Beneficial interactions between insects and gut bacteria

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Abstract Insects are amongst the most successful of animals, both in terms of diversity and in colonizing all ecological niches. Recent studies have highlighted the beneficiary roles that bacteria play in the success and establishment of insects. By adopting techniques like 16S rRNA sequencing we are now in a position to understand the diversity of bacteria present in insect guts. It has been shown that some of these bacteria, like *Wolbachia* and *Cardinium* are involved in manipulating insect populations and distorting their sex ratio. Attempts have been made to culture these bacteria in insect cell lines, as they are recalcitrant to culture under normal microbiological conditions. The diversity of bacteria associated with insects and the functional role played by them in the insect is discussed below.

Keywords Insect · Bacteria · Endosymbionts · Commensal

Introduction

The animal gut can be simplistically envisaged as a long hollow tube with its inside covered with an epithelium and surrounded on the outside by the longitudinal and circular musculature. The gut, as an organ, is one of the primary improvisations during evolution with animals deciding to extract and absorb their nutrients from the ingested food while plants deciding to produce their own food by photosynthesis. Actually, the importance of the gut is gaining appreciation and its enormous complexity is just beginning to be revealed [1]. The inside of this hollow tube (lumen) is lined by a continuous layer of epithelial cells arranged into finger like projections (microvilli) an arrangement which increases the surface area of the intestine drastically. While significant amount of information is available on human gut, the insect gut has largely been unexplored and only recently, insect gut analysis has started gaining significance. The microvilli structural design of the intestine is not a static entity but a highly dynamic architecture where in the cells lining the microvilli undergo a constant turnover rising from the intestinal stem cells at the base of the crypts and the ascending upwards to the tip of the villi where they are sloughed off by apoptosis [2]. Other than the intestinal stem cells (ISC), there are two other cell types which make up the insect gut epithelium – enterocytes, which make up the majority of cells and are mainly responsible for enzyme secretion and food absorption, and enteroendocrine cells, which secrete hormones like allatostatin, tachykinin etc. Further, these cells are connected by a network of neural crest cells, which maintain the peristalsis – churning movement of the gut [3].

The sequencing of the genome of insects like *Drosophila* and *Anopheles* [4] has led us to decipher that insects lack many of the metabolic repertoire to fully digest the food

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and extract energy. The gut is estimated to contain 10 times more microbes than total cells and 100 folds more microbial genes than animal genes. Such studies and statistics lead to questions, whether animals are ‘super-organisms’ [5] containing animal + microbial genomes or whether animals are just a successful microbial ensemble? The insect gut is a highly nutrient rich ecological niche in which various groups of microbes (viruses, bacteria, archaea as well as microscopic eukarya like protozoans, fungi and nematodes) survive and multiply. Among the microbes, the role and diversity of bacterial communities associated with gut have received some attention, while studies involving other microbial groups are sparse and far in between.

Microbial diversity in insects

Initial procedures employed for understanding the identity of bacteria in the gut relied on the conventional approach of dilution plating on a solid agar medium and subsequently characterizing the bacterial colonies. Many seminal studies were performed especially on cockroaches and locusts and there are excellent papers giving a gist of these studies [7, 8]. The normal agar based medium of nutrient agar, Luria Bertani agar or tryptic soy agar do not capture and present the dynamic physicochemical environment of the gut – with the variability of different sugars and nutrients, the availability and non-availability of oxygen, the various redox, pH and chemical gradients etc for the growth of all the different bacteria [9]. Instead, efforts to isolate some of these bacteria focused on the ability of these bacteria to infect and multiply in insect cell lines. Dipteran insect cell lines from *Drosophila* and *Aedes* provided the right environment for these bacteria where they infected and multiplied under *in vitro* conditions. By adopting this approach, isolates of *Wolbachia*, *Arsenophonus arthropodicus* and two aphid secondary symbionts viz. *Consessoris aphidicola* and *Adiaceo aphidicola* have been established. Importantly, these cultures are amenable to long term storage in liquid nitrogen thus prolonging their viability.

With the development of nucleic acid based approaches, it became possible to isolate DNA from environmental samples and subject them to polymerase chain reaction (PCR) based selective amplification using bacterial specific primers [10]. Purifying and ligating these PCR products to cloning vectors and their subsequent transformation followed by sequencing of these fragments in the library, it became possible to study the diversity of different bacteria present in the environmental sample. As like any other technique, nucleic acid based methods have their own limitations which stem from the difficulty of nucleic

acid extraction from certain bacterial groups, poor sample size and sampling strategies, over amplification of majority microbe and vice versa through increased PCR cycles resulting in skewed representation of the diversity.

Nevertheless, 16S rRNA based sequence diversity has revealed the bacterial distribution and diversity in many insects. In one of the earliest such studies O’Niel *et al.* [11] studied the bacterial diversity in many insects and found *Wolbachia* bacteria in all cases where cytoplasmic incompatibility was observed. It was also found that there were more than one species of *Wolbachia* present in insects. Around the same time, the endosymbiont bacteria associated with whitefly were discovered and found to differ from those of aphids [12]. Subsequently, a number of studies have brought out the diversity of gut bacteria in different insect groups.

Bacterial diversity of the soil dwelling collembolan insect *Folsomia candida* has been studied and the predominant bacteria associated were *Erwinia amylovora*, *Staphylococcus capitis*, *Pantoea agglomerans* and this insect preferred to feed on *Pseudomonas putida*. [13]. It was also found that various *E. coli* borne plasmids could be effectively transferred to different gut bacteria of *F. candida* indicating that insect gut is an important environment for horizontal gene transfer (HGT) [14].

Termite and cockroach gut house a complex of micro organisms ranging from protozoan spirochetes, gram positive and gram negative bacteria, archaea and yeast [15]. Similarly, coleopteran insects also have a highly diverse bacterial structure dominated by sugar fermenting bacteria belonging to *Lactobacillus*, *Clostridium*, *Bacillus* and members of CFB (Cytophaga, Flavobacterium, Bacteriodes) group. Members belonging to Actinobacteria are most numerous, besides Proteobacteria. Methanogenic archaea was exclusively identified only in the hindgut besides *Desulfovibrio* related bacteria were limited to the hindgut wall [16]. Interestingly, in both termites and coleopteran beetles, there is a clear compartmentalization of the gut into regions of acidic and alkaline pH, each harboring specific groups of microbes [17].

Studies on *Drosophila* have identified *Enterobacter* and *Enterococcus* as the most important and extensive bacteria present in the insect. Other bacteria species associated with *Drosophila* include Proteobacteria like *Gluconobacter*, *Acetobacter*, *Campylobacter*, *Pseudomonas*, *Serratia*, *Klebsiella* and *Comomonas*; Firmicutes like *Lactobacillus* and *Enterococcus*; and Bacteroidetes group besides *Wolbachia* [18]. Similarly Mediterranean fruit fly *Ceratitis capitata* is found consistently associated with *Klebsiella*, *Enterobacter*, *Citrobacter* and *Pantoea* [19].

In lepidopterans, the bacteria inhabiting the gut of polyphagous pest *Lymantria dispar* has been studied.

Enterobacter and *Enterococcus* were present in the insects isolated from all different crops. The other bacteria included *Pseudomonas*, *Staphylococcus*, *Paenibacillus*, *Serratia*, *Pantoea* and *Micrococcus* [20]. The bacterium *Bacillus thuringiensis* is an important pathogen of lepidopteran insects and one of its toxin proteins Cry1Ac has been deployed world wide in transgenic cotton, soybean, maize and canola plants, which has provided efficient insect pest control for the last 6–8 years [21]. As explained earlier, the gut is always in dynamic transition such that, intestinal cells move up the microvilli and as they reach the peak, they are sloughed off. Even if a cell is infected by bacteria, there is very little chance for it (pathogen) to enter the inside of the gut. This protective mechanism is exploited by *B. thuringiensis*. The insecticidal toxins made by Bt are deposited with the spores, which when consumed by the insect are solubilized in the alkaline pH of gut, activated, bind to receptors and form pores in the gut. *Enterobacter* which is a commensal bacterium in the insect is able to cross over to the inside of the gut lining through this pore formed by the Bt toxin protein, invade the haemolymph and cause death due to septicemia. Axenic insects, when fed Bt toxins, are not affected since the pores so formed are “stitched” up during the migration of the cells along the crypt to the apex [22]. This is an isolated example of a pathogen using commensal bacteria for its advantage; otherwise most studies have brought out the beneficial role played by the commensal bacteria.

Functional role played by bacteria in insects

Digestion and nutrition

Commensal bacteria play an essential function in the survival of any higher organism. Detailed studies in mouse have established these functions as digestion and metabolism of food, protection from invasion and infection by pathogens, absorption of nutrients and proper gut function, production of essential compounds including vitamins, xenobiotic degradation etc. Similar functions have been attributed in insects for bacteria, besides reproduction, and fitness which is not known to be affected in mammals by microbes.

In insects the digestion of food by gut associated bacteria has been demonstrated in crickets and cockroaches. Germ-free crickets have much less hydrolytic enzyme activity *vis a vis* their conventional cousins and these bacteria help in utilizing a wider range of food substrates [23]. Also, the bacterial population changes with change in the type of diet that crickets and cockroaches are exposed to. The protozoan population is enriched when the cockroaches are exposed to

a cellulose rich diet [24]. Similarly, it has been established that gut bacteria are responsible for the nitrogen metabolism of insect host and these microbes fix atmospheric nitrogen in fruit flies and termites [25].

These results amply support the view that bacteria associate with insects in providing essential nutrients, as against bacterial pathogens which depend on the insect/animal for its nutrient supply. Insects belonging to the order Hemiptera like aphids, white fly, mealy bug, plant hoppers feed exclusively on the plant sap – a freely available sugar rich diet. Since plant sap is very poor in nitrogen and amino acids, these insects have developed obligate symbiosis with bacteria like *Buchenra* (aphid) or *Portiera* (white fly), where in the bacteria supply all the essential amino acids required by the insect, while the insect accommodate these bacteria in specialized structures in their gut - mycetomes / bacteriocytes. These obligate bacteria are transmitted from mother to the offspring (trans-ovarially) [26]. Recently the genome of *Buchnera aphidicola* has been sequenced from different aphid hosts – *Baizongia pistacia* and *Achrythosiphon pisum*. *Buchnera* are Gram negative bacteria belonging to Enterobacteriaceae, bearing close resemblance to *E. coli* [27]. The genome size of *Buchnera* is approximately 0.7 Mb and bears around 700 genes compared to approx 5.0 Mb of *E. coli* with around 4200 genes. Importantly, *Buchnera* has not acquired any new genes during the process of obligate symbiosis, when compared to *E. coli*; but on the contrary has lost close to 3500 genes of those present in *E. coli*. Many of the so called essential genes of free living bacteria, like recombinase rec A, replication gene dnaA, various sigma factors and genes controlling transcription are either not present, or present in much lower numbers with respect to *E. coli* [28]. Contrastingly, they have retained all the genes governing the essential amino acid metabolism especially leucine and tryptophan which are retained in plasmids. Importantly, it seems that the control of the expression of these amino acid synthesis genes rests with the insect host, since none of the eubacterial control elements were identified (though this needs to be proven). *Wigglesworthia* and *Baumania* the primary symbiont in tsetse fly and sharp shooters respectively, have genes encoding for all the cofactors besides essential amino acids [29].

Immunity and protection

As detailed earlier, gut is a very dynamic environment, providing plenty of opportunity for the bacteria to flourish, thus resulting in extreme competition among bacteria to utilize the nutrients and survive within it. Many of the gut commensal bacteria have evolved to inhabit this environmental niche and thus are able to colonize the gut. In doing so, they

provide effective protection against invasion by pathogenic microbes- a process called colonization resistance (CR), the exact mechanisms are just about being revealed.

Axenic (germ-free) locusts were found to be extremely susceptible to infection by entomopathogenic fungus – *Beauveria* and *Metarhizium*. Certain phenolic components having antifungal activity were absent in germfree locust and introduction of one of the gut bacteria – *Pantoea agglomerans* resulted in the appearance of a part of the phenolic repository [30]. A similar effect on CR against *Serratia marsceans* was also observed [31]. Similarly, axenic silkworm (*Bombyx mori*) larvae were found to be more susceptible to *Serratia piscatorum* and baculo virus infection [32].

Thus the gut, especially its epithelial lining, is the site of major immune reactions - and how it recognizes beneficial microbes and accommodate them while monitoring a strong immune response is a subject of fascinating research of the day. The fruit fly *Drosophila melanogaster* is one of the best model organisms, and is also inhabited by various gut microbes [33]. Studies have shown that induction of innate immune gene products vary according to the type of gut microbe presented. It has been found that different strains of plant pathogenic fruit decay causing *Erwinia caratovora* differ in the induction of antibacterial peptide dipteracin. But normal residents of *Drosophila* gut do not incite anti microbial peptide (AMP) production, but their activity in the gut is controlled by the production of reactive O₂ species [34]. Though the microbes induce the AMP pathway, they are repressed by another protein Caudal that is involved in gut development and morphogenesis. Functional mutants of *Caudal* have a totally different microbial population than normal insects and have reduced fitness when infected by strain of *Gluconobacter*. Moreover, axenic flies were more susceptible to *Gluconobacter*, but flies having its normal gut micro flora could overcome the pathogenic potential of *Gluconobacter* [35].

Interestingly, it has also been found that some facultative endosymbiotic bacteria are able to protect the insect host from parasitoid attack. The facultative endosymbiont *Hamiltonella defensa* protects the aphid from attack by the parasitoid *Aphidius ervi* [36]. Similarly, the infection by another facultative symbiont *Serratia symbiotica* helps the aphid tolerate higher temperature [37]. The exact mechanism by which bacteria are able to bring about temperature tolerance is not very clear. Studies have shown that these endosymbiotic bacteria produce higher levels of heat shock proteins (HSPs). Transcript analysis of *Buchnera* genes has indicated that there is a higher constitutive expression of HSPs, especially GroEL that constitutes about 10% of the total proteins in *Buchnera* cells [38]. HSP are chaperon pro-

teins helping in proper refolding and stabilization of other proteins which would otherwise be disturbed at higher temperature. It is also suggested that since these endosymbionts have not gained any external DNA following their symbiosis with the insect, there is a strong possibility of deleterious mutations occurring over generations. Constitutive over expression of the GroEL chaperone could buffer the effects of such mutations and maintain the proteins structure and function. This hypothesis has been tested with *E. coli* by over expressing GroEL and this helps in maintaining the functions of mutated proteins under heat shock [39].

Many of the phloem feeding insects like whitefly and aphids are important as vectors of plant viruses. Whitefly *B. tabaci* transmits different Gemini viruses the world over while the aphids *Myzus persicae* and *Aphis gossypii* transmit more than 1400 different virus species [40]. The viruses have exploited the constitutive over expression of heat shock protein by the endosymbiotic bacteria in these insects by using GroEL as a receptor to anchor and enter the insect mid gut. Further, since GroEL of these endosymbionts is a secreted protein (while in normal free living bacteria, it is a cytoplasmic protein), the virus when bound to GroEL can easily be transported into the haemolymph up to the salivary gland [41, 42]. Thus, the increased level of GroEL, necessary for proper functional activity of the endosymbiont's protein, is high jacked by the viruses to gain entry into the insect.

Population changes and phenotype manipulation

The role of insect bacterial endosymbiont in phenotype manipulation has been one of the most important findings of last two decades. The most widely studied of bacterial phenotype manipulation has been performed by *Wolbachia*. This Alphaproteobacteria member is one of the most widely distributed microbes among invertebrates. The most common mechanism of *Wolbachia*'s action is by cytoplasmic incompatibility which occurs when uninfected females are devoid of offspring when mated with infected males. On the other hand, infected females produce viable offspring when mated with either infected or uninfected males. Thus, the uninfected female is reproductively less fit *vis a vis* an infected female. The other mechanism by which *Wolbachia* interfere is by inducing thelytoky parthenogenesis where in females give rise to diploid progeny even without fertilization, probably through non separation of chromosomes during meiosis thus leading to gamete duplication and totally homozygous population. Two other forms of phenotype manipulation by *Wolbachia* have been reported - feminization of males and selective killing of males and the exact mechanism by which these processes occur is still not clear [43].

Wolbachia has been detected in many insect orders, including hemiptera, lepidoptera, diptera, coleoptera, hymenoptera [44]. Besides *Wolbachia*, the other endosymbiont associated with phenotype manipulation is by the bacteria *Cardinium*, which belongs to the Bacteroidetes group causing mainly cytoplasmic incompatibility, though host feminization and parthenogenesis have also been observed [45].

Future prospects

The above studies have suggested that microbes have contributed a great deal towards the success of insects. Techniques developed for genome sequencing and analysis are now being applied to study insect gut microbes which have helped overcome the technical bottlenecks associated with the inability to isolate and culture them in normal microbiological media. With the recent success of culturing some of the insect associated bacteria in insect cell lines, the possibilities of understanding their genome sequences and the molecular process through which they affect the insect become bright.

As these bacteria can spread rapidly among the population and in the process affect the population structure – at times causing biological invasions by one strain of insect into the territory of another – across fields, districts, states, countries and even continents – understanding the spread and structure of these bacteria in insects become very important for insect control [46]. Some of the ways by which control of insect pest and disease transmission could be achieved are – to use *Wolbachia* / *Cardinium* to drive beneficial genes into the population such that mosquitoes do not transmit *Plasmodium* or dengue and other viruses; aphids and whitefly do not transmit plant viruses. Mass release of incompatible males, which would interfere with mating of females resulting in no progeny and introduce a male killing strain of bacteria especially for vector insects like whitefly and aphids, (where in haploids eggs become male and fertilized egg females) thus in the process females are eliminated are other imaginable ways of effecting population changes of pest insects [47].

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