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Research Article

RESEARCH ARTICLE

Virome-associated antibiotic-resistance genes in an experimental aquaculture facility

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One sentence summary: Aquaculture-associated ARGs are mobilized, together with other bacterial genes, from bacteria to phages or vice versa even in the absence of antibiotic treatment.

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ABSTRACT

We report the comprehensive characterization of viral and microbial communities within an aquaculture wastewater sample, by a shotgun sequencing and 16S rRNA gene profiling metagenomic approach. *Caudovirales* had the largest representation within the sample, with over 50% of the total taxonomic abundance, whereas approximately 30% of the total open reading frames (ORFs) identified were from eukaryotic viruses (*Mimiviridae* and *Phycodnaviridae*). Antibiotic resistance genes (ARGs) within the virome accounted for 0.85% of the total viral ORFs and showed a similar distribution both in virome and in microbiome. Among the ARGs, those encoding proteins involved in the modulation of antibiotic efflux pumps were the most abundant. Interestingly, the taxonomy of the bacterial ORFs identified in the viral metagenome did not reflect the microbial taxonomy as deduced by 16S rRNA gene profiling and shotgun metagenomic analysis. A limited number of ARGs appeared to be mobilized from bacteria to phages or *vice versa*, together with other bacterial genes encoding products involved in general metabolic functions, even in the absence of any antibiotic treatment within the aquaculture plant. Thus, these results confirm the presence of a complex phage–bacterial network in the aquaculture environment.

Keywords: virome; microbiome; aquaculture; antibiotic resistance genes; horizontal gene transfer

INTRODUCTION

According to recently released data (FAO - Fisheries and Aquaculture Department), world aquacultural production of fish for consumption has exceeded 60 million tons in 2011, with an increase of 6.2% over the previous year (FAO Fisheries and Aquaculture Department 2013). This large growth has been accompanied by an increased usage of a wide range of antibiotics (Armstrong, Hargrave and Haya 2005). In aquaculture, the main use for antibiotics is the prevention and treatment of bacterial infections in fish; the prophylactic use of antibiotics is a common practice as well. The necessity of antibiotic use in aquaculture is a consequence of lowered host defenses associated with high-density breeding under suboptimal hygienic conditions (Grave *et al.* 1999; Defoirdt *et al.* 2007; Cabello *et al.* 2013). Horizontal gene transfer among bacteria occurs by one of the three following mechanisms: conjugation, free DNA transformation or transduction through bacteriophages. Despite the potential importance of bacteriophages in transferring resistance genes

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from the environment to human and animal microbiomes, studies on this topic are limited (Modi *et al.* 2013). With respect to the virome associated with freshwater environments, studies only describe the taxonomic diversity and composition (López-Bueno *et al.* 2009; Rosario *et al.* 2009; Roux *et al.* 2011; Fancello *et al.* 2012; Tseng *et al.* 2013) or the viral and microbial community dynamics (Rodriguez-Brito *et al.* 2010).

In this work, we report a comprehensive characterization of viral and microbial communities in an experimental aquaculture sample using a metagenomics approach. The contemporary study of both communities resulted in the identification of different genes that are mobilized in the virome, with particular attention paid to antibiotic resistance genes (ARGs).

VIRAL TAXONOMIC CHARACTERIZATION

A water sample (20 L) was collected at the Edmund Mach Foundation – Technology Transfer Center (San Michele all'Adige, TN, Italy). The sample was collected from the wastewater of a fish breeding tank. Groundwater was initially pumped into a degasser tower for the removal of excess carbon dioxide prior to its use in the fish tanks. Tanks contained approximately 7 kg m^{-3} of salmonid (the fish density in a commercial aquaculture is close to 25 kg m^{-3}) with a water flow of approximately 83 m^3 h^{-1}. Antibiotic treatments had not been applied. The tank was used for the breeding of salmonids such as *Salmo carpio*, *Salmo trutta marmoratus* and *Salvelinus alpinus*. Water was treated to isolate virus-like particles (VLPs), from which DNA was extracted. The complete procedure is described in the Supplementary Materials and methods section. Metagenomic shotgun sequencing of viral DNA was determined using an Ion Torrent PGM platform (Life Technologies, Carlsbad, CA, USA). The MIRA program was used for *de novo* assembly of contigs, and ORFs were predicted by PRODIGAL (Supplementary Materials and methods).

According to several other studies (Fancello *et al.* 2012; Roux *et al.* 2012; Zablocki *et al.* 2014), the majority of the identified ORFs (accession number SAMEA3334740) originated from bacteria (86%) whereas those from viruses were the second most represented (13%). Thirteen families were identified (Supplementary Table S1), and prokaryotic viruses were the most abundant in the sample. *Caudovirales* accounted for more than 50% of the total taxonomic abundance. *Myoviridae* and *Siphoviridae* were the most represented families with 28% and 18% of the total ORFs, respectively. Analysis revealed the presence of viruses whose hosts include amoebae and algae; *Mimiviridae* and *Phycodnaviridae* were the main representatives of this group, at 13–15% of the total. Furthermore, the largest variety of phages identified interacts with *Proteobacteria*, which is consistent with the abundance of this phylum in the microbiome (Supplementary Fig. S1). The two main families of *Proteobacteria* represented in the water sample, *Sphingomonadaceae* and *Comamonadaceae*, and their related genera are typical constituents of freshwater environments, because bacterioplankton are opportunistic pathogens (Kallman *et al.* 2006; Kilic *et al.* 2007; Lin *et al.* 2010; Vaz-Moreira, Nunes and Manaia 2011; Chen *et al.* 2013). However, the most abundant ORFs identified in the virome were best matched with phages that infect *Bacillus*, *Synechococcus* and *Mycobacterium*, bacterial genera that have not been identified in the microbiome, thus suggesting that these genera were probably under-represented in the water sample because of a phage infection that resulted in a lytic cycle. In this context, the sharp reduction or local extinction of microbial taxa by viruses is a phenomenon that would be expected according to the 'Kill-the-Winner' dynamics hypothesis (Thingstad 2000). This dynamic model postulates a repetitive cycle in which an increase in prey population leads to an increase in the predator population that in turn decreases the prey population, thus causing its own subsequent decline.

DISTRIBUTION AND TAXONOMY OF ARGS IN THE VIROME AND MICROBIOME

Microbial cells were isolated to extract their DNA (Supplementary Materials and methods). 16S rRNA gene profiling (accession number SAMEA3333506) and metagenomics shotgun analysis (accession number SAMEA3334741) were performed on this sample. The ORFs obtained from shotgun metagenomic sequencing were analysed using the CARD database. The total number of ARGs identified was 950 (4.13% of the total ORFs identified with NCBI) in the microbiome and 214 (2.64%) in the virome. The distribution of the antibiotic resistance gene (ARG) identified in the two metagenomes (Supplementary Fig. S2) showed that the most abundant genes were those encoding proteins involved in the modulation of antibiotic efflux pumps (CARD nomenclature), with values ranging from 16 to 19% of the total identified ARGs. Among the different ARG classes, antibiotic efflux pumps had the most general function, acting on different target molecules and having a cell detoxifying activity (Pao, Paulsen and Saier 1998). Macrolide-resistance genes were the second most abundant group and included erythromycin, telithromycin and clarithromycin resistance genes. With few exceptions, the distribution of ARGs was similar between microbial and viral metagenomes. Glycopeptide efflux pump and lincosamide ARGs had higher values in the virome than in the microbiome (Supplementary Fig. S2), whereas gene modulating antibiotic efflux pumps and fluoroquinolone resistance genes were the most abundant in the microbiome. For each ARG identified, the taxonomy of the respective ORF was assigned based on the results obtained from the BLAST comparison with the NCBI refseq database. The taxonomy of the ARGs in the microbiome (Fig. 1) showed the presence of three main families (*Comamonadaceae* 26–37%, *Sphingomonadaceae* 18–24% and *Sinobacteraceae* 14–21%) equally distributed among the different drug classes, and a large number (15–26%) of the ARGs were shared among different microbial families, accounting for an amount lower than 4%. The virome analysis revealed that ARGs within this metagenome were mainly distributed between *Flavobacteriaceae* (38–53%) and *Methylophilaceae* (19–33%) families and that other bacterial families (7–13%) had a relative abundance below the 5% threshold (Fig. 1). None of the families present in both the virome and the microbiome, accounted for a relative abundance higher than 5%. From a taxonomic point of view, it is interesting to note that the microbial ORFs identified in the virome did not reflect the microbiome taxonomy, as determined by 16S rRNA gene profiling (Supplementary Fig. S3) or shotgun metagenomic analysis (Fig. 1), suggesting that microbial genes mobilized in the genome of viruses could be considered to be remnants of past recombination events rather than a picture of the current microbial diversity.

THE VIROME AND MICROBIOME SHARE ARGS

In order to identify possible gene mobilization events, ORFs located both in the microbiome and in the virome were identified (Supplementary Materials and methods). A total of 213 different ORFs were shared between the two metagenomes, and

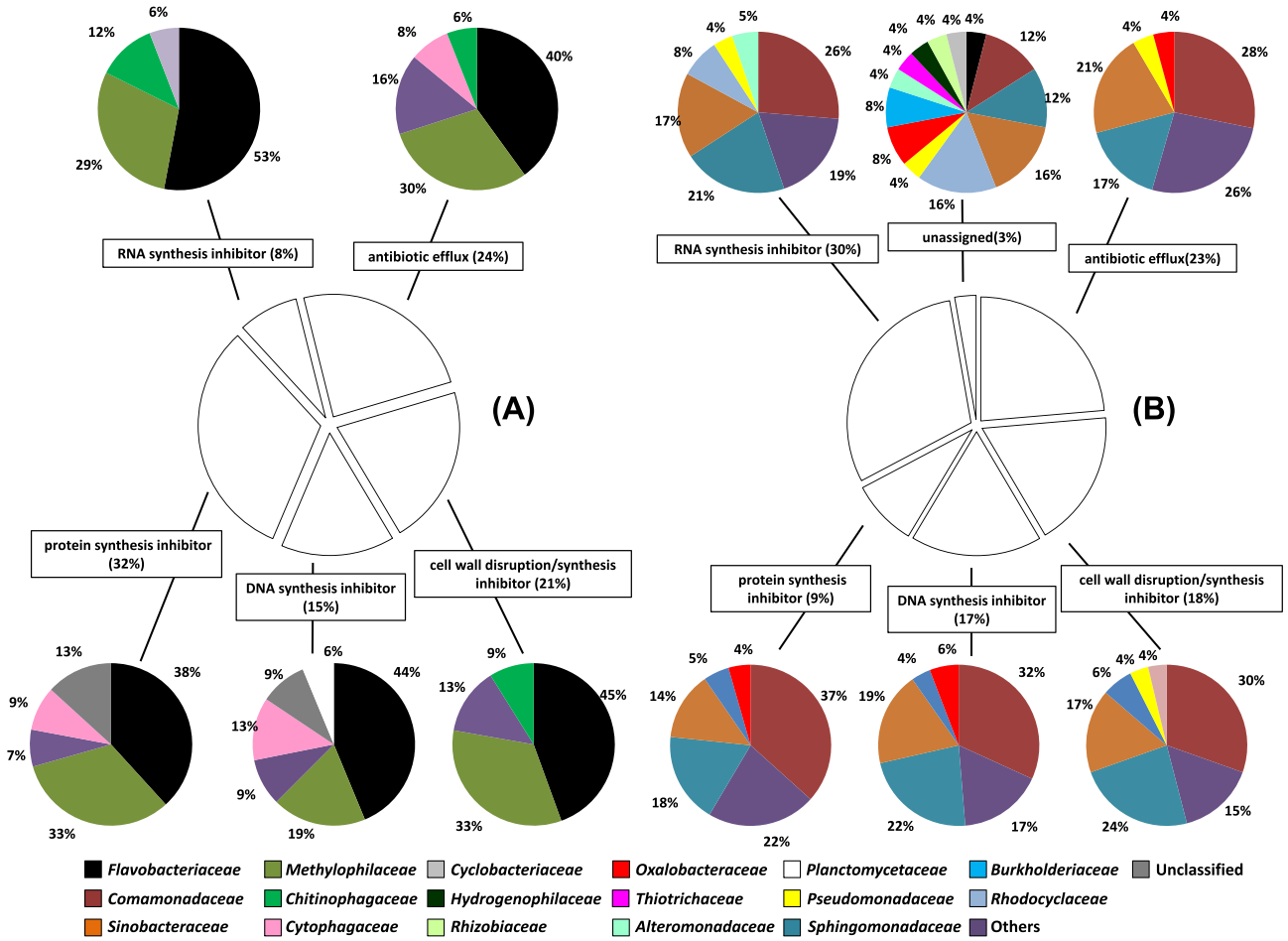

Figure 1. Comparison of the antibiotic resistance-related ORF distribution among viral (**A**) and microbial (**B**) metagenomes. ARGs have been divided into six different drug classes: RNA synthesis inhibitors, protein synthesis inhibitors, DNA synthesis inhibitors, cell wall disruption and synthesis inhibitors, antibiotic efflux modulators, and unassigned. Relative abundance (%) of each drug class was calculate with respect to the total of the classes. The taxonomic distribution at family level has been assigned to each drug class: data were expressed as relative abundance (%) with respect to the single drug class.

most of them were related to microbial metabolism. With respect to the ARGs identified in the virome, 4% were shared with the microbiome and had an amino acid sequence identity higher than 95%. Half of the identified ORFs encode genes modulating the antibiotic efflux (i.e. transcriptional regulator); ORFs that encode glycopeptide, tetracycline and β-lactam resistance genes were also identified. Among these genes, one was classified as a *Sphingopyxis alaskensis* β-lactamase encoding gene, which was putatively mobilized together with four other genes including a pseudogene coding for a β-lactamase of *Sphingomonas* sp. (Table 1). The other five genes shared between the virome and the microbiome are putatively involved in tetracycline and glycopeptide resistance and antibiotic efflux mechanisms. In all cases, shared ARGs were not transferred alone but with other genes hosted by the same contig (Table 1). Our data indicate that ARGs are present in the virome and some of these ARGs are mobilized from bacteria to phages or *vice versa*. These data suggest that ARGs may be mobilized even in the absence of selective pressure, i.e. in the absence of antibiotic treatment in aquaculture. Moreover, we showed that ARGs were mobilized together with other bacterial genes encoding products involved in more general metabolic functions, thus confirming the presence of a complex phage–bacterial network in the aquaculture environment.

ARGS PRESENT IN PUBLICLY AVAILABLE VIROMES

In order to compare our data with publicly available data, we calculated the relative amount of ARGs in the 17 viromes tested. Our sample showed a relative ARG abundance of 0.85%, the second highest value after that of El Barbera lake (Fancello *et al.* 2012). Interestingly, the relative amount of ARGs did not correlate with the presence of anthropic activities geographically located near the tested aquatic environments, because the highest ARG abundance was found in the El Barbera sample, which was collected from the Mauritanian desert. The presence of ARGs in an uncontaminated wild environment has been reported previously. Several studies have identified ARGs in different ecosystems, including soil and permafrost (D'Costa *et al.* 2006; Hallen *et al.* 2010), the human gut (Hu *et al.* 2013), and the microbiome of members of an isolated Yanomami Amerindian village (Clemente *et al.* 2015). Therefore, the diversity, taxonomic distribution and ecological role of antibiotic resistant genes in the environment are still far from being fully understood. Moreover, it is worth noting that the presence of ARGs in metagenomes does not directly represent a risk for human health, and a proper analysis of such risks should be carried out (Martinez, Coque and Baquero 2015).

Table 1. ARGs shared between viral and microbial metagenomes.

ARGs shared between viral and microbial metagenome	Coding function[a]	Identity[b] (%)	Mobilized ORFs hosted in the same contig	contig no.	
				Virome	Microbiome
β-Lactam resistant gene (*Sphingopyxis alaskensis*)	β-Lactamase (class C family)	100	β-Lactam resistant gene partial (*Sphingomonas* sp.) Hypothetical protein (*Sphingomonas* sp.) Peptidase (*Sphingopyxis alaskensis*) membrane protein (*Sphingopyxis alaskensis*)	c4174	c208
Tetracycline resistant gene (*Caulobacter* sp.)	Transcriptional regulator, subgroup of AraC transcriptional regulators having an N-terminal Type 1 glutamine amidotransferase (GATase1)-like domain	100	Integrase (*Novosphingobium* sp.) Sarcosine oxidase subunit β (*Novosphingobium* sp.)	c4836	c92
Gene modulating antibiotic efflux (Oxalobacteraceae bacterium IMCC9480)	Transcriptional regulator (repressor function), TetR family	100	Cyclohexanone monooxygenase (*Brevundimonas subvibrioides* Acyl-CoA ligase (*Brevundimonas subvibrioides*) TonB-denpendent receptor (*Brevundimonas subvibrioides*) L-Carnitine dehydratase/bile acid-inducible protein F (*Caenispirillum salinarum*) α/β-Hydrolase (*Deinococcus phoenicis*) Putative flavin-containing monooxygenase (*Rhodococcus wratislaviensis* NBRC 100605)	c4172	c321
Glycopeptide resistance gene (*Sphingopyxis baekryungensis*)	AAA+ (ATPases Associated with a wide variety of cellular Activities)	100	GTPase HflX (*Sphingopyxis baekryungensis*) RNA-binding protein Hfq (*Sphingopyxis baekryungensis*)	c4235	c143
Glycopeptide resistance gene (*Sphingobium baderi*)	HATPase_C (Histidine kinase-like ATPases)	100	ATPase AAA (*Sphingopyxis baekryungensis*)		
Gene modulating antibiotic efflux (*Limnohabitans* sp. Rim28)	REC (cheY-homologous signal receiver domain) HATPase_C (Histidine kinase-like ATPases)	97	—	c73	c713
		98	—		

[a]Function assigned by NCBI conserved domains database.
[b]Amino acidic identity between the ORFs in the virome and the ortholog in the microbiome.

In conclusion, this study addresses, for the first time, a complete description of the microbiome and virome in an aquaculture plant sample, relaying information on the presence of antibiotic resistance genes and their mobilization via transduction mechanisms.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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