Agriculture, Agribusiness and Biotechnology

Vol.60: e17160370, January-December 2017
http://dx.doi.org/10.1590/1678-4324-2017160370
ISSN 1678-4324 Online Edition

BRAZILIAN ARCHIVES OF BIOLOGY AND TECHNOLOGY

A N I N T E R N A T I O N A L J O U R N A L

Functional Genes of Microorganisms, Comprehending the Dynamics of Agricultural Ecosystems

Catiusca Reali[1]*, Harry Luiz Pilz Júnior[1], Jean Christophe Meile[2], Sabine Shorr Galindo[3], Lidia Mariana Fiuza[4].

[1]Universidade do Vale do Rio dos Sinos - Laboratório de Microbiologia e Toxicologia, São Leopoldo, Rio Grande do Sul, Brasil; [2]CIRAD – Qualisud, Montpellier, France; [3]Université de Montpellier, Montpellier, Languedoc-Roussillon, France; [4] Instituto Rio Grandense do Arroz, Cachoeirinha , Rio Grande do Sul, Brasil.

ABSTRACT

The microbial composition of different types,in ecosystems (including agro-ecosystems), has been investigated in a rapidly growing number of studies in the past few years. The importance of microorganisms, regarding the maintenance and stability of nutrients in agroecosystems, is a key to maintain the sustainability of a crop. Molecular tools to study microbial communities are possible through many methods such as RISA, DGGE, TGGE, clone libraries, T-RFLP, RAPD, SSCP and more recently NGS (Next-Generation Sequencing). DGGE is widely employed to characterize the diversity and the community dynamics of microorganisms in the environment, making possible to find out specific groups through functional genes, allowing access to data that cannot be obtained by cultural methods. The aim of this paper is to review the functional groups related to agroecosystems and to indicate the critical choice of DNA primers pairs and targeted DNA regions that may be used in PCR-based methods such as the DGGE technique in order to evaluate the microbial communities in a variety of environments.

Keywords: agroecosystems, functional genes, bacteria, DGGE, metagenomics, microbial ecology

*Author for correspondence: catiuscar@gmail.com

INTRODUCTION

Microorganisms play a major role in biochemical cycles, soil and water nutrient reposition, and they could be used as biological indicators of soil quality [1]. Molecular biology tools have become important to allow the identification of key species in ecosystems [2], or even for the identification of microbial communities with specific functions. The functions realized by the microorganisms can be related to: the fixation of atmospheric nitrogen, decomposers of organic residues or pesticides residues, promote the cycling of nutrients, fight diseases in plants caused by pathogens present in the soil and produce bioactive compounds like vitamins, hormones and enzymes that stimulate the growing of the plants [3].

As most bacterial species present in general ecosystems cannot be grown in laboratory conditions, the use of culture-independent, PCR-based techniques is very promising to explore microbial communities [4,5]. For example, the PCR-DGGE technique, besides from inferring communities that are present in soil and water, may also help to identify epiphytic [6] and endophytic bacterial communities [7,8], which are relevant in agroecosystems studies.

Agroecosystems provide different environments with a wide variety of microhabitats. Rice fields, for instance, remain irrigated during most of the culture cycle [9], and may have a quite complex bacterial community [10]. Culture management and farming systems also interfere with microorganisms composition. Crops in general are manipulated during the agricultural year receiving the addition of different nutritional inputs, besides irrigation and soil trundling, for instance. Moreover, the phenological phases of crops may also modify soil characteristics through the excretion or absorption of substances by plants [11,12].

Microorganisms, for many reasons, directly affect crop development. Microbiota in the soil and water provide nutrient cycling in rice paddy ecosystems [13]. The study of bacterial functional groups allows the identification of specific activities in these environments related to nutrient availability and methane consumption, which is a key function to decrease greenhouse gases production [14-16].

Ecological studies about bacterial functional groups present in the water and soil of dry or irrigated agroecosystems, allying techniques that are culture-independent or dependent through selective media may bring important data regarding the nutritional dynamics in these environments.

Microorganisms and the functionality of ecosystems

The functional diversity comprehends a wide range of microbial activities in the environment, assuming great importance in ecology studies [17], considered an important characteristic in biological assemblages [18], it supports many services to the ecosystem [19]. Species cannot provide the same effect above the ecosystem functioning, they can participate in different process [20], in other words, a variation on the microbial composition can represent a variation in the services provide by biological communities. Nevertheless, very little is known about the relationship between structural and functional diversities.

Authors have quoted some theories about the effect of species diversity regarding particular functions in the ecosystem. Some of them suggest that ahigher species diversity is beneficial to the functionality of ecosystems [21,22]. Relate the stability of ecosystems to species diversity, since they have fast growth and may occupy vague niches rapidly, may afford an efficient environment recovery after perturbation, or in other words, it returns to equilibrium condition very fast [17,22,23]. On the other hand, some authors claim that functionality relies more in species composition, therefore, in the ability of certain species to exert functions in ecosystems [24-26].

Over the past 50 years several studies has been trying to clarify questions about the diversity of species in relation to ecosystem functions [27-30]. This type of study is very used to communities of plants and animals [31]. However, for bacterial communities, it was only possible after the introduction of molecular biology methods, this happens due the difficulty imposed by conventional techniques in getting enough data for obtaining the diversity [27,32,33]. More information about methods used to obtain data through techniques for microbial ecology studies are described below.

The high functional divergence can be associated with a high degree of differentiation niches [20] and less competition for resources [18]. For species adapt well to a location, they need to tolerate the abiotic characteristics, species adapted to the same local tend to have similar functional characteristics [34], however,many species ecologically similar could not happen in a single place [24], thus, the competition can act producing functional divergence within communities.

Bacteria have a long evolutionary history, they are able to colonize the most varied environments, occupying many niches [35]. Nevertheless, the constant management in agricultural ecosystems may cause changes in the species composition caused by placing the rice straw on the soil [36], application of pesticides [37-39] or increase, in some species, benefit from exudates of plants. [40,41]. The management, in general, can benefit populations of most species adapted to the environment through the detriment of others, causing a decrease in species diversity, however, may benefit certain functional groups.

The reduction of species not always represent a loss in functions in the ecosystem, functional redundancy may occur or several species are capable of performing the same function [42]. However, despite carrying the same function, they cannot have the same efficiency, produce different metabolites as an end product or even may have lower growth rates and are not be competitive as the original community [27]. The exclusion of some species can influence the composition of other populations as a result, it causes changes in other global ecosystem functions, despite having kept the original function [43].

Techniques for the Identification of Functional Groups

Conventional techniques only provide partial data about diversity and functionality in ecosystems since they select groups that develop better in culture media and laboratory conditions. However, when they are allied to molecular biology techniques the results become more satisfying.

In order to access the diversity of species or functional groups through molecular techniques, a total DNA extraction must be performed from samples such as soil, water, sediments or even plants in the case of endophytic bacteria. Total DNA content is representative of the bacterial populations present in the environmental sample [44-46]. The advantage of using culture-independent techniques is that they do not present a series of methodological barriers to the growth and multiplication of the group to be approached, since they can be withdrawn directly from their natural habitat, from which total DNA may be extracted [47]. Studies indicate that around 99% of microorganisms present in the environment cannot be grown in the laboratory [48].

As for the bacterial diversity, many functional groups may be accessed by culture-independent techniques [32,33,49]. This sort of study facilitates the recognition of bacterial in specific environments, including agroecosystems [50]. Therefore, communities may be evaluated through time or even after disturbance simulations.

Several culture-independent techniques are utilized to obtain a profile of microbial communities: Ribosomal Intergenic Spacer Analysis – RISA [51], Denaturant Gel Gradient Electrophoresis - DGGE [52]. Temperature Gradient Gel Electrophoresis – TGGE [53], clone libraries [54,55], Terminal-Restriction Fragment Length Polymorphism

- T-RFLP [56-58], Random Amplified Polymorphic DNA – RAPD [59], Single-Strand Conformation Polymorphism – SSCP [60] and Next-Generation Sequencing - NGS [61].

Studies performed with bacteria in freshwater ecosystems are basically concerned with phylogenetic aspects, using sequence analyses of the *16S rRNA* gene polymorphisms and related techniques [32]. However, functional diversity has been studied by methods based on specific enzymatic activities. The use of functional genes brings a whole new perspective which is to access the microbial ecology. One of the main advantages in the use of functional genes is the possibility to restrict the study to the target functional group, indicating the phylogenetic relatedness of the carrying bacterium but gives few clues about its physiology [62].

Among the cultivation-independent methods, the fingerprinting is one of the most common techniques. Through them, it is possible to access the most abundant members of the microbial communities quickly, not involving high costs. DGGE is a technique widely employed to characterize microbial diversity and community dynamics in the environment, with the possibility to access specific groups through functional genes [63-66]. DGGE with amplification of PCR fragments from the *16S rDNA* gene was first employed by [52] to access biofilm-forming bacteria, but today it is used to access several functional groups in a variety of environments.

DGGE application is possible through total DNA extraction from environmental samples, that is, the mixture of bacterial communities present in those samples. The species present in the sample are separated through the denaturing gradient of a DGGE gel. The number of bands in the pattern corresponds to the number of predominant members in the community [53]. The band patterns are formed by the base pair sequences, not according to the DNA fragments size, which allow separation of species or bacterial species groups.

A variation of this technique is TGGE, which uses a thermal gradient to separate the groups maintaining constant urea and formamide concentrations.Band patterns can be evaluated by different softwares that normalize data and calculate abundance and richness of species through thickness and number of bands. Each band position is registered in a database where the comparison between DGGE gels is performed, but they must have the same denaturation gradient and migration time, that is, the gels must have a standardized methodology [67].

Species of communities are identified by band excision from the gel followed by sequencing or hybridization with specific DNA probes. Genes such as 16SrRNA (around 1,500 nucleotides) serve as clone libraries associated to many bacteria groups in a variety of environments. Soil and sediment alone comprise around 10,000 different bacterial species [68]. However, the necessity of more punctual studies led to the use of functional genes, which restrict the approach to the target group only, and not the whole environmental diversity.

Still, it is possible tocompare microbial populations or communities by next generation sequencing [69]. Moreover, sample sequencing costs tend to decrease through the application of new sequencing technologies, which are more efficient, and with higher competition between companies that provide such services [70].

Next-Generation Sequencing (NGS) methods, such as pyrosequencing, Ion Torrent, Illumina and SOLiD Systems, bring a large-scale information about the diversity of microorganisms, starting a new path in microbial ecology [71]. The challenge today is how to interpret that amount of results and information generated by these new technologies [70]. NGS methods has the advantage of generating, in a few hours, megabase sequences [72], and can be used to describe bacterial communities in various environments [35]. Pyrosequencing works through fluorescence detection, but also have some limitations. They are related to the sequencing of homopolymeric stretches, which may define the insertion or deletion of nucleotides by the intensity of the light signal, changing the results [73]. Some algorithms were designed to correct

this problem [74,75]. Ion Torrent [76] and Illumina [73] are the both "benchtop" sequencers most widely sold and has a relatively low cost; they use highly informative fractions of 16S rRNA gene [77]. Most sequencers using bases labeled with fluorophores, in the Ion Torrent the polymerase reaction generates a proton, modifying the pH of the medium. This pH change is detected by a transmitter and converted into an electrical signal.

Directing the study for the microorganisms who had roles in the ecosystem, GeoChip, developed amicroarray method [78], containing more than 24,000 probes and covers, 150 gene families involved in biogeochemical C, N and P cycling [79] an important tool in agricultural ecosystems. NGS use a more robust analysis and as a consequence, increase the analytical power of results, being more important in several projects involving genomics and metagenomics [70]. Bioinformatics software are fast and are in steady development, increasing the amount of data evaluated and contributing to the construction of megabases, increasing, as well, the amount of information on various ecosystems.

Functional genes

In agricultural environments many microbial activities are related to plant development and consequently to crop productivity. Functional biodiversity in agroecosystems is an ecological key to sustainable production, and microorganisms have a fundamental importance in this process [2,80]. Soils that are poor in microorganisms exhibit a higher demand on fertilizers and synthetic addiction of nutrients, which besides increasing production cost, may also increase the risk of contamination of nearby natural environments. Moreover, some nutrients rely on microorganisms to be absorbed such as mycorrhizal bacteria and fungi which are directly associated to nutrient absorption by plants.

Microbial characterization of specific environments such as agro-ecosystem soils or water from irrigated crops may be performed with the use of different target genes. Describing the microbiota of these sites brings a series of new insights into the functional roles of fungi and bacteria in those habitats. Among the functional groups present in the soil,with particular agricultural importance, are microorganisms such as diazotrophic, denitrifying and ammonifying bacteria. As well microorganisms capable of degrading complex polymers, methanogenic and methanotrophic bacteria and archaea participating in the carbon cycle. An overview of functional groups discussed in this paper can be found in Table 1.

The use of specific DNA primers to detect the related genes brings a rapid response regarding the presence and composition of functional groups. Today there are a few sets of oligonucleotides, which are used according to the group to be accessed (Table 2). A genetic region that is sufficiently conserved among the target group allows the design of primers used to the identification of such groups, but this does not mean this functional gene is actually being expressed by the community in the environment [50].

The urea is the principal nitrogen fertilizer utilized in rice crops, however occurs a great loss of nitrogen by the volatilization in ammonia (NH_3) [105]. The loss of nitrogen fertilizers in crops may be 20-40% of the nitrogen applied [106]. Ammonia oxidation is the key step in the nitrogen cycle [86,107] were the enzyme ammonia monooxygenase (AMO) oxidizes ammoniac to hydroxylamine and is encoded by *amoA* and *amoB* genes[108]. The functional group of ammonia-oxidizing bacteria can be accessed through the amoA-1F e amoA-2R primers 2R, describe by [99]. Hydroxylamine oxidoreductase (HAO) oxidizes hydroxylamine to nitrite [109] and is composed of subunits encoded by the *hao* gene [110-112].

A problem, resulting from the water of crops, is the production of methane, produced by aerobic bacteria [62,113]. In anaerobic environments, as flooded soil of crops, anaerobic bacteria [114,115] or archaeas [116] transform methane in nitrite, nitrate, sulfate and metal [83]. The pMMO enzyme is universally found in methanotrophic bacteria and is therefore used as a functional marker for these organisms [97,82,117]. The *pmoA* gene has been used as a marker for methanotrophic bacteria [14] and encodes a subunit of methane monooxygenase enzyme [118]. A189 and A682 primers are frequently used to profile communities that oxidize methane in the environment [119-121]. Moreover, other studies also bring the reverse primer mb661 and A650 with detection sensitivity for the *pmoA* gene [96]. However, the use of the A189 and A682 is limited to environments with high frequency of methanotrophic bacteria. The A189 and A650 primer set may not target all genus of methanotrophic bacteria but can bring satisfying results regarding community composition [97]. According to the same authors the A189 and mb661 primer set exhibited the highest number of genus and highest bacterial diversity of the *pmoA* gene. Nevertheless, the use of the three sets may be necessary in order to obtain the more complete composition.

The rhizosphere bacterial community may be accessed through the *nifH* gene. Diazotrophic bacteria promote nitrogen biological fixation through a highly conserved enzyme called nitrogenase [88,89]. The *nifH* gene is considered as a good marker for heterotrophic bacteria [122], although there are many others that are also employed such as *nifD* and *nifK* [123]. The *nirK* and *nirS* genes participate in the nitrogen cycle through denitrification with the action of the nitrite reductase enzyme [65,90,91], and so does the *nosZ* gene through the nitrous oxide reductase enzyme [92].

Table 1. Descriptions of functional groups and your attributions in agroecosystems.

Functional group	Description	Agricultural importance	Reference
Aerobic methanotrophic bacteria	Aerobic bacteria use methane as carbon source and energy through the action of the methane monooxygenase enzyme that oxidizes methane producing methanol and generating two molecules of water.	Aerobic oxidation of methane in aquatic environments such as rice fields. Participate in the carbon cycle. Reduce the emission of methane gas to the environment.	(81,82)
Methanotrophic Archaea	Transform methane in nitrite, nitrate, sulfate or metal.	Methane oxidation in strictly anoxic environments. Participate in carbon cycling. They are present in deeper layers of soil in rice crops because the soil layers covered by water create an anaerobic environment. Reduce the emission of methane gas into the atmosphere.	(14,83)
Ammonifying bacteria	First step of ammonia oxidation in nitrate, via nitrite. It occurs by the oxidation of ammonia to hydroxylamine, catalyzed by the ammonia monooxygenase enzyme.	Fundamental process in nitrogen cycling.	(84–87)
Nitrogen-fixing bacteria	Microorganisms make a enzymatic conversion of gaseous nitrogen to ammonia through a highly conserved enzyme called nitrogenase.	Promote the biological nitrogen fixation, reducing the use of nitrogenous fertilizers.	(88,89)
Denitrifying bacteria	Denitrification process through the action of the nitrite reductase enzyme or oxide reductase enzyme.	Assist in biological nitrogen fixation, promoting growth in plants.	(65,90–92)

Table 2. DGGE employment in several environments using the following genes of functional groups: *16SrRNA, pmoA, mmoX, amoA, nifH, nirK, nirS and nosZ.*

Groups	Gene	Primers	Sequence (5' - 3')	Annealing conditions (°C)	Amplicon lenght (bp)	Denaturing gradient and polyacrylamide concentration	Reference
Bacteria	16S rRNA	968f	AAC GCG AAG AAC CTT AC	53	434	40-80, 6% polyacrylamide	(93)
		1401r	CGG TGT GTA CAA GAC CC				(93)
		63F	CAGGCCTAACACATGCAAGTC	57	489	30-40/60-80, 8% polycrylamide	(94)
		338R	GCTGCCTCCCGTAGGAGT				(94)
		357	CCTACGGGAGGCAGCAG	55	586	40-80%, 6% polyacrilamide	(53)
		907rM	CCGTCAATTCMTTTGAGTTT				(53)
		341f	CCTACGGGAGGCAGCAG	55	194	15-30/60-70 8% or 10% polycrylamide	(53)
		518R	ATTACCGCGGCTGCTGG				(53)
Aerobic methanotrophic bacteria	*pmoA*	A189f	GGN GAC TGG GAC TTC TGG	56	525	35-80%, 6,5% polyacrilamide	(95)
		A682r	GAA SGC NAG AAG AAS GC				(95)
		mb661r	CCG GMG CAA CTG CYT TAC C		491		(96)
		A650r	ACG TCC TTA CCG AAG GT				(97)
Methanotrophic Archaea	*mmoX*	206f	ATCGCBAARGAATAYGCSCG	60	720	40-70%, 8% polyacrylamide	(98)
		886r	ACCCANGGCTCGACYTTGAA				(98)
Ammonifying bacteria	*amoA*	a*moA*-1F	GGGGTTTCTACTGGTGGT	55	491	40-70%, 8% polyacrylamide	(99)
		a*moA*-2R	CCCCTCKGSAAAGCCTTCTTC				(99)
Nitrogen-fixing bacteria	NifH	FGPH19	TAC GGC AAR GGT GGN ATH G	55	452	20-70%, 8% polyacrylamide	(100)
		PolR	ATS GCC ATC ATY TCR CCG GA				(101)
		PolF	GAC GAT GTA GAT YTC CTG		339		(101)
		AQER	TGC GAY CCS AAR GCB GAC TC				(101)
Denitrifying bacteria	nirK	FlaCu	ATCATGGT(C/G)CTGCCGCG	57	>400	60-80%, 8% polyacrylamide	(102)
		R3Cu	GCCTCGATCAG(A/G)TTGTGGTT				(102)
		nirK1F	GG(A/C)ATGGT(G/T)CC(C/G)TGGCA	51	>400	40-70%, 8% polyacrylamide	(103)
		nirK5R	GCCTCGATCAG(A/G)TT(A/G)TGG				(103)
	nirS	nirS-1F	CCT A(C/T)T GGC CGC C(A/G)C A(A/G)T	55	450	60-80%, 8% polyacrylamide	(103)
		nirS6R	CGTTGAACTT(A/GCCGGT)				(103)
	nosZ	nosZ-F	CG(C/T)TGTTC(A/C)TCGACAGCCAG	55	>400	60-70%, 8% polyacrylamide	(104)
		nosZ1622R	CGC(G/A)A(C/G)GGCAA(G/C)AAGGT(G/C)CG				(64)

The 16S rRNA is not directly used for access the functional groups, however can infer data about the composition of bacterial communities present in many environments [124], including crops [36,125,126]. Through this, is possible identified all species present in agroecosystems and verified the influence in crop management above which species and the utilization of pesticides [9], fertilizers [127], and root exudates [35]. For the16SrRNA gene, Sánchez [93] compared 6 sets of primers regarding their efficiency in obtaining profiles of bacterioplankton communities (63f and 518r; 357f and 907rM; 357f and 907r; 357f and 518r; 968f and 1401r; 1055f and 1392r) and the best result came from the 357f-GC and 907rM set.

The combinations to amplify nitrifying bacteria isolated from soil samples they were tested. Taking into account the number of amplifications, number of genus, number of environmental samples amplified and the amplicon quality, the set of primers that provided the best result for the *NirS* gene was cd3aF with R3cd. For the *nirK* gene, the best results were obtained with the FlaCu and R3Cu set. For the *nosZ* gene the best combination was nosZ-F with nosZ1622R [64]. There are also, primers based on 16S rRNA gene, however, for the identification of target species, as the growth promoters bacteria in plants. Species of *Pseudomonas* can be accessed through PsF, PsR [128], F311PS and R1459PS primers [129]. For the genus *Burkholderia* are cited the BurkR e Burk3 primers [130].

The set of primers listed on Table 1 for the identification of the *mnoX* gene was used for archaea characterization from estuaries water [131]. However,used genes such as *16S rRNA*[132]with 27F/1492R primers [133] and the *pmoA* gene through the a189f/mb661 set [97,96] to characterize bacterial methanotrophic activities, besides those primers previously cited for archaea.

The access ofthe diversity of microbial species can bring answers more effective about the crop management and soil impact, gas emission, as well as the relation between plants and microorganisms who benefits there development. Some problems as extraction and purification of nucleic acids may be an obstacle for the analysis that depend of PCR. Agricultural environments vary a lot in their chemical composition and there is also the presence of humic acids that is known to inhibits PCR amplification. Even so, the data generate by the utilization of methods from molecular biology, allowed in greater range of results, which do not depend of temperature, oxygen or any other limiting factor of growth, as method of cultivation of microorganisms.

Perspectives

The DGGE technique can be very promising in agricultural management assessments. The results are fast and have low coast. The cluster analysis of patterns generated by the bands shows the response of the bacterial species, including diversity analysis regarding to the treatment tested. Several Brazilian universities use the technique for various studies. Researchers at UNIOESTE and Unipar evaluated the effects of using wastewater to irrigate crops [134], comparing the effects of cover crops, evaluated by UFSC [135], the comparison between different types of management, conducted by UFU [136] and UNB, Embrapa Cerrado and UFRRJ [107], comparison of farming systems conducted by UEL, UEM and Embrapa Soja [137].The UFRJ together with Embrapa Solos and Embrapa Arroz e Feijão, also used the DGGE technique to evaluate treatment using biochar, which provides a reduction in CO2 emissions by agriculture and the promotion of plants growing [138].Assessments of ecology and soil dynamics or water in the case of irrigated crops, combine different views on agriculture in search of lower costs to farmers and also, lower environmental impact.

ACKNOWLEDGEMENT

This study was funded by fellowships and grants from Rice Irrigated Institute of Rio Grande do Sul (IRGA), Capes (Foundation, Ministry of Education of Brazil) and Microbiology and Toxicology Laboratory Vale do Rio dos Sinos University (UNISINOS).

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Received: February 03, 2016;
Accepted: July 14, 2016