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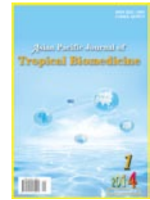

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Insect gut microbiome – An unexploited reserve for biotechnological application

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PEER REVIEW

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Comments

This work is interesting and contains novel data collection. Situated as a systematic review, this work can be a good data collection for further referencing in metagenomics that is relating to insect microbiology.
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ABSTRACT

Metagenomics research has been developed over the past decade to elucidate the genomes of the uncultured microorganisms with an aim of understanding microbial ecology. On the other hand, it has also been provoked by the increasing biotechnological demands for novel enzymes, antibiotic and signal mimics. The gut microbiota of insects plays crucial roles in the growth, development and environmental adaptation to the host insects. Very recently, the insect microbiota and their genomes (microbiome), isolated from insects were recognized as a major genetic resources for bio-processing industry. Consequently, the exploitation of insect gut microbiome using metagenomic approaches will enable us to find novel biocatalysts and to develop innovative strategies for identifying smart molecules for biotechnological applications. In this review, we discuss the critical footstep in extraction and purification of metagenomic DNA from insect gut, construction of metagenomic libraries and screening procedure for novel gene identification. Recent innovations and potential applications in bioprocess industries are highlighted.

KEYWORDS
Microbiome, Metagenomics, Non-cultivable microbes, 16S rRNA, Gypsy moth, Termite gut

1. Introduction

Insects are the most successful group of animals, both in terms of diversity and survivability in various ecological niches. The insect gut is estimated to contain 10 times more microbes than total cells of the insect and 100 folds more microbial genes than animal genes[1]. Microorganisms colonize the insect gut through food and plays a significant role in digestion and metabolism. While most of the gut microbes are commensals or parasites, some of them are known to play beneficial role for their hosts. Few insect gut symbionts are vertically transmitted and their association is mutually essential such as *Buchnera* sp. in aphid flies[2]. However, such extra cellular associations are thought to be vulnerable to invasion and replacement by transient microbes. Most of the studies were focused on

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understanding the interactions between host and symbiotic microbiota.

Recently, both basic and applied research in biotechnology is focused on the identification of novel genes and proteins/ enzymes from various natural sources. Soil and other environmental niches were considered to be the prominent sources of novel biomolecules. Metagenomic approaches allow us to access the genomes of all microorganisms referred as the microbiome. Metagenomics makes it possible to relate potential function of the specific microorganisms within the gut communities. This review describes insect gut metagenomic methodologies, approaches in novel protein/ enzyme discovery and their potential industrial applications.

2. Mining the gut microbiome

Experimental insects are washed in suitable sterile buffer and dissected to obtain the complete gut. The gut may be separated into three parts (foregut, midgut and hindgut) (Figure 1). Each part is suspended in extraction buffer and the metagenomic DNA extraction is performed. Cell lysis is a critical step in metagenomic DNA extraction. The enzymatic lysis is gentle and therefore used to lyse the insect gut cells. To access the enriched gut microbiome, the remaining intact microbial cells are washed, lysed again and subsequently DNA was extracted. The mechanical lysis methods such as thermal shocks, homogenization and bead beating can be used to attain complete lysis[3].

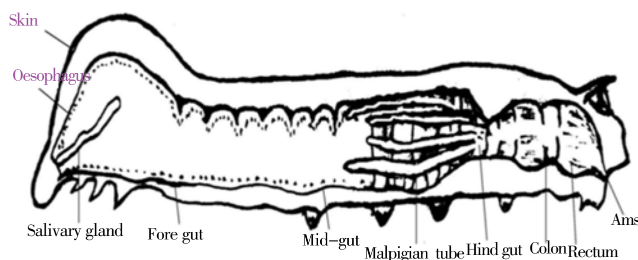

Figure 1. Image of the morphology and different regions of the insect gut.

3. Accessing genomes from cultivable microbes

The gut samples are suspended in phosphate buffered saline, serially diluted and plated on suitable nutrient media. Plates are incubated in a growth chamber at 28 °C for 48 h, and bacterial colonies are categorized based on morphology from the plates with three least countable dilutions. Pure cultures of bacterial isolates are subjected to screening for various enzymes. Subsequently, the DNA from cultures showing important enzyme activities is extracted and the genes coding for the enzymes are cloned and sequenced[4].

4. Accessing genomes of total microbiota

Till date, no specific method has been published, which is universally accepted for the isolation of metagenomic DNA from the insect gut. The major goal of the metagenomic DNA isolation should be to get unbiased access of all microbial communities. In addition, degradation and contamination of the isolated metagenomic DNA should be considered. During metagenomic DNA isolation, shearing or DNA damage should be taken care to obtain the high molecular weight DNA, so that it can be used for construction of metagenomic DNA library using BAC vectors. The metagenomic DNA must be free from other macromolecules without affecting the downstream application such as restriction digestion, PCR and cloning[5].

Most of the gut metagenomic DNA extraction procedure has been adopted from soil DNA isolation methods with slight modifications. In metagenomic DNA isolation, two major strategies have been employed; they are the cell recovery method and the direct lysis method[6].The cell recovery method isolates intact microorganism from the gut content prior to cell lysis, and the cell isolation is carried out either by frequent homogenization and differential centrifugation or by gradient centrifugation in media such as percoll or sucrose[7,8]. Some commercial kits are also available for the isolation of metagenomic DNA from uncultured organisms. However, the isolation protocol must be standardized because most of these kits are not designed for insect gut metagenomic DNA isolation.

In general, individual gut or pooled guts from 10 or 30 larvae or adult insects are placed in 1.5 mL microfuge tubes containing 50 μL PBS and maintained at 4 °C until DNA extraction. The tubes are gently mixed and centrifuged at low speed to pellet the insect gut and DNA is extracted from the bacteria in the supernatant. Cell lysis should be aimed to lyse microorganisms but not the insect gut tissues. Few reports are available for the selective isolation of microbes from soil and from plant tissues. A Nycodenz density gradient was successfully used to separate bacterial cells from soil particles[9]. Similarly, density gradient centrifugation has been applied to enrich microorganisms associated with plant tissues[10]. However, no reports are available for the selective lysis of microorganisms from the insect gut. Since the insect cells are considerably higher than the bacterial cells, the gut suspensions may be subjected to filtration with different pore sized filters. For example, if the suspension is passed through a 1 μm filter, insect cells will be in the retentant and the bacterial cells will be in the filtrate. Subsequently, the bacterial cells can be concentrated through a 0.2 μm filter.

5. Strategies for specific gene enrichment

During the hunt of signified genes, various strategies of gene enrichment were employed which inturn increased the efficiency of cloning prospect and also rushed the exploration of uncharacterized/unknown genes from a reservoir. A typical mode of gene enrichment can be achieved through exposing the microbes under selective pressure such as nutritional selective conditions. Those selective community microbes with preferred phenotype will yield a high responsive/boosted gene enrichment in the particular substrate of interest. The enrichment techniques include suppressive subtractive hybridization phage display and affinity capture[11,12].

6. Metagenome expression libraries (function based screening)

Metagenomic libraries based on the functional gene assessment are developed by inserting fragmented metagenomic DNA into cloning vectors based suitable host system. The gene expression was screened by functional assays. Recently, identified genes from the insect gut microbiome by functional screening are listed in Table 1. The advantage of direct screening of functional gene from metagenome libraries was that it enables the researchers to access previously unclassified/novel genes. Furthermore, the characteristics of functional gene such as enzyme activities are expressed with a proficient vector. Heterologous expression of a gene in the host cells is impeded by various steps such as transcription, translation and post translational process or maturation.

In a complex gene cluster, a core enzymatic activity was attenuated in one or more subunits found adherent in a single colony. At most of the circumstances, the clone from a metagenome will elevate a single subunit in a clone, to rectify this and to activate the core enzyme resolution, a suitable host vector carrying multiple fragments in a single clone was exhibited. Usually, the metagenomic library construction is splitted into two different modes: 1. Small fragment library with inserts between 2 and 10 kb are constructed in plasmids or in lambda expression vectors, and then screened for enzyme expression; 2. Larger fragment library preferentially necessitate expression libraries with inserts between 20 and 40 kb in cosmids and fosmids, and 100 to 200 kb in bacterial artificial chromosome vectors.

Though *Escherichia coli* (*E. coli*) host strains have relaxed necessities for promoter recognition and translation initiation, many genes from environmental samples may not be expressed efficiently in heterologous hosts. This is due to differences between transcription and/or translation initiation signals, protein-folding elements, post-translational modifications, such as glycosylation, or toxicity of the active enzyme. These obstacles could be conquered partly by selecting suitable vector systems including transcription and translation-initiation sequences at both ends and using suitable expression hosts, such as the *E. coli* Rosetta strains that contain the tRNA genes for rare amino acid codons[12], or co-expression of the chaperone proteins, such as GroES, GroEL, and DnaK *etc*[13]. The studies conducted by Martinez *et al.*[14] suggested the alternative heterologous gene expression systems used in the metagenomic study. Several modified function-based methods exist specifically for exploring metagenome libraries[15]. Uchiyama[15] have reported the screening based on substrate expression to rapidly identify clones that can be induced by a target substrate (substrate induced gene expression) and display catabolic gene expression, while metabolite-regulated expression detects clones generating Quorum-sensing gene-inducing compounds[16].

Several novel genes were reported for polysaccharide and plant cell wall biomass-degrading enzymes. In most of the colorimetric-based analysis employing dye-linked substrates of reacting products staining was used for preliminary screening. Only elected clones were then confirmed by enzyme activity assays. For screening the large metagenomic libraries, Guan *et al.*[17] have developed a high throughput intracellular screen technique called metabolite regulated expression where the metagenomic DNA and biosensor exist in the same cell. In this case, the biosensor detects compound that induce the expression of GFP from the bacterial quorum sensing promoter and this can be further detected by fluorescence activated cell sorting[16].

7. Metagenomic sequencing (homology based identification)

When compared to function-based methods, homology based identification can divulge target genes despite of the expression problems in the heterologous hosts. Sequence-based screening methods depend on the existing conserved sequences, and hence, may not help to identify brand new non-homologous enzymes[18]. Cloning and sequencing of entire microbiome by conventional Sanger's method is a tedious process because of its complexity and size. The development of next generation sequencing technologies such as illumina, solexa and 454 pyrosequencing, has changed the scenario of metagenome sequencing. Metagenome sequencing captures DNA from diverse organisms, and many sequence reads remain unassembled due to the variation in size. Even the fragments may be extensive to contain the full open reading frame of the gene of interest. Among the next generation sequencing strategies, the 454 pyrosequencing method using recently developed titanium kit, which yields more than 500 bp read length and appears more promising than other approaches[19].

Table 1

List of enzymes/genes from the insect gut microbiome by functional screening.

Insect gut source	Enzyme/Gene	Potential applications	Reference
Reticulitermes flavipes	RfBGluc-1 beta- glucosidase	Lignocellulose digestion	Mattiacci *et al.*(1995)
Rotschildia lebaeu (Lepidoptera)	Xylanase	Xylan degradation	Brennan *et al.* (2004)
Termites (Nasutitermitidae)	Endo-1,4-β-xylanase	Xylane degradation	Brennan *et al.* (2004)
Nasutitermes ephratae	Glycosyl hydrolase	Lignocellulose digestion	Warnecke *et al.* (2007)
Termites (*Nasutitermes takasagoensis*)	Bacterial glycosidase genes	Polysaccharide degradation	Chaffron *et al.* (2007)
Reticulitermes flavipes	esterase	Hemicellulose solubilization	Marsha *et al.* (2009)
Gypsy moth (*Lymantria dispar*)	Quorum-sensing compound	Communication within the microbial communities	Tartar *et al.* (2009)
Termites (*Reticulitermes flavipes*)	Beta-glucosyl ceramidase	Cellulose	Tartar *et al.* (2009)
Termites (*Reticulitermes flavipes*)	Trehalase	Trehalose	Tartar *et al.* (2009)
Termites (*Reticulitermes flavipes*)	Alpha-mannosidase	Mannose	Tartar *et al.* (2009)
Termites (*Reticulitermes flavipes*)	Endo-beta-N-acetylglusosaminidase	Oligosaccharides	Tartar *et al.* (2009)
Limnoria quadripunctata	glycosyl hydrolase genes	Lignocellulose digestion	Andrew *et al.* (2010)
Coptotermes formosanus	β-glucosidase	Cellulose degradation	Zhang *et al.* (2010)

The sequence-based search combined with efficient bioinformatics tools may result in the identification of novel genes in a higher rate than by the function-based methods. Bioinformatics tools have been developed for sequence mining, based not only on primary sequence homology but also on the basis of predicted protein structures (Table 2). With the betterment of protein sorting and modeling tools, the putative active sites, gene function can be predicted[20]. Gene-finding tools, like MetaGene, were used to predict 90% of shotgun sequences[21].

Several recent publications describe metagenome sequence databases searching in prospecting for genes and enzymes that will be useful for commercial production. For example, sequencing a metagenome library of hindgut microbiota from the largest family of wood-feeding termites generated 71 million base pairs of sequence data. By detecting complete domains using global alignment, more than 700 domains homologous to glycoside hydrolase catalytic site corresponding to 45 different carbohydrate-active enzymes families including a rich diversity of putative cellulases and hemicellulases were identified[22].

8. Recent insights and potential applications of insect gut microbiome

8.1. Cellulose and xylan hydrolysis

Termites are an extremely successful group of wood degrading organisms and hence they are the potential sources of catalysts for efforts aimed at converting wood into biofuels. Warnecke *et al.* have reported the metagenomic analysis of the bacterial community residing in the hindgut of a wood feeding higher *Nasutitermes* species and show the presence of a large, diverse set of bacterial genes for cellulose and xylan hydrolysis[22]. They have identified a number of previously uncharacterized protein families. Thus, degradation of lignocellulose does not occur by a single enzyme but due to the interaction of many macromolecular complexes that lead to its degradation. These macromolecular complexes have been termed as cellulosomes and are partially known in several microbes. The cellulose degradation of termite was long thought to rely only on microbial gut symbionts[23]. More recently, cellulase gene transcripts have been identified from the termite itself[24]. Similarly, three xylanases genes have been discovered from lepidopteran intestinal tract samples, and one from termite sample. The digestome of the insect gut comprising of microbial as well as termite coded enzymes act together to bring out the complete digestion of lignocelluloses. Many microbes have been identified and play important roles in the conversion of wood into a biofuel, such as ethanol, because of its potential for at least partially replacing fossil fuels in transportation and thereby lowering greenhouse gas emissions[25].

8.2. Vitamin production

The genome of *Wigglesworthia* sp., the mycetocyte symbiont of *Glossina brevipalpis* has been sequenced and the

Table 2

Online tools for the analysis of metagenomic DNA sequences.

Online tool	Website	Application	Reference
BRENDA	http://www.brenda- enzymes.org/	To find the comprehensive enzyme information system in the metagenomic library	Vineet K. Sharma *et al.* (2010)
CAMERA	http://camera.calit2.net	To observe the microbial communities in the ocean and their response to environmental changes	Rekha Seshadri *et al.* (2007)
DOTUR	http://www. plantpath.wisc.edu/fac/ joh /dotur.html	It assigns sequences to operational taxonomic units (OTUs)	Patrick D *et al.* (2004)
EnGenIUS	http://engenius.software.in former. com/	Provides a comprehensive metagenome research toolset specifically designed to accommodate the needs of large environmental genome sequencing efforts	Kaplarevic M *et al.* (2008)
EnvDB	http://metagenomics.uv.es /envDB	Classifies the environmental samples and their associated 16S rDNA sequences	Miguel Pignatelli *et al.* (2009)
GAAS	http://sourceforge.net/proj ects/gaas/	GAAS is used to calculate accurate community composition and average genome size in metagenomes	Angly FE *et al.* (2009)
HOMD	http://www.homd.org	Provides comprehensive set of analysis tools and maintains frequently updated annotations for all the human oral microbial genomes that have been sequenced and publicly released	Tsute Chen *et al.* (2010)
IMG/M	http://img.jgi.doe.gov/m	Provides comparative data analysis tools extended to handle metagenome data, together with metagenome-specific analysis tools	Markowitz *et al.* (2008)
MEGAN	www-ab.informatik.uni-tuebingen. de/software/me gan	To compute and interactively explore the taxonomical content of the dataset, employing the NCBI taxonomy to summarize and order the results	Daniel H *et al.* (2007)
Megx.net	http://www.megx.net/	To predict gene functions of metagenome sequences	Renzo Kottmann *et al.* (2009)
MetaBioME	http://metasystems.riken.jp/ metabiome/	To find novel homologs for known commercially useful enzymes in metagenomic datasets and completed bacterial genomes	Vineet K. Sharma *et al.* (2009)
MetaSim	http://www-ab.informatik.uni-tuebingen.d e/software/metasim.	To generate collections of synthetic reads that reflect the diverse taxonomical composition of typical metagenome data sets	Daniel C *et al.* (2008)
MG-RAST	http://metagenomics.nmpdr.org/	Provides a new paradigm for the annotation and analysis of metagenomes	F Meyer *et al.* (2009)
Proxygenes		Annotates the metagenome short Reads	Daniel Dalevi *et al.* (2008)
RDP database	http://rdp.cme.msu.edu/ http://rdp.cme.msu.edu	Provides ribosome related data and services to the scientific community, which comprises online data analysis and aligned and annotated Bacterial and Archaeal small-subunit 16S rRNA sequences	Cole JR *et al.* (2007)
SOrt-ITEMS	http://metagenomics.atc.tcs.com/ binning/SOrt- ITEMS.	To identify an appropriate taxonomic level	M. Monzoorul Haque *et al.* (2009)
UniFrac	http://bmf.colorado.edu/unifrac	Provides easy access to powerful multivariate techniques for comparing microbial communities in a phylogenetic context	Catherine Lozupone *et al.* (2006)

annotation has revealed the presence of genes encoding for the synthesis of pantothenate (Vitamin B_5), biotin (Vitamin B_7), thiamin (Vitamin B_1), riboflavin FAD (Vitamin B_2), pyridoxine (Vitamin B_6), nicotinamide (Vitamin B_3) and folate (Vitamin B_9)[26].

8.3. Nitrogen fixation and phenolics metabolism

Insects can absorb the atmospheric nitrogen only through the symbiotic association with gut associated bacteria because the ability to fix nitrogen is widely available among bacteria but apparently absent from all eukaryotes. Nitrogen fixing *Enterobacter* species have been isolated from the southern pine beetle, which together with some fungal associates, may concentrate nitrogen on developing larvae[27]. *Rahnella aquatilis*, *Klebsiella* species and *Pantoea* species were commonly found in southern pine beetle, and the pine beetle (*Dendroctonus frontalis*) larvae are known to fix nitrogen in other environments[28]. Another important role might be detoxification of conifer defensive compounds, which consists primarily of monoterpenes, diterpenes and phenolics groups known to be metabolized by bacteria[29].

8.4. Antibiotic resistance

Allen *et al.*[30] have reported that gypsy moth midgut microbial community harbors hitherto unknown antibiotic resistant genes. In particular, novel β-lactamases from gypsy moth midgut metagenome were identified. These genes were found to confer resistance in *E. coli*, illustrating that insects might play a role in disseminating important antibiotic resistance genes[30].

8.5. Signal mimics

Microbes produce metabolites with diverse chemical feature and biological activities[31]. Signal molecules have been reported from the uncultured microbial world through insect gut metagenomics. Guan *et al.*[17] applied the Matrix screen to a metagenomic library constructed from the microorganism associated with midgut of gypsy moth. They have reported the identification of a metagenomic clone of gypsy moth midgut microbiome that produce inducers of quorum sensing and that are chemically different from the earlier quorum sensing inducers. The clone harbored the gene coding for monooxygenase homologue that mediates a pathway of indole oxidation which resulted in the production of a quorum sensing compound.

9. Future perspectives

The metagenomic approach provided a thorough knowledge on microbial census in the insect gut. Identification of novel genes and the development of potential biotechnological applications is a great challenge due to the complexity of microbial species and the presence of diverse genes in their genomes. Many bioinformatics programmes are developed for collection and deposition of the metagenomic sequences composition, and data management. More sophisticated bioinformatics tools are expected to be developed to analyze the hitherto unexplored microbial genes of insect gut metagenomics. Even though, the new high throughput sequencing technologies generate the identification of novel candidate genes. Assay for protein function represents one of the most important and inimitable tool for identifying their targets genes. Hence, the development of high throughput functional screening methods will reduce the time in primary screening.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

This work is a review on novel aspect of metagenomics. The review focus on this new omics science and its application in specific micrology aspect of the insect. It can be future referenced in future papers.

Research frontiers

Although it is not an original article, this work has novelties and report on interesting clinical omics science. It is a good attempt and up-to-date to collect the data on metagenomics of insect gut microbiome in the era of worldwide studies on omics science.

Related reports

This work has an interesting novelty and report on the area that lacks for information and publication in the present literature. Few reports are available on this aspect, as metagenomics is specifically applied to the insect gut microbiome.

Innovations and breakthroughs

This work has novelty and report on rarely published of area of omics science, metagenomics. By nature, the review on this new area of omics, metagenomics, is limited and the specific article on microbiology of insect gut is extremely limited.

Applications

This work can be further applied in the field of genomics and microbiology. It can be further referenced in future studies on insect microbiology and might be extrapolated to

the study on pathology, physiology and pharmacology that are relating to human and animal health as well.

Peer review

This work is interesting and contains novel data collection. Situated as a systematic review, this work can be a good data collection for further referencing in metagenomics that is relating to insect microbiology.

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