FLÁVIA DE OLIVEIRA SOUZA

DIVERSITY OF VIRUSES PRESENT IN CATTLE RUMEN AND IN GENOMES OF *RALSTONIA* sp SPECIES COMPLEX

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Microbiologia Agrícola, para obtenção do título de *Doctor Scientiae*.

Orientadora: Poliane Alfenas Zerbini

Coorientadores: Fernanda Prieto Bruckner
Hilario Cuquetto Mantovani

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Assentimento:

Flávia de Oliveira Souza
(Autor)

Poliane Alfenas Zerbini
(Orientadora)

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A Deus;

À minha mamis Ester Sandra

Por seu amor incondicional;

Ao seu grande coração;

Com toda gratidão,

À irmã Hilda,

Seu trabalho árduo,

Apoio e compreensão,

Dedico!

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"É JUNTO DOS BÃO QUE A GENTE FICA MIÓ."
(JOÃO GUIMARÃES ROSA)

ABSTRACT

SOUZA, Flávia de Oliveira, D.Sc., Universidade Federal de Viçosa, September, 2019. **Diversity of Viruses Present in Cattle Rumen and in Genomes of *Ralstonia* sp Species Complex.** Adviser: Poliane Alfenas Zerbini. Co-advisers: Fernanda Prieto Bruckner and Hilario Cuquetto Mantovani.

Viruses are microorganisms present in all environments and can infect all types of organisms. Viral infection affects the dynamics and characteristics of its hosts, depending on the type of infection cycle the virus uses. Efforts have long been made to discover and characterize new viruses through techniques such as filtration, tissue culture, electron microscopy, serology, and vaccination under laboratory conditions. Advances in nucleic acid sequencing techniques, bioinformatics and independent laboratory culture maintenance techniques such as metagenomics have been used in studies to discover and characterize viruses in various environments. The ecological relationship between viruses and a given host group has been accessed through bioinformatics, identifying virus-derived sequences in already sequenced bacterial genomes. This analysis is possible because some bacteria-infecting viruses can integrate into the bacterial genome and remain in the form of a pest, replicating their genome along with the bacterial genome. In this sense, the growing number of complete sequences of bacterial genomes available in databases has made it possible to search for evidence of viruses integrated into these genomes on a global scale. *Ralstonia solanacearum* is the main phytopathogenic bacterium that causes bacterial wilt disease, capable of infecting about 200 cultures, distributed in about 50 different botanical families. In addition, *R. solanacearum* is recognized as a group of bacteria with genetic diversity, where strains can be phenotypically subdivided into four phylotypes. Despite current efforts to isolate infecting viruses, little is known about the occurrence and composition of pests in the genomes of *Ralstonia* spp. However, the importance of viruses in a complex environment has also been the focus of studies. In fact, viral diversity in different ecosystems has been accessed through independent laboratory culture methods and without the need to know the host of these viruses. The main environments surveyed are seawater, soil, feces and intestines of human and mammalian animals. The rumen is a component of the gastrointestinal system of ruminant animals that has as one of its main characteristics a complex population of microorganisms residing in this environment. Ruminate animals are unable to produce enzymes to degrade the fiber ingested in food. Ruminal

microorganisms, unlike animals, produce these enzymes and, therefore, are directly responsible for obtaining energy from the animal and, consequently, for its health and profitability. Therefore, rumen microbiota is the focus of research, but studies on rumen viroma are still scarce. Due to the complex relationships between viruses and their hosts, either in relation to a specific group of bacteria, such as plant pathogens, or in symbiotic environments such as bovine rumen, we proposed to analyze the presence of virus derived sequences in the species complex genomes. *Rasltonia solanacearum* (RSC) available from the National Center for Biotechnology Information (NCBI) database. We also accessed the viroma of two dairy cows from the Federal University of Viçosa and were able to isolate the first mimivirus-like virus from these rumen samples.

Keywords: Viral Diversity. Viral Ecology. *Rasltonia* species complex. Rumen virome.

RESUMO

SOUZA, Flávia de Oliveira, D.Sc., Universidade Federal de Viçosa, setembro de 2019. **Diversidade de Vírus Presentes em Rúmen de Bovinos Leiteiros e em Genomas do Complexo de Espécies de *Ralstonia* sp.** Orientadora: Poliane Alfenas Zerbini. Coorientadores: Fernanda Prieto Bruckner e Hilario Cuquetto Mantovani.

Os vírus são microrganismos presentes em todos ambientes e podem infectar todos tipos de organismos. A infecção viral afeta a dinâmica e as características de seus hospedeiros, dependendo dda estratégia de multiplicação que o vírus utiliza. Por muito tempo esforços foram realizados para descobrir e caracterizar novos vírus por meio de técnicas como filtração, cultura de tecidos, microscopia eletrônica, sorologia e vacinação, em condições laboratoriais. O avanço nas técnicas de sequenciamento de ácidos nucléicos, bioinformática e técnicas independentes de manutenção de culturas em laboratório tais como a metagenômica, passou a ser utilizada em estudos com o intuito de descobrir e caracterizar vírus em diversos ambientes. A relação ecológica entre vírus e um determinado grupo hospedeiro tem sido acessada por meio de ferramentas de bioinformática, identificando-se sequências derivadas de vírus em genomas bacterianos já sequenciados. Essa análise é possível devido ao fato de que alguns vírus que infectam bactérias podem se integrar ao genoma bacteriano e permanecer na forma de profago, replicando seu genoma juntamente com o genoma bacteriano. Nesse sentido, o crescente número de sequências completas de genomas bacterianos disponíveis em bancos de dados tornou possível a busca de evidências de vírus integrados nesses genomas em escala global. *Ralstonia solanacearum* é uma bactéria fitopatogênica causadora da murcha bacteriana, capaz de infectar cerca de 200 culturas de importância agrícola, distribuídas em cerca de 50 famílias botânicas diferentes. Além disso, *R. solanacearum* é reconhecida como um grupo de bactérias com diversidade genética, onde as cepas podem ser fenotipicamente subdivididas em quatro filotipos. Apesar dos esforços atuais para isolar vírus que infectam, pouco se sabe sobre a ocorrência e composição de profagos nos genomas de espécies de *Ralstonia* spp. A diversidade viral nos diferentes ecossistemas também tem sido acessada a partir de métodos independentes de cultivos laboratoriais e sem a necessidade de se conhecer o hospedeiro que desses vírus. Os principais ambientes pesquisados são água marinha, solos, fezes e intestinos de humanos e animais mamíferos. O rúmen é um componente do sistema gastrointestinal de animais ruminantes que tem como uma das principais características uma população de microrganismos complexa

residente nesse ambiente. Animais ruminantes são incapazes de produzir enzimas para degradar a fibra ingerido no alimento. Os microrganismos ruminais, produzem essas enzimas e, portanto, são diretamente responsáveispela obtenção de energia pelo animal e, consequentemente, por sua saúde e rentabilidade. Desta forma, muitas pesquisas tem se concentrado em estudar a microbiota ruminal, mas estudos sobre o viroma ruminal ainda são escassos. Devido às complexas relações entre vírus e seus hospedeiros, seja em relação a um grupo específico de bactérias, como as fitopagênicas, ou em ambientes simbióticos, tais como o rúmen bovino, propusemos analisar a presença de sequências derivadas de vírus nos genomas do complexo de espécies *Rasltonia solanacearum* (RSC) disponíveis no banco de dados do National Center for Biotechnology Information (NCBI). Também acessamos o viroma de duas vacas leiteiras da Universidade Federal de Viçosa e fomos capazes de isolar o primeiro vírus do tipo mimivírus dessas amostras ruminais.

Palavras-chave: Diversidade Viral. Ecologia Viral. Complexo de espécies de *Rasltonia* sp. Viroma ruminal.

SUMÁRIO

1 INTRODUCTION

1.1 Viral Ecology

The viruses can infect all types of microorganisms and can be found in every environment. They are the most numerous organisms on Earth (Wobus and Nguyen, 2012), and for a long time, approaches based on laboratory cultivation techniques, such as filtration, tissue culture, electron microscopy, serology and vaccination were the main techiniques used in viral discovery and ecological studies. Despite the knowledge obtained from these techniques it was agreed that less than a 1% of the viral universe had been discovered and explored. In the last decades, the advances in nucleic acid sequencing techniques, bioinformatic, and techniques independent from laboratory culture (metagenomic) have become applicable to studies of viral discovery and characterization in several environments (Mokili et al., 2012).

The first viral metagenomic study was performed by Breitbart et al., (2002). This study was important in showing the high prevalence of viruses and viral diversity in marine environment. In addition, this study also reported that most sequences found have no homology to any other sequences deposited in the current databases, and consequently being completely uncharacterized, showing that much remains to be known about viruses.

Since then, many studies have identified various viral sequences through metagenomics in the most diverse environments such as soil, fresh water, sediment, human gut and animal faeces (Aggarwala et al., 2017; Corinaldesi et al., 2017; Graham et al., 2019; Vibin et al., 2018; Vlok et al., 2019a). However, the vast majority of these studies have focused on marine environments (Ahlgren et al., 2019; Gregory et al., 2019; López-Pérez et al., 2019; Ponsero and Hurwitz, 2019; Vlok et al., 2019b).

One advantage of using metagenomic to study viruses is that unlike cellular organisms, viruses do not have a common shared gene, which can serve as a molecular and phylogenetic marker. Thus, metagenomics can be applied to all viruses, regardless of the nature of their genetic material. Besides that, it may be unviable and costly to cultivate and maintain viruses under laboratory conditions, since they are obligate parasites, which is unnecessary in metagenomic approaches. On the other hand, techniques based on bioinformatics presents as challenges the use of a large number of machines associated with the correct choice of which analyzes to use against the generated datasets (Nooij et al., 2018).

By interacting with the host, viruses can alter the ecosystem in several ways. One of the most studied contributions of bacterial-infecting viruses to bacteria evolution has been predicted to be the horizontal transfer of genes (HTG). HTG can alter the host lifestyle in a variety of ways, such as favoring host survival in an environment that the host would not be able to survive without association with the virus. Viruses may also mediate the acquisition of virulence genes for their hosts, or may even be the cause of hypovirulence in some cases (Allen and Abedon, 2014).

Currently, the relationship between viruses and procariote hosts has been found through bioinformatics in the search for virus-derived sequences integrated in bacterial genomes. Some bacteria-infecting viruses may integrate into the bacterial genome and remain then as a prophage. Prophages studies have been performed in bacteria such as *Yersinia* sp., *Bacillus thuringiensis* and *Staphylococcus aureus*, *Pectobacterium* spp. and *Dickeya* spp. and *Cronobacter sakazakii* (Czajkowski, 2019; Fu et al., 2019). These studies have shown that viruses can provide bacterial virulence factors such as antibiotic and heavy metal resistance, thus favoring the adaptability of these bacteria to a wider range of environments. In addition, analysis of the presence of viruses in bacterial genomes may explain the difficulties encountered in the practice of isolating lytic viruses in certain bacterial groups, thus directing future research (Liang et al., 2019; Tran et al., 2019; Zeng et al., 2017).

Moreover, the interest of the study of viruses that infects phytopathogenic bacteria is increasing due to the potential use as biological control agents. This becomes relevant due to the resistance of bacteria to routinely used chemicals and the demand for control techniques less harmful for the environment. The identification of an integrated phage in a genome can be challenging due to continued phage evolution after insertion. In addition, as most of the bacteriophages described today belong to Order *Caudovirales*, the tools developed to search viral genome in bacterial DNA are focused on this group of viruses, making it difficult to identify viruses belonging to another taxonomic group (Varani et al., 2013).

Viruses are known to make a major contribution to the functioning of the ecosystem in different environments, either by providing organic matter when lysing their hosts and by influencing the biogeochemical carbon cycle, or by producing enzymes such as hydrolases, which have the potential to degrade polymers as pectin, hemicellulose and starch. In a study on viral diversity in permafrost these proteins produced by virus have been predicted through bioinformatics and one hydrolase was expressed and functionally

assayed, confirming activity with specific cleavage, highlighting the ability of bioinformatics to predict what really happens in different environments (Graham et al., 2019)(Graham et al., 2019)(Graham et al., 2019; Dell'Anno et al., 2015; Emerson et al., 2018).

1.2 *Ralstonia solanacearum*

Ralstonia solanacearum is a soil -borne plant pathogenic bacteria, gram negative and classified as beta-proteobacteria belong to a species complex (*Ralstonia solanacearum* species complex-RSC). By definition, a species complex is a group of bacteria related isolates, where members can individually represent more than one specie (Gillings et al., 1993).

R. solanacearum is the main causal agent of bacterial wilt disease, being able to infect about 200 cultures, which are distributed in about 50 different botanical families and is recognized as a group of bacteria with genetic diversity where the strains can be subdivided into four phylotypes according to their geographical origin (Allen et al., 2005; Fegan and Prior, 2005).

According to their geographic origin phylotype I included strains originating primarily from Asia, phylotype II those from Americas, phylotype III those from Africa and phylotype IV those from Indonesia, Australia and Japan. Phylotype IV also contained the two close relatives of *R. solanacearum*: *Ralstonia syzygii* and the blood disease bacterium (BDB) strains (Peeters et al., 2013).

Ralstonia genome is organized in two replicons (a chromosome and a megaplasmid). For the type strain *R. pseudosolanacearum* GMI1000, these replicons have a mosaic structure containing numerous elements signaling the potential for evolution by recombination and horizontal gene transfer (Salanoubat et al., 2002). Because of these characteristics *Rasltonia* spp is said to be a highly able microorganism that can quickly adapt to environmental changes and new hosts, as well as overcoming plant resistance mechanisms (Wicker et al., 2009).

RSC genomes have several regions with alternative codon usage (designated as ACURs), which contain genetic mobile elements associated, such as prophages and insertion sequences. In addition, the virulence evolution of GMI1000 is attributed to the clusters of ACURs, since the most type III-dependent effectors appear to reside within these clusters (Salanoubat et al., 2002).

The great destructive power of RSC is because *R. solanacearum* accesses plant roots through wounds or natural openings and travels to the developing vascular bundles, quickly reaching susceptible root xylem vessels. Upon entering the xylem, *R. solanacearum* systematically spreads through the plant. Additionally, bacterial cells can adhere to the xylem, forming biofilm aggregates that can fill whole vessels and potentially obstruct the flow of water. Meanwhile, other cells move along the vessel walls. The bacterium is difficult to control because it survives in water and in residues of infected plants in the soil. (Lowe-Power et al., 2018). Additionally, the use and distribution of contaminated soil, irrigation water, farm equipment and asymptomatic plants, results in the establishment and permanence of this pathogen in numerous regions of the world. If identified, crop fields, gardens and greenhouses should be destroyed and materials should be decontaminated with the use of chemical bactericides which have been shown to have limited efficiency (Hayward, 1991).

The restricted efficiency of the control strategies used has made it necessary to develop new effective practices that optimize early detection and monitoring of pathogen presence. In this sense, several bacteriophages that infect *Ralstonia* sp. species complex, with potential to control bacterial wilt disease have been isolated and caractherized (Addy et al., 2012a; Effantin et al., 2013; Elhalag et al., 2018; Fujiwara et al., 2008; Kawasaki et al., 2016; Van Truong Thi et al., 2016; Xavier et al., 2018).

Despite the current efforts to isolate viruses that infect *Ralstonia* spp, little is known about the occurrence and composition of prophages in RSC. The growing number of complete bacterial genome sequences deposited in databases has made it possible to search for evidence of viruses integrated in these genomes in global scale.

Prophages can have a positive or negative effect on fitness of the most bacteria, affecting its lifestyle, genomic diversity and bacterial fitness (Brueggemann et al., 2017)(Brueggemann et al., 2017)(Brueggemann et al., 2017). Furthermore, prophages may carry several genes that benefit their host. Some very well-known prophages are responsible for the production of toxins that increase the virulence of pathogenic bacteria, such as *Escherichia coli*, *Streptococcus pyogenes*, *Salmonella enteric*a and *Staphylococcus aureus* (Fortier and Sekulovic, 2013). Prophages are also efficient vectors for horizontal gene transfer that can contribute to the emergence of plant-pathogenic bacteria variants (Varani et al., 2013).

Despite the great economic and environmental impact of phytopathogenic bacteria, few studies have focused on looking for viruses integrated into their genomes.

This strategy becomes interesting due to the low efficiency and high polluting power of chemicals used in unsuccessful attempts of pathogens in different cultures. In addition, knowing viruses and their influences on bacterial fitness can be useful in optimizing the development of virus-derived products to control bacterial spread.With this purpose, we screened all complete genomes of *Ralstonia solanacearum* complex (RSC) available in the National Biotechnology Information Center (NCBI) database, looking for virus derived sequences. Our analyzes revealed a large amount of viral sequences integrated into the RSC genomes, indicating that these viral sequences may contribute to the genetic diversity of these genomes and confer a better adaptability of these hosts in different environments.

1.3 Rumen Ecology

Rumen is a component of the gastrointestinal system of cattle and is characterized by a complex microbiota residing in this environment. Ruminant animals are unable to produce enzymes to degrade the fiber what is ingested as food. Ruminal microorganisms, unlike animals, are capable of producing such enzymes, and thus are directly responsible for making energy available to the animal, and consequently for its health (Li et al., 2012).

The rumen microbiota can be influenced by several host factors including sex, age, host genetics, feeding strategy, physiological state and environmental factors. Despite the differences in gut physiology among different species of ruminants, it is believed that the influence of the gastrointestinal microbiome in cattle is more relevant than in other mammals (Turnbaugh et al., 2008).

Due to this fact, the rumen microbiota has received attention from researchers for some time, in an attempt to describe which microorganisms reside in this environment. The main limiting factor for this research is the difficulty of cultivating ruminal microorganisms in laboratories, as they require an anaerobic environment, whereas the most easily cultivated organisms account for only about 11% of the total rumen bacteriome (Morgavi et al., 2013).

The development of new platforms and high throughput sequencing techniques has allowed to make profile analyzes of microbial communities independent of laboratory cultivation, thereby broadening the knowledge of phylogenetic relationships and broader characterizations of the gastrointestinal microbiota. Regarding the rumen environment, most studies focus on bacteria, which make up approximately 95% of the microbial community in this environment. (Yoon et al., 2015).

Viruses that infect prokaryotes (bacteria and archaea), are always present in the gastrointestinal tract of animals and humans. In rumen is no different and the few viruses that have been isolated from this environment are phages that infect ruminal bacteria. Thus rumen was a source of lytic viruses that infect well-characterised and predominant rumen bacteria, such as *Prevotella* (*Bacteroides*) *ruminicola* and *Streptococcus bovis* (Iverson and Millis, 1976; Klieve et al., 1989).

Gilbert and Klieve (2015), listed twenty-seven papers on rumen bacteriophage isolation. Most of these isolated viruses had the morphology of the order *Caudovirales*. Two of these viruses had *Inovirid*ae family morphology and four these viruses didn't have their morphology defined.

In recent years, rumen viruses have received attention in studies that use metagenomic approaches. Ruminal virome has been explored in different ruminant species and today it's known that there are more types of viruses present in this environment, besides bacteriophages. In addition to the families already described in previous studies, metagenomic studies showed evidence of the presence of virus from *Herpesvidae*, *Phycodnaviridae*, *Mimiviridae*, *Poxviridae*, *Baculoviridae*, *Iridoviridae*, *Polydnaviridae*, *Adenoviridae*, *Bicaudaviridae* families and also taxonomically unclassified viruses in rumen (Miller et al., 2012). Metagenomics of bacteriophages from thirteen lactating dairy cattle showed that estimations of rumen phage diversity using electron microscopy is extremely underestimated (Ross et al., 2013).

Given these complex relationships between viruses and their hosts, either in relation to a specific group of bacteria, such as phytopathogenic, or in an extremely symbiotic environment such as bovine rumen, we aim to analyze the genone-integrated viral composition in the *Rasltonia solanacearum* species complex (RSC) deposited in the National Center for Biotechnology Information (NCBI) database. We also analyzed through a metagenomic approach the viroma of two dairy cows from the Federal University of Viçosa. In addition, we were able to isolate a mimivirus like virus from one of the samples.

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CHAPTER 1

Widespread distribution of prophages signaling the potential for adaptability and pathogenicity evolution of *Ralstonia solanacearum* Complex genomes

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Summary

Prophages can have a positive or negative effect on the host cell, affecting its lifestyle, genomic diversity and bacterial fitness. However, many basic aspects of how these organisms affect the host cell remain poorly understood. *Ralstonia solanacearum* is a gram-negative plant pathogenic bacterium, encompassing a great diversity of ecotypes regarded as a species complex (*R. solanacearum* complex - RSC). *Ralstonia* genomes have a mosaic structure containing numerous elements, signaling to the potential for its evolution through horizontal gene transfer. In this context, we have made a screening in 120 RSC complete genomes from the NCBI database in order to identify prophage sequences integrated into RSC genomes. In total, 374 prophage-like elements were found in both the chromosome and megaplasmid. These elements encode several genes, including some related to host fitness, virulence factors, antibiotic resistance and niche adaptation that might contribute for RSC adaptability. Putative complete prophages belonging to the families *Inoviridae*, *Myoviridae* and *Siphoviridae*, were found, being the members of *Inoviridae* family the most abundant. Similar prophage-like elements are widespread into the complex at different species and/or geographic origin, suggesting that RSC phages are ancestrally acquired. Also, an analysis of CRISPR-Cas spacer sequences demonstrated the presence of viral sequences that indicates successive infection events during the bacteria evolution. Among the complete prophages, we found 14 novel putative viruses integrated into RSC genomes. These genomes have hallmark proteins from bacteriophages and might be active. Altogether, our results provide insights about the diversity of prophages in RSC genomes and suggest that these elements may deeply affect the shape of the genome evolution among the strains, impacting the virulence and host-range adaptation.

Introduction

Viruses are present in the most diverse environments and can infect all known microorganisms (bacteria, archaea, fungi), including other viruses (Berjón-Otero et al., 2019). It is estimated that there are 10^{31} viruses on Earth, most of which infect bacteria and are referred to as bacteriophages or simply phages (Graham et al., 2019). When phages contact their host, they can directly affect the shaping of bacterial communities based on their lifecycle (Erez et al., 2017).

Currently, in many bacterial genomes deposited in public database, it is possible to detect phage DNA integrated into the bacterial chromosome, called prophages. It is not difficult to find bacteria that contain several prophages in their chromosomes, which may constitute a considerable part of the total bacterial DNA (Calero-Cáceres et al., 2019). However, there is a lack of studies on viral DNA in several important bacterial species.

Prophages may have a positive or negative effect on the fitness of most bacteria and affect their habits, genomic diversity and bacterial fitness (Fillol-Salom et al., 2019). Furthermore, prophages may carry several genes beneficial to their host. Some very well-known prophages produce toxins that increase the virulence of pathogenic bacteria, such as *Escherichia coli*, *Streptococcus pyogenes*, *Salmonella enteric*a and *Staphylococcus aureus* (Wahl et al., 2019). Prophages are also efficient vectors for horizontal gene transfer, which favors the emergence of plant-pathogenic bacteria variants (Roossinck, 2019).

Ralstonia solanacearum is one of the most devastating worldwide phytopathogen, which causes bacterial wilt diseases in more than 250 plant species from 54 different botanical families (Pastou et al., 2019). This soil-borne pathogen enters into plant roots, multiplies into the xylem and collapses the water-conducting system, which leads to wilting and rapid plant death (Cho et al., 2019).

R. solanacearum encompasses a great diversity of ecotypes, separated into four phylotypes, according to their geographic origin: Asia (phylotype I), Americas (II), Africa (III) and Indonesia (IV) (Fegan and Prior, 2005; Wicker et al., 2012). Currently, they are regarded as a species complex (*R. solanacearum* Complex – RSC) composed of three species: *R. solanacearum* (strains of phylotype II), *Ralstonia syzygii* (strains of phylotypes IV, including the blood disease bacterium (BDB) strain), and *Ralstonia pseudosolanacearum* (strains of phylotypes I and III) (Safni et al., 2014; Prior et al., 2016).

Ralstonia genome is organized into two replicons (a chromosome and a megaplasmid). For the type strain *R. pseudosolanacearum* GMI1000, these replicons present a mosaic structure with numerous elements signaling the potential for evolution through horizontal gene transfer (Salanoubat et al., 2002). Its genome has several regions with alternative codon usage (designated as ACURs), which contain genetic mobile elements associated, such as prophages and insertion sequences. In addition, the virulence evolution of GMI1000 is attributed to the clusters of ACURs, since most types of III-dependent effectors seem to reside within these clusters (Wu et al., 2019).

Despite the current efforts to identify and characterize viruses that infect *Ralstonia* spp, little is known about the occurrence and composition of prophages in RSC. The increasing number of complete bacterial genome sequences deposited in databases has made it possible to search for evidence of viruses integrated in these genomes in global scale. In this study, we screened 135 complete genomes of *Ralstonia solanacearum* complex (RSC) available in the NCBI (National Biotechnology Information Center) database. We found prophages belonging to the known viral families *Myoviridae*, *Siphoviridae* and *Inoviridae*. In addition, we were also able to identify fourteen different putative novel phages. The genomes of these new viruses have low identity with the genomes present in NCBI. Members of the *Inoviridae* family proved to be the most abundant prophages. The CRISPR-Cas target analysis revealed the presence of phage derived spacer sequences integrated in RSC genomes. However, the same bacterial strain genome presented no spacer sequences corresponding to its own prophage-derived elements. In addition, analyzes indicate that viruses may provide bacteria important factors for adaptability and possible resistance to heavy metals. Together, these results highlight the importance of phage in evolution shaping and virulence modulation of RSC. A better understanding of these mechanisms could help the development of more efficient control strategies.

Results

Global analysis of prophage-like elements integrated in RSC genomes

A total of 120 genomes (80 complete and 40 drafts) of RSC and non-pathogenic species of *Ralstonia* spp. (*R. mannitolilytica, R. picketii* and *R. indisiosa)* available in the NCBI database were analyzed (Table S1). Virus-derived sequences have been detected in pathogenic and non-pathogenic *Ralstonia* species. In total, 374 prophage-like elements were identified by the PHASTER software system (Table S2). Among these elements, 314 are integrated into chromosomes and 60, into megaplasmids. We found that 90% (n= 108) of the *Ralstonia* genomes have at least one prophage-like element within the chromosome, while for the complete megaplasmids analyzed, 60% (n = 48) present evidence of prophage-like elements.

The size of these prophage-like elements ranged from 5 to 65kb in chromosome sequences and from 3 to 56 kb in megaplasmid sequences (Fig. 1A, Table S2). The GC content of most prophage-like elements was like that found in RSC genomes (65% CG content) (Fig. 1B). No correlation was observed between the host bacterial genome size and the prophage genome size. (R^2=0.03; Fig. 1C), indicating that the size of the bacterial genome does not influence the size of the genome integrated in it. In general, whole prophage-like element sequences constitute about 0.2% of *Ralstonia* chromosomes and 0.03% of its megaplasmids.

Whole prophage-like genomes comparison allowed us to identify clusters based on shared nucleotide pairwise alignment. Prophages-like elements are widely distributed throughout the *Ralstonia* spp. and RSC genomes. *R. pseudosolanacearum* is the main dominant species found. Within RSC, *R. pseudosolanacearum* mostly shared sequences similarity with *R. solanacearum* and then *R. syzygii*. In addition, *R. pseudosolanacearum* is the only RSC specie linked to the other *Ralstonia* spp. As result, this data suggesting evolutionary relationships between prophage-like elements found integrated in RSC genomes (Figure 2).

Most sequences described as *Ralstonia* genomes are deposited in draft assemblies and not as complete genomes, which makes them difficult to analyze. Evaluation of assessing genome assembly and annotation completeness with BUSCO program showed that these genome drafts have low collinearity and thus are not ideal for these types of analyzes. Since they were not included in the analysis, the number of prophage-like

elements may be underestimated. Aiming to verify this information, we built a local database with our predicted prophage-like elements and performed a BLASTn with incomplete genomes (Supplementary Table S3).

Analysis of Putative Intact Prophages integrated in the RSC genomes

We manually curated our dataset of 374 prophage-like elements identified by PHASTER and therefore performed a BLASTn analysis against the nucleotide viruses NCBI database to search for putative intact prophages integrated into RSC genomes. A total of 43 intact prophage integration events into chromosomes and 11 into megaplasmids of 44 *Ralstonia* strains were found.

Among these prophages putative intact prophages, twenty-seven showed best hit with viruses of the *Inoviridae* family (*Ralstonia phage* PE226, *Ralstonia phage* RSS0, *Ralstonia phage* RSS30, *Ralstonia phage* RSM3, *Ralstonia phage* RSM1*, Ralstonia phage* p12J*, Ralstonia phage* Rs551 and *Ralstonia phage* RS603) ranging in size from 5.4 to 8.8 kilobases (kb). Interestingly, the great majority of prophages in the RSC (61%) belong to the *Inoviridae* family. Thirteen intact prophages matched to members of the *Myoviridae* family (*Ralstonia phage* RSY1 and *Ralstonia phage* RSA1), ranging from 30.2 to 46.4 kb, and three, to the family *Siphoviridae* (*Ralstonia phage* RS138), all corresponding to the same phage, with the size of 41.0 kb (Table 1). Two *Ralstonia solanacearum* strains T60 and T78, have integration events of intact prophages on their chromosomes and megaplasmids. These two strains have the same virus integrated in their plasmids. Already the integrations in its chromosomes are of two different viruses. In addition, poly-lysogenesis events, a condition in which there is more than one virus integrated into a single bacterial genome, occurs in twelve *Ralstonia* sp strains and only one of these strains corresponds to a non-pathogenic species, as shown in table 2.

The relationships among the putative intact prophages of the *Inoviridae* family were analyzed by pairwise comparison of the amino acid sequences of morphogenesis (pI) protein, an ATPase that represented the only conserved marker gene among the viruses in this family (Fig. 3). Based on the similarities of the amino acid sequences of pI protein of the prophage and of all publicly available inovirus genomes described in NCBI database, the prophages of *Inoviridae* family were grouped into three clusters. It is noted that the inovirus integrated in the non-pathogenic specie *R. picketii* grouped with the prophages of pathogenic species R. *solanacearum* (strain CFBP 2957) *and* R.

pseudosolanacearum (strains 3103, FJAT 1458 and FJAT 91) and with viruses *Ralstonia phage* PE226 and *Ralstonia phage* p12J. Interestingly, only viruses integrated in pathogenic species of R *pseudosolanacearum* grouped with inoviruses RSS-type, a group of inovirus tha infects *Rastonia* known to increase the virulence of their hosts. Finally, viruses that are integrated into genomes of species of R. *solanacearum and* R. *pseudosolanacearum* also grouped with other kind of inoviruses, RSM-type viruses that are associated with decreased host virulence. However, the effects that viruses can have on bacteria probably have additional factors involved.

All strains with integrated prophages of *Myoviridae* family come from Asia, except the *R. pseudosolanacearum* GMI1000 strain, which is from South America. Comparative analyses of the prophage genomes of *Siphoviridae* family were carried out using the MAUVE software system. All prophages presented the same collinear blocks and the same genomic structure. All these viruses are integrated to the genome of bacteria originated from the Asian continent (not show).

Identification of novel putative phages in RSC genomes

During the general analysis of complete phages integrated in RSC genomes, we identified phages with low similarity with any nucleotide sequence deposited in the NCBI nucleotide collection database. These putative new phages were observed in 28 integration events predicted in 20 RSC genomes, all of them identified in chromosome replicons. These 28 integration events encompass 14 different phages. The analysis of these phages revealed DNA genomes ranging from 10 to 38 Kb, with GC content ranging from 60.1% to 66.4%. All these viruses exhibit the potential to encode the ORFs necessary to assemble their own replisome, including DNA ligase, primase, helicase, polymerase and DNA-binding protein, and ORFs involved in lysis, structural proteins, and transcription (Fig. 4). Thus, these viruses found could possibly be classified as belong to the order *Caudovirales*.

RSC prophage-like elements encoded putative host fitness factors

It is widely known that phages are intimately associated with the virulence and evolution of several important bacterial pathogens (Fortier & Sekulovic, 2013). Aiming to provide a glimpse of prophage involvement in RSC pathogenicity and evolution, we mapped the distribution of putative genes encoding host fitness factors in prophage-like elements integrated within the analyzed genomes. For this purpose, we considered genes

related to virulence, antibiotic resistance and host niche adaptation as host fitness factors. Our analysis revealed that prophage-like elements, widely distributed in the genomes of pathogenic and non-pathogenic bacteria, carry genes encoding putative proteins that may confer novel phenotypic properties for the RSC (Table S4). By grouping these key genes according to the RAST server annotation pipeline, we found different classes of fitness factors related to virulence, secretion system, cobalt-zinc-cadmium resistance, macromolecule metabolism, DNA metabolism, cellular division, membrane transport, motility and chemotaxis, mobile genetic elements, regulation and cell signaling and toxin-antitoxin systems (Fig 5A). The type III effector was the most important putative virulence factor found, since it is known to be directly involved in the virulence of bacteria. Other factors related to virulence, such as avirulence effectors and type III secretion injected protein were identified (Fig. 5B). Some factors were found in prophage-like elements, which may confer niche adaptation to the RSC by encoding advantageous properties, including the cobalt-zinc-cadmium resistance protein CzcA (Fig. 5B). *R. pseudosolanacearum* FJAT-1458 and *R. pseudosolanacearum* SEPPX05 held a group of prophage-like elements carrying a Methyl-accepting chemotaxis sensory transducer (Fig. 5B). We also found a putative component of Trs (taxis to serine and repellent), a chemoreceptor with high specificity for aspartate and serine. Additionally, a gene related to the biosynthesis of secondary metabolites was found in a *Ralstonia pseudosolanacearum* (strain RSCM) megaplasmid, encoding an antibiotic biosynthesis monooxygenase (Table S4).

Correlation between prophage integration and spacer occurrence in CRISPR-Cas systems of RSC genomes

In an attempt to understand the relation between the CRISPR-Cas system and the occurrence of prophages in RSC genomes, we identified putative spacer sequences by using the CRISPRCasFinder software system (Couvin et al., 2018). The spacer sequences were grouped in a database and used to look for putative targets in viruses and plasmids in ACLAME database and NCBI virus database, using the BLASTn tool. As shown in Fig 6A, we found that 29.95% (162 de 543) of the spacer sequences identified in the CRISPR-Cas system match with sequences from phages, which suggests the occurrence of successive infection episodes during RSC evolution. Besides that, 3.32% match with plasmids; and 66.73% are spacers from unknown sequences. We also attempted to correlate the number of sequences derived of viruses present in genomes to the number

of spacers in the CRISPR-Cas loci in bacterial genomes. We noticed that, for example, genomes having approximately 100 spacer sequences at the CRISPR-Cas locus presented a range of targets that matched to viruses ranging from 1 to 10 (Fig 6B). Thus, we do not establish a direct relation between the number of spacer sequences in the strain and the number of phages targeted by them in both pathogenic and non-pathogenic species.

Because most of the integrated viruses found in this study belong to the Inoviridae family, we analyzed the number of spacers targeting these viruses. Of the total viral spacers found, only 4.60% are derived from inoviruses. The prophages that have more spacers present on the CRISPR-Cas locus of *Ralstonia* sp genomes are in descending order: *Ralstonia phage Rs551*, *Ralstonia phage RSM1*, *Ralstonia phage RSM3*, *Ralstonia phage RS603*, *Ralstonia phage p12J*, *Ralstonia phage RSS0*, *Ralstonia phage RSS30* and *Ralstonia phage PE226* (Fig 6C). In addition, the CRISPR locus may have more than one motif sequence derived from the same phage inserted as spacer (Figure 6D). This is the case of the phage *Ralstonia solanacearum* FJAT1458phi2, which has 9 different sequences targeted by that system.

Discussion

In this study, we provide genomic insights into the diversity of prophage-like elements and complete phages integrated into distinct genomes of RSC. We performed genome mining and comparative genomic analysis in 135 sequenced *Ralstonia* strains. Our analyses identified a total of 374 prophage-like elements distributed in 95% of the chromosomes and 81% of the megaplasmid replicons, with variation in size and GC content. Most of the prophage-like elements presented GC content like that of RSC genomes, which implies the establishment of stable and long relationships between host and phage genomes (Canchaya et al. 2004; Howard-Varona et al. 2017).

Although most bacterial genomes of RSC deposited in databases have derived-phage DNA, little is known about how such viruses affect RSC and the extent to which they might be associated with virulence or the host range of this important group of phytobacteria. The presence of viruses integrated in *Rasltonia* genomes indicates that these viruses play important roles in bacterial adaptation and maintenance in the environment. It is known that phages can affect the host in many ways, when integrated: by shifting bacterial genes; modifying the expression levels of host genes; working as recombination hot spots; protecting from lytic infection by others phages; lysing the host

297 cells when induced and adding fitness factors to the host, such as lysogenic conversion
298 genes (Kang et al., 2017).
299 The prophages were identified by the program PHASTER used in virus searching
300 and manually inspected. Most existing bioinformatic tools created to analyze
301 bacteriophage genomes are based on known characteristics of virus classified of the order
302 *Caudovirales*. However, several inoviruses were identified, which proved that these tools
303 can identify viral genomes from diverse taxonomic groups.
304 When viruses integrate the bacterial DNA, they may alter that genome, changing
305 the host phenotype in different ways, including promoting virulence (Nanda et al., 2015,
306 Casas and Maloy, 2018). Several putative genes related to virulence factors, antibiotic
307 resistance and niche adaptation factors were found in prophage-like elements. We
308 identified avirulence effectors and Type III secretion effectors that might contribute to
309 bacterial pathogenicity. These genes are associated with prophage-like elements, which
310 points to their potential role in the horizontal dissemination of virulence factors
311 throughout RSC species. Regarding niche adaptation, we found prophage-like elements
312 encoding the protein CzcA, a cobalt-zinc-cadmium resistance factor, which could
313 contribute to resistance against heavy metals. This protein is part of a cation efflux system
314 that mediates resistance to copper and silver. In addition, a group of prophage-like
315 element carrying a Methyl-accepting chemotaxis sensory transducer was identified.
316 These genes play an important role in the virulence of *Ralstonia* in the early stages of
317 host invasion and colonization (Yao and Allen, 2006). The pathogenicity of RSC, like
318 many gram-negative bacteria, depends on the type III secretion system that provides
319 bacterial virulence proteins called effector proteins in cells of the infected plant. This type
320 of secretion system is capable of modulating and altering host processes such as
321 cytoskeleton disposition and rearrangements, protein trafficking and degradation, and
322 transcriptional regulation in order to suppress plant immunity and generate an enabling
323 environment for growth. of the pathogen (Nakano and Mukaihara, 2019). Altogether, the
324 results demonstrate a series of putative genes associated with prophage like elements that
325 may affect the RSC under environmental conditions and plant colonization. It
326 corroborates the concept that viruses can encode genes that can benefit bacterial fitness,
327 even without being directly involved in a viral replication and infection (Hacker and
328 Carien, 2001).
329 Phages and bacteria are always struggling against each other. Thus, bacteria need
330 to acquire or develop strategies to defend itself against viral infections. These strategies

include mechanisms such as the CRISPR-Cas system (Samson et al., 2013). We found that 29.95% of the spacer sequences identified in CRISPR loci from 120 RSC genomes are phage-like, while 66.73% are spacers of unknown sequences. Interestingly, it has demonstrated that, for *R. solanacearum* strains CFBP2957, CRISPR-Cas system is not the primary antiviral strategy during phage infection (Xavier et al., 2019). The authors suggested that the CRISPR-Cas system is involved in other processes besides defense, such as the regulation of gene expression, including that of genes involved in viral virulence. In this study, in fact for this isolate we found only one spacer sequence of viral origin, corroborating this hypothesis.

In this study, most intact prophages found belong to the family *Inoviridae*. When an inovirus infects the cell, the phage genome can integrate in the host genome to form a prophage, or alternatively, it can replicate in cell cytoplasm as an episome (Ilyina, 2015). It is interesting to observe that these viruses codify a secretion machinery that doesn't kill the the bacteria to release new viral particles, keeping the host viable for further replication cycles (Mai-Prochnow et al., 2015). Therefore, they can carry out the horizontal transfer of genes without affecting the bacterial population density. This might become advantageous for the bacteria, which can evolve and acquire new genes and maintain the required density. Inovirus that infects *Ralstonia* sp. has been classified into two groups: RSM-type and RSS-type phages. Generally, RSS-type viruses integrate with host chromossome and increases host virulence. RSM-type inoviruses usually does not integrate into the host bacteria chromosome and reduce the host virulence (Askora and Yamada, 2015). Viruses from these families have already been described infecting RSC strains and proved to be promising for controlling the bacterial wilt disease (Kawasaki et al., 2007; Fujiwara et al., 2008; Yamada et al., 2010; Murugaiyan et al., 2011; Addy et al., 2012b; Kawasaki et al., 2016; Elhalag et al., 2018 and Xavier et al., 2018).

Finally, our analysis identified 14 different putative novel phages in the *Ralstonia* genomes. These phages seem to have all core genes necessary for their structural assembly and replication, which indicates that they may be new complete phages. This result also highlights the occurrence of phages integrated in *Ralstonia* genomes. Altogether, our study brought some insight on prophage diversity, which contributes to RSC diversity and may play an important role in the genome evolution and adaptation of this important plant pathogen.

Methods

Screening of prophage-like elements in chromosomes and megaplasmids of *Ralstonia solanacearum* Complex species

We used two approaches to identify prophages: through the web server PHASTER and manual search for sequences related to prophage in annotated RSC genomes (Supplementary figure S2). 105 sequenced RSC genomes and 15 genomes for non-pathogenic species *R. mannitolilytica*, *R. picketii* and *R. indisiosa* sequences were downloaded from the National Center for Biotechnology Information database (https://www.ncbi.nlm.nih.gov/genome), in July 2018 (Table S1). The assembly quality of chromosome sequences was analyzed using BUSCO (Waterhouse et al., 2017) and genomes with collinearity above 90% were used in this study.

Prophage-like sequences were identified and selected by PHASTER (PHAge Search Tool Enhanced Release) (http://phaster.ca/) (Arndt et al., 2016). A pairwise identity matrix from the alignment of prophage-like sequences was calculated by Geneious® 11.1.5 (Biomatters Lts) using default parameters. Therefore, we selected the sequences with scores >70% of similarity of pairwise alignment to create an undirected network graph using the program Gephi 0.9.2 (https://gephi.org/).

Identification of prophage-encoded potential host fitness factors

The *Ralstonia* prophage-like elements were screened for virulence factors, antibiotic resistance, niche adaptation and macromolecule metabolism genes by BLASTn algorithm, against the NCBI database of annotated ORFs (Open Reading Frames), using default parameters (E-value 10^{-4} and nucleotide identity >90%). Virulence factors were classified according to the RAST server (http://rast.nmpdr.org/).

Genomic analysis of complete prophages

The genomes of putative complete prophages identified were analyzed on DNA level by comparison with virus database from NCBI using the BLASTn algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi), based on the percentage of sequence identity and coverage. The genomes were manually inspected using the Geneious software system and the direction of the nucleotide sequences and ORFs was adjusted. The ORF's of all putative complete prophages sequences were also predicted by GeneMarkS (http://exon.gatech.edu/GeneMark/genemarks.cgi) (Besemer, 2001) using the following

parameters: sequence type: virus and advanced options: genetic code 11 and annotated using the RAST server (http://rast.nmpdr.org/) (Aziz et al., 2008). For prophages belonging to the order Caudovirales, the occurrence of Head-neck-tail module genes was detected by the Virfam software system (http://biodev.extra.cea.fr/virfam/) (Lopes et al., 2014). For viruses belonging to the *Inoviridae* family, a pairwise comparison was carried out for the amino acid sequences of morphogenesis (pI) protein in MEGA 7 using Maximum Likelihood (1,000 bootstrap replicates) (Kumar et al., 2018). A phylogenetic tree was constructed using FigureTree (http://tree.bio.ed.ac.uk/).

CRISPR- Cas system analysis and determination of spacer matches

CRISPR/Cas system was detected in RSC chromosomes by using CRISPRCasFinder (https://crisprcas.i2bc.paris-saclay.fr.) (Couvin et al., 2018). All the identified spacer sequences were extracted and used to search for viral targets. The spacer sequences were compared with all viral genomes deposited in the NCBI database by the BLASTn tool. Sequences that share at least 84% of identity with the spacer sequences (minimum of 27/32 matching nucleotides) and e-value $\geq 10^{-1}$ were considered for putative target identification. We also performed a similarity search using the ACLAME database (http://aclame.ulb.ac.be) (Leplae et al., 2010).

Figure Ledends

Figure 1. Features of prophage-like element distributed among RSC genomes sequences. **(A)** General length distribution of prophage-like elements on chromosomal and megaplasmid sequences. **(B)** GC content of RSC genomes and prophage-like elements. **(C)** Correlation between genome sizes of *Ralstonia* and prophages. Shaded regions indicate the 95% confidence interval. According with the Pearson correlation coefficient, the plots have no linear relationship.

Figure 2: Pairwise Identity Matrix of the distribution and diversity of prophage-like elements among the genomes of *Rastonia* sp. A pairwise identity matrix from the alignment of prophage-like sequences was calculated by Geneious using default parameters. We selected the sequences with scores >70% of similarity of pairwise alignment to create an undirected network graph using the program Gephi.

Figure 3. Phylogenetic analysis based on the amino acid sequence of the pI protein of viruses found integrated into the *Rasltonia* sp genomes and innovovirus deposited in the NCBI database. The analysis was performed in MEGA 7 using Maximum Likelihood (1,000 bootstrap replicates).**Figure 4.** Heatmap representing the diversity of putative new viruses found integrated into the genomes of *Rasltonia* sp.

Figure 5. Putative genes associated to host fitness found in prophage-like elements. **(A)** Number of ORF's and its functions of prophage-like elements among the RSC. **(B)** Mapping the distribution of putative genes encoded host fitness factors in local prophage-like elements dataset.

Figure 06. Relation of CRISPR-Cas system and putative prophage-like targeted sequences. (A) Percentage of spacer sequences that are targets of CRISPR-Cas of RSC **(B)** Relation of the number of prophage-like elements and number of spacers in RSC genomes sequences. (C) Analysis of presence of spacer sequences derived from viruses belonging to *Inoviridae* family present at the locus of CRISPR-Cas system in genomes of *Ralstonia* sp genomes**. (D)** Schematic representation of CRISPR-Cas locus in *Ralstonia solanacearum* FJAT1458, showing the spacer sequence that targets the prophage *Ralstonia solanacearum* FJAT1458phi2. Other phage sequences that are target by spacers sequences are highlighted.

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Table 1. Summary of genomic features of intact prophages in RSC chromosomes and megaplasmids.

Host	Best Hit (Blastn)	Family	Prophage Size (Kb)
Ralstonia solanacearum			
	Halomonas phage QHHSV-1	*Myoviridae*	23.2
	Rastonia phage PE226	*Inoviridae*	5.0
	Burkholderia phage KS5	*Myoviridae*	37
	Ralstonia phage Rs551	*Inoviridae*	7.9
Ralstonia pseudosolanacearum			
	Ralstonia phage RSM1	*Inoviridae*	8.4
	Ralstonia phage RSA1	*Myoviridae*	39.0
	Ralstonia phage RS603	*Inoviridae*	7.5
	Ralstonia phage RSY1	*Myoviridae*	41.8
	Ralstonia phage RSS0	*Inoviridae*	7.3
	Ralstonia phage PE226	*Inoviridae*	5.4
	Ralstonia phage RSM3	*Inoviridae*	8.8
	Bacteriophage phiE125	*Myoviridae*	24.1
	Ralstonia phage RSS30	*Inoviridae*	8.5
Ralstonia syzygii			
	Burkholderia phage KS10	*Myoviridae*	31.3
	Ralstonia phage RS138	*Siphoviridae*	41.0
Ralstonia pickettii			40.6
	Ralstonia phage 12J	*Inoviridae*	7.1
	Ralstonia phage PE226	*Inoviridae*	7.1
Ralstonia sp			
	Ralstonia phage RSY1	*Inoviridae*	38.3
	Ralstonia phage RSA1	*Inoviridae*	43.5
	Burkholderia cenocepacia phage	*Myoviridae*	40.4
	Ralstonia phage RS603	*Inoviridae*	7.4
	Ralstonia phage RSS0	*Inoviridae*	6.9

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Table 1. Summary of genomic features of intact prophages in RSC chromosomes and megaplasmids (contin.)

Host	Best Hit (Blastn)	Family	Prophage Size (Kb)
Ralstonia solanacearum			
	Halomonas phage QHHSV-1	*Myoviridae*	23.2
	Rastonia phage PE226	*Inoviridae*	5.0
	Burkholderia phage KS5	*Myoviridae*	37
	Ralstonia phage Rs551	*Inoviridae*	7.9
Ralstonia pseudosolanacearum			
	Ralstonia phage RSM1	*Inoviridae*	8.4
	Ralstonia phage RSA1	*Myoviridae*	39.0
	Ralstonia phage RS603	*Inoviridae*	7.5
	Ralstonia phage RSY1	*Myoviridae*	41.8
	Ralstonia phage RSS0	*Inoviridae*	7.3
	Ralstonia phage PE226	*Inoviridae*	5.4
	Ralstonia phage RSM3	*Inoviridae*	8.8
	Bacteriophage phiE125	*Myoviridae*	24.1
	Ralstonia phage RSS30	*Inoviridae*	8.5
Ralstonia syzygii			
	Burkholderia phage KS10	*Myoviridae*	31.3
	Ralstonia phage RS138	*Siphoviridae*	41.0
Ralstonia pickettii			40.6
	Ralstonia phage 12J	*Inoviridae*	7.1
	Ralstonia phage PE226	*Inoviridae*	7.1
Ralstonia sp			
	Ralstonia phage RSY1	*Inoviridae*	38.3
	Ralstonia phage RSA1	*Inoviridae*	43.5
	Burkholderia cenocepacia phage	*Myoviridae*	40.4
	Ralstonia phage RS603	*Inoviridae*	7.4
	Ralstonia phage RSS0	*Inoviridae*	6.9

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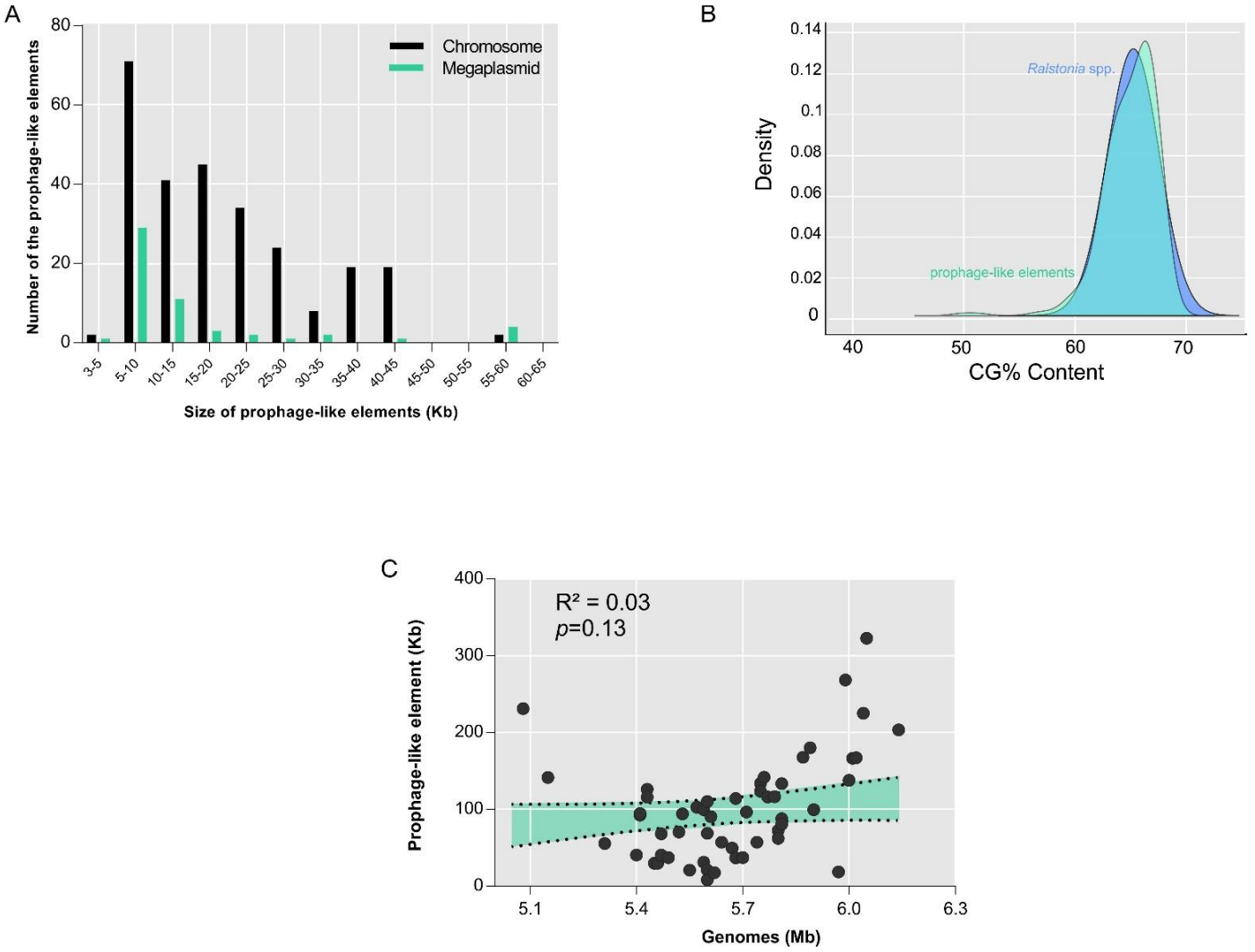
Table 2. Poly-lysogenesis events of putative intact prophages found in genomes of RSC.

Strain	Coordinates in bacterial genome	Best Hit (Blastn)	Family	Prophage Size (Kb)	Identity (%)	Coverage (%)
CQPS-1	1820133-1857237	*Ralstonia phage RSA1*	*Myoviridae*	37.1	95.7	96
	2608471-2642553	*Ralstonia phage RS603*	*Inoviridae*	7.5	91.2	90
	3071478-3113234	*Ralstonia phage RSY1*	*Myoviridae*	41.7	90.8	86
	3525060-3549703	*Ralstonia phage RSS0*	*Inoviridae*	7.9	98.7	85
EP-1	1099477-1143047	*Ralstonia phage RSA1*	*Myoviridae*	43.5	88.8	55
	1249959-1293296	*Ralstonia phage RS603*	*Inoviridae*	7.7	92.7	97
	1689423-1724621	*Ralstonia phage RSY1*	*Myoviridae*	35.2	99.8	100
FJAT91	1776443-1801011	*Ralstonia phage PE226*	*Inoviridae*	5.4	99.9	100
	2599202-2620542	*Ralstonia phage RS603*	*Inoviridae*	7.4	91	94
FJAT-1458	426995-463041	*Ralstonia phage RSA1*	*Myoviridae*	36.0	95.9	94
	1622955-1666443	*Ralstonia phage RSY1*	*Myoviridae*	46.4	98.1	84
	2168497-2190188	*Ralstonia phage PE226*	*Inoviridae*	5.4	99.8	100
OE1-1	2105472-2145520	*Ralstonia phage RSY1*	*Myoviridae*	40	99.8	100
	2696191-2739761	*Ralstonia phage RSA1*	*Myoviridae*	43.5	88.8	55
SEPPX05	3326547-3367009	*Burkholderia cenocepacia phage*	*Myoviridae*	40.4	74.7	60
	3670755-3689193	*Ralstonia phage RS603*	*Inoviridae*	7.4	91.1	94
SL3755	1145217-1167343	*Ralstonia phage RSM1*	*Inoviridae*	8.6	99.8	100
	2009326-2032661	*Ralstonia phage RSS0*	*Inoviridae*	7.2	99.8	95
SL3882	1278383-1299727	*Ralstonia phage RSM3*	*Inoviridae*	8.8	98.5	89
	2528368-2575489	*Ralstonia phage RSY1*	*Myoviridae*	47.1	85.3	57
YC40-M	393572-430273	*Ralstonia phage RSA1*	*Myoviridae*	36.7	96.5	92
	2080244-2091283	*Ralstonia phage RSS0*	*Inoviridae*	6.7	99.9	100
RS488	1126815-1163841	*Burkholderia phage KS5*	*Myoviridae*	37	81.3	02
	1218739-1246295	*Ralstonia phage Rs551*	*Inoviridae*	7.9	99.9	100
RSCM	399957-442146	*Ralstonia phage RSA1*	*Myoviridae*	40.0	96.1	85
	2229300-2253491	*Bacteriophage phiE125*	*Myoviridae*	24.1	67.7	18
Ralstonia pickettii 12J	1480034-1500270	*Ralstonia phage p12J*	*Inoviridae*	7.1	99.9	100
	2649220-2659409	*Ralstonia phage PE226*	*Inoviridae*	7.1	73	27

893

894 **FIGURE 01**



895

FIGURE 02

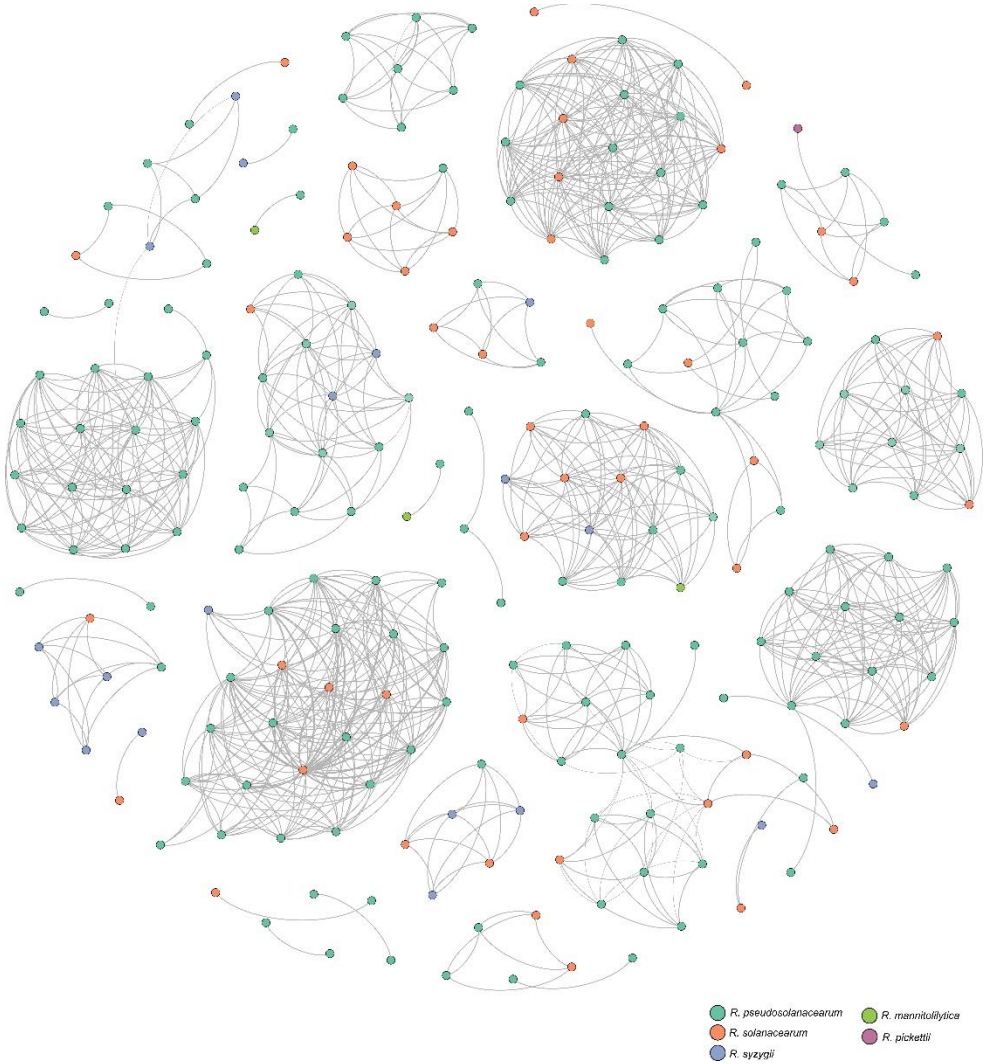
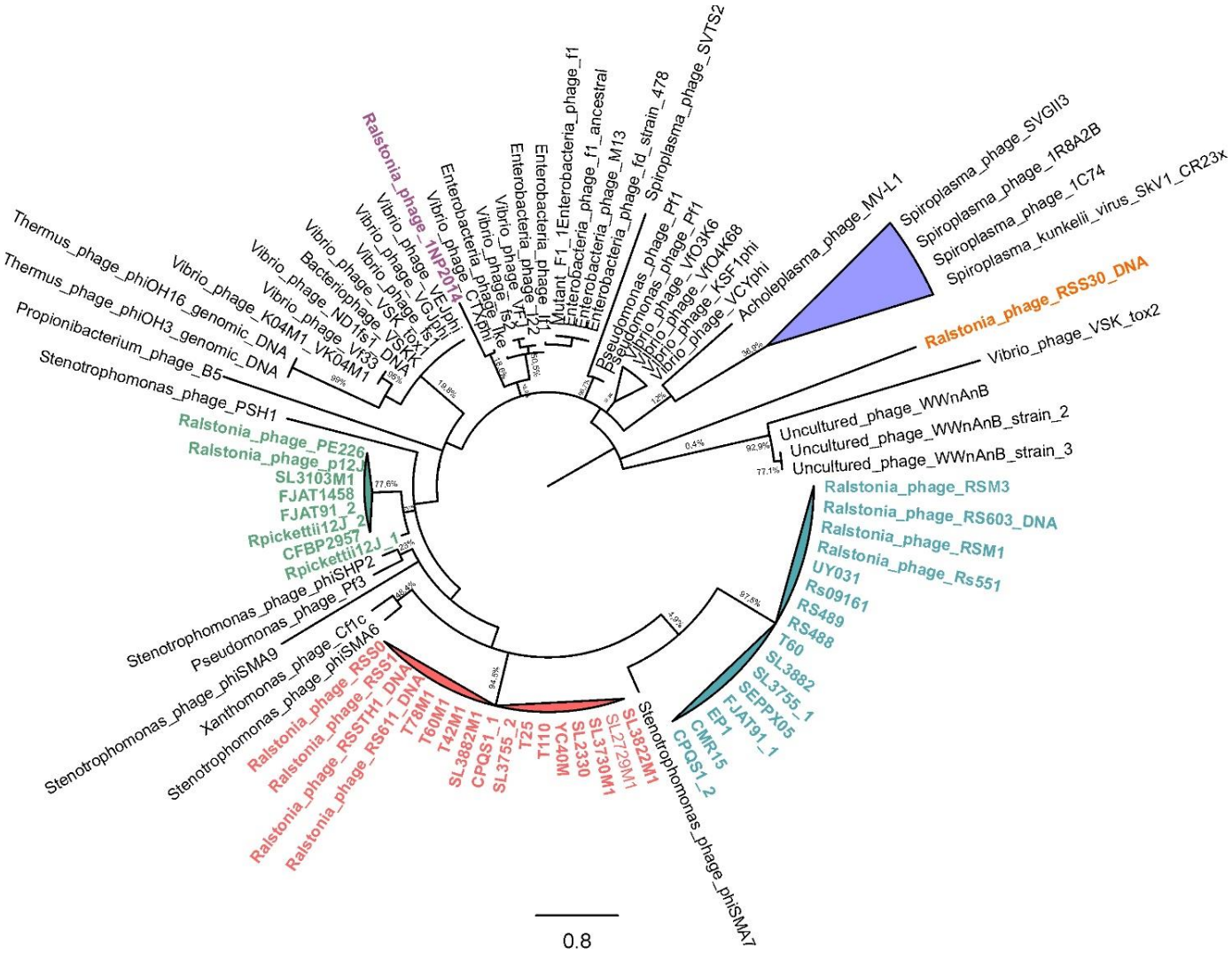
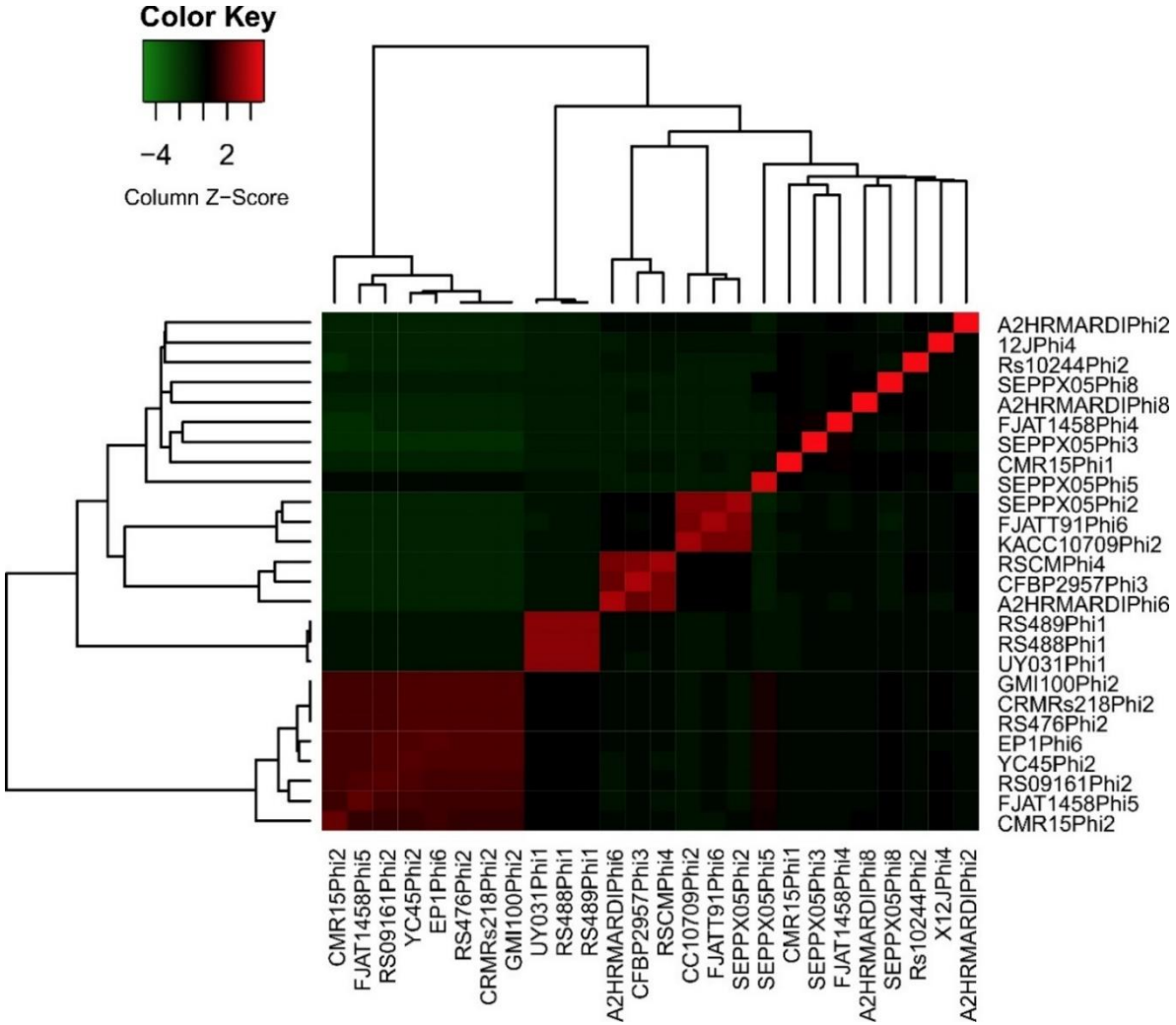

R. pseudosolanacearum
R. solanacearum
R. syzygii
R. mannitolilytica
R. pickettii

FIGURE 03

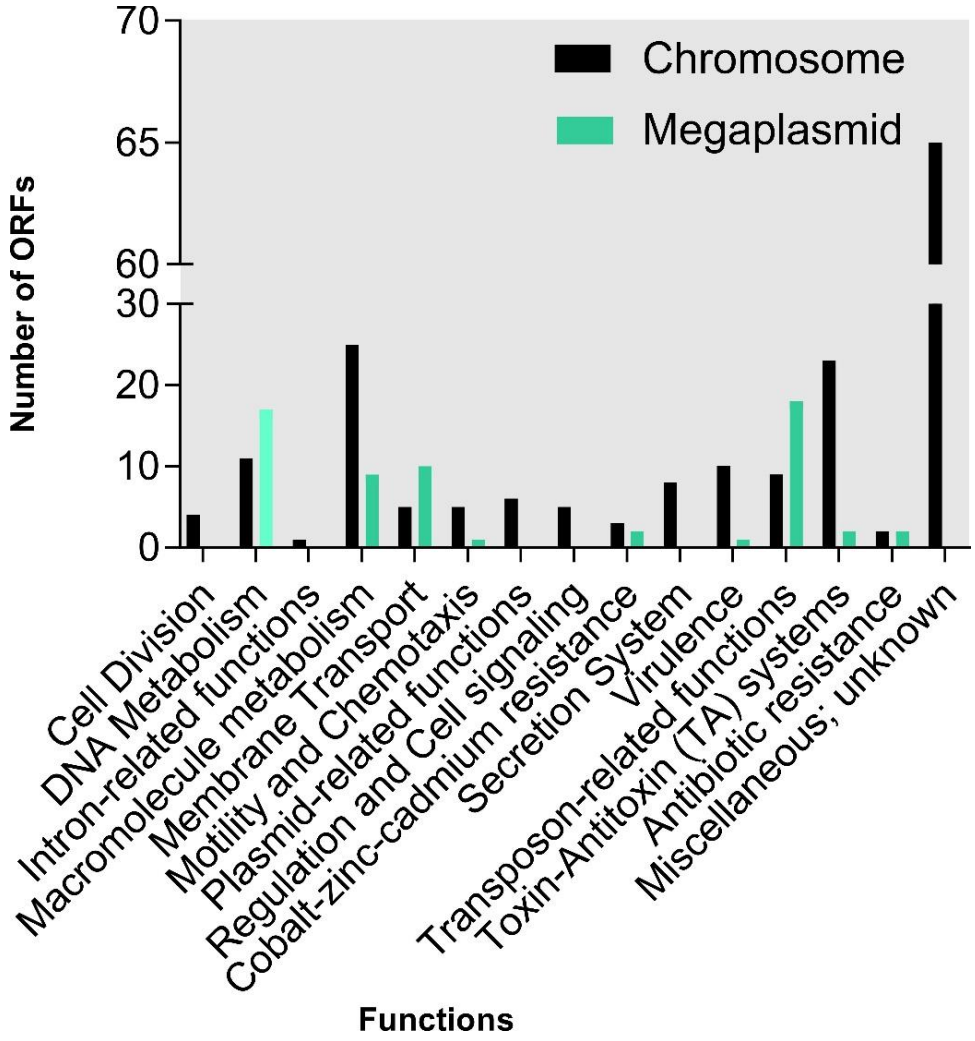

Ralstonia_phage_1NP2014
Enterobacteria_phage_C-1Xphi
Vibrio_phage_fs1_like
Enterobacteria_phage_fs2
Enterobacteria_phage_fs1
Mutant_F1_1Enterobacteria_phage_f1
Enterobacteria_phage_f1_ancestral
Enterobacteria_phage_M13
Pseudomonas_phage_fd_strain_478
Spiroplasma_phage_SVTS2
Pseudomonas_phage_Pf1
Vibrio_phage_VfO4K68
Vibrio_phage_VfO3K6
Vibrio_phage_KSF1phi
Vibrio_phage_VCYphi
Acholeplasma_phage_MV-L1
Spiroplasma_phage_SVGII3
Spiroplasma_phage_1R8A2B
Spiroplasma_phage_1C74
Spiroplasma_kunkelii_virus_SkV1_CR23x
Ralstonia_phage_RSS30_DNA
Vibrio_phage_VSK_tox2
Uncultured_phage_WWnAnB
Uncultured_phage_WWnAnB_strain_2
Uncultured_phage_WWnAnB_strain_3
Ralstonia_phage_RSM3
Ralstonia_phage_RS603_DNA
Ralstonia_phage_RSM1
Ralstonia_phage_Rs551
UY031
Rs09161
RS489
RS488
T60
SL3882
SL3755_1
SEPPX05
FJAT91_1
EP1
CMR15
CPQS1_2
Stenotrophomonas_phage_phiSMA7
SL3822M1
SL2729M1
SL3730M1
SL2330
YC40M
T110
T25
SL3755_2
CPQS1_1
SL3882M1
T42M1
T60M1
T78M1
Ralstonia_phage_RSS11_DNA
Ralstonia_phage_RSSTH1_DNA
Ralstonia_phage_RS611_DNA
Ralstonia_phage_RSS01
Stenotrophomonas_phage_phiSMA6
Xanthomonas_phage_phiSMA9
Stenotrophomonas_phage_Cf1c
Pseudomonas_phage_Pf3
Stenotrophomonas_phage_phiSHP2
Rpickettii12J_1
CFBP2957
Rpickettii12J_2
FJAT91_2
FJAT1458
SL3103M1
Ralstonia_phage_p12J
Ralstonia_phage_PE226
Stenotrophomonas_phage_PSH1
Propionibacterium_phage_B5
Thermus_phage_phiOH3_genomic_DNA
Thermus_phage_phiOH16_genomic_DNA
Vibrio_phage_K04M1_VK04M1
Bacteriophage_ND1fs1_DNA
Vibrio_phage_Vf33
Vibrio_phage_VSK_tox1
Vibrio_phage_VSKK
Vibrio_phage_VEJphi
Vibrio_phage_VGJphi
0.8

900 **FIGURE 04**



901

902 **Figure 05A**



903

FIGURE 05B

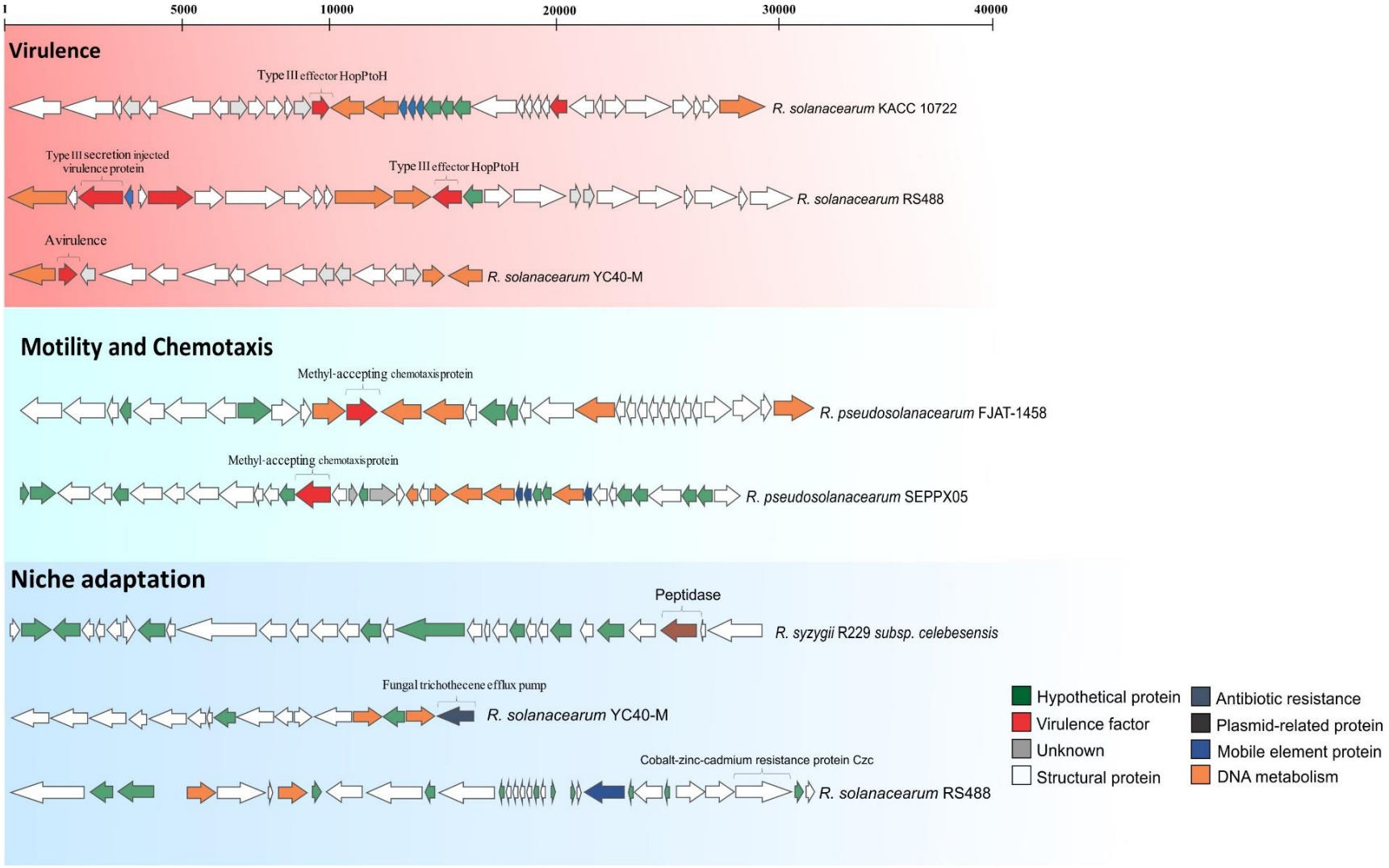
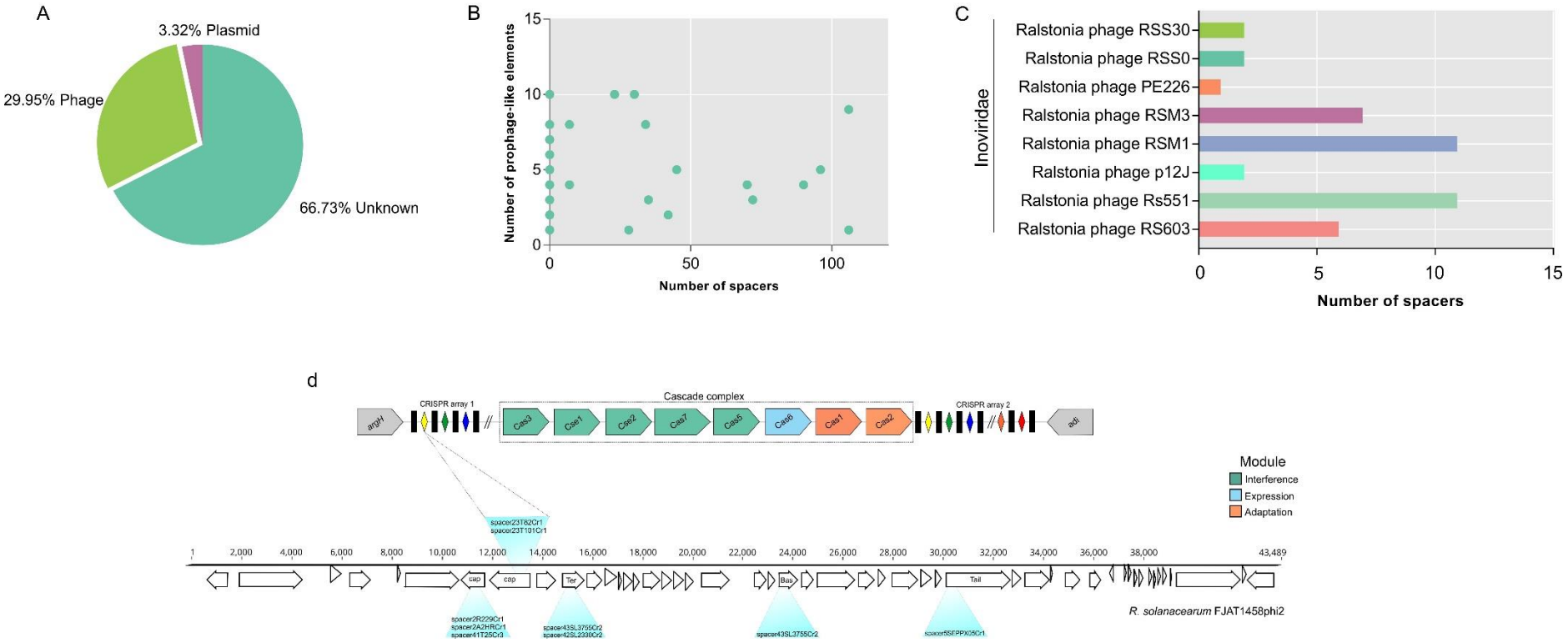

1
5000
10000
20000
30000
40000
Virulence
Type III effector HopPtoH
R. solanacearum KACC 10722
Type III secretion injected virulence protein
Type III effector HopPtoH
R. solanacearum RS488
Avirulence
R. solanacearum YC40-M
Motility and Chemotaxis
Methyl-accepting chemotaxis protein
R. pseudosolanacearum FJAT-1458
Methyl-accepting chemotaxis protein
R. pseudosolanacearum SEPPX05
Niche adaptation
Peptidase
R. syzygii R229 subsp. celebesensis
Fungal trichothecene efflux pump
R. solanacearum YC40-M
Cobalt-zinc-cadmium resistance protein Czc
R. solanacearum RS488
Hypothetical protein
Virulence factor
Unknown
Structural protein
Antibiotic resistance
Plasmid-related protein
Mobile element protein
DNA metabolism

FIGURE 06


A
3.32% Plasmid
29.95% Phage
66.73% Unknown
B
Number of prophage-like elements
Number of spacers
C
Inoviridae
Ralstonia phage RSS30
Ralstonia phage RSS0
Ralstonia phage PE226
Ralstonia phage RSM3
Ralstonia phage RSM1
Ralstonia phage p12J
Ralstonia phage Rs551
Ralstonia phage RS603
Number of spacers
d
CRISPR array 1
Cascade complex
CRISPR array 2
Module
Interference
Expression
Adaptation
R. solanacearum FJAT1458phi2

CHAPTER 2

Metagenomics of the Viral Community in Ruminal Fluid of Two Cattle Dairy Samples

Souza, Flávia de Oliveira[1], Rezende, Rafael Reis de[1]; Vidigal, Pedro Marcus Pereira[2]; Silva, José Cleydson Ferreira[3], Arantes, Thalita Souza[4]; Abrahão, Jonatas Santos[4], Cascardo, Renan de Souza[1]; Alfenas-Zerbini, Poliane[1]

Manuscript to be submmited to Virology

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Summary

The microbiota of bovine rumen is complex, including organisms of the three domains of life, potential hosts of numerous viruses. The viral population of the rumen plays an important role in maintaining the balance of the bacterial population and in horizontal gene transfer. In addition, bacteriophages may be used as a possible strategy for methane mitigation. This requires the identification of bacteriophage species that infect the dominant methane-producing archaea in the rumen. In this study we characterized the rumen virome at dairy cattle from the University Federal of Viçosa (Brazil) using a metagenomic approach. A high level of viral diversity was observed with the presence of viruses of several families. The most of viral sequences did not have any match with previously described viruses in database. Functional metabolic identification based on SEED database revelead predominance of functions related to nucleic acid metabolism. Moreover, we find sequences of enzymes hydrolases, which are involved in carbon metabolism, an important source of energy in this environment. Additionally, we were able to isolate for the first time a giant, mimivirus-like virus from one of the rumen samples.

Introduction

Rumen is a structure of the digestive system of ruminant animals directly involved in the health and efficiency of the convertion of vegetable fiber eaten by the animal into profitable products such as meat and milk. This efficiency is related to the rumen microbiome, that is, all microorganisms present in this environment, as well as genetic elements that may be associated with these microorganisms and their interactions, since it is an extremely symbiotic environment. It is believed that the amount of microorganism species present in a single rumen may vary from hundreds up to thousand microbial species (Li, 2015).

In the past, the rumen microbiome had been the focus of studies, but the fact that it is an anaerobic environment makes most rumen microorganisms non-cultivable. In fact, apparently only 11% of ruminal bacteria can be grown and kept under laboratory conditions. Because of this, most of the traditional molecular techniques become ineffective in characterizing geneticaly the structure and function of the rumen microbiome because they are dependent on good and large quantity of genetic material, which is often unviable (Edwards et al., 2004).

The development of new platforms and high throughput sequencing techniques has allowed profile analyzes of microbial communities in ways independent of laboratory cultures, thereby broadening knowledge of genetic diversity, phylogenetic relationships and characterizations of the gastrointestinal microbiota. Regarding the rumen environment, most studies focus on bacteria, which make up approximately 95% of the microbial community in this environment. (Yoon et al., 2015).

Viruses that infect prokaryotes (bacteria and archaea), often also referred to as bacteriophages, are always present in the gastrointestinal tract of animals and humans. In rumen is no different and the few viruses that have been isolated from this environment are phages that infect ruminal bacteria. Thus, rumen was a source of litics viruses that infect well-characterised and predominant rumen bacteria, such as *Prevotella* (*Bacteroides*) *ruminicola* and *Streptococcus bovis* (Iverson and Millis, 1976; Klieve et al., 1989).

Gilbert and Klieve (2015), listed twenty-seven papers on rumen bacteriophage isolation. Most of these isolated' viruses had the morphology of the *Myoviridae*, *Siphoviridae* and *Podoviridae* families, in order *Caudovirales*. Two of these isolates

viruses presented the *Inoviridae* family morphology and four these isolates didn't have their morphology defined.

Nowadays, metagenomics can analyze the composition of microorganisms community in an environment without the need that microorganisms growing in the laboratory, due to the modernization of sequencing techniques in recent years associated with fast progress in number of sequences added to reference databases and the development bioinformatic tools (Galbraith et al., 2004).

The most of metagenomic studies have focused on ruminal bacteria in different ruminate species, which makes sense, since bacteria are predominant in this environment, with approximately up to 10^{11} viable cells per gram of rumen contents, representing more than 200 genera (Li et al., 2012).

In this study we characterized the rumen virome from two Brazilian dairy cattle animals from the University Federal of Viçosa using a metagenomic approach. A high level of viral family diversity was observed with viruses infecting organisms from all domains life. The majority of the viral sequences didn't have any match with previously described viruses in database. Functional metabolic classification based on SEED database revelead predominance of functions related to nucleic acid metabolism. Morover, we find sequences of enzymes hydrolases, which are involved in carbon metabolism, an important source of energy in this environment. Additionally, we were able to isolate a giant, mimivirus-like virus from one of the rumen samples.

Material and Methods

Rumen fluid collection and processing

Ruminal fluid (RL) was collected from two fistulated dairy cattle at the Unidade de Ensino, Pesquisa e Extensão em Gado de Leite da Universidade Federal de Viçosa, Brazil. One of the animals, identified as 11, was in the pre-partum period and its diet consisted of 3 kilograms of feed and corn silage. The other animal (08) was in the period of low milk production (dry period) and its feeding consisted of one kilo of feed added with corn silage. The collections were performed according to animal availability on the day of collection.

Purification and Viral Concentration of Particles in Liquid Fraction

Immediately after collection, the liquid fractions heated at 75°C for 20 minutes to eliminate possibly nuclease activity present in the rumen fluid. The protocol for purification and the concentration of viral particles present in the liquid fraction was adapted from the method described by Klieve and Swain (1993), as described: after heating, samples were centrifuged twice at 20,000xg for 30 minutes at 4°C and the supernatant was filtered on sterile 0.45 µm pore size membranes.

After filtration the viral particles were concentrated by ultracentrifugation at 51,000xg for 2 hours at 4°C. The supernatant was discarded, and the precipitate resuspended in 1ml SM buffer (50mM Tris-HCl, pH 7.5, 100mM NaCl, 10mM $MgSO_4$ and 0.01% gelatin) and incubated overnight at 4°C. Then, each tube was filled in to 25 ml with TE and the sample was once again ultracentrifuged using the same parameters described above. The viral precipitate was resuspended in 500 µl PBS buffer (0.13 M NaCl, 2mM KCl, 9mM Na_2HPO_4 and 1mM KH_2PO_4). At the end of the purification procedure the sample was filtered on sterile 0.22 µm pore size membranes. The viral particles were stored in sterile tubes at 4°C.

Viral DNA Extraction

To minimize contamination of the viral particles with non-viral nucleic acid, prior to nucleic acid extraction, the samples were treated with 2 ul of RQ1 RNase-Free Dnase I (Promega) and incubated at 37 ° C for 30 minutes. DNAse inactivation occurred in the presence of 2 µl of Stop Solution for 30 minutes at 65°C. Then the sample was treated with RNAse A (Sigma-Aldrich) using a final concentration of 10µg/ml. Samples were incubated at 37°C for 1 hour, followed by incubation for 30 minutes at 65 ° C. Samples were stored at 4°C.

Extraction of viral DNA was performed by the Proteinase K method, as described by Sambrook and Russell (2001). Quality analysis of extracted nucleic acid was performed by agarose gel electrophoresis (0.8%) and concentration was measured using NanoDrop 2000 spectrophotometers (Thermo Scientific).

Genetic Material Sequencing and Assembly

Sequencing of DNA from virus-like particles was extracted from the rumen fluid was performed using the Illumina platform HiSeq2000 system (Macrogen Inc.-Korea).

Quality analysis and assembly of reads

Quality control of reads generated by sequencing was performed using Fastqc (Andrews, 2010). The reads were trimmed, filtered, and adapters removal was performed using Trimmomatic (Phred ≥ 28 and reads ≥ 50 bases) (Bolger et al., 2014). Then, the reads were assembled into contigs using Trinity (Grabherr et al., 2011).

Analysis of viral diversity in rumen

To access viral diversity, BLASTn and BLASTx analyzes were performed by comparing assembled contig sequences with viral nucleotide and proteins sequences deposited in the National Center for Biotechnology Information-NCBI database (https://www.ncbi.nlm.nih.gov). Comparison with sequences from other metagenomas sequences was made by BLASTn against nucleotide sequences of viruses of Integrated Microbial Genomes & Microbiomes dataset (Chen et al., 2019). The parameters used for both analyzes were percentage of alignment ≥ 100 nucleotides, identity ≥ 70% and e-value $\leq 10^{-5}$.

In addition, the viral family diversity indices identified in this study were calculated using the standard parameter of PAST program (https://folk.uio.no/ohammer/past/).

Functional Classification of Viral Metagenome Sequences

To predict the functional profile of ruminal virome the reads sequences of each rumen was uploaded into MG-RAST and predicted metabolic profiles for virome were generated by comparison to the SEED subsystems database the e-value $\leq 10^{-5}$ as cutoff (Overbeek et al., 2014).

Presence of hydrolases analysis

A BLASTx analysis of the rumen virome contigs sequences was made against the hydrolase protein sequences deposited in the Carbohydrate Active Enzymes (CAZy) database using a cutoff value of e-value $\leq 10^{-5}$ (Lombard et al., 2014).

Viral isolation and purification of a giant virus

The rumen fluid was diluted until 10^{-3} and all dilutions were added to *Acanthamoeba castellani*i (ATCC 30010) cultures in 96-well plates. The plates

visualization of cytopathic effects for further isolation and purification of giant viruses, as described in Campos et al., (2014).

After confirmation of the presence of mimivirus-like particles in the ruminal liquid, total nucleic acids was extracted according to Dellaporta et al., (1983) and the confirmation of viral presence was performed through PCR for helicase gene .

The primers sequences were: 5'-ACC TGA TCC ACA TCC CAT AAC TAA A-3'and 5'-GGC CTC ATC AAC AAA TGG TTT CT-3 . The PCR conditions were: 95°C for 2 min, followed by 35 cycles of 94°C for 45 seg, 53°C for 45 seg and 72°C for 30 seg. The final extension was of 72°C for 10 min and PCR was hold a 12°C.

Primers specific to B-family DNA polymerase were designated from nucleotide sequences of that gene available in the database. A fragment approximately 1000 base pairs in size was amplified. The primers sequences were: MimiLinAF: 5'-GCC TAG TGA GAC AAT TGA TTC-3' and MimiLinAR: 5'-AAT CTG GTC TCA ATT CGG C-3'. The PCR conditions were: 95°C for 2 min, followed by 34 cycles of 94°C for 45 seg, 56°C for 45 seg and 72°C for 1 min. The final extension was of 72°C for 10 min and hold a 12°C.

Transmission electron microscopy

After 7 hours of infection, *A. castellanii* were washed twice with 0.1 M phosphate buffer (pH 7.4) and fixed with 2.5% glutaraldehyde (grade I) in 0.1 M phosphate buffer (pH 7.4) (Electron Microscopy Sciences, Germany) for one hour at room temperature. The amoeba monolayer was scraped from the plates and recovered by centrifugation at 900 g for 5 minutes. The amoebae were postfixed with 2% osmium tetroxide and embedded in EPON resin. Ultrathin **sections** were stained with 2% uranyl acetate and examined using a Tecnai G2-Spirit FEI 2006 transmission electron microscope operating at 80 kV at the Microscopy Center, UFMG, Brazil.

Results and Discussion

Diversity of rumen virome

We sequenced virus like particles from the rumen fluid of two dairy cattle using HiSeq2000 (Illumina platform). After checking the quality of sequences, were generate 49.783.046 and 49.587.176 reads in pair end for the samples 08 and 11, respectively. *De novo assembly* was performed, and we identified 294.757 contigs for the sample 08 and

193.262 for the 11. The general profile of the contigs present in the two ruminal samples was accessed using BLASTn and BLASTx analyzes against virus sequences deposited in National Center for Biotechnology Information (NCBI). Assessing the diversity of viral families in the rumen environment, we found the same a group of viruses belonging to the following viral families: *Baculoviridae*, *Bicaudaviridae*, *Herpesviridae*, *Iridoviridae*, *Marseilleviridae*, *Mimiviridae*, *Myoviridae*, *Phycodnaviridae*, *Podoviridae*, *Poxviridae*, *Rudiviridae* and *Siphoviridae* besides unclassified and uncultured viruses in both samples. In sample 08, there are virus sequences belonging to the *Adenoviridae* and *Tectiviridae* families that were not found in another sample. On the other hand, in sample 11 were found sequences of viruses classified in the *Ascoviridae*, *Flaviviridae*, *Inoviridae* and *Retroviridae* viral families, that were unique to this sample (Figure 1).

Together, *Myoviridae*, *Siphoviridae* and *Podoviridae* were the most detected families as previously reported in others viral metagenomic studies (Cantalupo et al., 2011; Fernandez-Cassi et al., 2018; Guerrero-Latorre et al., 2018; Tamaki et al., 2012). This is probably because the vast majority sequences deposited in the databases are viruses belonging to the order *Caudovirales*. Thus, this prevalence of sequences makes this virus more characterized in a much higher proportion in metagenomic studies. *Ascoviridae* and *Baculoviridae* are viruses that infect arthropods.

Baculoviridae can be used for pest insect control as they have been shown to be as effective at controlling the effects as the commonly used chemical agents, thus decreasing environmental contamination with such agents and presenting little or no risk for infecting other hosts. (Kamita et al., 2017).

Bicaudaviridae and *Rudiviridae* are viruses that infect hyperthermophilic archaea. Archaea are organisms known to inhabit extreme environments and appear to have characteristics of prokaryotic and eukaryotic organisms. In the rumen, archaea are related to the production of methane by ruminants. Thus, isolating viruses that infect ruminal archaea may be a useful to develop strategies for methane mitigation. On the other hand, the development and establishment of these strategies is also challenging because of the difficulties of conducting *in vitro* experiments with archaea systems (Patra et al., 2017).

Amoeba-infecting giant viruses (*Marseilleviridae*, *Mimiviridae*, *Pandoravirus* and *Pithovirus*) have been recently discovered and sinc then have been isolated from various environments. In addition, metagenomics has also detected a large distribution of these giant viruses in wide range of environmental and host-associated samples (Halary et al., 2016).

The viral diversity was analyzed in the two ruminal samples. Diversity analyzes showed that t in sample 11 the distribution of viral families is more diverse than in sample 08, according to Shannon indices. Comparison of sample richness was done by rarefaction analysis which showed that it was possible to access the richness of the viral families in sample 08. In the sample 11 there is still more to be explored. Beta diversity (principal coordinate analysis-PcoA) showed that the diversity in both samples is heterogeneous with respect to each other (Figure 2).

Thus, accessing viral diversity by metagenomics in different environments has become relevant in several areas. Bibby et al., (2019) points out that the use of viral metagenomics in direct pathogen detection has been a promising idea and that human-associated viruses are a rich reservoir for developing future tools for managing water quality, as well as regulating wastewater reuse and monitoring. agricultural and recreational activities. In the rumen environment, unraveling the viral population profile can be useful in defining strategies for controlling undesirable organisms such as methanogenic archaea and favoring microorganisms involved in improving the production and welfare patterns of these animals.

Comparison to other viromes

As only about 15% of the contigs analyzed had homology with some viral sequence deposited in the NCBI database, we attempted to increase this percentage of viral contigs identification through Blastn against nucleotide sequences of others metagenomes deposited in the Integrated Microbial Genomes and Microbiomes (IMG/VR -Viral) which has viral fragments derived from metagenomic samples. About 23% of the contigs from sample 08 and 21.4% of the contigs from sample 11 had match with some sequence from this database. The sequences that had some homology come from metagenomas of host-associated mammals' digestive system as stomach, foregut, large intestine, cow and rumen. These findings may be indicative that these mammalian gastrointestinal tract-related environments may have a viral community core that is always present in multiple hosts and may be necessary to ensure host health.

Functional Classification of Viral Metagenome Sequences

To elucidate the functionality of the rumen virome and access the function of the proteins there in, we compared reads processed with the SEED database using the

Metagenome Rapid Annotation using Subsystem Technology (MG-RAST) to generate a possible rumen metabolic profile. A small percentage of the sequence reads (2.25% for the sample 08 and 1.95% for the sample 11) had some significant hits to the SEED subsystems database. This result profile has been repeated, since most viral proteins in the database are “hypothetical proteins”, ie, they are neither characterized nor functionally classified. Thus, in these analyzes most reads have low or no homology and are not considered in the bioinformatics parameters (Edwards and Rohwer, 2005).

As most viruses isolated and described in rumen are bacteriophages, therefore a larger number of protein sequences of this virus group are characterized. This larger number of described proteins probably explains the fact that most of the proteins identified in the rumen virome in both samples are derived from bacteriophage and phage derivatives, which are bacteriophages integrated into the bacterial genomes. The remaining identified proteins are related to DNA, RNA and protein metabolism and membrane transport, probably due to the fact that these proteins are usually conserved. A low number of proteins were identified with functions related to virulence, clustering-based subsystems, fatty acids and derivates and carbohydrates metabolism were summed and grouped like “others”, as illustrated in figure 03. The increased presence of proteins from rumen virome involved in nucleic acid metabolism is consistent with the metabolic virome profiles described for other environments (Dinsdale et al., 2008).

The fact that we identified a few carbohydrate-related proteins in analyzes based on the SEED database and the importance of these protein in rumen digestion, we investigated the possible presence of proteins related to carbon metabolism, such as hydrolases. A BLASTx analysis of the rumen virome nucleotide sequences was made against the Glycosyl hydrolase (GH) protein sequences deposited in the Carbohydrate Active Enzymes (CAZy) database. This analysis did not reveal the presence of this type of protein in sample 11. A small number of reads derived from these proteins was detected in sample 08, in a total of 03 GH’s proteins were detected in this sample. The general characteristics of hydrolases found are summarized in table 1. The GH’s identified in this study belong to families 05, 06 and 13. GH 05 comprises enzymes with various activities described such as: endoglucanase, beta-mannanase and xylanase. The activities associated with GH of family 06 are endoglucanase and cellobiohydrolase. Ultimately,

GH 13 are associated with many activities: α-amylase, maltogenic amylase, cyclomaltodextrin glucanotransferase, among others (http://www.cazy.org/).

Several studies have focused on isolate these enzymes that can degrade rumen microbiome polymers, especially hydrolases. Morgavi et al., (2013) listed 16 studies of rumen metagenomics in different species that identified this type of enzyme. However, Studies showing the contribution of viruses to the biosynthesis of these proteins are lacking. In permafrost soil, it has been identified that viruses produce proteins of the hydrolase family. In addition, its functionality has been evaluated and proven in vitro, showing that viruses contribute to hosts and play a central role in the carbon cycle in this environment (Emerson et al., 2018).

The importance of studying the activity of enzymes that degrade the cellulosic biomass present in the rumen goes beyond understanding how viruses can benefit and contribute to better animal energy conversion. Metatranscriptomics and heterogeneous gene expression were used to explore and evaluate novel cellulase genes from sheep rumen microbiome. It is important to highlight that the increasing energy demand has required abundant and renewable sources of cellulose, which makes the discovery of enzymes that degrade this type of material necessary (He et al., 2019).

Isolation of a virus mimivirus-like from rumen

Due an evidence of the presence of sequences derived of giant viruses in metagenomic analyzes, we attempted to isolate this virus by cell culture with *Acanthamoeba castellanii* .We isolate a mimivirus-like virus from a rumen fluid sample after an amoebal co-culture after 7 days of incubation. Transmission Electron Microscopy showed mimivirus-like particles with icosahedral capsid, with a diameter of about 400nm, typical of *Mimiviridae* particles, showed in figure 4. Viral factories were observed occupying a large portion of the amoeba cytoplasm. Furthermore, distinct steps of virus morphogenesis were detected in association with the viral factories. To infer some phylogenetic characteristic of this virus, we amplified, and we sequenced a fragment by PCR of the B-family DNA polymerase gene. BLASTx analysis of this sequencing fragment showed similarity with this gene with *Niemeyer virus* (Query cover 99% and % Identity 98.36%).

Mimivirus are viruses that has genome consisting of double-stranded DNA with genome and particles sizes on the same order of magnitude of small bacteria. Since its discovery, three lineages (A, B and C) have been represent among these viruses, based

on their B-family DNA polymerase gene sequences. These lineages correspond to group A (which includes *Mimivirus* and *Mamavirus),* group B (*Moumouvirus*) (Yoosuf et al., 2012), and group C (*Megavirus chiliensis*) (Arslan et al., 2011). *Acanthamoeba* sp. it has been used as a bait host for the isolation of this virus in several environments (Yutin et al., 2013).

The discovery of giant viruses is relatively recent and has certainly changed the classical view of virology. The idea that viruses are always smaller than bacterial and filterable organisms probably led to the delay in isolation of this group of viruses. The first giant virus of amoebae, *Acanthamoeba polyphaga* mimivirus (APMV) and the circumstances behind its isolation and discovery was reported in 2003 and has a 0.5 μm large icosahedral capsid and a 1.2 megabase pair (Mbp) genome with approximately 1,000 genes. This virus has a larger genome than some bacteria, such as *Mycoplasma genitalium*, *Ureaplasma urealyticum*, *Buchnera* sp., and *Wigglesworthia brevipalpis*, and can be visualized by light microscopy (Scola et al., 2003).

In addition, genome and protein analyzes of giant viruses have shown that they do not have a ribosome, but they have a much larger number of genes than other viruses, including genes related to RNA translation, such as a peptide chain release factor, GTP-binding elongation factor, translation initiation factors and aminoacyl-tRNA synthetases (Sharma et al., 2016). Currently, there are more complex mimiviruses such as *Tupanvirus*, a mimivirus that contains the most complete translational apparatus of the known virosphere. These findings coupled with the widespread distribution of giant viruses raise questions about these giant viruses that still need to be investigated (Abrahão et al., 2018).

Giant viruses have other characteristics of their own, they can be infected by other parasitic viruses called virophages, and the DNA of virophages can integrate into the mimiviral host genome as ‘pro-virophages’. Besides that, they may contain a diverse mobilome, such as DNA elements (transpovirons) and they can have a defense system against virophages called MIMIVIRE (Mimivirus virophage resistance element) (La Scola et al., 2008; Desnues et al., 2012; Levasseur et al., 2016).

Viruses of *Mimiviridae* family have been detected in several environments both by co-cultivation techniques and bioinformatics approaches, showing the broad distribution of these viruses in various environments, such as marine, water and soil (Dann et al., 2016; Emerson et al., 2018; Francis et al., 2019; Schulz et al., 2018).

Rumen viruses has not received much attention mainly because it is an anaerobic environment and the cultivation of microorganisms becomes difficult and expensive.

Some metagenomics studies showed evidences of presence of mimivirus in dairy catlle rumen through some sequence detection (Miller et al., 2012).

In this work we analyze the diversity of ruminal viroma, detecting sequences of a wide variety of viruses that infect microorganisms from all domains of life. Identification of hydrolase family protein sequences may indicate viral contribution to carbon biogeochemical cycle in this environment. Finally, we were able to isolate the first *Mimiviridae* from rumen fluid. This virus showed close relationship with *Niemeyer Virus*, a mimivirus isolated from water samples of an urban lake in Brazil.

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Table 01

Table 01-BLASTx comparison of reads from the sample 08 to the Carbohydrate Active Enzyme (CAZy) database.

	Sample 08		
CAZy Family	**Number of Reads with hits**	**Query cover (%)**	**E-value**
GH 05	864	80	1^{-07}
GH 06	1346	72	1^{-07}
GH 13	1023	56	1^{-06}

Analyzes were done for both samples, but no significant results were found for sample 11.

Figure Legends

Figure 1

Heatmap representing the diversity of viral families in two-liquid ruminal samples (08 and 11). The low row indicates the samples and the host of family viral detected in this study. The right columm correspond to the count's sums of viral families on each sample and left column indicates the classification of viral host in the tree of life. Data spanned from green (low detected) to red (high relative abundance), as illustrated by the colour scale.

Figure 2

Diversity indices of viral families present in two liquid-rumen samples obtained through PAST (PAleontological Statistics Version 3.25).

A) Rarefaction analysis of two rumen samples for diversity richness analysis **B)** General diversity analysis using Shannon H index

Figure 3

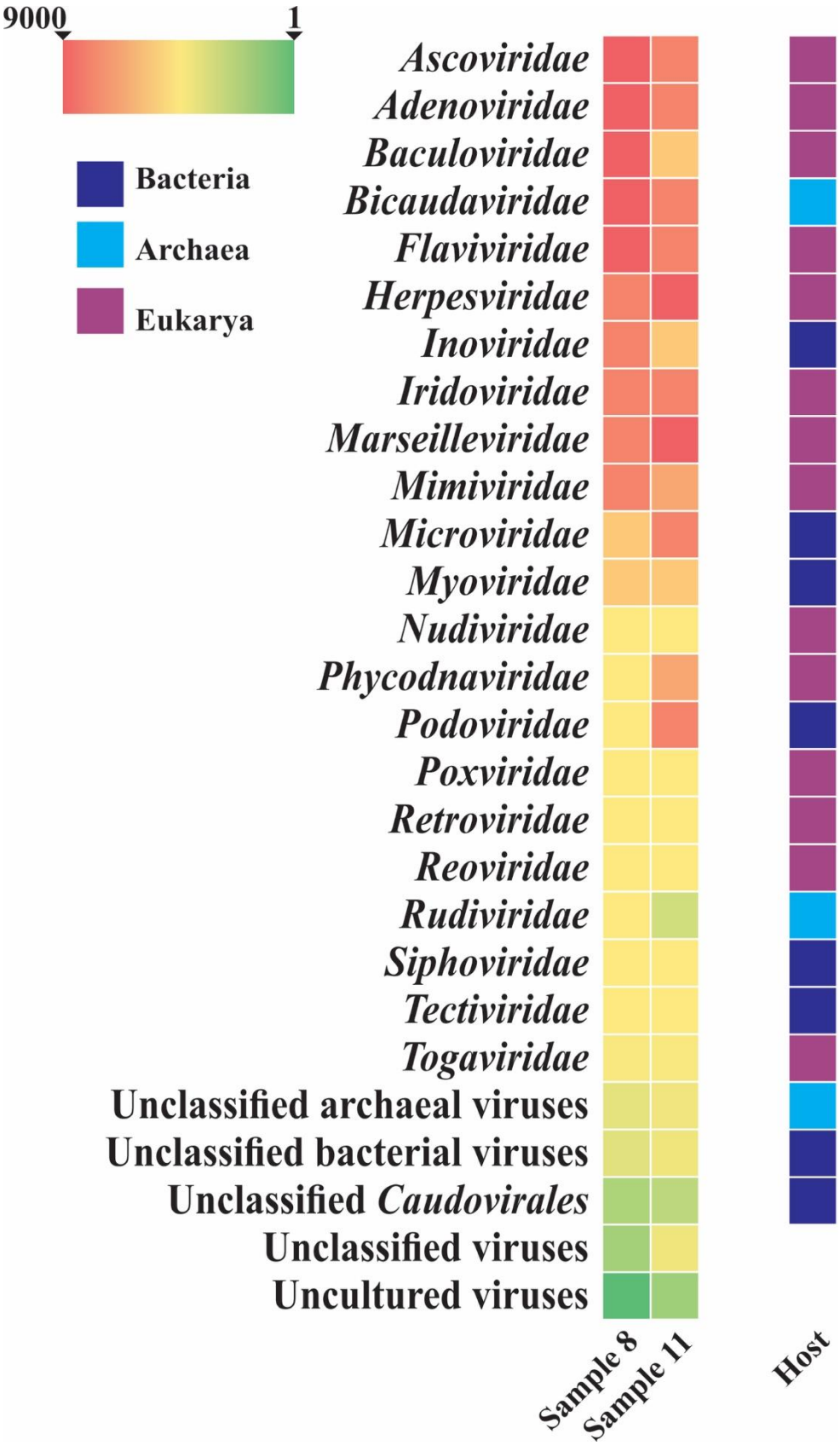
Functional classification into SEED subsystems of ruminal virome sequences from two dairy cattle. Predicted metabolic profile of two virome ruminal based on comparison of sequence reads from each viral metagenome to the SEED subsystems database (e-value $\leq 10^{-5}$). The predict metabolic profile for the sample 08 is shown in orange and for sample 11 is indicated in blue. The percentage of reads in each identified category is indicated on the right of the bars.

Figure 4

Replication of a mimivirus-like in Acanthamoeba castellanii observed by transmission electronic microcopy. Giant viral factories are present within the amoebal cytoplasm and morphology of virus near the viral factory can be viewed: early morphogenesis (red arrows), intermediate morphogenesis (green arrows) and late morphogenesis (blue arrows)

884 **FIGURE 01**

885



886

887 **FIGURE 02**

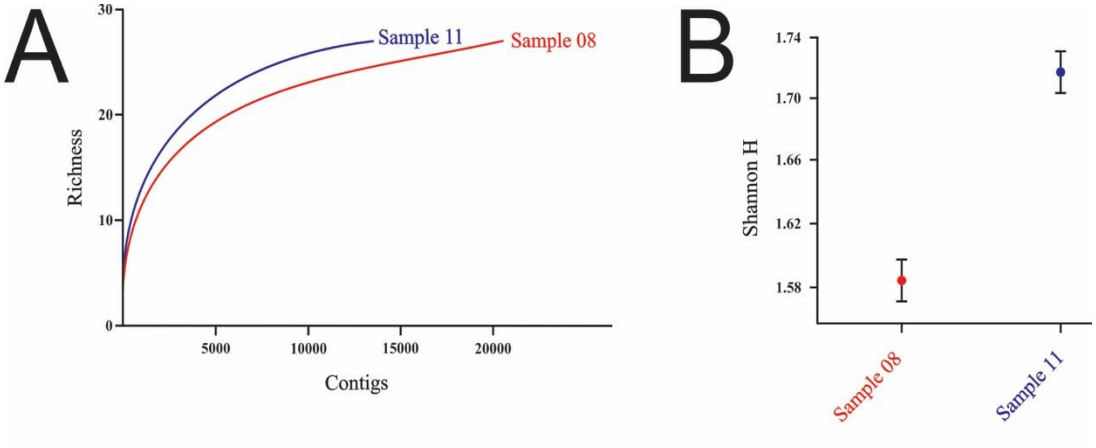


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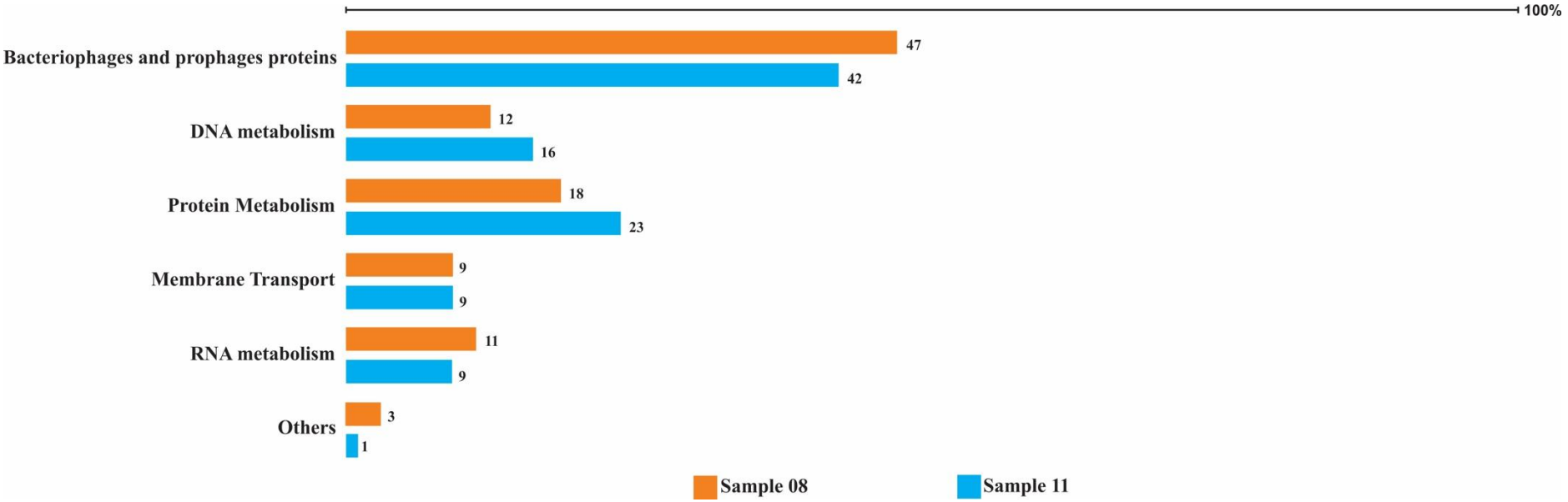
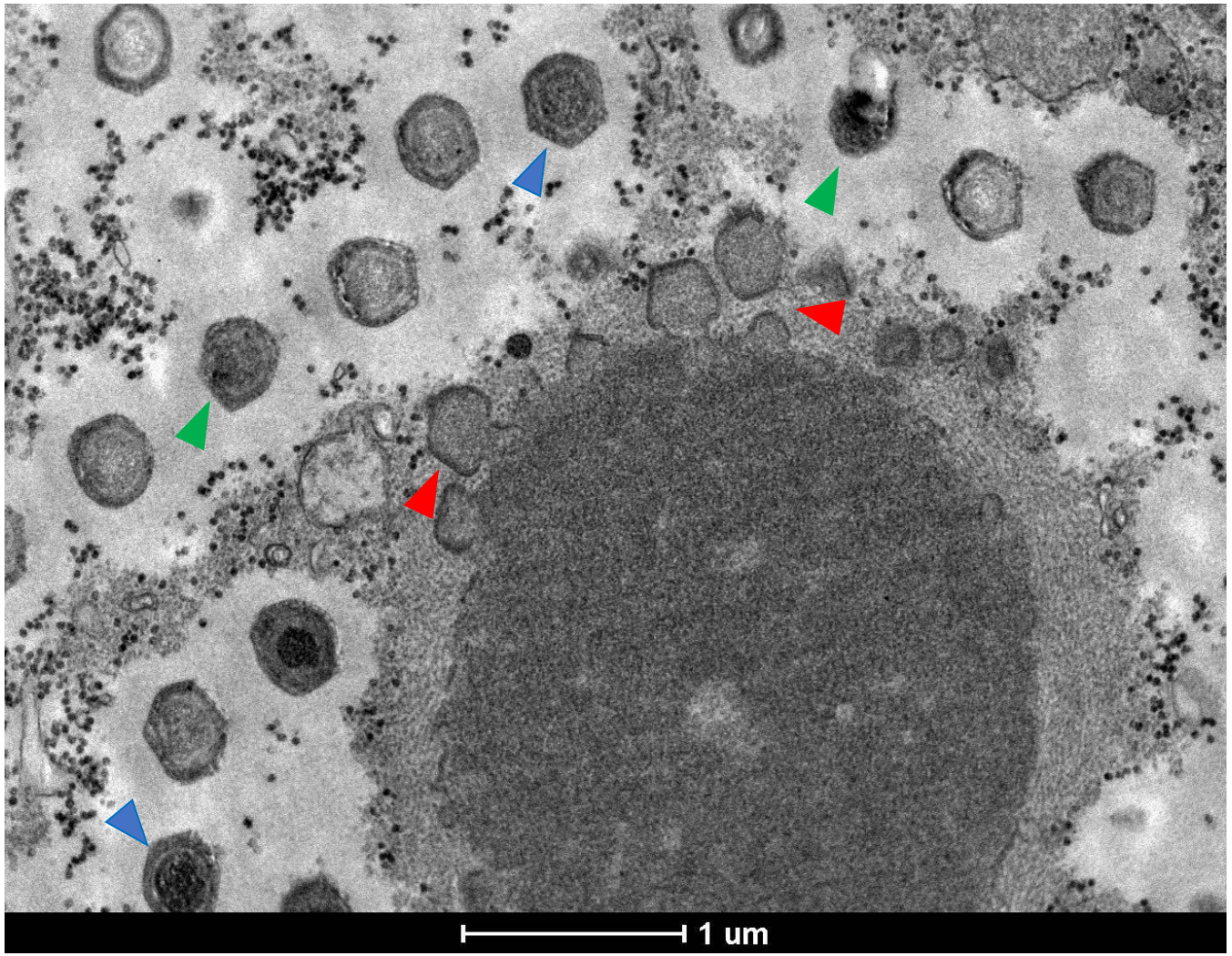
FIGURE 03


100%
Bacteriophages and prophages proteins
47
42
DNA metabolism
12
16
Protein Metabolism
18
23
Membrane Transport
9
9
RNA metabolism
11
9
Others
3
1
Sample 08
Sample 11

894 **FIGURE 04**

895



CHAPTER 3

EXPLORING THE VIRAL DIVERSITY OF SINGLE-STRANDED (SS) DNA VIRUSES IN DAIRY CATTLE RUMEN

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EXPLORING THE VIRAL DIVERSITY OF SINGLE-STRANDED (SS) DNA VIRUSES IN DAIRY CATTLE RUMEN

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Summary

Ruminant animals stand out for their ability to efficiently convert food into milk, meat and derivatives. This efficient use of ingested energy is directly linked to the symbiotic relationship of the microorganisms present in the rumen of these animals. Microorganisms are essential because they can produce enzymes to degrade the the fiber ingested by the animal. It is well known that rumen is not only important for digestion, but it also plays a central role in ruminant growth, high production performance and health in general. The studying and promoting strategies that optimize the functioning of the rumen has been the objective of researches. There are several studies about rumen microbioma. Recently, interest in rumen virome (groups of viruses in the rumen) has been increasing due to the important ecological role of viruses in different environments such as soil and seawater. Metagenomic approach associated with the advent of high throughput sequencing of animal faecal matter have been disseminated as a method to investigate the virome associated with gut of human and animal species. This approach has proven to be efficient in identifying new as well as previously described viruses from various animals. Among these newly identified viruses are single-stranded(ss) DNA viruses. Viruses with a ssDNA genome are found associated with all organisms. Here, we used a metagenomic approach associated with the enrichement for circular viral DNA using rolling circle amplification (RCA) to recover ssDNA genomes from ruminal fluid collected from dairy cattles. Analysis of BLASTn and BLASTx against the sequences of ssDNA viruses deposited in NCBI revealed genomes of *Anelloviridae*, *Inoviridae*, *Microviridae, Parvoviridae* and *Pleolipoviridae* families in the both samples. Diversity analyses, found that the most diverse viral ssDNA viruses family in the rumen environment was *Parvoviridae*. Clonning and sequencing of partial fragments showed sequences of capsid and replication initiator proteins related to *Microviridae* family. Before the advances in modern sequencing technology, the real diversity of ssDNA viruses was unknown. It seems that these ssDNA viruses are common and ubiquitous in nature. Thus, studies are needed to evaluate the impact and the relationship of these viruses with their hosts.

Introduction

Ruminant animals stand out for their ability to efficiently convert food into milk, meat and derivatives. This efficient use of ingested energy is directly linked to the symbiotic relationship of the microorganisms present in the rumen of these animals. Microorganisms are essential because they can produce enzymes to degrade the fiber ingested by the animal, unlike mammalian animals that are not able to produce these enzymes (Gruninger et al., 2019).

It is well known that rumen is not only important for digestion, but also plays a central role in ruminant growth, high production performance and health. Thus, the studying and promoting of strategies that optimize the functioning of the rumen has been the objective of several researches (Diao et al., 2019).

There are several studies about rumen microbioma, mostly focused on bacteria. Recently, interest in rumen viroma (studies on the groups of viruses in the rumen) has been increasing due to the important ecological role of viruses in different environments such as soil and seawater (Suttle, 2007). Studies using the metagenomic approach associated with the advent of high throughput sequencing of animal faecal matter have been disseminated as a method to investigate the virome associated with various animal species. This approach has proven to be efficient in identifying new and previously described viruses from various animals (Graham et al., 2019). Among these newly identified viruses are single-stranded(ss) DNA viruses, that are found associated with all types of organisms.

Nowadays, ssDNA viruses are classified into thirteen families, of which eleven contain circular genome and two families, *Parvoviridae* and *Bidnaviridae* have a linear single stranded DNA genome. It is believed that seven of these families infect eukaryotic hosts: *Anelloviridae*, *Bacilladnaviridae*, *Circoviridae*, *Geminiviridae*, *Genomoviridae*, *Nanoviridae* and *Smacoviridae*. ssDNa viruses that infecting bacteria belong to the families *Microviridae* and *Inoviridae*. *Pleolipoviridae* and *Spiraviridae* viruses infect archaea (ICTV, http://ictv.global/report).

Most of ssDNA viruses have a genome of circular DNA genome encoding a replication-associated protein (Rep). Because of this protein they are also called CRESS DNA (circular Rep-encoding ssDNA viruses). In addition to the Rep protein, the genome of these viruses also encodes a capsid protein (CP). Furthermore, it is suggested that these viruses have a common ancestor because they all use the rolling circle replication

mechanism. It is noteworthy that viruses of the *Parvoviridae* family a little in the initiation mechanism of replication compared to others ssDNA viruses. Parvoviruses replicate your genome through a hairpin mechanism initiated by a CRESS-DNA virus-like Rep protein (Malathi and Renuka Devi, 2019).

With the aim to identify the diversity of circular DNA viruses associated with ruminal fluid we use two associated methodologies. First we explored the general diversity using an metagenomics approach from total DNA extracted from this environment. In addition, we used rolling circle amplification (RCA) on two samples in an attempt to enrich and retrieve sequences derived from ssDNA viruses. The RCA obtained was digested by restriction enzymes and fragments were cloned> These fragments were sequenced and correspond to sequences similar to capsid and replication initiator proteins characteristics of M*icroviridae* family. Back to back primers were designed based on the sequences of the recovered clones aiming to recover the complete genome of these *microvirus*.

Analysis of BLASTx against the sequences of ssDNA viruses deposited in in NCBI database revealed sequences of genomes of *Inoviridae*, *Microviridae* and *Pleolipoviridae* families in both samples. In addition to those previously mentioned, in sample 08 we found sequences of genomes of *Anelloviridae* and *Parvoviridae* families. Before the advances in modern sequencing technology, the real diversity of ssDNA viruses was unknown. It seems that these ssDNA viruses are common and ubiquitous in nature. Thus, studies are needed to evaluate the impact and the relationship of these viruses with their hosts.

Materials and Methods

Ruminal fluid collection

Ruminal fluid (RL) was collected from two fistulated dairy cattle at the Unidade de Ensino, Pesquisa e Extensão em Gado de Leite da Universidade Federal de Viçosa, Brazil . The liquid fraction of the rumen were collected of the two animals on the same day, according to the availability of the animals on the day of collection.

Purification and Viral Concentration of Particles in Liquid Fraction

Immediately after collection, solid fractions were stored at -20° and liquid fractions heated at 75°C for 20 minutes to eliminate possibly nuclease activity present in

the rumen fluid. The protocol for purification and the concentration of viral particles present in the liquid fraction was adapted from the method described by (Klieve and Swain, 1993). After heating, samples were centrifuged twice at 20,000xg for 30 minutes at 4°C and the supernatant was filtered on sterile 0.45 µm pore size membranes (Millipore) to remove intact bacteria and other large cellular debris.

After filtration the viral particles were concentrated by ultracentrifugation at 51,000xg for 2 hours at 4°C. The supernatant was discarded, and the precipitate resuspended in 1ml SM buffer (50mM Tris-HCl, pH 7.5, 100mM NaCl, 10mM $MgSO_4$ and 0.01% gelatin) and incubated overnight at 4°C. After incubation, each tube was filled in to 25 ml with TE and the sample was once again ultracentrifuged using the same parameters described above. The viral precipitate was resuspended in 500 µl PBS buffer (0.13 M NaCl, 2mM KCl, 9mM Na_2HPO_4 and 1mM KH_2PO_4). At the end of the purification procedure the sample was filtered on sterile 0.22 µm pore size membranes (Millipore). The purified viruses were stored in sterile tubes at 4°C.

Nucleid Acid Extraction, **amplification, cloning and sequencing**

The viral purification was treated with 10U RQ1 RNase-Free Dnase (Promega) and with RNAse (Sigma-Aldrich) at final concentration 10µg/ml. The misture was incubated at 37°C for 90 minutes to remove unprotect nucleic acids. Total acid nucleic extraction from the samples was performed by the Proteinase K method, as described in Sambrook and Russell (2001). Quality analysis of extracted nucleic material was performed by agarose gel electrophoresis (0.8%) and concentration was measured using NanoDrop 2000 spectrophotometers (Thermo Scientific). The extracted viral DNA was enriched for circular viral DNA using rolling circle amplification (RCA) using phi29 DNA Polymerase (New England Biolabs) at 30°C for 20 hours. The RCA products products were digested with four restriction enzymes (EcoRI, Hind III, PstI and XhoI). The digestion products were cloned into pBluescript (pKS+) vector and transformed into chemically competent *Escherichia coli* (DH5α) cells through electroporation. Bacteria were plated on LB agar plates containing ampicillin, X-Gal and IPTG. The clones were sequenced by the sanger method using the M13F and M13R universal primers and primer walking methods. Back-to-back primers method was performed to amplify the whole inserts sequences using the partial sequences obtained by sequencing as a reference for primer design.

The sequence of back-to-back primers using in this article was: MicroV01F: 5'-TTT GGA TCC AGG TTG AAT GGT GAT ATG-3', MicroV01R: 5'-TTT GGA TCC ATT CAA CCT GGG TG-3', MicroV06F: 5'-TTT GGA TCC GCC ACA ATT AAT GAA CTG C-3', MicroV06R: 5'-TTT GGA TCC GGC GGC TGT GAA AT-3', MicroV07F: 5'-TTT GGA TCC GTA TCG CCG CTT ATC TC-3' and MicroV07R: 5'-TTT GGA TCC GGC GAT ACT TAC GGC-3'.

Bioinformatics Analysis

The sequencing of virus-like particles total nucleic acids extracted from the rumen fluid was performed using the Illumina platform HiSeq2000 system. Data generated by sequencing was computationally analyzed for contig assembly using Trinity program (Grabherr et al., 2011). We performed BLASTx analysis to comparing the generated contigs with the sequences of ssDNA viruses deposited in NCBI database with an e-value cutoff of $\leq 10^{-5}$ according with Wang et al., (2018). The sequencing was performed by Macrogen Inc. (Seoul, Korea).

General diversity analysis of ssDNA families in the rumen environment.

In addition, we performed general diversity analyzes in the rumen environment of the families found in this study. We first ranked the viral species found as best hits in Blastx analysis and ranked this virus at the genus level according to the NCBI database. Viruses that were deposited only up to the taxonomic family level were categorized as unclassified within their respective family. Once categorized, the members of the same family were pooled and classified according the genus within each family for further diversity analysis. Diversity analyzes were performed using the Shannon H index in the Past (PAleontological Statistics Version 3.25)program (https://folk.uio.no/ohammer/past/).

Results and Discussion

Overview of ssDNA viral sequences in ruminal fluids

The two ruminal fluids (samples 08 and 11) from dairy cattles generated unique sequences reads using the Iluminna HiSeq 2000 run with 100 bases paired ends. Sequence reads were de novo assembled using the Trinity program (Grabherr et al., 2011) and compared to the NCBI nucleotide and protein databases using Blastx analysis. Results indicated both samples have sequences of viruses from the *Inoviridae* and *Microviridae* (viruses known to infect bacteria) and *Pleolipoviridae* (infects archaea). In addition to these viral families, in sample 08 we also identified *Anelloviridae* (infects vertebrates) and *Parvoviridae* that infects vertebrates and insects. Sequences of *Anelloviridae* and*Parvoviridae* families were not recovered in sample 11 as shown in figure 1. Viruses classified in these two families have as host human and non-human vertebrates. The viral species from the different ssDNA viruses' families found as best hits by BLASTx analysis are listed in table 1.

To access the general diversity of ssDNA viruses in the rumen environment, we performed viral family diversity analyzes in this environment. The best hits from each family have been ranked at the genus level through their taxonomy described in the NCBI database. Viruses that were classified in the database only up to family level were categorized as "unclassified" within their respective family. The total genera within each sample family 08 and 11 were summed into one pool. using the SHANNON H index through the Past program. Interestingly, among ssDNA families found in this study, the *Parvoviridae* family was the most diverse in the rumen environment, followed by the *Microviridae* and *Inoviridae* families. Finally, the *Pleolipoviridae* family was the least diverse. As only one individual was found for the *Anelloviridae* family, this was excluded from this diversity analysis.

We carry out enrichment of genomes made up of ssDNA using rolling circle amplification (RCA). The RCA result was then digested with restriction enzymes and cloned into pKSII+ vector. Viral sequence of clones was then sequenced in Macrogen (Korea). Two clones with 1346 bp (clone 1) and 1111 bp (clone 2) were recovered. BLASTx analyzes of clone's sequences show that clone 1 has putative conserved domains of F protein, a family of proteins from single-stranded DNA viruses. The sequence of this clone has 84% of query cover, 50% of identity and e-value of 4e-115 with the major capsid protein of *Tortoise microvirus 3*. The second clone also has a possible conserved

domain between the ssDNA viruses, the putative replication initiation protein with 55% of query cover, 43.24% of identity and e-value of 5e-32 with *Capybara microvirus* Cap3_SP_581, a diverse ssDNA viruses associated with Capybara (Hydrochoerus hydrochaeris) in Brazil (Unpublished). Due to these strong evidences of the presence of *Microviridae* viruses in the rumen environment, back to back primers were constructed to amplify the total genome of these ssDNA viruses in the rumen environment.

The discovery of CRESS DNA viruses has increased drastically in recent years due to the broad application of metagenomics approaches. However, the majority of these viruses' genomes remain uncultivated in laboratories and unclassified due to their high diversity and lack of host information (Fontenele et al., 2019).

In prokaryotic organism a strategy for assigning a virus to some host is Clustered Interspaced Short Palindromic Repeats (CRISPR-Cas systems) a widespread prokaryotic immune system against foreigner nucleic acid sequences (Díez-Villaseñor and Rodriguez-Valera, 2019). The spacers sequences inside CRISPR locus make it possible to connect virus and host pairs in prokaryotes in a highly reliable way when there is an identical match between a spacer and a protospacer (Jansen et al., 2002).

In contrast to the nuclear genomes of animals, many DNA virus genomes have circular topology, which allows selective enrichment through rolling circle amplification (RCA) methods. We then used RCA to enrich circular genomes and we recovered sequences of viruses belonging to *Microviridae* family.

Microvirus are viruses that infect bacteria and are divided into two subfamilies, *Gokushovirinae* and *Bullavirinae* (International committee for virus taxonomy: 2018 release; https://talk.ictvonline.org/taxonomy/). Viruses into subfamily *Bullavirinae* infect enterobacterias and viruses into *Gokushovirinae* consists of viruses infecting obligate parasitic bacteria (ICTV, 2018).

Microviruses have been reported in various fecal and gut microbiome studies of various organisms such as bees, capybaras, arthropods and protocordates (Creasy et al., 2018; Fontenele et al., 2019; Kraberger et al., 2019a, 2019b). It was reported the presence of microvirus sequences integrated in genomes of species of Bacteroidetes, a phylum which until then was not associated with microviruses. The integrated sequences were highly similars to sequences in human gut microbiome. This study was the first report indicating that viruses of the *Microviridae* can lysogenize bacteria (Krupovic and Forterre, 2011). Viruses can have several roles in a complex ecosystem like the gut and

283 many factors influence the phage function beyond abundance and diversity of phages and
284 hosts (Yatsunenko et al., 2012).

285 In this work, we accessed a diversity od ssDNA viruses in rumen. Mostly viruses
286 are found that have bacteria as possible hosts, according to the information that the rumen
287 microbiome is composed in its mastery of anaerobic bacteria. Future efforts are needed
288 to recover the total microvirus genomes from this environment.

289 Despite the large number of studies reporting the large presence of ssDNA viruses in
290 various environmental samples and associated with various organisms, the real
291 ecological role of these viruses remains unknown and its necessary to investigate
292 mechanisms of phage infection in rumen and gut virome of dairy cattles, such as
293 *Microviridae*.

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734

735 **Table 01: Owerview of ssDNA viruses on two ruminal liquid and the best hits accessed by Blastx analyses.**

	VIRAL FAMILIES				
Sample 08	**Anelloviridae**	**Inoviridae**	**Microviridae**	**Pleolipoviridae**	**Parvoviridae**
Best Hits	Torque teno douroucouli virus	Enterobacteria phage Ralstonia phage RSM1 Ralstonia phage RSM1 Ralstonia phage RSM3 Ralstonia phage 1 NP-2014 Ralstonia phage 12 J Ralstonia phage RSS0 Ralstonia phage RSS20 Ralstonia phage RSS30 Spiroplasma phage Vibrio phage Uncultured phage WWnAnB	Microviridae phi-CA82 Chimpanzee faeces associated microphage Gokushovirinae Bog Microviridae Fen Cellulophaga phage phi12:2 Marine gokushovirus Parabacteroides phage Chlamydia virus Chp1	His2 virus Halogeometricum pleomorphic virus 1	Fenneropenaeus chinensis Parvoviridae Rat bocavirus Porcine parvovirus Danaus plexippus plexippus iteravirus Bufavirus 3 Sea otter parvovirus 1 Acheta domestica mini ambidensovirus Primate Parvoviridae 1
Sample 11					
Best Hits	Not found	Ralstonia phage RSM1 Ralstonia phage RSM3 Ralstonia phage 1 NP-2014 Enterobacteria phage If1 Spiroplasma virus SkV1CR23x Spiroplasma phage 1-C74 Spiroplasma phage SVTS2 Spiroplasma phage 1-R8A2B Vibrio virus CTXphi Vibrio phage VFJ Stenotrophomonas phage SMA9	Chimpanzee faeces associated microphage Cellulophaga phage phi12a:1 Cellulophaga phage phi12:2 Gokushovirinae Fen Parabacteroides phage YZ Gokushovirinae Bog Chlamydia phage 2	Halogeometricum pleomorphic virus 1	Not found

736

737 **Figure Legends**

738

739 **Figure 01**

740 Diversity of ssDNA viruses in two rumen fluid samples accessed by Blastx analysis. The
741 percentage of viral families is indicated by the size of the bars. The colors representing
742 the respective families are indicated at the bottom of the table.

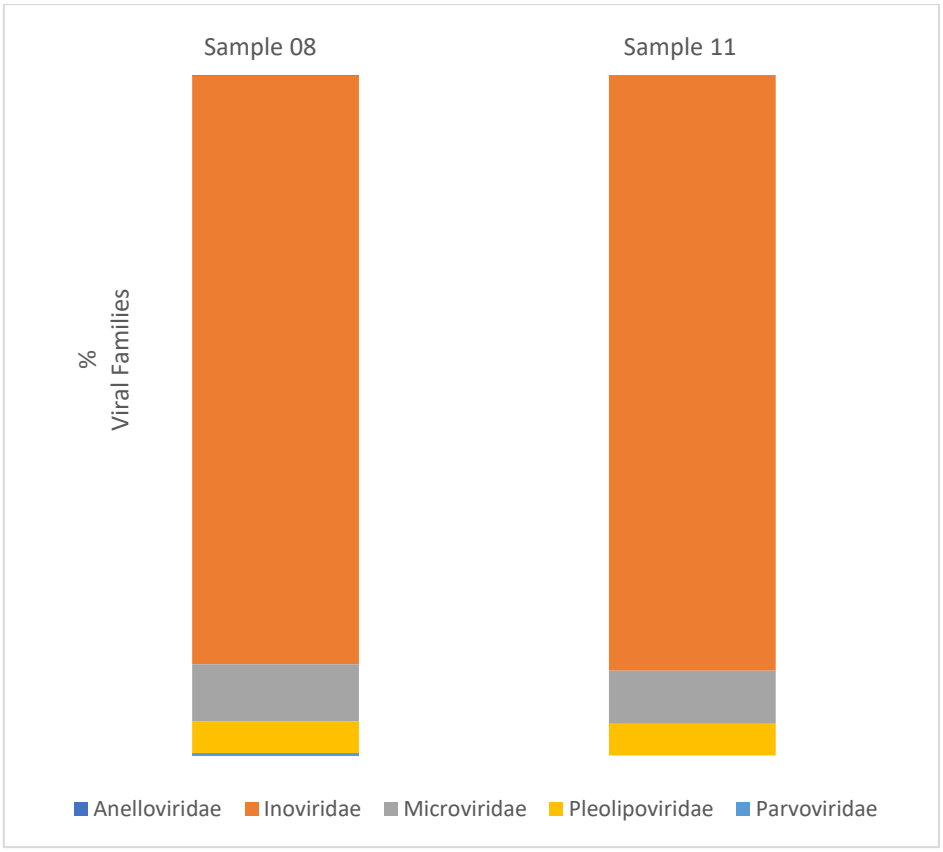
743

744 **Figure 02**

745 Analysis of total diversity of ssDNA families in the rumen environment. The sum of all
746 families presents in both samples (08 and 11) were used to analyze the diversity of ssDNA
747 families found in this study. The x axis indicates the viral family and y axis indicates the
748 diversity index used. The analysis was performed in the PAst program.

749 **FIGURE 01**

750



751

752

753 **FIGURE 02**

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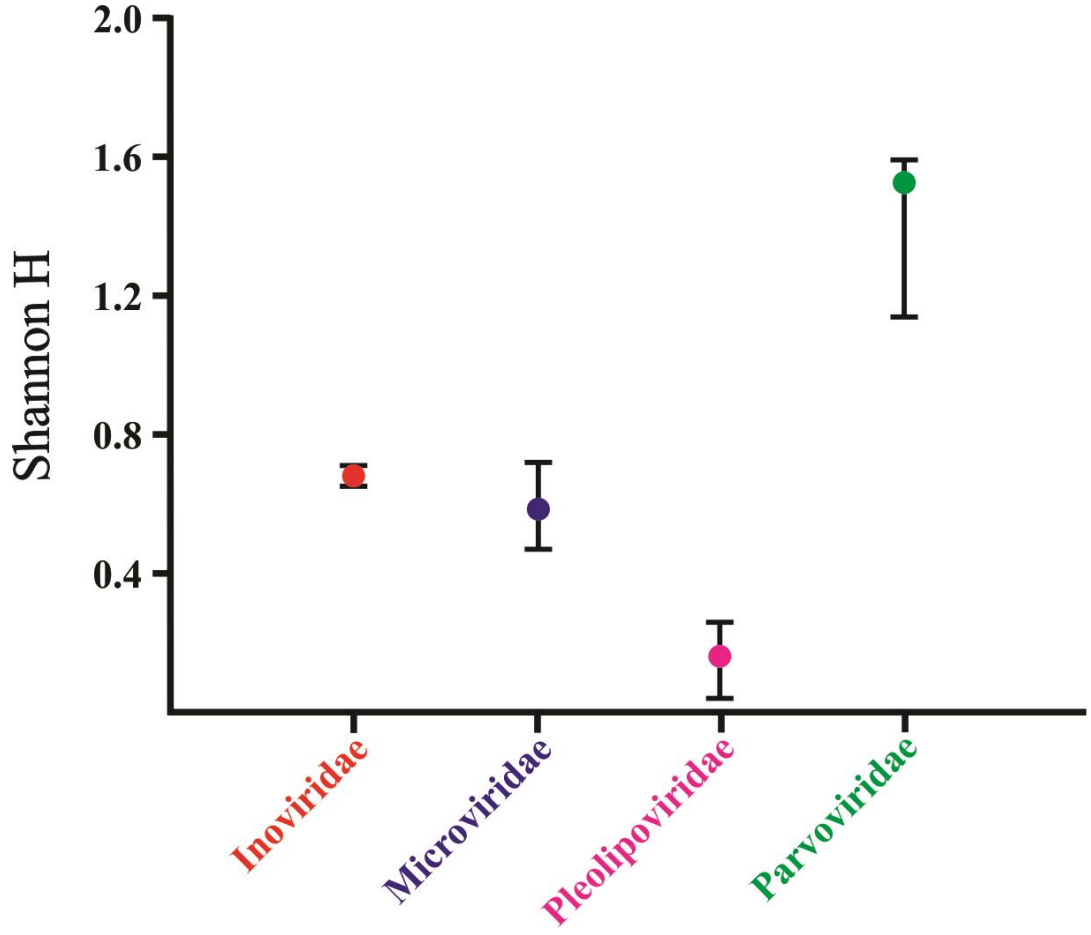

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CONCLUSION

The fact that the total amount of viruses present in a marine environment is greater than the total abundance of prokaryotic organisms, and that a large amount of these prokaryotes may be infected with bacteriophages has spurred interest in further research into ecology. viruses and what role they play in different ecosystems. Most of the data on viral ecology is from studies in aquatic environments and no doubt the viruses that have the genetic material formed by double stranded DNA (dsDNA) are the most commonly found. A consequence of host lysis due to viral infection is the efficient release of carbon from bacterial biomass that becomes available and dissolved organic matter. In addition, viral lysis may also contribute to the release of myronutrients, especially Fe. Importantly, prophages (viruses that remain integrated into the host genome) may also contribute to the host that acquires important bacteria, such as virulence factors or resistance to antibiotics or heavy metals, for example.

In this work we evaluated the presence of prophages in *Rasltonia* sp. species complex (RSC) genomes available in National Center for Biotechonology Information (NCBI) database. We found prophages belonging to the known viral families *Myoviridae*, *Siphoviridae* and *Inoviridae*. In addition, we were also able to identify fourteen different putative novel phages. The genomes of these new viruses have low homology with the genomes present in NCBI. Members of the *Inoviridae* family have been shown to be the most abundant prophages. CRISPR-Cas target analysis demonstrated the presence of phage derived spacer sequences integrated in RSC genomes.

We also accessed the viroma of two dairy cows from the Federal University of Viçosa and were able to isolate the first mimivirus-like virus from these rumen samples. In addition, we evaluated the diversity of single strand (ss) DNA viruses in the rumen environment. Thus, this work highlights the wide distribution of viral sequences present in bacterial genomes available in current databases. In addition, in the rumen environment we found a great diversity of viruses in this environment, especially ssDNA viruses and were able to isolate a virus mimivirus-like.