrifluens* (AJ233415), *Serratia marcescens* (AF286874), *Salmonella enterica* (AF276989), *Escherichia coli* (AE000452), *Acyrthosiphon pisum*-S (M27040), *Bemisia tabaci*-S (Z11926), *Yersinia pestis* (X89573), *Paratrioza cockerelli*-S (AF286127), *Planococcus citri*-S (AF322016), *Aphalaroide inermis*-S (AF263556), *Glossina austeni*-P (L37340), *Glossina morsitans*-P (L37339), *Glossina brevipalpis*-P (L37341), *Camponotus castaneus*-P (AJ245594), *Camponotus balzani*-P (AJ245596), *Camponotus silvicola*-P (AJ245592), *Camponotus socius*-P (AJ245595), *Acyrthosiphon pisum*-P (M27039), *Rhopalosiphum maidis*-P (AF275248), *Melaphis rhois*-P (M63255), *Siphnius phillyreae*-P (Z11927), *Trialeurodes vaporariorum*-P (Z11928), *Bemisia tabaci*-P (Z11925), *Paratrioza cockerelli*-P (AF286119), *Trioza magnoliae*-P (AF077604), *Psylla pyricola*-P (AF286118), *Planococcus citri*-P (AF322017). Reproduced, with permission, from Ref. [83].

Euprymna scolopes acquires the luminescent bacterium *Vibrio fischeri* from ambient seawater using an elaborate set of tissues that appears to function solely to ensure symbiont colonization [20,21]. In yet another example of an environmentally acquired symbiont obligate to the host, the reduviid bug *Rhodnius prolixus* acquires its symbiont *Rhodococcus rhodnii*, a soil-associated nocardiform actinomycete, through coprophagy (in this case, feeding on the feces of adult conspecifics) by emerging nymphs. In the absence of this symbiotic association, the nymphs are unable to complete their sexual development [22–24].

In addition to such obligate mutualists, many insects also harbor commensal associations that do not display extensive evolutionary histories with host species (S for secondary symbionts). These symbionts might represent either recent independent acquisition by each host species or, alternatively, multiple horizontal transfer events between species [25]. Interestingly, comparative phylogenetic analysis of these symbionts from distant insect taxa, including weevils (*Sitophilus* species), aphids (*Acyrthosiphon pisum*), whiteflies (*Bemisia tabaci*) and tsetse flies (*Glossina* species) indicates that they share a recent ancestor within *Enterobacteriaceae,* a taxon that also includes important pathogens such as *Salmonella*, *Yersinia* and *Shigella* (Figure 2). It has not been possible to cultivate these organisms *in vitro* (with the exception of the tsetse symbiont described below), indicating that they

might have also undergone genome reductions. These commensals could offer a unique opportunity to study the effects of transitioning into an obligate mutualistic lifestyle on microbial genomes. In contrast to the obligates, the functional roles of the indigenous commensal microflora in host biology have been less apparent. However, it is suggested that these associations could also enable insect hosts to potentially exploit novel ecological niches by allowing expansion of physiological capabilities [26]. Functional roles such as increased temperature tolerance [27,28] and resistance against parasitoid development [29] have also been attributed to the commensal symbionts in aphids.

Symbiosis in tsetse

Tsetse flies (Diptera: Glossinidae) harbor three distinct microorganisms that exhibit different forms of symbiotic relations [30]. Two of the organisms harbored in the gut tissue of all tsetse flies analyzed are members of the *Enterobacteriaceae*: the obligate mutualist genus *Wigglesworthia glossinidia* [31,32] and the commensal genus *Sodalis glossinidius* [33,34]. The third symbiont present in some tsetse populations is related to the parasitic microbe *Wolbachia pipientis*, an α-Proteobacteria [35,36]. Tsetse flies have a viviparous reproductive biology; an adult female produces a single egg at a time, and this hatches and develops *in utero* (Figure 3). After a period of maturation and sequential molting within the mother, a fully mature third instar larva is deposited and pupates shortly thereafter in the soil. Unlike most insects, which typically produce many offspring on multiple occasions, each tsetse female can only deposit six to eight larvae during her 3–4 month average life span. During intrauterine life, the mother's milk gland secretions transmit to the progeny the gut symbionts, along with nutrients [37–39]. Given the unique reproductive biology of the tsetse fly, all three symbionts are in essence maternally acquired by the progeny.

Obligate mutualist *Wigglesworthia*

Wigglesworthia cells lie free within the cytoplasm of bacteriocytes that make up the bacteriome structure located in anterior midgut (Figure 3). Recently, the genome of *Wigglesworthia* was completely sequenced and found to be 697 724 base pairs in size, encoding 621 predicted protein coding sequences (CDSs) [15]. Analysis of the CDSs indicate that *Wigglesworthia* has retained the ability to synthesize various vitamin metabolites, including biotin, thiazole, lipoic acid, flavin adenine dinucleotide (riboflavin, vitamin B2), folate, pantothenate, thiamine (vitamin B1), pyridoxine (vitamin B6), protoheme, and nicotinamide, which are known to be low in the single diet of tsetse flies: vertebrate blood. Hence, as suspected from dietary supplementation experiments, providing vitamins appears to play a central role in the functional basis of this mutualistic relationship. One of the unusual findings in the *Wigglesworthia* genome is the absence of the important gene coding for the DNA replication initiation protein, DnaA – an observation previously unprecedented in eubacteria. The recently sequenced genome of the taxonomically related symbiont *Blochmannia* from the carpenter ant also lacks the *dnaA* locus [8]. Hence, lack of robust DNA replication machinery in both of these symbionts could be one of the mechanisms by which their hosts regulate symbiont cell numbers and functions. Another unanticipated finding in *Wigglesworthia* is the presence of a complete flagellar biosynthesis capability, despite the fact that a flagellum structure has never been shown to be associated with *Wigglesworthia* in the adult bacteriocyte. It is possible that flagella might function to mediate the transmission of cells from the adult bacteriome to the intrauterine larva, or during the invasion of larval bacteriocytes.

Commensal *Sodalis*

In addition to harboring *Wigglesworthia*, all tsetse flies analyzed from the field harbor the gut commensal *Sodalis* (for *companion* in Latin) [40]. Members of the facultative symbiont genus *Sodalis* from distant tsetse species do not exhibit significant phylogenetic differences based on their 16S rDNA sequence [25], suggesting a relatively younger symbiotic establishment. *Sodalis* has a wide tissue tropism, harbored both inter- and extracellularly, principally in the midgut tissue, but also in the muscle, fat body, hemolymph, milk gland and in the salivary gland tissue of certain species [37], and its overall abundance varies in the different host species and sexes analyzed [35,41].

The genome-wide sequencing of *Sodalis* is near completion (Aksoy and Hattori, pers. commun.). In the absence of such information, a previous study that hybridized *Sodalis* DNA to *Escherichia coli* macroarrays provided a broad understanding of the functional aspects within this symbiont's genome [42]. The *Sodalis* genome has apparently retained a high proportion of the genes necessary for all of the amino acid biosynthetic pathways, transcription and translation processes and for nucleic acid biosynthesis. Many genes involved in the biosynthesis of cofactors, replication and transport functions are also present, as well as most of the DNA repair and recombinase orthologs of *E. coli* involved in direct damage reversal, base excision repair, mismatch repair and recombinase pathways. However, the *Sodalis* genome might have lost genes involved in some metabolic processes, and these restrictions could represent early genome reduction events during the course of symbiosis as the organism adapts to its host physiological environment and nutritional ecology. Nevertheless, *Sodalis* can grow *in vitro* in cell-free media, further supporting retention of many free-living capabilities, presumably because of its recent symbiotic establishment [3,43]. Undetected by the heterologous hybridization approach was the presence of a functional invasion mechanism in *Sodalis*, a type III secretion system reminiscent of *Salmonella* [7], suggesting that this organism might have had a parasitic association with the flies in the past that evolved into a commensal relationship through an attenuated adaptive process.

The heterologous macroarray approach was similarly used to infer the genomic contents of *Sitophilus oryzae* primary endosymbiont (SOPE), a symbiont of the rice weevil (Coleoptera; *Sitophilus oryzae*), which is a close relative of *Sodalis* [44]. Significant differences between the two genomes were found in the retention of genes involved

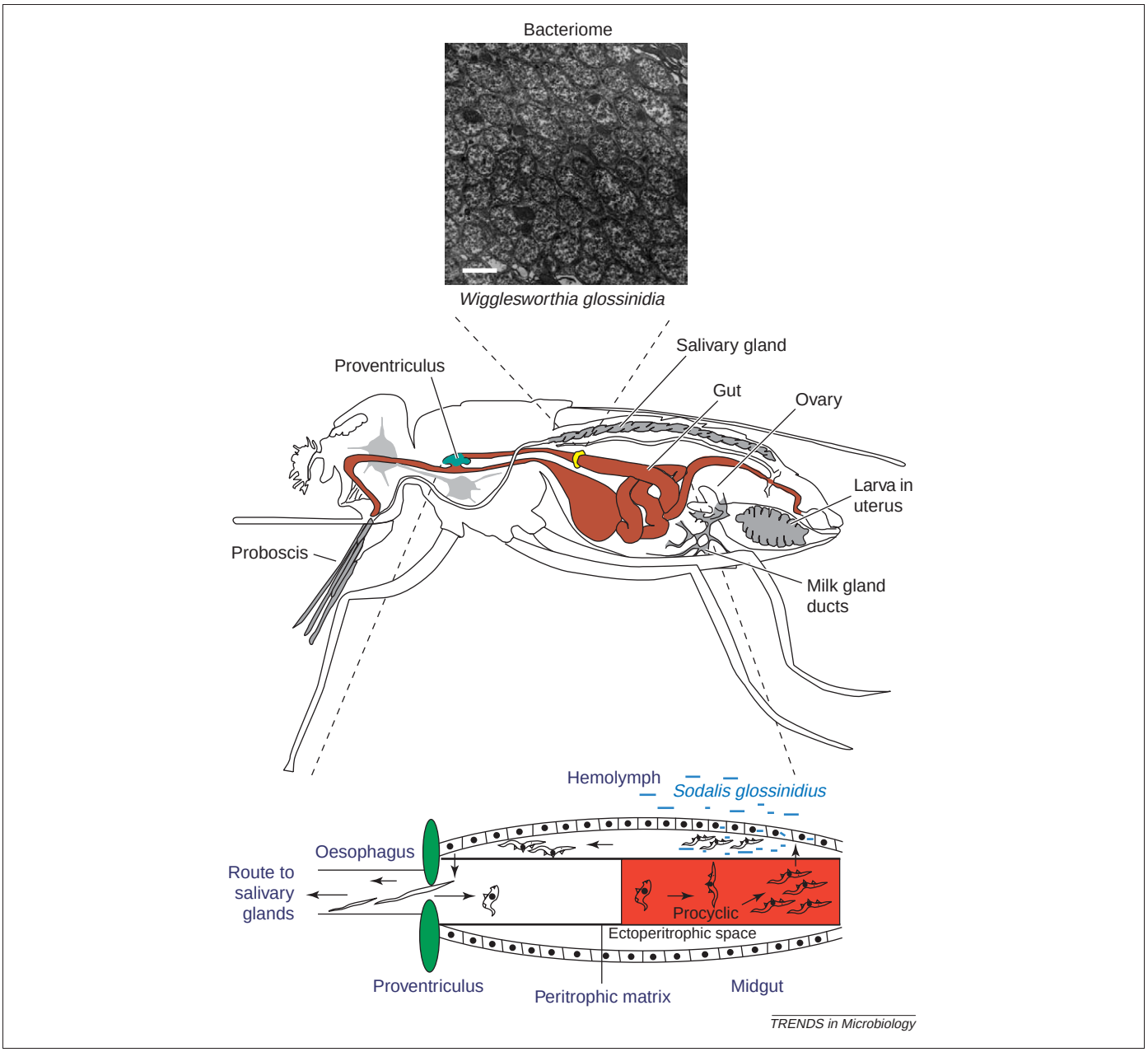

Figure 3. The diagrammatic representation of a female *Glossina*. The long coiled pair of salivary glands (one is shown here), proventriculus organ at the foregut/midgut juncture and bacteriome organ in the gut are shown. The ovary and the larva in the uterus are depicted. Milk gland ducts that provide nutrients to the intrauterine larva can be seen surrounding the uterus. *Wigglesworthia* is housed intracellularly exclusively in the bacteriome structure. The micrograph shows *Wigglesworthia* lying free within the bacteriocyte cytoplasm. *Wolbachia* resides intracellularly in the ovaries, whereas *Sodalis* is primarily associated with gut cells and hemolymph, both intra- and extracellularly. The diagram located below this depicts the differentiation and migration pathways during the establishment phase of the trypanosome lifecycle in the midgut and foregut. Upon entry, bloodstream parasites transform to procyclic cells by day three and enter into the ectoperitrophic space of the gut, escaping the chitinous barrier (peritrophic matrix) that separates gut epithelium from the blood bolus. Initially, the trypanosomes undergo attrition, with the majority succumbing to various insect defenses. In a few of the flies, the cells that survive continue to replicate and establish infections in the midgut. The midgut symbiont *Sodalis* resides in close proximity to where the parasites replicate. After six to ten days, trypanosomes begin to congregate in the proventriculus region and presumably penetrate through the soft peritrophic matrix produced there; they then re-enter the gut lumen to complete their progression from epimastigotes to metacyclics (maturation) either in the salivary glands of the fly, or the mouthparts, depending on the parasite species (not shown here). Early in the infection process, parasite numbers are at their lowest in the ectoperitrophic space after ~3–4 days, and hence effector products produced and secreted by rec*Sodalis* can have an impact on parasite viability.

in carbon compound catabolism, energy metabolism, fatty acid metabolism, cell structure and transport. Despite their close taxonomic relatedness, the genome contents of SOPE and *Sodalis* display differences suggestive of adaptations to their host ecologies, in particular to the different metabolites provided in their host diets. Both symbionts are believed to be anchored tightly to their host biology through restricted metabolic capabilities and therefore might not be able to readily undergo horizontal transmission and establishment in distant insect taxa.

Development of transgenic technologies for vector-borne disease control

In addition to housing beneficial microbes, many insects (vectors) also transmit disease-causing organisms to the animals and plants upon which they feed. Control of these diseases, such as malaria, dengue, African trypanosomiasis,

Chagas disease and leishmaniasis, has re-emerged as an important priority for the medical and scientific community. Development of efficacious vaccines has been difficult, and the emergence and rapid spread of resistance in parasites to the commonly used and affordable drugs has further exacerbated the problems associated with the control of infections in the mammalian host. Similarly, agricultural losses resulting from the various disease agents transmitted to plants by insects continue to threaten the already restricted nutritional resources worldwide. Although control of the insect hosts was traditionally achieved by the use of chemical-based strategies, the emergence of insecticide resistance in many vectors now threatens to further restrict disease management tools. Affordable and environmentally acceptable vector control strategies are needed.

Recent years have seen major scientific advances pertinent to insect cell biology, immunology and developmental biology, and important vector genomes are being sequenced, with *Anopheles gambiae* [45] completed and *Aedes aegypti* in progress. This new knowledge provides an opportunity to strengthen some of the existing approaches and also forms the basis for future vector control strategies. One approach in particular that has received much effort is the development of DNA transformation systems for important vectors, with the goal of vector competence modulation. The success of this approach depends on the development of reproducible and efficient tools to introduce and express foreign genes in insects (genetic transformation system), the characterization of genes (transgenes) whose products could confer pathogen-refractory phenotypes when expressed under appropriate spatial and temporal regulation and, lastly, mechanisms that are environmentally safe and acceptable to society, so that these laboratory-engineered refractory insects can be spread into natural populations (gene driving systems), where they can replace their susceptible counterparts, thereby resulting in disease reduction.

Genetic transformation systems

The process of genetic transformation in many insects has been achieved by the microinjection of various transposable elements (plasmid or viral vectors) into synctial embryos (germline transformation) [46]. The transposable elements insert themselves randomly into insect DNA, resulting in germline transformation, whereby the transgene is passed on to every individual cell of the genetically modified organism. Marker genes carried by the transposable element help to identify transgenic individuals. It has now been possible to reproducibly introduce foreign genes into the important mosquito vectors transmitting malaria [47–49]. By using tissue-specific expression systems, transgene products can be synthesized where they can affect parasite viability; i.e. in the gut [50], salivary gland or hemolymph [51]. Recently, a molecule that interferes with *Plasmodium* development in mosquitoes has been successfully expressed in transgenic *Anopheles* mosquitoes and its adverse impact on malaria transmission has been demonstrated [52].

The viviparous reproductive biology of tsetse flies has hampered the application of the germline transformation technology. However, it has been possible to exploit the commensal *Sodalis* that lives in close proximity in the gut to pathogenic trypanosomes, to express foreign gene products that could affect parasite viability (symbiont-based transformation system known as paratransgenesis) [37]. Similarly, it has been possible to exploit *Rhodococcus* to express foreign gene products in triatomine bugs [22]. Through such an approach, the insect cells are not transformed, as in the germline transformation approach, but instead transgenes are expressed in the symbiotic bacteria (somatic transformation). The specific associations the symbionts have established with host populations, their physical proximity to the developing pathogenic agents in insect tissues and the vast information available on prokaryotic transformation and gene expression mechanisms make the beneficial symbionts highly desirable expression vehicles that can be exploited for the control of a variety of vector-borne diseases. Box 1 describes some of the requirements for a successful symbiont-based insect transformation approach for disease control and its specific application in tsetse flies (Figure 4).

The availability of an *in vitro* culture system for *Sodalis* has allowed for the development of a genetic transformation system to introduce and express foreign gene products [3,43]. The broad host range replicon *oriV* derived from a *Pseudomonas aeruginosa* plasmid was used to construct a shuttle vector, which was then introduced into *Sodalis*, where it was found to replicate extrachromosomally and confer resistance to multiple antibiotics. Experiments in progress with the *Sodalis* expression system aim to increase the level of the transgene product by the use of endogenous promoters and incorporation of

Box 1. Requirements for efficacious application of the symbiont-based insect transformation approach for disease control

- The availability of naturally harbored symbionts that can be isolated and cultured.
- Knowledge on the transmission mode as well as the population dynamics of the symbiont throughout the host lifecycle.
- An efficient transformation system that produces stable phenotypes.
- Availability of effective anti-pathogenic products (transgenes) such as antimicrobial peptides or transmission-blocking monoclonal antibodies.
- Expression of such transmission-blocking molecules by symbionts without compromising fitness.
- Ability to repopulate hosts with the modified symbionts.
- Synthesis of the transgene products in the tissues or insect compartments that can impede pathogen lifecycle.
- Evaluation of the potential emergence of resistance by target pathogens to the transgene products.
- Lack of any fitness cost for the repopulated insect host in comparison to their wild-type counterparts.
- Availability of effective gene-driving mechanisms to replace field populations with engineered lines.
- An ecological assessment of potential spread of modified symbionts and barriers of dispersal to non-target organisms.
- An in-depth analysis of the potential harmful effects of symbionts or transgene products to humans and animals.
- An environmentally sound implementation project for delivering insects repopulated with modified symbionts into the field.

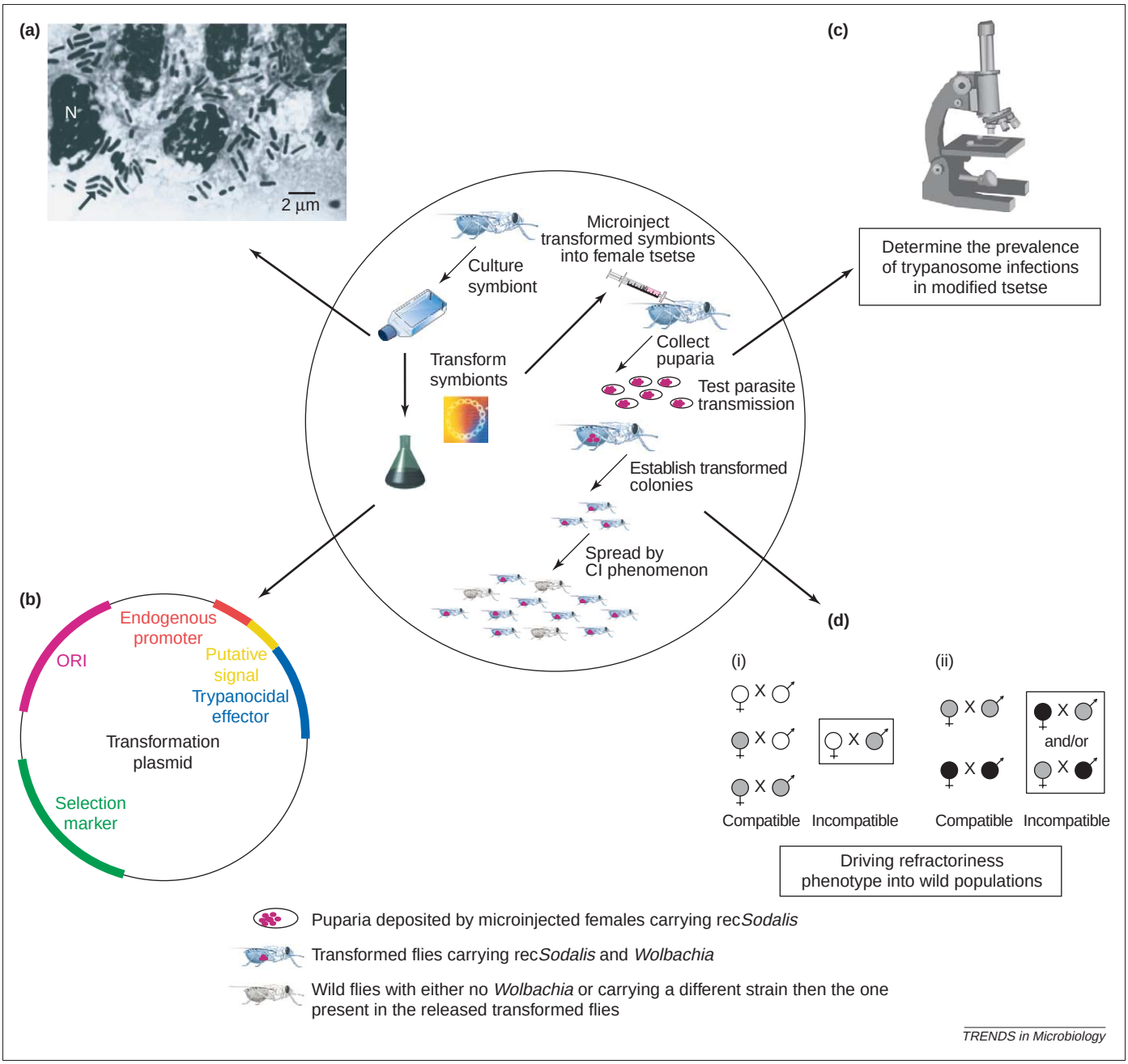

Figure 4. An approach depicting the symbiont-based transformation system and its potential application in tsetse flies for reducing African trypanosomiasis. **(a)** *Sodalis* were isolated from hemolymph and cultured initially *in vitro* on a feeder cell layer (*Aedes albopictus* C6/36). Subsequently, *Sodalis* was grown in cell-free medium, as well as plated on solid media under microaerophilic conditions. **(b)** A plasmid containing the trypanocidal effector that is expressed and secreted by an endogenous control element with spatial and temporal specificity along with a selection marker gene can be used to transform *Sodalis*. Recombinant *Sodalis* (rec*Sodalis*) can be microinjected into pregnant mothers, where it is vertically transmitted. **(c)** The offspring harboring modified symbionts can be challenged with trypanosomes. Microscopic examination of fly tissues can determine the prevalence of parasite infections and the extent of the refractoriness conferred by the transgene product. **(d)** Colonies harboring the rec*Sodalis* can be established to replace their susceptible counterparts in nature by virtue of the cytoplasmic incompatibility (CI) phenomenon conferred by *Wolbachia* symbionts. This approach requires that either the established colony should have a *Wolbachia* infection (depicted here with blue flies), with the native population being uninfected, or that the colony carries a different *Wolbachia* strain to that found in the field population. The cartoon describes the expected outcomes of these crosses. In the CI phenomenon, the sperm modification in infected males can only be rescued by infected females. In (i), CI is expressed when uninfected females (open circles) mate with infected males (hatched circles), resulting in loss of progeny, (unidirectional incompatibility). This allows the spread of the infected (hatched) phenotype by virtue of the reproductive advantage it confers to the infected type. In (ii), CI is also expressed when infected females mate with males that are infected with a different *Wolbachia* strain or with multiple *Wolbachia* strains (bidirectional incompatibility). This will allow for spreading genes into a population already infected with *Wolbachia* or when a second wave of spread is required over an existing infected population. By inflicting these mating incompatibilities, *Wolbachia*-infected insects can replace naïve populations while simultaneously driving other maternally inherited elements such as rec*Sodalis* into the tsetse fly populations.

native secretion signal sequences into the expression construct. The ongoing genome project of *Sodalis* now provides the opportunity to explore such endogenous transcription regulatory elements for foreign gene expression. In the *Rhodococcus* expression system, a shuttle plasmid was constructed using the origin of replication from a plasmid naturally harbored in these cells [22]. To improve the stability and environmental safety of the symbiont transformation approach, direct insertion of the transgene into the bacterial chromosome is

desirable, as has been achieved for *Rhodococcus rhodnii* [53], to eliminate the possibility of the extrachromosomal construct moving into non-target organisms.

The *in vitro* manipulated recombinant *Sodalis* (rec*Sodalis*) was found to be successfully acquired by progeny when microinjected into the female parent hemolymph. The recombinant symbionts have been shown to be passed on to multiple generations, where they successfully express the marker gene product, green fluorescent protein [37]. In yet another insect system, the facultative accessory symbionts of aphids has also been shown to be maternally transmitted to offspring with high fidelity between different host species using a similar hemolymph microinjection approach. For reintroduction of the engineered symbiont into *Rhodnius*, nymphs were reared in sterile chambers from surface-sterilized eggs, and the transformed symbionts were delivered to aposymbiotic nymphs by membrane feeding [54]. Symbiont transgenics are also currently being investigated to inhibit the spread of plant diseases such as Pierce's disease of grapevines in California. Several sharpshooter species (Homoptera: Cicadellidae) transmit the bacterium *Xylella fastidiosa*, the causative agent of Pierce's disease. A potential transgene vehicle *Alcaligenes* sp., a nonpathogenic plant endophyte, has been isolated from the cibarium of the sharpshooter [55]. Studies in progress show that the bacterium can be transformed to express foreign marker gene products and be inoculated into host plants, where it establishes infections (T. Miller, pers. commun.).

Characterization of antipathogenic gene products

The discovery of gene products that can have an adverse effect on pathogen development (effector genes) when expressed in a vector background is a crucial step in transgenic technology. The identification of monoclonal antibodies (mABs) with parasite-transmission blocking characteristics, and their subsequent expression as single-chain antibody gene fragments, provides a vast array of potential antipathogenic effectors. Towards this goal, transmission-blocking antibodies affecting the major surface protein procyclin of the insect-stage procyclic trypanosomes have already been reported [56]. The feasibility of expressing single-chain antibody fragments, which retain their functional activities, has been demonstrated in *Rhodococcus* [57], as well as in human commensal flora [58–60].

Alternatively, insect immunity genes that naturally confer resistance to pathogens can be pursued as potential effectors. Because most insects mount significant immune responses to the presence of pathogens and can effectively clear these infections, the immunity genes are good candidates to be further explored. During an infection process, it is thought that these natural immunity responses might be elicited either too late to combat the parasites, or might not be expressed in the correct compartments, where they can effectively clear the pathogens, resulting in the successful development and transmission of parasites in a few of the exposed insects. In tsetse flies, the complex lifecycle of the parasite might take from a few days for *Trypanosoma vivax* to three to four weeks for *Trypanosoma brucei* subspecies [61]. During their initial stages of development, mammalian-stage trypanosomes differentiate to procyclic cells and replicate in the posterior midgut in close proximity to where the *Sodalis* symbionts reside. Not all flies exposed to a parasite-infected blood meal will eventually transmit the parasite; in fact, in only a few exposed flies do parasites actually successfully achieve their transmission to the next mammalian host [62]. In experiments where the immune system of the newly emerged adult tsetse fly was stimulated by microinjection of either *E. coli* or lipopolysaccharide before providing the infectious blood meal, the prevalence of trypanosome infection rates in the tsetse midgut was substantially lowered [63]. This indicated that tsetse fly immune components could interfere with parasite development. Further characterization of the induced responses in such immune-challenged tsetse flies suggested that the antimicrobial peptide attacin could be playing a role in parasite clearance. Attacin has been well characterized in *Drosophila melanogaster* as an inducible immune peptide with specificity against some Gram-negative bacteria and protozoa [64]. To examine the feasibility of expressing attacin in the Gram-negative *Sodalis*, we expressed *Glossina* attacin in a *Drosophila* cell line and showed that the recombinant protein did not interfere with *Sodalis* viability (S. Aksoy, unpublished). In fact, the sensitivity of *Sodalis* to diptericin, another immune peptide with known antimicrobial activity, was also found to be significantly lower than that of *E. coli* [63]. The greater resistance of *Sodalis* to its host immune molecules might reflect its constant exposure to these products.

Constitutive and abundant expression of another immunity molecule (the antimicrobial peptide cecropin isolated from the cecropia moth) in *Rhodococcus* has been found to reduce the development of *Trypanosoma cruzi* infections in hindgut [65]. It is now important to undertake experiments to evaluate the fitness of both the recombinant symbionts and the insects harboring these modified symbionts. Any cost of fitness for either the symbiont or insect biology would not be desirable, as they will not be able to compete with their natural counterparts and hence be eliminated in nature over time. Equally vital, no matter which transformation approach is used, is to consider whether over time the pathogens being targeted could develop resistance to the transgene product(s). With transmission blocking mABs, it is possible to have a multitude of potential antiparasitic effectors, which can be sequentially expressed in bacteria to inhibit the development of resistance. Alternatively, several transgenes can potentially be simultaneously expressed in the symbiont expression system to deter resistance against any one individual target.

Possible gene driving systems

An important applied aspect of all transgenic work is the ability to spread the laboratory-engineered phenotypes into natural populations. The *Wolbachia* symbiont, which has infected a wide-range of invertebrate hosts [66], including several tsetse fly species, provides one potential drive mechanism. The functional presence of *Wolbachia* has been shown to result in a variety of reproductive

abnormalities in the various invertebrate hosts they infect. One of these abnormalities is termed cytoplasmic incompatibility (CI), and when expressed, commonly results in embryonic death because of disruptions in early fertilization events [67]. In an incompatible cross, the sperm enters the egg but does not successfully contribute its genetic material to the potential zygote, and in most species this results in none or very few hatching eggs. The infected females have a reproductive advantage over their uninfected counterparts, as they can produce successful progeny with both the imprinted and normal sperm eventually allowing the *Wolbachia*-infected insects to spread into populations. As *Wolbachia* spreads itself into populations, it will also spread other maternally linked traits and organelles such as mitochondria [68] and *Sodalis* [69].

Although in a field survey many tsetse fly populations have been found to be infected by different strains of *Wolbachia* [35], the extent of the CI phenomenon needs to be evaluated to assess the utility of this gene-driving system in the tsetse fly. Analysis of the laboratory colonies has shown that 100% of the sampled individuals carry *Wolbachia* infections, making the analysis of *Wolbachia*-mediated effects impossible by traditional mating experiments [35]. Most functional studies in other systems have involved curing insects of their *Wolbachia* infections by administering antibiotics in their diet. These cured uninfected lines were then used to test for CI with the original infected individuals. This approach, however, has not been feasible in tsetse flies because the antibiotic treatment of flies results in the clearing of all bacterial symbionts, including *Wigglesworthia*, which results in fly sterility. In the different field populations examined, however, the infection prevalence shows variability unlike colony flies [35]. Perhaps *Wolbachia* infected and uninfected lines can be developed from these polymorphic field populations and used to elucidate the functional role of this organism in tsetse fly biology. Although no naturally occurring horizontal transfer of *Wolbachia* has been observed, it has become increasingly common to experimentally transfer *Wolbachia* between different hosts and even into insects with no prior infection history [69]. Hence, the introduction of different *Wolbachia* strains into the tsetse fly colonies provides an alternative means to test the CI effects.

For spread of symbionts that are not maternally linked but acquired environmentally, such as the symbionts of the reduviid bugs, alternative approaches will need to be developed. One approach that has had considerable success in cage settings is a simulated fecal paste, termed CRUZIGARD, comprised of an inert guar gum matrix impregnated with the modified symbiont. Studies have shown that aposymbiotic nymphs exposed to a preparation of CRUZIGARD reached sexual maturity at a rate comparable to similar nymphs exposed to natural feces [70]. Additional experiments are in progress to evaluate the environmental implications for such a release strategy.

Potential roles of trypanosome-refractory tsetse flies in disease control

The eventual replacement of parasite-susceptible vector populations with the engineered refractory flies could provide an additional strategy to reduce disease. If *Wolbachia* infections in tsetse flies do express strong CI phenotypes, the two symbiotic systems can be coupled to mediate the spread of the refractory phenotypes conferred by the rec*Sodalis* into natural populations. It is important that the fidelity of the maternal linkage between the two symbiotic systems remains high, to ensure that the transgene will not be uncoupled from the driving system. A strong linkage has been demonstrated between *Wolbachia* and the maternally associated mitochondria in *Drosophila* [68]. At the present time, little is known about *Sodalis* dynamics in natural tsetse fly populations, or about the different ecological factors that might affect its biology, such as its density. Other environmental questions with respect to the potential for non-maternal transfer of *Sodalis* and its presence and transmission in non-target insects also need to be investigated. It is, however, encouraging that the *Sodalis* genome appears to have been tailored to subsist on its host diet, vertebrate blood, as a comparative genome-wide hybridization study with its phylogenetic relative SOPE (symbiont of weevil) has shown. This genome adaptation suggests that *Sodalis* has a restrictive host range, although it still needs to be demonstrated if it can establish infections in other hematophagous hosts.

The availability of engineered trypanosome-refractory tsetse flies can also enhance an existing disease control strategy. One such strategy is the sterile insect technique (SIT), a genetic population suppression approach, involving sustained systematic releases of irradiated sterile male insects into the wild population. The insects to be released are mass reared in large-scale insectaries and males are sterilized by irradiation before their release in a selected area. Releasing sterile males in high numbers over a period of three to four generations, after having reduced population density by other techniques (i.e. trapping or insecticide spraying), can lower the reproductive capacity of the target population, eventually leading to its eradication [71,72]. The recent successful eradication of *Glossina austeni* from the island of Zanzibar by SIT has demonstrated the feasibility and applicability of this technology in integrated tsetse fly control programs [72]. Tsetse flies that are incapable of transmitting trypanosomes have the potential to improve the current SIT technology by enhancing its efficacy and safety and reducing its cost in future programs. Because the large numbers of male flies released can potentially contribute to a temporary increase in disease transmission, the incorporation of refractory traits into the SIT release strains will greatly enhance the efficacy of this approach, especially in human disease endemic foci. The use of *Wolbachia*-mediated CI as a method of inducing sterility, as an alternative or in addition to irradiation, would further enhance this technology. With CI, the released strain of tsetse fly would carry a *Wolbachia* infection that would be incompatible upon mating with wild females. The competitiveness of these males would be expected to be higher than in irradiated males, and, as a result, fewer insects would need to be released, thereby decreasing the cost of the approach significantly, given that the released flies are refractory [73].

Concluding remarks

The advancements in the field of symbiosis bring forth new avenues that can lead to a broad range of applications, ranging from the protection and treatment of their partners against harmful agents (invading pathogens) to the manipulation of undesirable host traits (i.e. pathogen transmission in the case of vector insects). For the many insect-borne diseases of animals and plants, for which adequate control is currently lacking, these strategies promise novel control tools. The commensals of insects can be genetically manipulated to express products that impede the development and transmission of various harmful pathogens [1]. Understanding effective gene-driving mechanisms, the ecological consequences of the replacement strategies and the safety aspects of both the transgene products and the engineered insect phenotypes are important questions for future work. The principles of this approach are being widely debated among scientists at large [74], and to evaluate the efficacy and feasibility of this strategy, various international committees are currently being established [75].

Approaches that exploit natural commensal flora have therapeutic applications in diverse hosts, including humans. The 'hygiene hypothesis' postulates that the frequency of allergy has increased because of decreased microbial exposure early in life, leading to the delayed maturation of immunity [76]. It has also been found that early colonizing commensals, by altering host gene expression [77] and possibly through interspecific communication (i.e. quorum-sensing), can have a profound impact on the microbial ecology of their particular hosts by helping to determine what other bacterial species will be tolerated and, furthermore, be able to colonize [78,79]. Recently, alterations in the host microflora have been explored for the prevention and management of pathophysiological disorders such as inflammatory bowel diseases [80]. In addition, therapeutic antibodies, vaccine antigens and cytokines have been expressed *in vivo* through the genetic engineering of normally occurring microflora for the delivery of passive immunity [60,81,82]. The success of these applications requires a good understanding of host–symbiont–pathogen interactions, including their cellular and developmental biology as well as information on symbiont genomics and genetics. The manipulation of the pre-existing microbial flora might form the basis of future control strategies that can be integrated into the existing arsenal in our fight against devastating diseases.

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