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Insect-symbiont systems: from complex relationships to biotechnological applications

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Abstract

Microbial symbiosis is a ubiquitous aspect of life and was a major element in the ability of insects to explore several adverse environments. To date, the study of symbiosis in insects has been impaired by the unculturability of most symbionts. However, molecular methods have demonstrated to be powerful tools to understand insect-microorganism associations and to disclose new symbiont-host systems. Beyond playing an essential role in nutrition and development of the insects, symbionts can produce bioactive compounds that protect the host against adverse environmental conditions, predators and/or direct competitors. Since the search for natural bioactive products and new enzymes is a developing area, understanding the diversity and nature of symbiont-host relationships pave the way for the exploitation of new resources in biotechnology. Furthermore, genetic transformation of the symbionts with genes that code for compounds that are toxic for pathogenic and phytopathogenic agents is also a promising area of application of the insect-symbiont relationships. The search for new bioactive compounds, the use of symbionts for pest and disease control and the molecular strategies applied for these purposes are issues particularly interesting for innovative biotechnological applications and will be addressed in the present review.
1 Introduction

1.1 General aspects of symbiosis

Many microorganisms have established strict and persistent interactions with higher organisms in a relationship termed symbiosis. According to the fitness effects on the partners, the symbiotic association can be referred as parasitism (relationship between two species in which one benefits at the expense of the other), commensalism (relationship in which one species benefits from the interaction, while the host is neither positively nor negatively affected) or mutualism (interaction that is beneficial to both species). Depending on the location of the symbiont in host cells, the relationship can be classified as endosymbiosis (symbiont is located inside the host cells) or ectosymbiosis (symbiont is located outside the host cells). Regarding the degree of dependence between the partners, symbionts can also be classified as primary (P-symbiont or P-endosymbionts) or secondary (S-symbionts). The first ones are intracellular and have a codependence relationship with the host. Usually they share a long evolutionary history with it and are vertically transmitted to the progeny. S-symbionts seem to have established more recent associations with their hosts and frequently retain the ability to return to a free-living condition. They can be located both inside and outside the host cells and can be transferred vertically or horizontally. In some cases S-symbionts can evolve to become obligate partners and, if a P-symbiont already exists, a microbial consortium can be established, working to fulfill host needs [1]. Invertebrates show a huge diversity of symbiotic relationships and, in the particular case of insects, it was estimated that 15-20% of them have established symbiotic associations with microorganisms. These interactions probably played a central role in their evolutionary success, allowing...
1.2 Nutritional basis of microorganism-insect symbiosis

The relationships between P-symbionts and insects are mostly nutritionally based. The microorganism provides nutrients that are scarce or unavailable in the insect diet and the symbiont gets a permanent supply of several metabolites produced by the host. To date, only bacteria were identified as P-symbionts.

Most insect endosymbionts that have been studied can provide essential amino acids, vitamins and cofactors to the host and contribute to nitrogen and sulfur metabolism [2, 3]. These symbionts are located in specialized host cells named bacteriocytes that constitute the bacteriome [4]. This structure enables the host to control symbiont spread and transmission and favors the exchange of nutrients and metabolites between both partners [3]. Endosymbionts have smaller genomes than their free-living relatives, as a result of the symbiont-host relationship [5]. Although the mechanisms involved in genome reduction are not clear, the consequences are the massive loss of genes and functions that become unnecessary in the “new” intracellular environment. However, genes required for host survival are maintained [5]. \textit{Buchnera, Blochmannia, Wigglesworthia, Baummania} and \textit{Carsonella} that are symbionts of aphids, carpenter ants, tsetse flies, sharpshooters and psyllids, respectively, are the most studied examples [1, 4]. All these symbionts have similar roles in the relationship with their hosts, providing vitamins and cofactors (\textit{Wigglesworthia} and \textit{Baummania}) or essential amino acids (\textit{Buchnera} and \textit{Blochmannia}) [2, 4, 6].

Genome sequencing shows a comparable pattern of genomic reduction, with the
maintenance of the functional genes that allow the fulfillment of the host
nutritional requirements. Insect-microorganism interactions are usually very
complex, especially when the nutrient source of the insect is highly deprived or
unbalanced. The recent molecular studies of symbiotic associations allowed the
detection of stable microbial consortia associated with insects and the evaluation
of the contribution of each member to the relationship. Each insect can harbor up
to six different symbionts working as a consortium to complement all the host
needs [7]. One consortium that has been described includes Buchnera aphidicola
BCc and Serratia symbiotica SCc from the aphid Cinara cedri [8]. Although S.
symbiotica is considered a secondary symbiont, its presence in the bacteriome
was observed and there are evidences that this bacterium has potential to
replace B. aphidicola. Another reported example includes two P-symbionts
(Sulcia muelleri and Baumannia cicadellinicola) that have complementary
functions in the biosynthesis of several essential nutrients in xylem-feeding
insects. Sulcia muelleri is a typical nutritional symbiont from the phylum
Bacteroidetes. Phylogenetic analysis indicated that this association may
represent the oldest example of bacterial symbiosis [9]. This bacterium is
associated with several clades of insects from the group Auchenorrhyncha,
sharing an evolutionary history with their hosts. In the xylem-feeding
sharpshooter Homalodisca coagulata, Sulcia muelleri is present in the
bacteriome, together with Baumannia cicadellinicola [6]. Genome analysis
indicated that a significant number of B. cicadellinicola genes are assigned to the
biosynthesis of vitamins and cofactors and that most amino acids biosynthetic
pathways are lacking. On the other hand, S. muelleri has retained most genes
involved in the production of essential amino acids. A similar association occurs
with the obligatory S-symbiont *Sodalis glossinidius* of the tsetse fly *Glossina morsitans*. This bacterium is responsible for the synthesis of several essential amino acids, complementing the function of the P-symbiont *Wigglesworthia glossinidia* which is the supplier of most vitamins and cofactors [10].

### 1.3 Other functions associated with microbial symbionts

Although many microorganism-insect associations are based in nutrient and metabolite exchange, there are evidences of the involvement of symbionts (particularly S-symbiont) in other functions. Among the possible beneficial roles that have been associated to S-symbionts are the rescue from heat damage (aphids that have acquired "*Candidatus Hamiltonella defensa*" or "*Candidatus Regionella insecticola*" showed an improved survival when exposed to high temperatures [11]) and the contribution to host specialization, fitness and genomic differentiation [12, 13, 14]. Several examples of host protection from predators, pathogens and parasites are also reported [15], as in the case of the bacterial symbiont of *Paederus* beetles, which produces a polyketide toxin that protects the larvae against wolf spiders [16]. "*Candidatus R. insecticola*" is involved in aphid resistance to the pathogenic fungus *Pandora neoaphidis* [17]. In *Anopheles gambiae* there are evidences that the production of anti-plasmodium factors in the mosquito gut is induced by its natural bacterial flora [15]. *Serratia symbiotica* and "*Candidatus H. defensa*" protect the host *Acyrthosiphon pisum* from the parasitoids *Aphidius ervi* and *Aphidius eadyi*, apparently by causing high mortality in parasitoid larvae [18, 19]. Additionally, symbionts can produce enzymes involved in host digestion of food material and detoxification [20].
Nonetheless, not all symbiotic relationships are beneficial to the host. One of the best characterized examples is *Wolbachia* which is a symbiont of many arthropods. This bacterium is maternally transmitted with high efficiency, but also goes through extensive horizontal transfer, sometimes between distantly related hosts. It is present in many insect species and probably played an important role in their evolution [21]. *Wolbachia* invades insect populations by manipulating their reproductive systems and reducing the fecundity of uninfected females that mate with infected males. In other cases, infected females may be transformed to go through parthenogenic reproduction, producing only infected progeny. Surprisingly, in filarial nematodes, *Wolbachia* is an obligate mutualistic symbiont, playing a fundamental role in their reproduction [22, 23]. Many complex mechanisms of insect-microorganism interactions are unclear mostly because symbiont cultivation and maintenance is frequently difficult or impossible. However, the production of bioactive metabolites is involved in several symbiont-host systems, representing an unexplored source of new compounds. The recent introduction and generalization of genomic and molecular approaches is disclosing many practical aspects of symbiosis, opening a new biotechnological research area with many application fields.

2 Molecular methodologies in the study of insect symbionts

2.1 Genomic analysis

To date, culture procedures have been unsuccessfully attempted for primary symbionts [1, 2]. Furthermore, many secondary symbionts are also considered uncultivable [1, 8]. In such a scenario, molecular methods have demonstrated to be powerful tools to study microorganisms associated with insects. In the last few
years, genome sequencing and culture independent methods, namely metagenomics and transcriptomics, have brought new insights to the study of symbiosis. DNA sequence data have been the first information source in the study of symbiotic relationships, allowing the identification of symbionts, the reconstruction of host-symbiont phylogenies and the analysis of symbiont genes and genomes. Presently, genome sequences are available for several bacterial symbionts (Table 1), but this was only possible after overcoming several technical hindrances. Intracellular symbionts are usually present in high numbers in the host cells and are restricted to the bacteriome, facilitating the isolation of their DNA without substantial contamination with host DNA. However, some secondary symbionts are not located in specific tissues of the host and/or are present in low numbers. Therefore, DNA isolation can become a hard task with low recovery of genetic material. Some advances in DNA technology have enabled the use of improved methods to overcome this problem. Multiple displacement amplification (MDA) or whole genome amplification (WDA) is a strategy based on the use of phi29 DNA polymerase. This enzyme obtained from phage Phi29 of *Bacillus subtilis* is a highly processive polymerase which allows a highly efficient isothermal DNA amplification [24, 25]. Phi29 DNA polymerase also possesses a proofreading activity that ensures a high fidelity [26]. This strategy has been successfully applied for genome amplification of secondary symbionts, using minimal amounts of template DNA recovered from PFGE gels or from DNA extracts obtained from insect hemolymph [27, 28]. The use of phi29 DNA polymerase based techniques may allow a rapid progress in genome sequencing of secondary uncultured symbionts.
Whole genome sequencing approaches and sequence analysis were essential to clarify some issues related with symbiosis and unveil the molecular pathways that regulate symbiont-host relationship [1, 2]. Functional analysis has disclosed the nutritional role of P-symbionts and provided groundwork for further physiological studies. Through their genome-reduction process, P-symbionts like *Buchnera*, *Baumannia*, *Blochmannia* or *Wiggeworthia*, have retained the genes implicated in the biosynthetic pathways of essential metabolites absent in the insect diet (amino acids, vitamins, cofactors, etc.) [2]. Genomic studies also enabled the reconstruction of symbiosis history, contributing to explain some coevolutionary aspects of these complex relationships and disclosing the existence of multiple symbiotic associations with complementary metabolic and genomic functions [6, 7, 29]. Since uncultured microorganisms are frequently detected in these consortia [7], suitable molecular methods are required for their study. Metagenomics (defined as the use of genome based methods to sequence DNA fragments from microorganisms in environmental samples) can be a valuable tool to analyze the genomic and functional aspects of these complex systems. Sequencing procedures can be directed for selected regions (e.g. those containing interesting rRNA genes or functions) or used in a shotgun approach [30]. This strategy has a large potential to be applied in complex insect-symbiont systems and was already used to study the relationship between the segmented worm *Olavius algarvensis* and its bacterial consortium that provides essential nutrients [31].
2.2 Gene expression and regulation

Genomic data have revolutionized the perception of symbiotic relationships and brought into light questions concerning gene regulation and expression in endosymbiotic systems. Transcriptomic analysis has been applied to clarify some of these aspects. Symbiont gene regulation was studied in *Buchnera aphidicola* using a full genome microarray and quantitative reverse transcriptase PCR (qRT-PCR) for examining the transcriptome responses to amino acid dietary changes [32]. The results confirmed that irreversible genome losses occur during the symbiont adaptation to intracellular life, affecting transcriptional regulators and restricting the symbiont ability to control gene expression in response to environmental fluctuations. Regarding the role of the bacteriocyte in the symbiotic relationship, expressed sequence tags (ESTs) and qRT-PCR were used to identify bacteriocyte specific expressed genes in *Acyrthosiphon pisum* [33]. The results revealed that most of these genes are involved in functions that complement the symbiont activity, namely non-essential amino acid metabolism, transport and lysozyme production. PCR-subtracted cDNA and reverse Northern analysis were applied in a similar study in *Sitophilus zeamais*, and demonstrated that the bacteriocyte increases sugar uptake and metabolism in response to the presence of the symbiont [34]. In addition, metabolic coordination between *A. pisum* and *B. aphidicola* was observed under heat shock stress by a dual-genome microarray [35], indicating that this strategy is a useful tool to understand the functional mechanisms of symbiotic systems.
3 Bioactive compounds

3.1 Evidences of the microbial origin of bioactive compounds

Natural compounds have always been a main target for new drug and chemical exploration. The search for bioactive compounds (antitumor and antimicrobial), as well as new enzymes, is a developing area in a context of constant new challenges. Microorganisms are an important source of these compounds, mainly due to their interactions with a huge number of other organisms and environments. Symbiotic associations have been very promising for this purpose. In several groups of organisms there are strong evidences that such compounds are produced by endosymbionts [36]. Neurotoxins and diarrheic toxins that have high impact in human health are believed to be produced by dinoflagellate associated bacteria. Nevertheless, sponges and their microbial symbionts are considered the most productive group with thousands of new compounds being reported every year [37] and some of them already included in drug trials. Many of these compounds are similar to complex polyketides or nonribosomal peptides, typical from bacteria [36]. Some examples include: (i) halichondrin B, firstly isolated from the sponge Halichondria okadai, and presenting a strong antitumor activity; (ii) mycalamide B, an antiviral compound obtained from a sponge of the genus Mycale; and (iii) jaspamide, obtained from Hemiastrella minor, which presents antifungal activity. Although less studied, tunicates are comparable with sponges in their natural product chemistry [36]. A remarkable example is ecteinascidin, which is one of the most advanced antitumor drug candidate from a marine source. Closely related metabolites have been found in the bacterial groups actinomycetes (safracin) and pseudomonads (saframycin Mx1), as well as in sponges (renieramycin). One of the most promising
compounds is bryostatin, obtained from the marine bryozoan *Begula neritina* and currently is an advanced phase of clinical trials. The polyketide structure of this compound indicates a bacterial origin [36] and its production was associated to the presence of a new \( \gamma \)-proteobacterium, “*Candidatus* Endobugula sertula”, discovered in this bryozoan. In addition, treatment of *B. neritina* with antibiotics resulted in a consistent reduction of bryostatin content and “*Candidatus E. sertula*” cell number. These findings suggest a link between production of the polyketide and the presence of the bacterium. Many other examples arise from protozoa, plants, crustacea and vertebrates [36].

Due to the large number of symbiotic associations in the group of insects, these systems are highly promising for the search of new compounds with biotechnological application. This potential is supported by several examples (Table 2). The planthopper *Nilaparvata lugens* lives in association with *Enterobacter* sp. which synthesizes andrimid, a compound also found in marine bacteria from sponges and tunicates. Andrimid is a strong antibiotic against some pathovars of the rice plant pathogen *Xanthomonas campestris* [36]. Antimicrobials were also found in the locust *Schistocerca gregaria* harboring the symbiont *Pantoea agglomerans* but were absent in axenic individuals of this species, suggesting the bacterial origin of these compounds [38]. One of the most studied examples of a bioactive metabolite produced by an insect symbiont is the toxin pederin, due to its high activity in antiviral and antitumor assays. This toxin was found in the hemolymph of rove beetles from the genera *Paederus* and *Paederidus*, which use it as a defense mechanism against predators. Pederin is structurally almost identical to mycalamides, onnamides, theopederins and icadamines that have been isolated from sponges and belong to the polyketic
family [39]. The unexpected detection of pederin-like compounds both in insects and sponges is an additional evidence of their bacterial origin, and the production has been attributed to members of the genus *Pseudomonas* [16]. Among attine ants, a new antifungal compound was detected as being produced by a bacterial symbiont of the family *Pseudonocardiaceae*. [40]. These ants have the ability to cultivate their own food by producing fungal gardens in their colonies, but this garden fungus is frequently infected by another fungus (*Escovopsis* sp.) that destroys entire colonies. In fact, ants from the genera *Acromyrmex* and *Atta* have *Pseudonocardiaceae* bacteria associated with specific ventral portions of their cuticle, which produce a substance promoting the growth of the garden fungus and inhibiting the pathogenic one. Although not yet characterized, this substance is promising as a selective antifungal.

Besides bioactive compounds with clinical or environmental interest, the search for new enzymes is of most importance for industrial purposes. The functional screening of DNA libraries from microbial DNA recovered from intestinal tracts of insects of the order Isoptera (termites) and Lepidoptera (moths) indicated the presence of unusual microbial xylanases. Sequence analysis of these hydrolytic enzymes revealed the presence of new domains that can correspond to unknown functions and increased activities [20].

### 3.2 Advantages of bioactive compounds of microbial origin

Natural bioactive compounds of several organisms are difficult to obtain in sufficient amounts to perform the assays required to evaluate their biological activity. If the real producers are microorganisms involved in symbiotic systems, this limitation can be easily overcome. On one hand, secondary symbionts that
can be cultivated without losing the ability to produce the active metabolite may be subjected to fermentation technology to produce large amounts of the compound. On the other hand, when culture is not possible, alternative molecular methods can be exploited since, unlike their hosts, genomes of microorganisms are small. Additionally, genes coding for biosynthetic pathways in prokaryotes are usually arranged in operons. These traits greatly facilitate cloning and expression of these pathways. Technology for cloning and expression of prokaryotic genes is well advanced and is totally feasible, allowing the production of large amounts of the target metabolite in a culture system. Furthermore, the structural similarity of the bioactive metabolites is likely to be related to coding genes that share a high degree of homology (e.g. transacyltransferases – PKS – that are responsible for synthesis of the core carbon of polyketides or nonribosomal peptide synthetases – NRPS). Individual mutations in these genes may originate new compounds [41]. Pederin was the first product from a symbiont for which the biosynthetic operon was sequenced [39] and can be referred as an example. Sequence analysis of this operon predicted a larger compound, more similar to onnamide, previously isolated from sponges. However, one of the PKS genes was interrupted, probably leading to a smaller peptide – pederin [41]. Therefore, sequence similarity enables the search for new compounds in other symbionts, using genomic based approaches and bioinformatic tools (PCR with specific primers, Southern blot hybridization, sequencing, metagenomics, sequence homology search and phylogenetic analysis) [36, 42, 43].

Even though prokaryotes present several advantages for gene manipulation and expression, fungi are also a highly promising microbial group. Their ability to produce extracellular bioactive compounds such as antibiotics or hydrolytic
enzymes is well known and symbiotic associations with other organisms are also reported [20, 44]. Among sponges a high diversity of fungi has been found associated with this marine group [37]. Several antibiotics, antifungal and cytotoxic substances were identified and showed to be produced by sponge associated fungi [45]. Among insects, obligate fungal gut endosymbionts (most of them yeasts) are known in planthoppers, aphids and some families of beetles. These symbionts probably play an important role in insect nutrition by supplying enzymes for degradation and detoxification of plant material [46]. The yeast Symbiothaphrina kochii occurs in the gut of the tobacco beetle Lasioderma serricorne and detoxifies the host from the plant material. Several species of fungi were also identified associated with leafcutter ants of the genus Acromyrmex [44]. Recently, an yeast of the genus Pichia was isolated from the gut of the wood-ingesting beetles Odontotaenius disjunctus and Verrus sternbergianus. Phylogenetic analysis of its ribosomal genes showed a close relatedness with xylose fermenting yeasts [47], reinforcing the hypothesis of a symbiotic association.

Cooperation between organisms has led to a vast diversity of bioactive natural products with multiple biotechnological applications. In addition, evidence that symbiotic microorganisms are the real producers of these compounds is accumulating. Until recently, marine organisms have been the main source of bioactive products. With the high number and diversity of symbiotic relationships among insects, their associations with microorganisms can provide new and promising exploration avenues, especially when combined with culture independent molecular tools.
4 Symbionts as vectors of pest and disease control

4.1 Insects as disease agents and vectors

Many insects are pests or disease vectors (transmitting the disease agent to the final hosts upon which they feed) with a high impact in public health and also promoting important economic losses in agriculture. The accomplishment of rapid and efficient methods to control these insects is one of the major scientific challenges in the present days. The control of insects that can destroy plant crops and transmit animal and plant diseases has been achieved through the use of chemicals (insecticides). However, the development of resistance to these chemicals, the concern on the impact of its use in the environment and human life and the new pest and vector emerging scenarios (due to human mobility, climate changes, etc.) require new solutions. Strategies including biological control agents and sterile-insect techniques (systematic releases of irradiated sterile insect males) provided good results, although too limited. Recent studies highlighted that biotechnological exploration of insect-symbiont relationships can be promising for insect and insect-borne disease control.

4.2 Symbionts as resources for pest and disease control

Symbiont-based control vs. paratransgenesis

Two different strategies have particular potential for application in pest and disease control (Figure 1). The first – the symbiont-based control strategy – involve the change of symbiont-insect relationships. It can be supported by one of two specific traits: (i) the insect dependence on its microbial symbiont for survival or (ii) the ability of non-essential microorganisms to alter insect characteristics that are important to their pest status. The second – the
paratransgenesis approach – is based on the interactions between disease transmitting vectors, bacterial symbionts and the pathogenic agents [49].

Symbiotic bacteria are isolated and genetically transformed in vitro to interfere with the pathogen viability or transmission. The genetically modified symbionts are then introduced into the host vector, where the expression of introduced genes affects the insect ability to transmit the pathogen. The purpose of this strategy is not the suppression of the insect populations, but the elimination of their harmful effects. Its greatest relevance is where the eradication of the pest is not economically or environmentally acceptable.

4.3 Symbiont-based control

Disruption of symbiont-host relationship

This strategy is suitable for situations requiring the suppression of insect pest populations over periods of days or weeks, but not the immediate insect death. It is based on the dependency, obligatory maternal transmission and specificity of the relationships between insects and their P-symbionts. Insects deprived on their symbionts fail to obtain essential nutrients that are absent in their diet. Disruption of the relationship has dramatic effects in growth rate, reproductive output and lifespan of the insect [48, 50]. This strategy must include two complementary approaches. The first one comprises the identification of specific targets suitable for the disturbance of the relationship (disruption of the symbiont transmission from the maternal bacteriocytes to the eggs and/or disruption of the system for the release of microbial nutrients to the host). The second is focused in screening for compounds that can be active against the symbiont. The research in this area was already initiated, but the increasing available
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18

information about genomic, transcriptomic and proteomic data for symbionts and
their hosts [3, 33, 50] will provide grounds for rapid advances.

3 Manipulation of insect traits

Facultative bacterial symbionts may largely affect several ecological traits of
insects. They can play important roles for their hosts as temperature tolerance
[11], resistance to pathogens [17, 18, 19], host range [14] and ability to transmit
pathogens [51]. These features can support the definition of strategies based on
symbiont-mediated manipulation, reducing the damaging effect of the insect
without suppress their populations. The control of pathogen transmission could
be achieved by depressing the incidence of the S-symbionts that are responsible
for the maintenance of the biological characteristics that are crucial for host pest
status. Although data is still sparse, the few systems studied to the date are
promising [17, 18, 19, 51], specially considering the high diversity of microbial
effects suitable to be valuable in pest management.

4.4 Paratransgenesis

This is a promising control strategy focused in removing the harmful effect of the
insect instead of eliminating the insect itself. It can be particularly important for
the control of insect vectors of diseases as malaria, African trypanosomiasis,
Chagas disease, plant infections, etc. This strategy is mainly based in the
development of transgenic technologies. The process of genetic transformation in
many insects has been achieved by microinjection of transposable elements in
germline cells [52], allowing a tissue-specific expression of transgene products
that affect the parasite viability. The work of Ito et al. [53] can be referred as an
example. These authors successfully expressed a transgenic molecule that
interferes with *Plasmodium* development in *Anopheles* mosquitoes resulting in a decrease of malaria transmission. However, this germline approach is not always applicable. Therefore, genetic transformation mediated by symbionts has become a powerful alternative for manipulation of insect traits and for its rapid dissemination. Through this strategy (originally termed paratransgenesis [54]) the insect cells are not transformed but the desired genes are expressed in the symbiotic microorganism. The specificity of symbiont-host association, the frequent closeness of pathogenic agent and symbiont in the insect tissues and the available technology for genetic transformation in prokaryotes make this strategy highly advantageous. Nevertheless, some important requirements must be fulfilled for a successful application of this approach: (i) the existence of naturally associated symbionts that can be manipulated; (ii) the availability of an efficient transformation system that allow the establishment of stable phenotypes; (iii) the knowledge about the insect-symbiont relationship (transmission mode, population dynamics, fitness alterations, expression of the transgene in the appropriate tissues, etc.); and (iv) the ability to repopulate hosts with the modified symbiont [55]. P-symbionts do not fulfill these requirements, but some commensals living in the insect gut (the same location of parasite) have been successfully exploited for this purpose. One example of paratransgenesis is the transformation of the bacterial S-symbiont *Rhodococcus rhodnii*, present in the blood-feeding bug *Rhodnius prolixus*, which is the vector of *Trypanosoma cruzi*, the causing agent of Chagas disease. The earliest experiment was performed by Beard *et al.* [56]. These authors constructed a shuttle plasmid containing a replication origin from a natural plasmid of this bacterium and capable of replicating both in *E. coli* and *R. rhodnii*. The next step consisted on the
expression of an antiparasitic compound active against *T. cruzi* in *R. rhodnii* that
turn the insect unable to transmit the parasite. The strategy was based on the
insect immune response against invading organisms, which is mainly based on
immune peptides. Cecropins are a group of these peptides. In particular,
ecropin A was studied and the corresponding gene was previously isolated and
expressed [48]. Since *T. cruzi* showed to be susceptible to 150-240 µM of this
peptide and *R. rhodnii* was resistant to concentrations up to 500 µM, the
symbiont was transformed with a recombinant plasmid containing the cecropin A
coding gene. Aposymbiotic nymphs of *R. prolixus* were colonized with cecropin A
transformed symbionts. The parasite was eliminated in 65% of the insects
carrying the transgene and in the remaining 35% a significant reduction was
obtained [48]. Furthermore, a low plasmid decay of 0.5% per generation was also
observed. The successful dissemination of the transformed symbiont was
guaranteed using a strategy based on the coprophagic behavior of insect
nymphs, which feed on adult feces [57]. This approach was based on the
development of a fecal paste named CRUZIGARD, composed by an inert guar
gum matrix impregnated with the modified symbionts. Nymphs of *R. prolixus*
were fed with CRUZIGARD, allowing the establishment of the transformed *R.*
rhodnii and the expression of the desired gene. Finally, an important
improvement in the stability and environmental safety of this symbiont
transformation system was achieved through transgene insertion directly in the
bacterial chromosome by means of an integrative plasmid [58]. This improvement
prevents the lateral transfer of extrachromosomal constructs to non-target
organisms. Recently, the genetic transformation of a corynebacterial symbiont of
another vector of *T. cruzi* (*Triatoma infestans*) was successfully achieved by
Durvasula et al. [59], strongly suggesting that this approach can be applied to other insect-symbiont systems.

The control of sleeping sickness in humans, caused by African trypanosomes, can be referred as a similar example. Tsetse flies (Glossina morsitans morsitans) are the vector of these parasites and have a gut bacterial S-symbiont, Sodalis glossinidius. This bacterium could be cultured and allowed the development of a genetic transformation system based on a shuttle vector containing the extrachromosomal broad range replicon oriV [56]. On the other hand, attacin, an antimicrobial peptide expressed in the fat body tissue of Glossina showed to have an inhibitory effect against Trypanosoma brucei at concentrations that are not harmful to Sodalis. Furthermore, attacin could be expressed in Drosophila cells in vivo [60]. These two findings together with the available genome sequence of this symbiont and its vertical transmission, that allows an easy spread to the progeny, constitute a powerful tool for the future control of the transmission of Trypanosoma brucei. A final example is found in several sharpshooters (Hemiptera, Cicadellidae) that transmit Xylella fastidiosa, a bacterium causing Pierce disease in grapevines. The insect Homalodisca coagulata is one of the vectors of this grape disease [61]. On the other hand, Alcaligenes sp. is a nonpathogenic endophytic bacterium that has potential to become a transgene vehicle of H. coagulata. The work of Bextine et al. [61] demonstrated that it was possible to establish Alcaligenes sp. in the cibarium of H. coagulata by feeding it with medium supplemented with the bacterium and using artificial plants. These authors also transformed Alcaligenes sp. with a marker gene and demonstrated the expression of this foreign gene in insects that acquired the transformed bacteria.
4.5 *Wolbachia*-mediated pest and disease control

*Wolbachia* is a vertical transmitted symbiont of a wide-range of invertebrates, including tsetse flies, fruit flies and aphids. The presence of this symbiont induces a variety of reproductive abnormalities in the hosts. Perhaps the most prominent is known as cytoplasmatic incompatibility (CI) ([for review see](#) [62, 63]). *Wolbachia* is responsible for modifying host spermatozoa, so that when an infected male mates an uninfected female, karyogamy fails causing a disruption in the early fertilization events and progeny is not viable [22, 65]. CI confers a reproductive advantage to infected females, promoting *Wolbachia* spread and ensuring the bacteria maintenance in insect populations, even when it causes detrimental effects on host fitness and reproductive output [22]. In current years, there has been increasing interest in *Wolbachia* biology as recent work indicates that CI can be used as a tool for pest and disease control [64]. Several promising approaches have been exploited. First, CI phenomenon can be directly applied to control insect populations. It was demonstrated the possibility to promote *Wolbachia* infection in insect species in which this symbiont is not naturally present, as well as the induction of CI in the new hosts [65, 66]. Zabalou et al. [65, 67] could established *Wolbachia* infection in a new host (the Mediterranean fruit fly, *Ceratitis capitata*) using a related species (*Rhagoletis cerasi*, the cherry fruit fly) as donor, and induced CI in these new host and the consequent population suppression. This work clearly demonstrated that the *Wolbachia*-induced cytoplasmatic incompatibility can be used for insect population control, in way analogue to the Sterile Insect Technique and, at the present, called Incompatible Insect Technique. Other powerful strategy is the use of this induced CI to spread desirable insect genotypes into natural populations [49, 66].
Considering that a crucial aspect of all transgenic work is the ability to spread the insects holding the desired genomic traits, CI represents a potential mechanism to increase the number of transgenic insects in the environment. This allows the replacement of the initial insect populations by the engineered insects that refractory to the disease vector [66]. Finally, the recent work of McMeniman et al. [68] has open new perspectives for Wolbachia applications. Most pathogens are transmitted by insects (e.g. mosquitos) only after a significant period of development in the insect vector before they can be transmitted to the final host (e.g. humans). Insect survival is therefore considered a critical aspect for disease transmission. A Wolbachia strain from D. melanogaster was described as reducing the life time of its natural host. This strain was successfully introduced into the mosquito Aedes aegypti, the vector of dengue’s agent, and the same reduction in life time was observed [68]. Together with the ability of Wolbachia to spread into insect populations and persist over time, this trait may provide a successful approach for pest and disease control.

A large amount of work must be done to solve the practical issues concerning the transfer of all these lab experiments to the field. However, the referred approaches are promising tools for insect pests and disease control. Although paratransgenesis involves several questions related with the use and release of genetic modified organisms into the environment, a rational assessment of the risks and an adequate research investment can contribute to the safe implementation of an important control tool for several insect-borne diseases.
5 Future perspectives: the potential of insect-symbiont systems

Symbiosis is a widespread phenomenon in nature and symbionts are preserved
due to their major effects on the hosts \textit{\textcolor{red}{(for subject review and historical evolution see [69, 70, 71, 72)}}. Genomic and molecular approaches have extensively
contributed to understand these relationships and to recognize microbial
symbionts as the real producers of many bioactive metabolites previously
detected in hosts. Phylogenetic related symbionts have established symbiotic
associations with unrelated hosts, but assume equivalent roles in host survival
and produce similar metabolites. Marine organisms, especially sponges, have
been the main suppliers of natural products with pharmacological interest,
including many polyketic compounds that are of bacterial origin. Similar
metabolites have been identified in insect symbionts supporting the arguments
for exploitation of this group of organisms as an alternative source of new drugs
and enzymes. A similar perspective can be applied to the control of pests and
insect-born diseases. Symbionts have demonstrated to be a powerful tool to
manipulate the genomic characteristics of insects and their reproduction patterns
in order to control the transmission and spread of infections \textit{\textcolor{red}{(for review see [69, 70, 71, 72)}}. New symbiotic systems can constitute an excellent opportunity to
study other mechanisms that can be used as models to find new strategies and
targets.

More recently, a novel application of insect-symbiont models have been explored
in a complete different perspective. Minimal cells and synthetic biology are
research areas that are expecting to define a minimal genome and express it in a
suitable support, either derived from a cell (top-down strategy) or a chemical
system like a liposome (bottom-up approach) \cite{1}. The first one is also termed
genome-driven cell engineering or minimal cell project and is expected to provide methods to produce manipulated cells which will have a large impact in biotechnology and in the research on basic life. *Escherichia coli* is one of the best-understood microorganisms and the first choice for research and production of metabolites with industrial or pharmaceutical interest. *E. coli* evolved in animal intestines or in natural environments. Therefore, several regions of its genome are unnecessary for many laboratory purposes. The process of genome reduction may decrease the redundancy at genomic and regulatory levels, improving metabolic efficiency [73]. The result will be a strain that is more efficiently transformed, propagates and expresses unstable products more accurately [74]. However, it is not easy to predict which genes can be deleted without disadvantageous effects. Insect endosymbionts show a natural genome downsizing as an adaptation to intracellular life. Nevertheless, they must retain the essential genes that are involved in housekeeping functions and in a minimal number of metabolic pathways required for cell survival and replication. So far, *Carsonella ruddii* and *Sulcia muelleri* are amongst the smallest known genomes. Comparison of these naturally reduced genomes with the genomes of their free living relatives (e.g. *E. coli*) may be a valuable tool to select which genes can be removed to accomplish the minimal genome. Another important aspect is that several possible sets of minimal genes may exist, depending on the environmental characteristics (chemical richness, carbon and energy source, etc). Comparative and evolutionary genomics of endosymbionts give insights into different alternatives, since these microorganisms have overcome several challenges for their own maintenance under diverse environmental conditions that arise from their different host lifestyles [74, 75].
Being less explored, the insect-symbiont systems already characterized are certainly promising in many biotechnology research areas. However, there are many symbiotic associations between insects and microorganisms that are unknown and completely unexplored. A significant example is the group of insects that feed on xylem sap. Little work has been developed concerning their symbionts, mainly due to the apparent lack of economical or clinical interest. However, a few examples are indicative of their potential. Cicadas are included in a small group of xylem-feeding insects. Some species have been used for a long time in traditional Chinese medicine to prepare extracts for treatment of certain diseases [76]. A potent antifungal peptide (cicadin) was isolated from the cicada species *Cicada flammata* [76]. Cicadin N-terminal sequence shares some similarity with proteins of bacteria and yeasts. Furthermore, *Cordyceps cicadae* is a parasitic fungus of *C. flammata* that is thought to produce bioactive compounds with antitumor, anti-*Plasmodium* and immunomodulatory activities [77]. The number of homologous compounds found in very different hosts (*e.g.* sponges and beetles [36]), the continuous search for better engineered biologic systems and the risk for the emergence of new plagues and diseases makes the study of insect-microorganism relationships very promising for the search of new pharmacological and biotechnological products. Xylem-feeders have a particularly scarce source of nutrients, containing only water, salts and a few amino acids [9]. It is expected that complex microbial consortia arise, playing an undisclosed role in host survival. Alternative biosynthetic pathways can be present and, as a consequence, new metabolites may be synthesized. These unexplored symbiotic systems involving xylem feeders can open new perspectives and opportunities for biotechnology.
1 Acknowledgments

2 Sandra Chaves is a post-doctoral fellow from Portuguese Foundation for Science and Technology (FCT BPD/17391/2004).
References


[28] Mavingui, P., Van, V. T., Labeyrie, E., Rancès, et al., Efficient procedure for purification of obligate Intracellular *Wolbachia pipiensis* and representative...


Legends

Figure 1. Symbiont based strategies for pest and disease control.
Table 1. Available genomes sequences for mutualistic symbionts of insects (adapted from [1])

<table>
<thead>
<tr>
<th>Organism</th>
<th>Host</th>
<th>Genome size (Kb)</th>
<th>CDS number</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baumannia cicadellinicola</td>
<td>Homalodisca coagulata (sharpshooter)</td>
<td>686</td>
<td>595</td>
<td>CP000238</td>
</tr>
<tr>
<td>Buchnera aphidicola</td>
<td>Acrithosiphon pisum (aphid)</td>
<td>652</td>
<td>574</td>
<td>BA0000003, AP001070</td>
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<tr>
<td></td>
<td>A. pisum (aphid)</td>
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<td>574</td>
<td>BA0000003, AP001070</td>
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<td>Buchnera aphidicola</td>
<td>Schizaphis graminum (aphid)</td>
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<td>556</td>
<td>AE013218, AF041836, Z21938</td>
</tr>
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<td></td>
<td>S. graminum (aphid)</td>
<td>653</td>
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<td>AE013218, AF041836, Z21938</td>
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<td>Buchnera aphidicola</td>
<td>Baizonia pistaciae (aphid)</td>
<td>618</td>
<td>507</td>
<td>AE016826, AF492591</td>
</tr>
<tr>
<td></td>
<td>B. pistaciae (aphid)</td>
<td>618</td>
<td>507</td>
<td>AE016826, AF492591</td>
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<tr>
<td>Buchnera aphidicola</td>
<td>Cinara cedri (aphid)</td>
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<td>CP000263, AY438025</td>
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<td>C. cedri (aphid)</td>
<td>422</td>
<td>362</td>
<td>CP000263, AY438025</td>
</tr>
<tr>
<td>Blochmannia floridanus</td>
<td>Camponotus floridanus (carpenter ant)</td>
<td>706</td>
<td>583</td>
<td>BX248583</td>
</tr>
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<td>C. floridanus (carpenter ant)</td>
<td>706</td>
<td>583</td>
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<tr>
<td>Blochmannia pennysylvanicus</td>
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<td>610</td>
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<tr>
<td></td>
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<td>610</td>
<td>CP000016</td>
</tr>
<tr>
<td>Carsonella ruddii</td>
<td>Pachypsylla venusta (psyllid)</td>
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<td>182</td>
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</tr>
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<td></td>
<td>P. venusta (psyllid)</td>
<td>160</td>
<td>182</td>
<td>AP0009180</td>
</tr>
<tr>
<td>Sodalis glossinidius</td>
<td>Glossina morsitans (tsetse fly) (S-symbiont)</td>
<td>4,171</td>
<td>2,516</td>
<td>AP008232,33,34, 35</td>
</tr>
<tr>
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<td>G. morsitans (tsetse fly) (S-symbiont)</td>
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<td>2,516</td>
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<td>GenBank Accession(s)</td>
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<tr>
<td>Sulcia muelleri</td>
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<td>Wigglesworthia</td>
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<td>698</td>
<td>BA000021, AB063523</td>
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</table>
Table 2. Examples of bioactive metabolites produced by microbial symbionts of insects.

<table>
<thead>
<tr>
<th>Insect</th>
<th>Symbiont</th>
<th>Metabolite</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nilaparvata lugens (planthopper)</td>
<td>Enterobacter sp.</td>
<td>Andrimid (antibiotic)</td>
<td>[36]</td>
</tr>
<tr>
<td>Scistocerca gregaria (locust)</td>
<td>Pantoea agglomerans</td>
<td>Antibiotics</td>
<td>[38]</td>
</tr>
<tr>
<td>Paederus spp. and Paedericus spp. (beetles)</td>
<td>Pseudomonas sp.</td>
<td>Pederin (antiviral and anti-tumural)</td>
<td>[16, 39]</td>
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<tr>
<td>Acromyrmex spp. and Atta spp. (attine ants)</td>
<td>Bacterium of the order Pseudonocardiaae</td>
<td>Selective antifungal</td>
<td>[40]</td>
</tr>
<tr>
<td>Order Isoptera (termites)</td>
<td>Unidentified bacterium</td>
<td>Xylanases</td>
<td>[20]</td>
</tr>
<tr>
<td>Order Lepidoptera (moths)</td>
<td>Unidentified bacterium</td>
<td>Xylanases</td>
<td>[20]</td>
</tr>
<tr>
<td>Lasioderma serricorne (beetle)</td>
<td>Symbiothapherina kochii</td>
<td>Uncharacterized enzymes involved in host detoxification</td>
<td>[46]</td>
</tr>
<tr>
<td>Odontotaenius disjunctus and Vernus stembergianus (beetles)</td>
<td>Pichia sp.</td>
<td>Potential xylanases</td>
<td>[47]</td>
</tr>
</tbody>
</table>
Figure 1. Symbiont based strategies for pest and disease control.
190x275mm (96 x 96 DPI)