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The molecular basis of bacterial-insect symbiosis

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Abstract

Insects provide experimentally tractable and cost-effective model systems to investigate the molecular basis of animal-bacterial interactions. Recent research is revealing the central role of the insect innate immune system, especially anti-microbial peptides and reactive oxygen species, in regulating the abundance and composition of the microbiota in various insects, including *Drosophila* and the mosquitoes *Aedes* and *Anopheles*. Interactions between the immune system and microbiota are, however, bidirectional with evidence that members of the resident microbiota can promote immune function, conferring resistance to pathogens and parasites by both activation of immune effectors and production of toxins. Antagonistic and mutualistic interactions among bacteria have also been implicated as determinants of the microbiota composition, including exclusion of pathogens, but the molecular mechanisms are largely unknown. Some bacteria are crucial for insect nutrition, through provisioning of specific nutrients (e.g. B vitamins, essential amino acids) and modulation of the insect nutritional sensing and signaling pathways (e.g. insulin signaling) that regulate nutrient allocation, especially to lipid and other energy reserves. A key challenge for future research is to identify the molecular interaction between specific bacterial effectors and animal receptors, and to determine how these interactions translate into microbiota-dependent signaling, metabolism and immune function in the host.

Keywords

bacteriocyte; endosymbiont; gut microbiota; nutrition; immunity

Introduction

Insects are the most successful animals, accounting for >90% of known animal species and dominating a variety of terrestrial habitats. Many insect lifestyles are founded on associations with microorganisms. These include the termites, which thrive on a diet of wood or soil through the metabolic capabilities of microorganisms in the hindgut "paunch"[1]; the leaf-cutting ants, whose apparent herbivory is based on the fungal gardens maintained in

their nests[2]; and the plant sap feeding habit of many hemipterans (aphids, whiteflies etc.), made possible by nutrient exchange with intracellular microorganisms that have been transmitted faithfully from mother to offspring for up to 100–200 million years[3]. More generally, all insects investigated to date bear resident microorganisms and, although some taxa are not obligately dependent on their microbiota, there is increasing evidence that these microorganisms influence many insect traits.

The ubiquity of microbial associations is not peculiar to insects. Rather, it is the normal condition for animals and other eukaryotes[4]. The important implication is that the capacity to interact with microorganisms has ancient evolutionary roots, even though the taxonomic composition of the microbiota can vary widely. We should therefore expect that various aspects of the signaling pathways and regulatory circuits controlling animal function have evolved, and function optimally, in the context of continual interactions with microorganisms, and that the fundamental molecular principles dictating interactions with resident microorganisms are likely to be conserved across the animal kingdom. Thus, the study of insect interactions with the resident microbiota is not only of intrinsic interest, but can also shed light on equivalent processes in other animals, including humans. In other words, just as studies on *Drosophila* and other insects have revealed the fundamental principles of many processes from embryonic development and hormone function to innate immunity and gene expression, so research on the relationship between insects and microorganisms has the potential to identify the basic ground-rules of how animals negotiate with their resident microbiota.

This article provides an overview of recent advances in our understanding of the molecular basis of insect interactions with resident bacteria. Although these associations have been investigated from the perspectives of morphology and whole-insect physiology for many decades, it is only in recent years that the underlying molecular processes have started to be dissected. Much of the molecular research has been conducted on *Drosophila melanogaster*, but important insights have also been obtained from studies of other insect species, including mosquitoes and aphids (Fig. 1). This review addresses how the microbial populations are regulated, and the molecular basis of their contributions to insect nutrition and defense against natural enemies.

Regulation of the microbial populations associated with insects

The resident microbiota of insects is regulated, meaning that the abundance, composition and location of the microorganisms fall within certain bounds. Most of the research on the molecular mechanisms that determine the abundance and composition of the insect-associated microorganisms have focused on the role of insect immunity, especially in *Drosophila* and mosquitoes. The fundamental question posed by this line of research is: How is the immune system structured, such that pathogens are generally eliminated and other microorganisms are spared? In principle, the microbial community may be dominated by taxa that are variously resistant to host immune effectors, lack immune elicitors, or trigger negative regulators of the immune response. The data are fragmentary and often contradictory, but instances of all three modes of interaction with the host immune system

have been identified. The key immune effectors that have been studied are anti-microbial peptides (AMPs) and reactive oxygen species, and they are considered below.

Drosophila is an amenable system to study interactions between AMPs and the gut microbiota because the profile of AMPs and the regulation of their production are well-understood. The expression of genes for two complementary sets of AMPs are induced by the IMD (immune-deficiency) and Toll signaling pathways, with activity predominantly against Gram-negative and Gram-positive bacteria, respectively[5]. Only the IMD pathway is expressed in the midgut of the adult fly, and genetic deletion of this pathway results in a ten-fold increase in numbers of gut bacteria[6]. The simplest interpretation of these data is that AMPs suppress, but do not eliminate, the populations of symbiotic bacteria. In apparent contradiction with these results, the bacterial populations are also elevated in flies with chronically activated IMD pathway, as obtained by RNAi-knockdown of expression of PGRP-SC2, a negative regulator of IMD that is strongly expressed in the midgut[7]. The underlying mechanisms are not understood, but one possibility is that the AMPs have differential effects on different members of the microbiota, and the suppression of susceptible taxa could result in the loss of community stability and overgrowth by resistant taxa[8]. Interestingly, the expression of AMP genes is increased in flies with either mutations or RNAi-expression knockdown of various transcription factors[9; 10; 11], and this is associated with changes in the abundance and composition of the gut microbiota for the two transcription factors tested, Caudal and ATF3[8; 11]. These effects have been attributed to the role of transcription factor Caudal in the negative regulation of the IMD immune signaling pathway[8] and to a generalized perturbation of gut homeostasis caused by imbalance of transcription factors that regulate gut structure and compartmentalization[10]. Taken together, these data are consistent with the possibility that the interplay between the suite of AMPs expressed by the host and profile of AMP susceptibility of different community members may play an important role in shaping the composition of the microbiota, as has also been suggested in other animal-microbial systems[12].

A second important immune effector is reactive oxygen species (ROS). ROS are produced in the midgut of *Drosophila* and mosquitoes by dual-oxidases (DUOX), enzymes with both NADPH oxidase and peroxidase domains[13; 14; 15]. DUOX-derived ROS may play a central role in the control of the gut microbiota in the mosquito *Aedes aegypti*, as indicated by the significantly elevated gut bacterial populations in individuals with the *duox* gene silenced by RNAi[16]. Furthermore, the gut bacteria increase 100–1000-fold over 12 h after the insect takes a blood-meal, and this pattern has been linked to reduced DUOX activity, by a mechanism that involves the activation of protein kinase C by heme in the blood-meal[16]. Paradoxically, DUOX in the mosquito *Anopheles gambiae* has been implicated in protecting gut microbiota. The site of the likely protective effect is the peritrophic membrane, which separates the ingested food and microbes from the epithelial cells of the midgut. DUOX functions in conjunction with a peroxidase to reduce the permeability of the peritrophic membrane, probably by catalyzing dityrosine cross links in the mucin proteins; and this has been suggested to reduce the traffic of microbial immune elicitors from the food bolus to the epithelial cells and, in reverse, the transfer of immune effectors produced by the epithelial cells to the microorganisms in the food bolus[17]. Yet another pattern of response to DUOX-

derived ROS is displayed in *Drosophila*. In this system, the gut DUOX activity is high in flies that have ingested live food yeasts or certain bacterial pathogens, but not symbiotic bacteria[18; 19]. The activation of the DUOX by bacteria in this system has been linked to bacterial release of uracil, a trait displayed, for example, by the pathogen *Erwinia* caratova, but not by most of the symbiotic bacteria in the gut[19]. Further research is required to establish why uracil release is apparently a trait restricted to pathogenic bacteria, and how the uracil interacts with the DUOX activation system.

Various insects possess intracellular bacteria within specialized cells, known as bacteriocytes, whose sole function appears to be to house and maintain the bacteria[20]. These associations are maintained by obligate vertical transmission, usually by transfer from the bacteriocytes to the eggs developing in the female ovary, and the association is required by both the insect and bacterial partners. The bacteriocyte symbioses have no parallel in mammals, for which all described intracellular bacteria are pathogens. (The only known beneficial intracellular microorganisms in vertebrates are algal cells in the embryos of *Ambystoma* salamanders[21].) Nevertheless, the bacteriocyte symbioses can provide valuable general insights into host mechanisms that regulate the resident microbiota. The bacteria in insect bacteriocytes are very tightly regulated, with uniform densities in replicate insects of a given developmental age, and consistent patterns of variation in abundance with host age, sex, morph etc[22; 23; 24; 25]. Insect immune effectors have been implicated in the regulation of the bacteria in bacteriocytes of the weevil *Sitophilus*. The *Sitophilus* bacteriocytes express a cationic AMP, coleoptericin-A, at high levels. When coleoptericin-A expression is suppressed by RNAi, the bacteria overgrow the bacteriocytes and colonize the insect hemocoel (body cavity), suggesting that this AMP plays a crucial role in controlling the bacterial populations[26]. However, the negative regulator of the IMD pathway, PGRP-LB, is strongly expressed in both the *Sitophilus* bacteriocytes and also the symbiosis between the tsetse fly *Glossina* and its bacteriocyte symbiont *Wigglesworthia*[27; 28], suggesting that IMD-dependent immune effectors are suppressed in the bacteriocytes.

Research on the interactions between the insect immune system and resident microbiota is increasingly being complemented by analyses of among-microbe interactions and their impacts on community composition. These studies are revealing a diversity of interactions. For example, when bacteria of the genera *Acetobacter* and *Lactobacillus* are administered in pairs to *Drosophila*, their abundance can be increased, decreased or unaffected, depending on the identity of the partner bacterium[29]. *Asaia* and *Acinetobacter* promote each other's growth in the gut of the mosquito *Aedes albopictus*[30], but the interaction between *Serratia* and other gut bacteria in the locust *Schistocerca gregaria* is antagonistic[31]. Many of the interactions may be direct, involving competition or cross-feeding of nutrients, competition for or facilitation of micro-site colonization in the insect gut, or the production of biologically-active molecules, such as antibiotics. How these interactions are integrated to shape the abundance of different members of the microbial communities is an important topic for future research. Nevertheless, interactions between resident bacteria and invading taxa have been addressed in a few systems. For example, ROS produced by a resident gut bacterium *Enterobacter* in *Anopheles* mosquitoes function to depress *Plasmodium*

acquisition by the insect[32]; and possible competition between *Wolbachia* and RNA viruses for limiting cholesterol in *Drosophila* may block RNA virus replication[33].

Interestingly, not all interactions among the bacteria in an insect host are direct. There is increasing evidence that some interactions between microorganisms are indirect, mediated via the host, particularly the insect immune system[34]. For instance, one microorganism may stimulate (or suppress) the production of immune effectors with high activity against other microorganisms, so depressing (or promoting) the abundance of the latter. A vivid example is provided by the demonstration that elimination of the bacteriocyte symbiont *Wigglesworthia* from its tsetse fly *Glossina* host results in major perturbation of the immune system, including a dramatic reduction of hemocytes, and susceptibility of the insect to opportunistic infection by *E. coli*. When either hemocytes or *Wigglesworthia* cell extract were administered to the treated insects, hemocyte titers rebounded, together with recovery of resistance to *E. coli*[35], demonstrating that these effects cannot be assigned to generalized malaise of the insects deprived of *Wigglesworthia*. These data indicate that a *Wigglesworthia* product (to be identified) activates host immune function against other microorganisms. This type of interaction can contribute to microbial protection the insect host against pathogens, which is considered next.

Molecular basis of microbial protection of insects against their natural enemies

Resident microorganisms can reduce the susceptibility of their insect hosts to natural enemies by multiple mechanisms. As well as symbiont-mediated stimulation of host immune effectors active against nature enemies (discussed above), symbionts can competitively exclude pathogens and parasites from microsites in the host, and produce toxic secondary compounds that complement the insect immune effectors. Most available information relates to the role of symbiont toxins. For example, *Pseudomonas* sp. in *Pederus* rove beetles synthesizes the polyketide “pederin” which protects the beetles from predation[36]. Another bacterium, *Hamiltonella defensa*, can protect its aphid host from the parasitic wasp *Aphidius ervi* but, in this case, the protective effect is perfectly correlated with a bacteriophage bearing a gene for a proteinaceous toxin, e.g. Shiga-like toxin, cytolethal distending toxin, and YD-repeat toxins[37; 38]. Various other protective functions of resident microorganisms have been identified but the molecular basis of these interactions is not yet fully established. Examples include the protective role of *Spiroplasma* for both *Drosophila hydei* against parasitic wasps[39], and *D. neotestacea* against *Howardula* nematode parasites[40], and protection of aphids from entomopathogenic fungi by multiple bacteria[41].

Some of the most exciting recent discoveries in the field of bacterial protection of insects against invading microorganisms are emerging from research on insect competence as vectors of medically-important pathogens. This area is founded on the observations that *Wolbachia* protect *Drosophila melanogaster* from viruses[42,43]. When the *Wolbachia* is experimentally transferred from *Drosophila* to mosquito species that do not naturally bear this bacterium, transmission of *Plasmodium* and viruses (e.g. dengue virus, Chikungunya virus, yellow fever virus) is reduced[9; 44; 45;46]. The depressed vector competence of the

Wolbachia-infected mosquitoes may be attributable to heightened immune function, including induction of AMPs, melanization and ROS[47; 48]. The interactions can, however, be multifaceted. For example, the negative interaction between *Wolbachia* and dengue virus in *Aedes aegypti* has also been linked to *Wolbachia*-mediated induction of a microRNA that suppresses expression of cytosine methyltransferase gene, with consequent global reduction in genome methylation[49; 50]. These studies raise important questions about how the impacts of *Wolbachia* on the methylation status of the genome, gene expression patterns and antiviral response of the host mesh together to depress vector competence.

This section has focused principally on microbial traits that confer protection against natural enemies by complementing or activating host immune function. However, insect capacity to resist and tolerate pathogens and parasites is strongly influenced by other physiological factors, particularly nutrition: nutritional health generally promotes resistance to pathogens and parasites[51], although reverse effects are known[52]. These considerations raise the expectation that microbiota-dependent effects on insect nutrition may contribute to the host immunological response to natural enemies. Dissection of the interactive effects of microbiota, immune function and nutrition is an emerging research priority that will be facilitated by a sure foundation of understanding of microbiota effects on insect nutrition, which is reviewed in the next section.

Molecular basis of microbial impacts on insect nutrition

The resident microbiota can influence insect nutrition by two processes, which are not mutually-exclusive. Microorganisms can, first, be a source of nutrients, made available to the insect by lysis of the microbial cells (this applies particularly to microorganisms in the digestive tract) and by the specific release of metabolites from living microbial cells. Second, microorganisms can modulate the host signaling networks that regulate nutrient allocation. Microbial impacts on nutrient acquisition and nutrient allocation are considered in turn, below.

The evidence for microbial provisioning of nutrients is particularly persuasive for bacteriocyte symbioses. It has been appreciated for decades that these associations are largely restricted to insect taxa living on nutrient-poor or unbalanced diets, notably plant sap (deficient in essential amino acids) and vertebrate blood (deficient in B vitamins), and multiple studies conducted over many decades have demonstrated that the nutritional status of these insects is compromised by experimental elimination of the microorganisms[20; 53]. These interactions involve the two-way transfer of multiple metabolites between the bacteriocyte cytoplasm and intracellular bacteria.

The molecular basis of these interactions has been investigated in the symbiosis between the pea aphid *Acyrthosiphon pisum* and bacterium *Buchnera*, facilitated by fully sequenced genomes for both partners. The interface between the *Buchnera* and the surrounding cell cytoplasm is metabolically very dynamic, with the transport of 58 metabolites between the partners inferred from *in silico* genome-scale metabolic modeling[54]. These metabolites include all the nutritional requirements of the *Buchnera*, the essential amino acids synthesized by the *Buchnera*, and also metabolic intermediates in essential amino acid

synthesis. The last arises from evidence that the synthesis of 5 of the 10 essential amino acids is shared between the *Buchnera* and the bacteriocyte[55], through the evolutionary loss of *Buchnera* genes coding for reactions that are also coded in the genomes of animals, including the pea aphid host. Fig. 2 provides an exemplar: *Buchnera* lacks the gene *ilvE*, coding for branched chain amino acid aminotransferase (BCAT), which mediates the terminal reaction in the synthesis of the branched chain amino acids (BCAs: isoleucine, leucine and valine), and this reaction is mediated by aphid BCAT in the bacteriocyte cytoplasm[56–58]. Although coupled metabolism through shared metabolic pathways has been demonstrated experimentally only in the aphid-*Buchnera* symbiosis, it may be widely distributed, at least among bacteriocyte symbioses, because the same genes are known to be missing from phylogenetically different bacteria in some other plant sap feeding insects, including whiteflies and mealybugs[59; 60].

As well as providing nutrients, resident microorganisms can influence the nutrient allocation patterns of insects. These effects are studied most readily by investigating the effects of eliminating the microbiota on host nutrient content, especially of insects that do not depend on their microbiota; for insects with high dependence, it can be difficult to disentangle the effects of microbial elimination and generalized malaise on the nutrient profiles and underlying signaling circuits. To date, most research has been conducted on *Drosophila*, which can be raised from the egg under sterile conditions with minor effects on growth and developmental rates[61]. The resultant axenic insects have elevated levels of lipid and glucose, together with reduced basal metabolic rates, indicative of enhanced energy harvesting[29; 62]. These effects have been linked to altered insulin signaling[63], including reduced expression of key insulin-like peptides (*dilp-3* and *dilp-5*) in neurosecretory cells of the brain[64]. Although the detail of the mechanisms are unclear, acetic acid produced by *Acetobacter* gut bacteria has been implicated in promoting insulin signaling and reduced lipid storage in this system[64].

Concluding comments

The study of the molecular mechanisms underlying the interactions between insects and their resident microorganisms is a “young” field. As this review illustrates, this research is facilitated by the small size and rapid generation time of many insects, enabling large sample sizes, sophisticated experimental designs and rapid data throughput. For example, the impact of multiple factors on host-microbial interactions can be quantified with sample sizes of insects that would be prohibitive in a mammalian biomedical model. *Drosophila* is emerging as a model system, especially for the study of interactions with the gut microbiota. Other insects can also make important contributions to understanding the molecular basis of many aspects of animal-microbial interactions, and they will become increasingly tractable to study with the rapidly expanding insect genomic resources[65] and improving technologies for insect genetic manipulation[66].

Nevertheless, the field faces multiple challenges. The literature on insect-bacterial symbioses is replete with pattern at the whole-insect level, i.e. how elimination or perturbation of the microbiota affects multiple insect traits, ranging from insect morph determination and dispersal behavior to body color, mate choice and food

choice[67; 68; 69; 70;71]. An important priority is to understand the molecular interactions between the microbiota and host that underpin these diverse phenotypes. This understanding can then be used to test and illuminate the core prediction that the fundamental principles of animal-microbial interactions are highly conserved, such that insect-bacterial symbioses can come to offer a valuable model for the mammalian, including human, symbioses.

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Highlights

- Insects, like other animals, are colonized by benign and beneficial microorganisms
- Insect immune factors affect the composition and abundance of the microbiota
- Microbial effectors can both promote and complement host immune function
- Microbiota provides nutrients and modulates insect nutrient allocation patterns

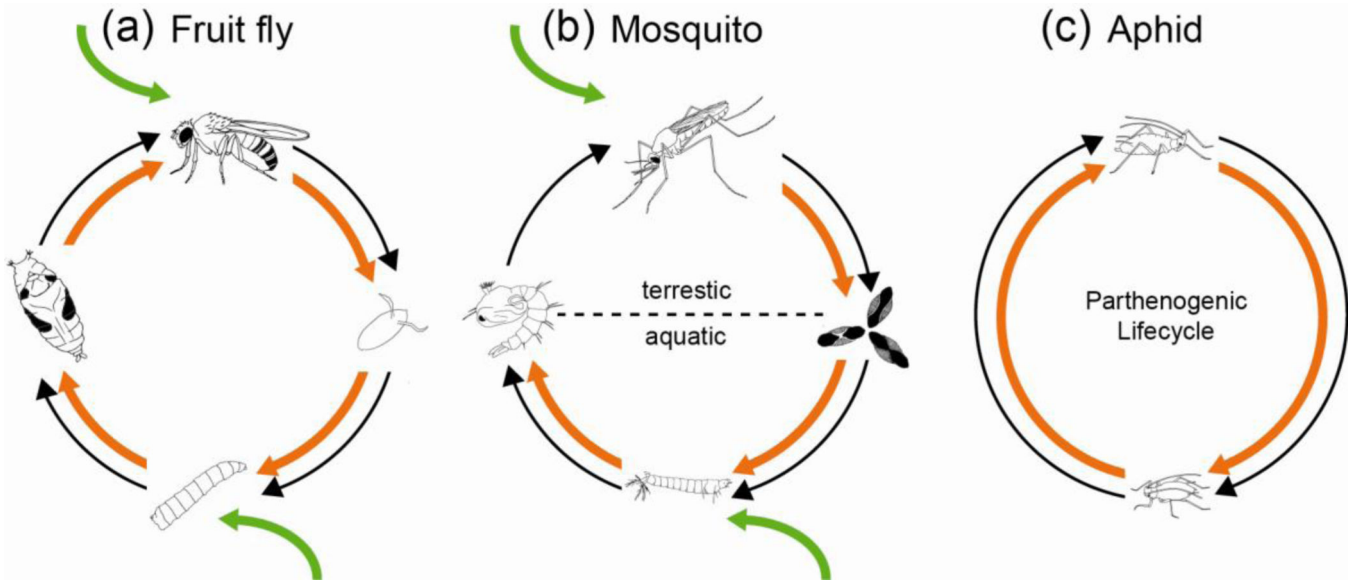

Fig. 1.

Interactions between insects and resident microbiota. (a) Gut microbiota of *Drosophila melanogaster*, an open system in which both the larval and adult stages gain microorganisms from the environment and previous life stages.(b) Gut microbiota of the mosquito, comparable to *Drosophila*, except that the microbiota is not retained in the pupal stage. (c) Intracellular bacteria in the aphid, an example of a bacteriocyte symbiosis (see text) in which the microorganisms are transmitted vertically to the female ovaries and inserted into the developing embryo; aphids have no pupal stage and summer generations (depicted) are viviparous. Black arrows, insect life stages; orange arrows, transmission of bacteria across life stages; green arrows, acquisition of microorganisms from the environment. [Figure prepared by S. Villareal and S. Franzenburg]

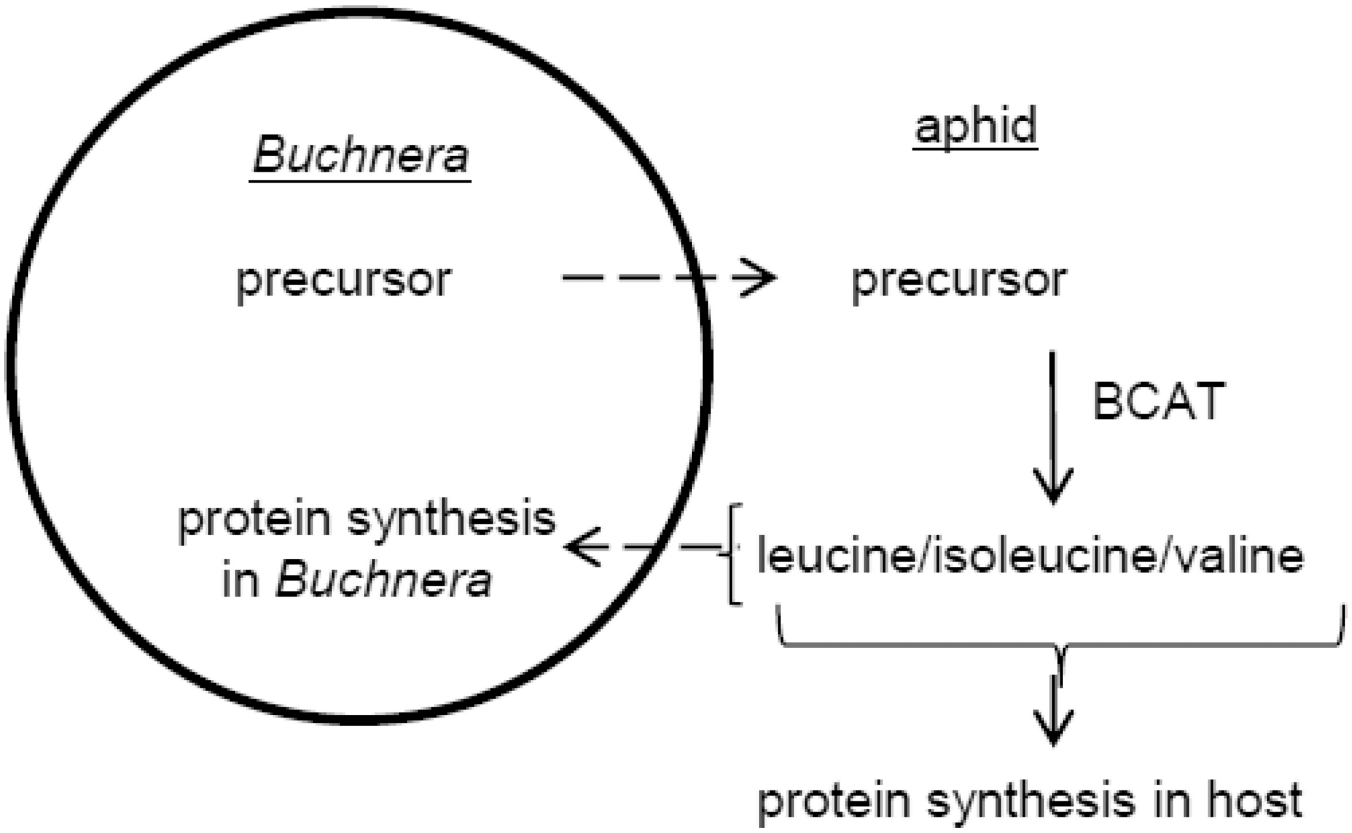

Fig. 2.

Metabolite exchange between the bacteriocyte (host cell) and intracellular bacterium *Buchnera aphidicola*. Insect-mediated terminal reaction in synthesis of branched chain amino acids (BCAs) from immediate precursors: (S)-3-methyl-2-oxopentanoate to isoleucine, 4-methyl-2-oxopentanoate to leucine and 2-oxoisovalerate to valine). The reaction catalyzed by BCAT is reversible (terminal reaction in BCA synthesis /first reaction in BCA degradation) such that, although BCAT functions in most animal tissues in the degradation of BCAs, it is recruited to the bacteriocyte to mediate the final reaction in BCA synthesis.