Chapter 5

The Microbiome of *Spodoptera littoralis*: Development, Control and Adaptation to the Insect Host

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Additional information is available at the end of the chapter

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Abstract

The symbiotic microbial consortium in the gut of *Spodoptera littoralis* shows dramatic, but reproducible changes in line with the development of the insect from the egg via six larval instars to the pupa. Since the food is kept constant during development, factors from the insect host and certain microbial symbionts are assumed to control the composition of the microbiome. A GFP-tagged *Enterococcus mundtii*, one of the major players of the consortium, easily integrates into the microbiome and can be monitored in all gut segments at all developmental stages. The reporter organism can be recovered from the gut using a preparative flow cytometry allowing subsequent RNA extraction for transcriptomic analyses. The transcriptomic profile from the fluorescent *Enterococcus* cells provides information on the adaptation of the reporter organism to the local gut conditions. The concept of using a fluorescent reporter organism that can be recovered at any time from any area of the intestinal tract will allow a holistic analysis of adaptation strategies used by the microbes to adapt to the insect gut. In combination with the analysis of transcript patterns from the gut membranes, a first insight into the molecular interaction between the insect host and the microbiome can be expected.

Keywords: *Enterococcus mundtii*, *Spodoptera littoralis*, gut microbiome, transcriptomics, flow cytometry

1. Introduction

The development of a gut in multicellular organisms is an evolutionary achievement of the highest order. The gut allows the host to exploit the metabolic and catabolic abilities of a multitude of microbial inhabitants to degrade and digest recalcitrant and complex organic matter.

The symbionts may also be involved in the detoxification of poisonous metabolites in food [1, 2]. The membranes of the intestinum carefully separate the bacterial symbionts from the host organism and prevent infection by invasive and deleterious members of the microbiome. Accordingly, the gut membrane is a complex structure that allows the exchange of nutrients with both high- and low-molecular weight (signaling) compounds and, on the other, blocks the entry of microbes and many of their macromolecular components [3]. The flux of nutrients and even more complex metabolites across the membrane is controlled by transport proteins expressed in the gut membrane [4, 5]. The microbiome also defends against parasites or pathogens [6–8]. The diverse functions provided by the microbial partners are vital for the insect's survival, especially in adverse ecological niches.

Although almost all organisms rely on core microbiomes [9], in many cases the gut community changes according to the insect's developmental stage. In early instars of *Spodoptera littoralis,* several *Enterococcus* spp. dominate, whereas in late instars, Clostridia contribute significantly (ca. 50%) to the microbial population [10]. A core community, consisting of Enterococci, Lactobacilli and Clostridia was revealed in the insect larvae. These bacteria were always present in the digestive tract at a relatively high frequency; although developmental stage and diet have a great impact on shaping bacterial communities, clearly the insect gut selects for particular bacterial phylotypes. Enterococci are also prominent in the gut of insects such as *Drosophila*, ground beetles and desert locusts [11, 12]. The strong dependence of the gut community on the developmental stage of the insect host suggests that unknown low- and high-molecular weight factors control symbiotic interactions among the partners. For example, in *Drosophila melanogaster*, the immune system not only plays a central role in preventing pathogen infection, but also controls the resident bacterial population. The intestinal homeobox gene *Caudal* regulates the resident gut microbial community by repressing the antimicrobial peptide genes that are dependent on the nuclear factor kappa B. Silencing the *Caudal* gene by RNAi resulted in the overexpression of antimicrobial peptides, which in turn reduced the microbial population in the gut [13].

To monitor such developmentally controlled changes in the microbiome of *S. littoralis,* a fluorescent member of the gut symbionts—in particular, the dominant *Enterococcus mundtii*—appeared to be an ideal reporting organism. A GFP-labeled *E. mundtii* [14] would easily integrate into the gut community and survive adverse conditions embedded in the community of enterococci, bacteria which are largely resistant to environmental stresses, such as antibiotic exposure, disinfection, desiccation and starvation [15]. As the transgenic *E. mundtii* are fluorescent, their presence can be monitored in all gut areas of the larvae and at developmental stages such as the pupa and the adult. Moreover, the reporter organisms can be easily recovered from the gut and used for transcriptomic analyses. By comparing transcriptomes from adjacent gut tissue and from the microbes, a "dialog" between the insect host and the symbiotic bacteria could be unraveled. This concept is generally applicable and can be used to holistically analyze host microbial interactions. The protocol of the approach based on the use of a fluorescent reporter organism—for example, using GFP-tagged *E. mundtii*—is described in this chapter.

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2. Fluorescent reporters and their applications for *in vivo* imaging in microbiomes

The green fluorescent protein (GFP) isolated from the jellyfish, *Aequorea victoria*, is widely used as a reporter for studying gene expression [16], and the localization and structure of living cells [17]. The GFP has a major excitation peak at about 395 nm and an emission peak at about 508 nm. The GFP contains 238 amino acids with a molecular weight of 26.9 kDa. It emits green fluorescence when exposed to light in the blue to ultraviolet range [18]. The GFP requires only oxygen as a cofactor for chromophore formation, which gives it an advantage over other reporter proteins [19]. It is sensitive and non-toxic, and does not affect cell growth [20, 21]. In addition, the GFP is stable at temperatures below 65°C and pH 6–11 [22]. Since the GFP was discovered, many mutants have been developed with modification in spectral and folding properties, or enhanced fluorescence intensity [23–26]. The choice of a GFP variant depends on several factors, such as pH, environmental temperature, toxicity, multimerization and photostability [26]. The first *gfp* gene was cloned in 1992 [27], and 2 years later, the gene was successfully expressed in both eukaryotes and prokaryotes [20]. Apart from GFP, many variants of red fluorescent proteins, such as mCherry and tdTomato have been developed based on DsRed originally isolated from *Discosoma* sp. [28]. Since then, over 40 coral fluorescent proteins with different colors, from cyan to chromo-red, have been described [29].

The reporter proteins provide important tools with which to monitor gene expression from within the cells in real time and in the *in vivo* environment, such as the gastrointestinal tract. For a gene to be selected as a reporter, it must be able to easily detect signals secreted by the expressed reporter gene in the cells [30]. The *lux* gene derived from bacteria, and luciferase from the firefly and click beetle (*luc*), are two other common reporter genes used in bioluminescence imaging. Fluorescence imaging is commonly associated with the use of green and red fluorescence proteins [31, 32]. Rats and mice are popular model organisms which study the proliferation and colonization of lactic acid bacteria (LAB) [33, 34]. LAB has been tagged with green fluorescent protein (GFP) and mCherry to study their colonization of the intestinal tract of chickens, mice and zebrafish [35–39].

2.1. Construction of a GFP fluorescent system for *E. mundtii*

LAB is widely used as probiotics due to the benefits they bring to human and animal health by balancing the gut microbiome and by eliminating pathogenic microorganisms through the production of antimicrobial peptides [40, 41]. Due to the importance of LAB in many applications, it is essential to study how they survive and colonize by monitoring their metabolic activities *in vivo* through the development of fluorescent reporter microorganisms. It is important that the reporter gene in the fluorescent bacteria is stably expressed [42].

Plasmids are present in most of the members of LAB, including *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, *Streptococcus*, *Pediococcus, Leuconostoc*, etc. Plasmids found in LAB

vary in size (0.87 kb to more than 250 kb), copy number (1 or more per cell) and gene content [43–46].

Enterococci harbor plasmids that are resistant to a wide range of antibiotics, including erythromycin, tetracycline, gentamicin and vancomycin [47–50]. Some of these plasmids encode bacteriocins [51–53], virulence factors [54, 55], toxins [56] and sex pheromones [57]. Plasmids replicate via rolling circle replication (RCR) and theta replication [58]. Theta-replicating plasmids can carry large DNA fragments and are more stable than RCR plasmids [59]. The enterococci plasmid pAMβ1 replicates via theta mode. In the early 1990s, shuttle vectors in the pTRK family of high and low copy number carrying the origin of replication of pAMβ1 for LAB and p15A for *E. coli* were developed [60]. The plasmids carrying the replicon pAMβ1 isolated from *Enterococcus faecalis* [61] have been reported to replicate in Gram-positive bacteria [62].

The choice of a reliable expression vector depends on several factors, such as the mode of replication, copy number and stability [63]. The expression vector used in this study is derived from pTRKH3 plasmid with a broad host range. pTRKH3 is a shuttle vector for *E. coli*, *Enterococcus*, *Lactococcus*, *Streptococcus* and *Lactobacillus* [60]. The vector has a copy number (30–40) in *E. coli*, and a somewhat higher copy number (45–85) in *Lactococcus* and *Streptococcus* species [64]. It carries a gene for erythromycin resistance, which is expressed in *E. coli* and LAB. In this chapter, we report the expression of mutated *gfp* (mgfp5) on a pTRKH3 plasmid controlled by a strong constitutive promoter, erythromycin ribosomal methylase (*ermB*) [61], in *E. mundtii* (**Figure 1A**). The lactate dehydrogenase (*ldh*) promoter from *Lactobacillus acidophilus* [65] has also been used to control the expression of GFP. In contrast, the use of a surface-layer (*slp*) promoter from *L. acidophilus* [66] was not able to induce the expression of GFP [14]. Accordingly, the selection of an appropriate promoter to achieve a high level of GFP expression is crucial. Nisin-inducible promoters have been used for heterologous gene expression in lactobacilli [67, 68]. Nisin that can be degraded within the intestinal environment is a drawback of this inducible expression system [69, 70].

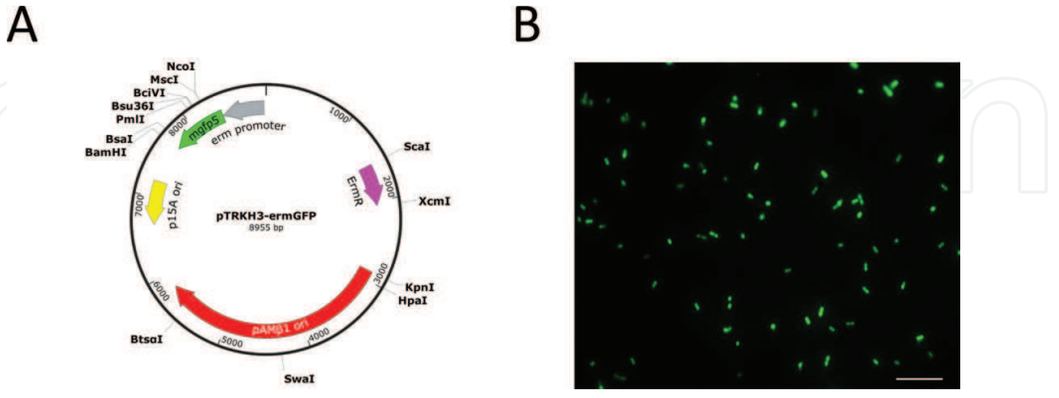

Figure 1. Construction of GFP-tagged *E. mundtii* by electroporation. (A) Plasmid map of pTRKH3 harboring the *mgfp5* gene regulated by an erythromycin ribosomal methylase (*ermB*) promoter. The plasmid is an *E. coli*-LAB shuttle vector with p15A and pAMβ1 as the origins of replication. (B) The *ermB* promoter was used to increase the expression of GFP from *E. mundtii* grown in Todd-Hewitt Bouillon (THB) broth culture. Scale bar: 10 μm [14].

Therefore, the use of constitutive or native-based promoters would be favorable, as these promoters could ensure the constant production of the target protein, especially in the gut environment. Several studies using homologous promoters have been reported to achieve efficient gene expression [71, 72], as the transcriptional signal induced by native promoters is recognized by the host bacteria. Bacteria with the *gfp* gene cloned downstream of a native constitutive promoter express GFP efficiently in broth culture (**Figure 1B**).

2.2. Transformation of *E. mundtii* KD251 using electroporation

Several methods have been used to introduce exogenous DNA into microbial cells; these include chemical treatment, electroporation, the use of a biolistic gun, ultrasound, polyethylene glycol, microwave and hydrogel [73]. Of all the methods, electroporation most efficiently transforms a broad array of microorganisms [74] by introducing foreign DNA-like plasmid into bacteria. Electroporation is one of the transformation techniques for rapid introduction of foreign DNA-like plasmid into bacteria. The method uses an electric pulse that forms pores on the bacterial cell walls so that DNA can pass into the cell. In recent years, numerous lactic acid bacteria have been transformed using electroporation [75]. The success rate of electro-transformation depends on the cell wall becoming sufficiently permeable to allow DNA to enter. In some cases, to improve a cell's electro-transformation efficiency, the cell wall is pre-treated with chemicals such as lysozyme [76, 77], threonine [78, 79], penicillin G [80], ethanol [81] and glycine [82, 83]. These weaken the cell walls only for certain bacteria species. It has been shown that the efficiency of electro-transformation of *Lactococcus lactis* was affected by several parameters, such as the cell's growth phase and density, the medium, the plasmid concentration and the strength of the electrical field [84].

The choice of method in the preparation of the competent cells is important for a successful transformation. Although competent *E. coli* cells have reportedly been prepared with ice-cold calcium chloride [85], the transformation achieved with this method is less successful than that achieved using the electroporation method [86]. The electrocompetent cells, the equipment and the washing buffers all have to be prepared at cold temperatures [87, 88].

In this chapter, we report the use of a conventional method to transform *E. mundtii* based on the modified protocol of *Escherichia coli* [89]. The electrocompetent cells and electroporation protocol for *E. mundtii* have been published [14]. Briefly, the bacterial cells were grown to the exponential phase and then washed with ice-cold water for two rounds to remove salts from the growth medium. Glycerol at a final concentration of 10% was added to the bacterial suspension so that the cells could be preserved and stored frozen. A concentration of plasmids between 0.15 and 0.2 μg worked fine for us. The competent cells were mixed with the plasmid DNA and then transferred to a 0.2 cm plastic cuvette for electroporation at a pulse of 1.8 kV, 600 Ω parallel resistance and 10 μF capacitance. The pulsed cells were recovered with fresh broth medium, and the cell suspension was incubated at 37°C for 2 hours before plating on plates containing antibiotic erythromycin. After 2 days, the bacterial transformants were screened for the plasmid-containing *gfp* gene.

2.3. Colonization of GFP-tagged bacterium in the gut of *S. littoralis*

The fluorescent reporter *E. mundtii* has been integrated into the gut microbiome across all developmental stages of *S. littoralis* [14], indicating its symbiotic relationship with the insect host. Microorganisms have the ability to face environmental stresses, particularly those within the gastrointestinal environment. Constructing the fluorescent reporter *E. mundtii*, we explored the mechanisms these bacteria use to adapt to stress; we recovered the reporter bacteria from the gut of *S. littoralis* using the state-of-the-art technology of flow cytometry. The dominance and persistence of *E. mundtii* in the gut motivates us to look deeper into their gene expression system. Therefore, it is important to unravel the mechanisms used by microorganisms living within the gastrointestinal environment. Construction of the fluorescent reporter *E. mundtii* is one of the strategies to find out those mechanisms, since it has been possible to recover the reporter bacteria from the gut of the very same insects using the *state-of-the-art* technology of flow cytometry. Fluorescence-activated cell sorting (FACS) enabled us to pick out the GFP-tagged reporter *E. mundtii* from a mixture of insect and other bacterial cells.

3. Fluorescence-activated cell sorting

Flow cytometry separates cells based on their intrinsic physical and chemical characteristics, integrating electronics, fluidics and optics. The sample, from which the cells of interest are to be sorted, is passed through a flow cell. The sheath fluid escorts the cells down the channel, where they encounter a laser beam. Light beams of specific frequencies and wavelength are emitted. Detectors measure the forward scatter (FSC) and the side scatter (SSC) based on cell size and granularity. FSC and SSC are unique for every particle. A combination of the two can differentiate among cell types within a cohort of cells. This way, the qualitative and quantitative data of a particular kind of cell can be assessed.

Fluorescence-activated cell sorting is an application of flow cytometry. The cells of interest are fluorescently tagged and sorted by the machine. Here the GFP-tagged fluorescent *E. mundtii* is isolated from a mixture containing insect gut homogenate with other bacteria. The solution is delivered to the flow channel and carried by the sheath fluid. The pressure from the compressor, which is adjustable, forces the solution through a laser beam using hydrodynamic focusing. Then monochromatic beams of high intensity interrogate cells one at a time. Depending on the excitation wavelength of the fluorophore, the laser wavelength is chosen. The scatters are then recorded. The forward scatter (FSC), which refers to light that is refracted by the cell and continues in the same direction, tells us about the size of the cell. In contrast, the side scatter (SSC), which refers to light that is refracted by the cells and travels at right angles to the excitation axis, tells us about the fluorescence and granularity of the cells. The more granular a cell, the more scattered light it produces. Furthermore, each cell enclosed in a droplet is assigned a charge, depending on the extent of the cell's deflection [90]. After passing through an electrical field, the cells are deflected to the collection tubes and the uncharged droplets are directed to the waste. The detector system consists of

a set of photo multiplier tubes that have specific filters to select for certain wavelengths of the beam and are set at the excitation range to view GFP.

Once the larvae are fed with the fluorescent *E. mundtii*, the number of larvae that survive can be determined and eventually recovered for further studies. The *E. mundtii* cells are sorted and their transcriptomes can be studied. This technology has made it possible to focus on a single cell or cells of interest, to study their function or their physiological state.

4. RNA extraction

The GFP-tagged *E. mundtii* are sorted by the flow cytometer and collected in a RNA-protective reagent (RNAlater®). The role of such reagents is twofold: first, they preserve the integrity of RNA, which has a very short half-life, for a few minutes. We need the RNA to be intact and of good quality in order to process it for sequencing. Second, addition of protective reagents minimizes subsequent changes from being introduced when the cells are handled. As soon as the cells are collected in a Falcon tube filled with the protective reagent (RNA Protect or RNA Later), the reagent percolates into the cells and prevents an alteration in the gene expression [91]. Additionally, the entire process is maintained at 4°C, as all metabolic activities slow down at low temperatures. The Falcon tube is centrifuged at a high speed to pellet down the cells, and care is taken not to disturb it while draining the supernatant.

RNA is very sensitive to exogenous and endogenous RNases. The entire extraction procedure is done in an area free of RNase. Moreover, RNase inhibitors are used to clean all equipment, ranging from gloves to microcentrifuge tubes to get rid of RNase. *E. mundtii* is a Gram-positive bacterium with a cell wall containing a thick layer of peptidoglycan and lipoteichoic acid, followed by a single lipid membrane. The cell wall is anchored to the membrane by diacylglycerols. To release the nucleic acid from the cell, it has to be detached from its peptidoglycan-containing cell wall and membrane. Lysozyme is a glycoside hydrolase that hydrolyzes the 1,4-beta linkages between *N*-acetylmuramic acid and *N*-acetyl-D-glucosamine residues of the peptidoglycan. Additionally, guanidium thiocyanate, beta-mercaptoethanol and a detergent called dithiothreitol help in cell lysis and deproteinization. Proteinase K frees the RNA from the bound proteins and endogenous RNase.

Following lysis, the RNA is separated by density gradient centrifugation using phenol, chloroform and isoamyl alcohol, and further precipitation with ethanol. The RNeasy® Mini Kit (Qiagen) based on silica-matrix RNA extraction was used in our work. Several studies have reported on extraction of high quality bacterial RNA using this kit [92–94]. Thus, RNA is obtained from the cells of the sorted *E. mundtii* [95].

The extraction of total RNA from the low number of bacterial cells that remain after sorting by the flow cytometer is challenging. The concentration of RNA was as low as a few picograms to 50 ng. The minimum threshold quantity for a successful RNA library preparation is 100 ng. This is too low an amount to proceed with RNA sequencing. Hence, the total RNA must be amplified before we can use it further.

4.1. Amplification of RNA

Amplification of RNA is required if the aim is to create an effective transcriptomic profile from a very low starting quantity of RNA. MessageAmp II aRNA amplification kit (Ambion) was used for amplification [96–99]. The principle is based on *in vitro* transcription. The steps are as follows:

4.1.1. Polyadenylation of RNA

Bacterial RNA is devoid of a poly (A) tail. The *E. coli* poly (A) polymerase enables a poly (A) tail to be added at the ends of RNA. This stretch is required for cDNA synthesis.

4.1.2. Synthesis of first-strand cDNA

Primers against the poly (A) stretch are used to synthesize the first strand of cDNA by reverse transcription. The primers are anchored with a bacteriophage promoter sequence: T7 oligo(dT) sequence, T3 or SP6. dNTPs are added to the reaction mix.

4.1.3. Synthesis of second-strand cDNA

RNaseH is used to degrade the RNA from the RNA-cDNA pair; DNA polymerase is required to synthesize the second strand of cDNA. The result is a double-stranded cDNA fragment with a T7 promoter sequence.

4.1.4. Purification of cDNA

cDNA is cleaned by removing the fragmented RNA, enzymes and salts, all of which could hinder *in vitro* transcription.

4.1.5. In Vitro transcription

Multiple copies of antisense RNA are generated using DNA-dependent RNA polymerase. Linear amplification is employed for this. Depending on the bacteriophage promoter sequence attached to the cDNA, a polymerase is selected. Promoter-specific dNTPs are added to the reaction mix. 37°C is optimum for this reaction. The reaction time depends on the extent to which one wants to amplify the RNA.

4.1.6. Purification of amplified RNA

The residual enzymes, salts and unincorporated dNTPs must be removed from the final product [100–104].

At this point, the RNA has been amplified several fold: 1–2 ug.

RNA amplification procedures have a drawback. When the concentration of RNA is brought to a point at which successful transcriptomic profiling is possible, certain biases are unavoidably introduced.

Certain amplified transcripts may be misunderstood as duplicates and *vice versa*, which could give a false positive read [105]. In PCR-based amplification procedures, duplicates that can arise from sample handling may have features such as fragmentation, sequencing depth or library complexity; unfortunately, these cannot be distinguished from PCR duplicates. Removing duplicates does not improve the accuracy of quantification or the power; rather, makes it worse [106]. The *Taq* polymerases used for the PCR-based approach are more prone to introduce errors than the RNA polymerases for *in vitro* transcription. Thus, *in vitro* transcription is favored over PCR-based amplification [105], although premature transcription termination can occur in low complexity sequences [107]. Nevertheless, *in vitro* transcription is an efficient method to follow when the starting quantity is limited [107].

5. Transcriptomics

At this point, we have enough RNA to get a transcriptomic profiling of the bacterial cells done. The transcriptome is the entire set of genes expressed in a type of cell at a particular time point and/or condition. This is in contrast to a genome, which refers to the full complement of genes in a cell-type. Not all genes are constitutively induced. Information about transcripts, or genes expressed, may shed light on the developmental or physiological state of the cell. It also talks about other species of RNA, small RNAs and non-coding RNAs, novel transcripts, the transcriptional start sites, splicing regions, post-transcriptional modifications, and 3′ and 5′ ends. Another purpose of transcriptomic profiling is to quantify the expressed genes. One can judge the extent of regulation of a particular gene in the given conditions. As compared to one situation, when cells behave differently in another, one can now say which genes are differentially regulated to bring about the same.

In this chapter, our aim has been to investigate the survival and adaptation strategies of *E. mundtii* living inside the gut of *S. littoralis* as compared to in the laboratory. This unraveling has been done by cataloging the genes of *E. mundtii* which are differently regulated and which make it as one of the dominant bacterial species in the gut.

5.1. RNASeq

Transcriptome sequencing has improved dramatically over the past few years, starting with EST-based Sanger sequencing. The early method was mainly useful with the most abundant transcripts, whereas subsequent next-generation sequencing has been successfully carried out on all transcripts with sensitivity and accuracy even allowing the identification of low expressed genes. The situation has ameliorated with the advent of deep sequencing, which can increase the average number of times a nucleotide is sequenced. The deeper the sequencing is, the better the probability of detecting the less abundant transcripts. Next-generation sequencing has several hierarchies of its own. These days, RNA-seq is more widely used than the microarrays. The former gives us a base-pair level of resolution. Whereas microarrays can be used only when the reference genome sequence is available, RNA-seq can build the transcriptome *de novo*. Also, background noise is taken better care of in the case of RNA-seq. These

days, sequencing is not confined to a larger number of cells. It is possible to obtain resolution up to a single cell. Naturally, the amount of RNA obtained from one single cell is in picograms and must be processed as discussed above. Among all the increased sensitivity of next-generation technologies, so far, Illumina allows us to start with the smallest amount of RNA.

The fragmented and adapter-ligated cDNA is allowed to flow through a flow cell of the sequencer, which has oligonucleotides that complement the adapter sequences embedded in them. After hybridization, the oligonucleotides prime the polymerization process with the provided dNTPs and DNA polymerase. Each of the dNTPs is tagged with a fluorophore. As the nucleotide is incorporated, the resulting fluorescence is detected. With the addition of each nucleotide, the fluorophore is released, regenerating the 3′ hydroxyl group for the next nucleotide to join. This way, the fluorescent intensity is recorded and converted into nucleotide identity using an algorithm.

The amplified RNA from the fluorescent *E. mundtii* cells sorted by flow cytometry went through deep sequencing (Hiseq) to detect as many genes as possible to tell us the story of their adaptation to the gut environment of *S. littoralis* (**Figure 2**).

The complications arising from several different forms of RNA, alternate splicing, removal of introns, that is, the ones that are profound in eukaryotes are not required to be considered in the case bacteria. Although, there are several regulatory and non-coding RNAs in bacteria, but this particular case dictates one to follow a rather straightforward approach of unraveling the upregulated and downregulated transcripts only.

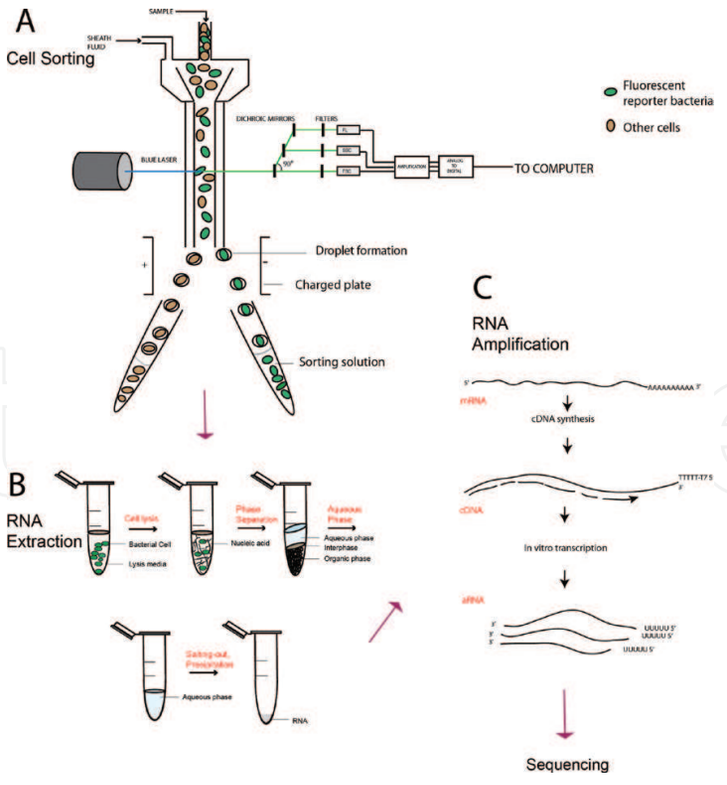

Figure 2. Overview of the workflow for bacterial RNA-seq. (A) Flow cytometry to sort fluorescent bacteria from gut homogenates. (B) Extraction of total bacterial RNA. (C) Amplification of the total RNA by *in vitro* amplification (unpublished).

5.2. Adaptation and survival strategies of *E. mundtii* in the gut of the insect

The GFP-tagged *E. mundtii* was fed to the *S. littoralis* larvae at early instars. The bacterial reporter was able to colonize the gut at various stages of the insect's life cycle, as seen in the fluorescent microscopic images (**Figure 4**).

The production of antimicrobial substances from insects or their resident symbionts is a survival strategy to keep pathogens at bay. The dominant gut bacterium *E. mundtii* has been shown to produce an antimicrobial peptide called mundticin KS, which is a stable class IIa bacteriocin. It establishes a chemical barrier, which prevents colonization by competitors [108]. If allowed to persist, the early colonizers of the *S. littoralis* gut, *Enterococcus faecalis* and *Enterococcus casseliflavus*, could be potential pathogens for the insects. Successful antimicrobial activities against them have been shown in the presence of *E. mundtii* [108].

The larvae were allowed to grow until the fifth instar, at which stag the guts were homogenized to retrieve the fluorescent *E. mundtii* by flow cytometry. The RNA of these sorted bacteria was used to probe their differential behavior inside the gut. RNA sequencing and analysis of differential gene expression were performed later.

Numerous genes are differentially regulated in the *E. mundtii* obtained from the gut, when compared to the *E. mundtii* grown in bacterial culture under lab conditions (**Table 1**, **Figure 3**). Reactive oxygen species, such as superoxide radicals, hydrogen peroxide or hydroxyl radicals, from metabolic activities may cause oxidative stress and damage macromolecules. To survive the stress, resident bacteria have to come up with means to fight it. Superoxide dismutase and catalase are effective enzymes, over-produced by *E. mundtii* when inside the gut, as compared to the broth culture.

Gene/protein	Pathway	Function
Superoxide dismutase (*SOD*)	Oxidative stress management	Quenching reactive oxidation species by partial reduction of O_2^-
Catalase	Oxidative stress management	Quenching reactive oxidation species, converting hydrogen peroxide to water and oxygen
LPxTG-motif cell wall anchor domain protein	Cell surface adhesion	Signal peptide cleaved by sortase for cell surface adhesion
WxL domain surface cell wall-binding protein	Cell surface adhesion	Cell surface adhesion and adaptation
Accessory gene regulator (*Agr*)	Two-component system	Virulence factor
General stress protein	Adaptation	Various stress management
Universal stress protein	Adaptation	Adaptation to diverse stress sources
Ferric (Fe^{+3}) ABC superfamily ATP binding cassette transporter (*fetC*)	Iron transport	Iron transporter permease
Phosphotransferase systems	Sugar transport	Regulates carbohydrate metabolism in diverse sources and adaptation

Table 1. Upregulation of genes and pathways in *E. mundtii* living in the gut of *S. littoralis*.

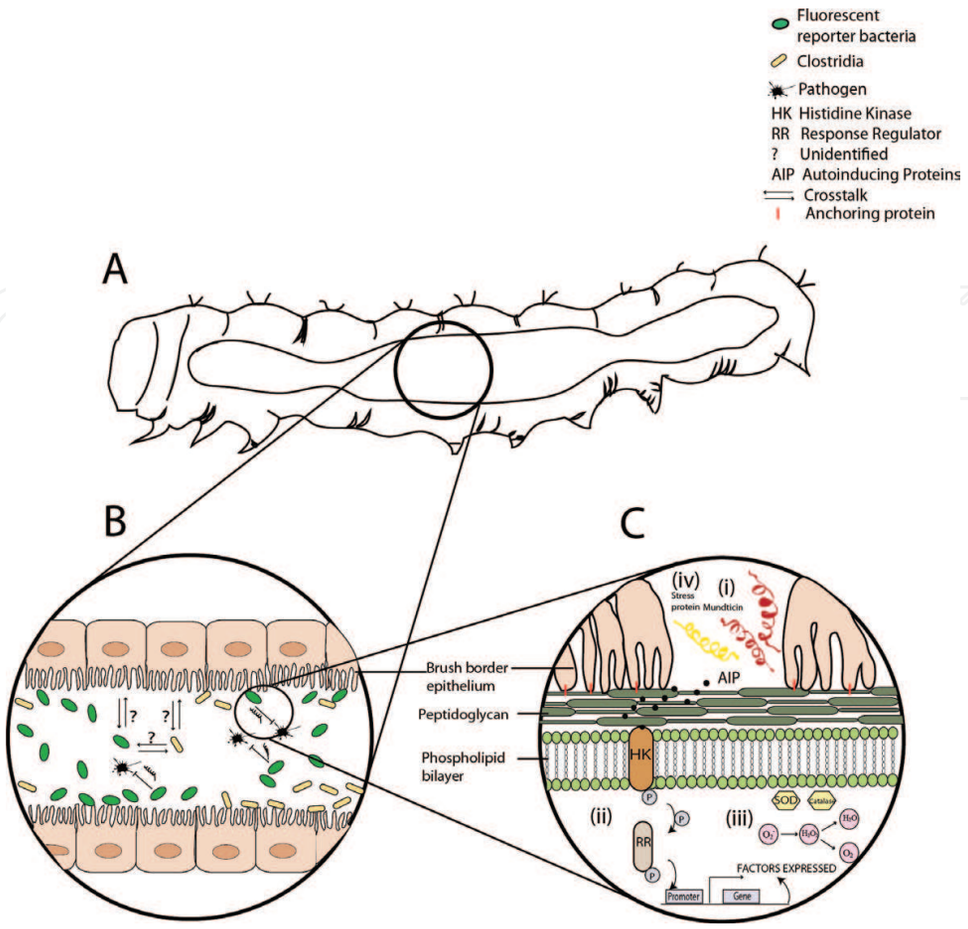

Figure 3. The gut microbiome of *S. littoralis* was dominated by *E. mundtii* and *Clostridia* sp. (A) Overview of the gut structure of fifth-instar larva of *S. littoralis*. (B) Illustration from within the gut space, which harbors major symbionts *E. mundtii*, *Clostridia* sp. and other bacteria. Bacteria adhere to the mucus layer of insect gut epithelium. Unknown interactions occur between microbe-microbe and host-microbe. (C) Illustration of some major expressed pathways *E. mundtii* used for survival in the gut. (i) Secretion of mundticin, an antimicrobial peptide, keeps pathogens at bay and helps the *E. mundtii* dominate the colonization process. (ii) A two-component system involving the accessory gene regulator (*agr*) system, which directs a histidine kinase to phosphorylate the response regulator, leads to the activation of transcription factors required for adaption. (iii) The induction of superoxide dismutase and catalase to manage oxidative stress leads to the conversion of superoxide radicals to water and oxygen. (iv) General or universal stress proteins help to overcome different kinds of stresses, such as oxygen starvation, heat or oxidative stress (unpublished).

Adhesion to the host gut epithelial surface is another key to successful colonization. Endosymbionts employ certain proteins (motifs and domains) for this purpose. These are mostly surface proteins associated with the cell wall and employing certain motifs, which act as the signal peptide for attaching to the cell wall. For example, the motif called LPXTG is a sorting peptide. The endopeptidase sortase cleaves it at the site between threonine and glycine residues, and links the peptide covalently to the peptidoglycan of the cell wall [109]. There is up-regulation in the genes encoding this motif and also in the sortase enzymes, indicating attachment of *E. mundtii* to the insect gut wall and biofilm formation. The up-regulation of the WxL domain hints at the increased colonization of the bacteria by their adherence to the gut epithelium. The WxL domain proteins are also crucial for adapting to varying environmental conditions [110].

The ability to adapt to variable living conditions is very much attributed to "two-component systems." These systems form a class of signal-transduction mechanisms that are induced when the insect senses stress in the environment. The main players in the system are auto-inducing proteins (AIPs), histidine protein kinases (HPKs) and response regulators. AIPs, which interact with the HPKs, are produced in response to stress. The signal is relayed to the response regulators. This cascade ultimately produces certain factors or proteins that aid *E. mundtii* to survive in the stressful environment [111]. Accordingly, the agr family of genes was found upregulated in *E. mundtii* living in the insect gut.

Quorum sensing is a phenomenon where the bacterial cells interact and communicate with one another for survival. AIPs are also key players for quorum sensing. In addition, also several quorum-sensing strategies are two-component systems. AIPs accumulate in response to increases in bacterial cell density; these increases are followed by a signaling cascade and lead to cooperative gene expression by the bacteria [112].

Stress proteins are adaptive factors that are induced when living conditions become stressful. There exist general and universal stress proteins. General stress proteins help bacteria deal with oxidative stress, heat stress, salt stress or oxygen limitation [113]. Universal stress proteins are induced in response to temperature fluctuations, heat or oxidative stress and hypoxia. Both of these protein classes were upregulated in *E. mundtii* in response to the insect gut's living conditions [114].

The type of sugar transport system expressed by bacteria depends on the types of carbon sources available. Phosphotransferase systems form a class of sugar transporters that sense the sugar source available in the environment and allow the respective transporters for fructose, glucose, mannose or cellobiose to act on it. Using energy from phosphoenolpyruvate, the transport system utilizes a cascade of cytoplasmic protein components with an accompanying phosphorylation of each component [115]. These transporters are generally sugar specific and because they help bacteria to survive in presence of complex carbohydrate conditions, they are said to help in their adaptation. Several of these PTS systems are upregulated by *E. mundtii* living in the gut of *S. littoralis*.

6. Discussion

Lactic acid bacteria are important in the production of fermented foods, such as dairy products. LAB is potential probiotics that provide benefits to human health [116]. Modified LAB could also be used as live vaccines or vaccine delivery systems [117]. It has been shown that the genetically modified *L. lactis* can survive and colonize the digestive tract of humans [118] and gnotobiotic mice [119]. In this chapter, we report the use of GFP to tag *E. mundtii* to monitor the bacteria's survival and activities in the intestinal tract of cotton leafworm, *S. littoralis*.

It has been shown that spatial and temporal distribution of fluorescent *E. mundtii* was observed across all developmental stages (**Figure 4**), as well as in the foregut, midgut and hindgut of *S. littoralis*. Data from the colony forming units (CFUs) show that the midgut houses the most

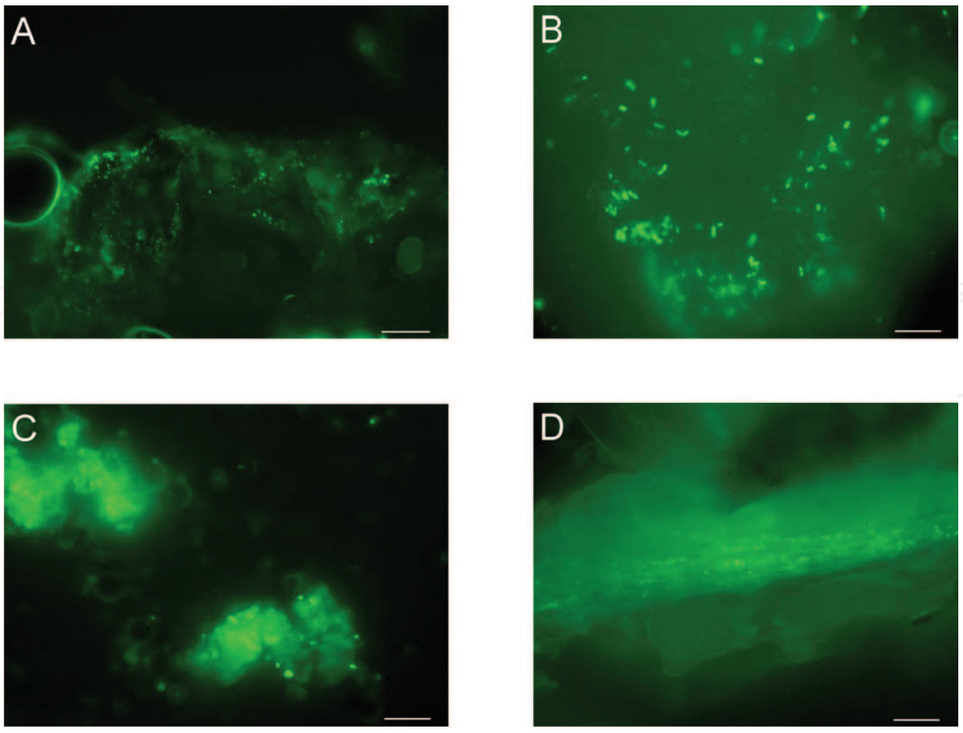

Figure 4. Photo showing the localization of fluorescent *E. mundtii* in the intestinal tract of *S. littoralis* at different life stages. (A) Bacterial cells accumulate on the peritrophic matrix separated between gut lumen and epithelium of fourth-instar larvae. (B) Bacteria cluster in the gut of fifth-instar larvae. (C) Fluorescent bacteria are visibly colonizing the tissue of pupae, although no gut tissue has been formed. (D) Vertical transmission of symbiont is evident as fluorescent *E. mundtii* survive first-generation and colonize second-generation first-instar progeny. Scale bars: 10–20 μm [14].

abundant bacterial counts, followed by the hindgut and foregut. Interestingly, the fluorescent *E. mundtii* were also detected in the eggs of *S. littoralis* [14], supporting a direct symbiont transmission from one generation to another. Other studies have shown that fluorescent bacteria were transmitted from the gut to the eggs in *Tribolium castaneum* [120]. The symbiotic *E. mundtii* was transmitted to the second-generation progeny, suggesting that the bacteria co-evolve with the insect host (**Figure 4D**). In addition, the fluorescent bacteria were detected in fecal samples of the larvae, indicating they had traveled successfully along the intestinal tract of *S. littoralis* (data not shown). The details of how a bacterial symbiont is transmitted from one generation to the next remain to be clarified. The symbiont that co-evolves with a host has a great chance to secure vertical transmission, for example, a symbiotic relationship exists between the aphid and its endosymbiont *Buchnera aphidicola*. It has been shown that the GFP-tagged *Asaia* strain is vertically transmitted from the mother to the offspring in *Anopheles stephensi* [121]. Bacterial symbionts can be horizontally transferred via "egg smearing," a phenomenon that involves a female stinkbug covering the surface of its eggs with symbiotic bacteria during oviposition. The newly hatched juveniles acquire the symbionts by ingesting the egg case [122].

Several factors, including the pH, redox potential, oxygen availability, and the nutrient and immune systems, can shape the microbial composition of the gut of insects [123]. Furthermore, constant change in gut contents due to molting and metamorphosis can affect the colonization of microorganisms. Many insects have an intestinal pH in the range of 6–8, and some

lepidopteran larvae have an even higher pH (11–12) in their midguts [124, 125]. The hindgut harbors high bacterial diversity and density in several insects, such as cockroaches, crickets and termites [126–128].

Microorganisms that live in the hindgut benefit from the metabolites and ions transported from the malpighian tubules into the hindgut. The hindgut, which stores nitrogenous and food waste, may contain nutrients for insect gut bacteria [123]. The hindgut is involved in water resorption [129]. The microbiota in the ileum of the hindgut of scarab beetles metabolizes plant polysaccharides into components that can be used by the insect [130]. In contrast to the hindgut, the midgut is an unfavorable environment for microorganisms. Many antimicrobial peptides [131] and digestive enzymes (lysozymes) [132] are secreted by the midgut epithelium cells of *D. melanogaster*. The peritrophic matrix secreted by midgut epithelial cells tends to accumulate digestive enzymes and to serve as a barrier to separate food particles, toxins and microorganisms [133]. The high alkaline pH in the gut of lepidopteran insects could kill many microorganisms. However, alkaline conditions favor the dominance of Firmicutes-related bacteria in the midgut of the beetle *Pachnoda ephippiata* [134]. Both culture-dependent and culture-independent methods have detected the presence of *Enterococcus* in the alkaline midgut of the gypsy moth larva [135].

The mechanisms of bacterial colonization in specific regions of the gut are not well understood. The gut of *S. littoralis* does not possess specialized structures called bacteriomes that contain endosymbionts, such as are found in aphids, whiteflies and other insects. How *S. littoralis* houses *E. mundtii* remains unknown, as no compartmentalized structures exist to protect the bacterium; for example, the gut of the pupae has been strongly reduced. Several mosquito species, especially newly emerged adults, that undergo metamorphosis eliminate their gut bacteria [136]. The host organism selects its own microorganisms as it depends on these for growth and development. As an example, see the case of the honeybee, whose bacterial symbionts were unable to survive in the gut of bumble bees [137].

Only a few of the important survival strategies of *E. mundtii* have been mentioned. There are several other pathways that are meant for their adaptation to the differential living conditions inside the gut. We anticipate that further RNA sequencing will help explain some of the other mechanisms that help the bacteria to survive in the gut.

E. mundtii is clearly a successful and a major symbiont in the gut of *S. littoralis.* The method that we have developed here can be used to investigate an indigenous bacterial species within the whole community. With further improvements and modifications, this kind of reporter system may be useful in many other species-specific interaction studies.

7. Future prospects

The survival strategies of *E. mundtii* in the gut of *S. littoralis* have been unveiled, yet the mechanisms employed by host insect to control the bacterium remain poorly understood. Transcriptomic analyses of the reporter organism indicated already a pattern of relevant

enzymes allowing the microbes to adapt to the harsh conditions of the insect gut. The studies can be extended to the very special conditions in the pupae where fluorescent bacteria could be observed. Thus, the concept of using a fluorescent reporter organism that can be recovered at any time from any area of the intestinal tract will allow a holistic analysis of adaptation strategies used by the microbes to adapt to the different developmental stages of the insect, as well as to study the impact of food-ingested plant toxins. In combination with the analysis of transcript patterns from the gut membranes, a first insight into the molecular interaction between the insect host and the microbiome can be expected. In conjunction with CRISPR/CAS9-created specific knock downs of defined metabolic capacities of the insect, detailed questions concerning the molecular dialog between the insect host and the microbial consortium can be answered.

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