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Research review paper

Prospects for viruses infecting eukaryotic microalgae in biotechnology

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A B S T R A C T

Besides being considered pathogens, viruses are important drivers of evolution and they can shape large ecological and biogeochemical processes, by influencing host fitness, population dynamics, and community structures. Moreover, they are simple systems that can be used and manipulated to be beneficial and useful for biotechnological applications. In this context, microalgae biotechnology is a growing field of research, which investigated the usage of photosynthetic microorganisms for the sustainable production of food, fuel, chemical, and pharmaceutical sectors. Viruses infecting microalgae have become important subject of ecological studies related to marine and aquatic environments only four decades ago when virus-like-particles associated with bloom-forming algae were discovered. These first findings have opened new questions on evolution and identity. To date, 63 viruses that infect eukaryotic microalgae have been isolated and cultured. In this short review we briefly summarize what is known about viruses infecting eukaryotic microalgae, and how acknowledging their importance can shape future research focussed not only on marine ecology and evolutionary biology but also on biotechnological applications related to microalgae cell factories.

1. Introduction

Microalgae are a large polyphyletic group of photosynthetic unicellular eukaryotes, counting several hundred thousand species, and populating fresh and marine water environments, and terrestrial systems. Since decades, they are investigated as promising sustainable biotechnological platforms for food, fuel, cosmetic and pharmaceutical sectors (Ibañez and Cifuentes, 2013; Khan et al., 2018). Due to their phylogenetic diversity, microalgae show differences in genetics, as well as morphology (e.g. unicellular, colonial, filamentous, motile), chemical composition, and taxonomic lineages (Borowitzka, 2018). Perfectly mirroring their hosts, viruses that infect eukaryotic microalgae show quite intricate diversity. The first viruses infecting eukaryotic microalgae were reported in the 1980s in the green filamentous algae *Uronema gigas* (Allen Dodds and Cole, 1980) and *Chlorella* spp. (Van Etten et al., 1983). Only several decades later, discoveries on virus-like-particles associated with important bloom-forming microalgae were made, addressing their ecological importance (Coy et al., 2018; Milligan and Cosper, 1994). These findings shaped the research of many marine biologists and ecologists, by raising new questions regarding the identity and evolutionary relationships within these virus-host systems. To date, 63 viruses are isolated and cultured in the laboratory and most of them contain a dsDNA genome (Coy et al., 2018). However, these numbers represent only a small percentage of the thousands known populating, for example, the marine environments (Hingamp et al., 2013). In fact, the advancement of genetic technologies (e.g. genome sequencing, meta-transcriptomics) has made the identification of viruses from environmental data more accessible, and it will help in elucidating the microalgae virosphere in a semi-quantitative ecological context in the near future. Recent reports put a magnifying glass on what is known about the virosphere populating the oceans, and a nice example of this was recently presented (Gregory et al., 2019). While the interest in having viruses in culture collection and the number of studies on this topic continue to increase (Nissimov et al., 2020; Sadeghi et al., 2021), the great abundance of marine viruses not only provides new perspectives for more in-depth ecological and evolutionary studies, but it also offers opportunities for biotechnological applications (Gil et al., 2021). In this short review we provide a brief look on isolated viruses infecting eukaryotic microalgae, and how they could be used in future biotechnological applications related to microalgae.

2. A brief look on what is known about viruses infecting microalgae

Despite their small size, viruses play an important role in aquatic ecosystems and food webs. The induced mortality in their hosts contributes to the releasing and recycling of carbon and other nutrients (Fuhrman, 1999; Pourtois et al., 2020; Wilhelm et al., 2006). Viruses are

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capable of reprogramming host metabolism including photosynthesis, as well as central carbon metabolism, nitrogen, phosphorus and sulphur cycling (Maat et al., 2016; Roux et al., 2016; Weitz and Wilhelm, 2012). Recently, they have been proposed as central players of the ocean "biological pump" by which CO_2, after being converted into organic carbon via photosynthesis and next exported through sinking particles, is sequestered in the deep ocean (Guidi et al., 2016). Moreover, viruses also have been considered responsible for gene transfer and driving microbial diversity, deeply influencing the genetic evolution of their hosts (Weinbauer and Rassoulzadegan, 2004).

Viruses infecting eukaryotic microalgae are extremely diverse and their phylogeny is still under consideration, as well as their classification. Up to date, the virus isolated and cultured contain either a DNA or RNA genome (linear or circular, double-stranded or single-stranded, segmented or non-segmented) with a total size ranging from 4.4 to 638 kb. To date, 63 viruses have been isolated and cultured, and among them, 32 are reported to have a dsDNA genome, 10 a ssDNA-, 1 a dsRNA- and 14 a ssRNA genome (Coy et al., 2018; Sadeghi et al., 2021) (Table 1).

The isolated viruses infecting microalgae containing a dsDNA genome are mainly classified in the family of Nucleocytoplasmic large DNA viruses (NCLDVs). NCLDVs are divided into 2 families, *Mimiviridae* and *Phycodnaviridae*, mainly based on the phylogenetic attribution of their Polymerase B gene (Koonin and Yutin, 2019). NCLDVs are highly diverse and abundant in aquatic environments, infecting a variety of different host organisms, particularly phytoplankton groups (marine microalgae) but also other eukaryotic lineages, including non-photosynthetic organisms. While the classification of viruses infecting microalgae is still under consideration (Eukaryotes and Claverie, 2018; Gallot-Lavallée et al., 2017; Jeanniard et al., 2013; Quispe et al., 2017), the development of high-throughput sequencing and bioinformatic technologies is expected to provide a more in-depth view of the evolutionary relationship and classification of this large class of viruses. Meanwhile, thanks to ocean global expeditions (Sunagawa et al., 2020), 6783 draft genomes of NCLDVs were constructed from environmental sequences, and associated with the *Mimiviridae* family (5091 phylotypes), and *Phycodnaviridae* (981 phylotypes) (Endo et al., 2020), suggesting the presence of way more broader number of viruses infecting eukaryotic microalgae among these families. While NCLDVs are to date the most studied class of viruses and still under discovery, the presence of other types of viruses (RNA, ds or ss, segmented or non-segmented) also highlights that the virosphere of microalgae is an underexplored field and is likely going to further expand (Sadeghi et al., 2021). Moreover, only 10 viruses have been isolated and cultured from freshwater species so far, and they are reported infecting three genera of microalgae: *Chlorella*, *Micratinium,* and *Chrysochromulina* (Jeanniard et al., 2013; Mirza et al., 2015).

To date, 10 ssDNA microalgae-infecting viruses have been isolated and they all infect diatoms (Coy et al., 2018). In particular, one of them infects *Thalassionema nitzschioides*, and the remaining 9 are infecting different species of the cosmopolitan genus *Chaetoceros*. Interestingly, species from the latter genus have also been reported hosts for 6 other ssRNA viruses that have been isolated. Diatoms are also the major phytoplankton population of oceans (counting >20,000 distinct species), and it seems reasonable to think that if an underexplored variety of viruses exists, it could be easily found in this host group. So far, viruses infecting microalgae have shown to possess a very narrow host specificity (Coy et al., 2018; Horas et al., 2018), which offers prospects of safety if used for the establishment of powerful biotechnological applications in microalgae.

Table 1
Graphic representation of distribution and list of isolated viruses depending on genome type (dsDNA, ssDNA, dsRNA, ssRNA), host genus and water environment (marine vs freshwater).

Virus genome type	Host genus	Number of viruses identified	Environment (M = Marine; FW = Freshwater)
dsDNA	*Aureococcus*	1	M
	Bathycoccus	1	M
	Chlorella	3	FW
	Chrysochromulina	4	FW
	Emiliania	1	M
	Haptolina	1	M
	Heterocapsa	2	M
	Heterosigma	2	M
	Micratinium	1	FW
	Micromonas	3	M
	Ostreococcus	3	M
	Phaeocystis	4	M
	Prymnesium	3	M
	Pyramimonas	1	M
	Tetraselmis	2	M
	total	**32**	
ssDNA	*Chaetoceros*	9	M
	Thalassionema	1	M
	total	**10**	
dsRNA	*Micromonas*	1	M
	total	**1**	
ssRNA	*Asterionellopsis*	1	M
	Aurantiochytrium	1	M
	Chaetoceros	4	M
	Heterocapsa	1	M
	Heterosigma	1	M
	Rhizosolenia	1	M
	Nitzschia	1	M
	Guinardia	4	M
	total	**14**	
ND	*Chaetoceros*	1	M
	Gymnodinium	1	M
	Heterosigma	1	M
	Skeletonema	1	M
	Stephanopyxis	1	M
	total	**5**	

ND stands for not determined, meaning virus isolated but not further genetically characterized. Information was collected from recent reviews and reports (Arsenieff et al., 2019; Coy et al., 2018; Sadeghi et al., 2021; Toyoda et al., 2019).

3. Microalgae biotechnology

Microalgae are unicellular eukaryotes with photosynthetic abilities. Like plants, they use water, sunlight and fix carbon dioxide for energy, making these unicellular microorganisms attractive sustainable options compared to heterotrophic biotechnological production hosts such as bacteria, yeasts, insect and mammalian cells. Moreover, microalgae cultivation is undemanding. Agricultural land is not needed, as microalgae can grow in liquid medium, and in various cultivation formats (e.g. reactor vessels, ponds, bags) (De Vree et al., 2015). Furthermore, marine species of microalgae grow in salt water and thus do not compete for fresh water. The vast range of bioactive compounds, including antioxidants, carotenoids, proteins, polysaccharides, polyunsaturated fatty acids, sterols and vitamins have made these microorganisms commercially exploitable in food, pharmaceutical and cosmetic industries (Levasseur et al., 2020). However, to date, a fully commercial exploitation is hindered by the relatively low photoautotrophic biomass concentration reached and the relatively high biomass processing requirements. Therefore, microalgae are currently not yet suitable for economically viable production of commodity chemicals, but they have a growing market as single cell food or for food ingredients (e.g. carotenoids, edible oil) for human and animal consumption, and niche markets. (Kusmayadi et al., 2021; Ruiz et al., 2016). In the past few decades, advances in genetic and synthetic biology tools have seen important breakthroughs in microalgal engineering, making these organisms subject of successful genetic interventions aimed at increasing product titres (Ajjawi et al., 2017; Haslam et al., 2020; Perozeni et al., 2020; Poliner et al., 2018), and with some of these photosynthetic hosts becoming attractive candidates also for the production of other high-

value biotechnological products such as isoprenoids, and biopharmaceuticals (Barrera and Mayfield, 2013; D'Adamo et al., 2018; Fabris et al., 2020; Lauersen, 2018; Wichmann et al., 2018).

In this regard, successful genetic engineering strategies of microalgae are still under optimization. This principally concerns the establishment of efficient toolboxes for genetic and synthetic biology to overcome the currently poor expression of heterologous genes, and on the other side obtaining more knowledge on metabolism and metabolic fluxes, in order to make targeted interventions (Ibnu et al., 2021; Work et al., 2012).

Viruses can be looked upon as an exceptional and exploitable resource for microalgae biotechnological applications and thus deserve to be explored. In the following sections, we give an overview on how viruses can be exploited in biotechnology, and we provide the first and recent examples of the usage of viruses infecting microalgae in applications that can help a successful establishment of these alternative and more sustainable platforms.

4. General virus-mediated applications in biotechnology

The identification of viruses and the study of molecular mechanisms essential to the completion of viral cycles have greatly contributed to deciphering fundamental processes in biology, in ecology, and in epidemiology. Although often considered frightening pathogens, nowadays, viruses have outstandingly entered the biotechnological era and numerous applications have already been developed. They have received increasing attention as outstanding resources for recombinant protein expression, viral vaccine production, as well as in nanotechnology, e.g. nanocarriers for drug delivery (Abrahamian et al., 2020; Felberbaum, 2015; Gil et al., 2021; Ibrahim et al., 2019; Roldão et al., 2019). In fact, viruses and viral-derived tools, because of their strong and universally-adaptable qualities, are used to manipulate genetic information, detect, diagnose, control and cure infectious diseases, produce molecules, or even design new structural assemblies. Viral subunits (i.e. proteins, enzymes, envelopes) and elements (e.g. transcriptional promoters, −terminators, internal ribosomal entry sites) have been often used and are now part of the molecular toolbox of geneticists/biologists, some of which have even been commercialized (Henry and Debarbieux, 2012). In this context, viral subunits coming from viruses infecting extremophiles can be used as molecular biology tools (e.g. polymerases, reverse-transcriptases), and some potential biotechnological applications for viruses infecting organisms living in extreme environments have been recently reviewed (Gil et al., 2021). Considering the increasing interest in microalgae as biotechnological platforms, here we resume some of the prospects for the viruses infecting microalgae (Fig. 1).

4.1. Viral elements as tools for genetic engineering

Viruses have evolved as successful pathogens that can invade their host cells and multiply to produce and disseminate viral progeny. During this process viruses hijack the host cellular machinery to express their genes needed for replication and particle assembly, and to defend against host responses (Rampersad and Tennant, 2018). For the replication and transcription of RNA/DNA genomes, viruses rely on viral-encoded genetic elements, many of which have been well characterized. Some of those are nowadays used as (strong/constitutive) promoters/enhancers to drive high expression of heterologous genes. This has (already) offered many advantages over classic systems, such as the ability for application in species belonging to different kingdoms (insects, animals, and plants, including microalgae), reduced gene-to-product time and high yields (Ibrahim et al., 2019). Among the most widely used elements are the strong and constitutive Cauliflower mosaic virus (CaMV) 35S (RNA polymerase type II) promoter and terminator, the SV40 promoter of the Simian virus (SV) 40, the strong and constitutive immediate early gene promoter of cytomegalovirus (CMV) and to certain extent the Omega (Ω) translational enhancer element from Tobacco mosaic virus. Historically adopted for testing transformation methods, these elements are still widely used for protein expression in many microalgae genera (Chen et al., 2001; Muñoz et al., 2019; Ruecker et al., 2008; Sakaue et al., 2008; Zou et al., 2018). While some of these elements are functional cross-kingdom, i.e. may be used in cells from species of different kingdoms, others are more cell-type/tissue specific or lead to lower expression levels when used in other cell types. Hence, to achieve higher levels of heterologous gene expression in microalgae, the use of viral promoters from viruses that naturally infect microalgae is likely leading to the highest chance of success (Fig. 1.1). Recent papers have reported on the very first examples of such exploitation. Kadono et al. identified five new promoter regions within the genomes of four

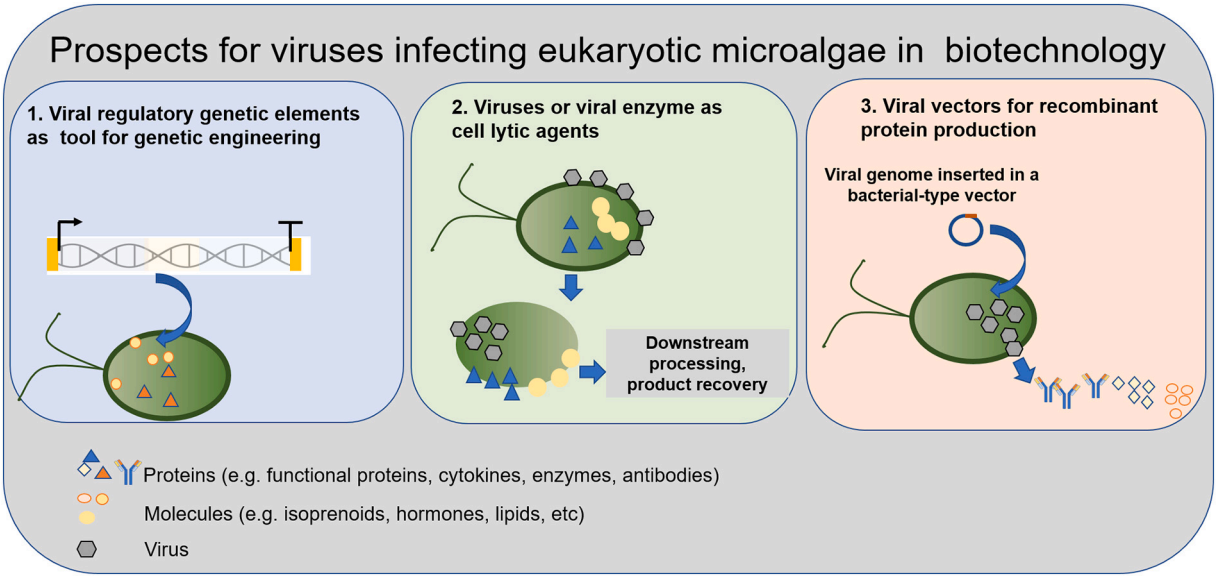

Fig. 1. Schematic representation of the major prospects for viruses infecting microalgae in biotechnology. (1) Viral genetic regulatory elements (e.g. promoters, terminators, ribosome binding sites, transcription enhancers, etc.) can be predicted and isolated from the viral genomes, and used as gene expression tools in genetic and metabolic engineering; (2) viruses or viral enzymes capable of degrading cell walls can be used as lytic agents, helping the recovery of cellular products in biorefinery; (3) viral vectors can be generated and engineered for the production of recombinant protein and biopharmaceuticals.

different viruses infecting microalgae of the diatom group (named DIVs, diatom infecting viruses) of the genera *Chaetoceros* and *Thalassionema* (Kadono et al., 2020, 2015). Three of these promoters were isolated from putative replication-associated protein (VP3) of the *Chaetoceros lorenzianus*-infecting DNA virus, *Thalassionema nitzschioides*-infecting DNA virus, and *Chaetoceros debilis*-infecting DNA virus (named by the authors CdP1, ClP1, TnP1, respectively), while the remaining two by the putative structural protein (VP2) encoding genes of the *Chaetoceros lorenzianus*-infecting DNA virus and *Thalassionema nitzschioides*-infecting DNA virus (named by the authors ClP2, TnP2, respectively). These five promoters were used to drive the expression of an enhanced green fluorescence protein encoding gene (*egfp*) in the model pennate diatom *Phaeodactylum tricornutum*. The *efgp* expression levels were compared with the ones driven by the endogenous, strong, and most widely used promoter of fucoxanthin chlorophyll *a/c*-binding protein A gene (Pt_FcpA), and by the promoters coming from the plant virus CaMV and mammalian virus CMV. The highest activity was reported for ClP1. Compared to the expression level of the endogenous Pt_FcpA promoter, ClP1 showed five times higher mRNA expression, and doubled in eGFP fluorescence level, while CdP1 and ClP2 had comparable expression levels. All five promoters exhibited higher activity than the heterologous viral promoters tested in the same study (CaMV 35S and CMV) (Kadono et al., 2015).

Being this the first example, it is expected that with the increase in molecular knowledge on virus infecting microalgae and in their isolation, many other genetic elements can be exploited to greatly enhance gene and protein expression, providing new important tools for microalgal genetic and metabolic engineering, and synthetic biology.

Genetic engineering of microalgae is a relatively young field and it has been hampered by the lack of efficient tools for the expression of genes and proteins (Fu et al., 2019). In the last decade, new cloning and transformation methods, promoter libraries and tools for genetic and metabolic engineering in microalgae are rapidly growing and expanding (Sproles et al., 2021). Moreover, several modular cloning systems are now available, high-throughput transformation and screening methods for positive transformant lines are reported, as well as episome-based transformation approaches, which enables faster and more reproducible screening studies than random chromosomal integration (Crozet et al., 2018; Fabris et al., 2020; Kumar et al., 2020; Muñoz et al., 2018; West and Patron, 2018). Therefore, screening for novel viral parts for genetic engineering can be carried nowadays in a relatively shorter time. Bioinformatic analysis and prediction tools for regulatory elements can be employed for mining the viral genome and identify the genetic parts (Hölzer and Marz, 2017); those can be then synthetized by commercial DNA custom suppliers, without the need to have the virus isolated.

4.2. Viruses and viral enzymes as lytic agents for biorefinery purposes

Biomass lytic mechanisms for the extraction of several commercially exploitable cell components are at the base of a successful biorefinery strategy in microalgae, and it is still a current bottleneck for the economic viability of microalgal biotechnological processes (Gifuni et al., 2019). Typically, cell disruption is a necessary step to break down the rigid and complex microalgae cell walls to recover microalgal components, such as proteins, lipids, and polysaccharides. Cell lysis can be carried out by physical, chemical, and biological cell disruption methods. Physical disruption methods include bead-beating, high-pressure homogenization, ultrasonication, microwave, and electroporation, while chemical lysis can be carried out by solvents, and biological disruption methods are usually based on enzymatic degradation of cell walls under mild reaction conditions (Günerken et al., 2015).

Viruses may play a favourable role in this context, as they have developed specific ways for entry and exit from cells that could be exploited also for biorefinery purposes (Fig. 1.2). Despite the little information available, the most studied NCLDV members show an entry mechanism into the host cells similar to those of bacteriophages and animal viruses (Sobhy, 2017). For example, the model virus *Paramecium bursaria Chlorella virus* (PBCV-1) infecting *Chlorella* seems to attach to host cells via a single spike protein, and like bacteriophages, it degrades the host cell wall at the site of attachment for entry of its genetic material and leaving an empty shell at the cell surface (Cherrier et al., 2009). Moreover, its genome encodes chitinases, chitosanase, β −1,3-glucanase, and alginase enzymes that likely catalyse cell wall lysis, and a potassium ion channel protein, which has a putative role in entry (Sun et al., 2000a; Thiel et al., 2010; Van Etten et al., 2017). Another virus of this family, *Emiliania huxleyi* virus 86, is thought to enter host cells via endocytosis or by fusion of the outer lipid membrane surrounding the capsid, similar to the entry mechanism of several animal-like infecting viruses (Mackinder et al., 2009). Virus-mediated entry and lysis offers the prospect of mild disruption conditions and avoids the usage of chemicals and energy-intensive equipment, thereby solving some inherent constraints of high energy demand and related costs. In this respect, the first examples of viruses used for such purposes were reported for the PBCV-1, a virus that infects the green and most commercially exploited genus of microalgae, *Chlorella*. Cheng et al. performed a biomass saccharification for bioethanol production using either starch degrading enzymes or PBCV-1 (Cheng et al., 2013). In this work, the authors incubated cell concentrate with starch degrading enzymes, PBCV-1 at a multiplicity of infection (MOI) of 5, and both simultaneously, and they monitored glucose, non-glucose sugars, ethanol and acetic acid release for 5 days. Same hydrolysed extracts were then used as feed for *E. coli* fermentation. The highest percentage of hydrolysed carbohydrates and subsequently *E. coli* fermentation ethanol yields were observed when virus and enzymes were used simultaneously (Cheng et al., 2013). Very recently and for the first time, Sun and Zhou evaluated the feasibility of performing a virus-assisted cell disruption of *Chlorella* sp. for lipid extraction. In their study, infected *Chlorella* sp. biomass yielded a lipid recovery as good as the one achieved after sonication. In this case, the viral infection was conducted for 5 days with multiplicity of infection (MOI) of 0.01. Considering that sonication is one of the most efficient methods for microalgal cell disruption (Prabakaran and Ravindran, 2011), this work shows that virus-mediated lysis is efficient, and it does not require chemical and energy usage, compared to sonication (Sun and Zhou, 2019).

Besides the usage of the virus, viral genomes can be mined to discover the presence of specific (and highly-efficient) microalgae cell-wall degrading enzymes (Agarkova et al., 2021; Sun et al., 2000b; Van Etten et al., 2017), which in turn can be exploited as a biological disruptive method (by expressing their encoding gene into a microalga or by using recombinant purified enzymes), offering the advantage of very efficient and selective lysis. In this regard, just recently, Agarkova et al. reported the identification and the usage of a cell wall degrading protein from the chlorovirus PBCV-1, named A561L^{D4} (Agarkova et al., 2021). In this study, the authors have developed an assay for cell wall degrading activity, where PBCV-1 was incubated at a MOI of 5 for 15 min, and chlorophyll release was monitored. Such chlorophyll-release method was also used to test the in vitro activity of A561L^{D4}, expressed in *Escherichia coli* and incubated at different concentration for 1 h with *Chlorella* cells. Twelve strains of *Chlorella*, including eight non-native hosts for such chloroviruses were tested, showing that A561L^{D4} was able to release chlorophyll for all strains (Agarkova et al., 2021). This study provides another example on how viruses and/or viral enzymes can be used as agents for cell lysis. Future research needs to be done in order to regulate and optimize such virus-based lytic approaches. For a successful establishment of this approach in biorefinery, it is important to gain more knowledge on the infection mechanism, and more studies elucidating the life cycle of viruses are needed in order to control a virus-mediated lysis. Metabolic rearrangements that may occur upon viral infection, and that will depend on the type of virus (Ziv et al., 2016), may need to be taken into consideration, as they can alter the biochemical composition of the virus-treated biomass. Knowledge on the timing of latent phase of the virus and propagation, and virus

quantification post-infection are also important parameters for a design of such virus-mediated lysis. As a best practice and at larger scale, virus-mediated lysis could involve the usage of a two-stage cultivation, and separate vessels dedicated for the viral infection; likewise, virus propagation could be carried out in a separate building or facility, as described for virus-mediated process in other cell systems (Buckland et al., 2014). Depending on the application, product value and the economics of the overall process, dedicated plastic and/or disposable bioreactors, which have been already described, could be employed for such purpose (Bergmann et al., 2013). Usually virus inactivation for processes at large scale can be achieved by low pH, or by heat (60–70 °C), as described for other virus-mediated biopharmaceutical production using virus infecting mammals and insects, and it will need to be assessed for new processes (Esmeralda et al., 2015; Gillespie et al., 2019; Klutz et al., 2015).

4.3. *Viral vectors for the production of recombinant proteins, biopharmaceuticals and vaccines*

Many viruses are being exploited, by genetic engineering, to heterologously produce human and animal therapeutic proteins (Ibrahim et al., 2019; Roldão et al., 2019). To this end, the most widely used approach is to insert a copy of the viral genome into an expression vector downstream a cell-type specific promoter and generating the so-called viral vector. Next, the coding sequence of a heterologous gene is inserted into the viral genome sequence (either as part of a viral polyprotein or downstream a subgenomic promoter), after which this construct is transfected into host cells for *in vivo* transcription by the host RNA polymerase machinery. Once first transcripts of the viral genome are generated, these will become translated and boost virus replication and (heterologous) gene expression. The heterologous gene products (e.g. recombinant proteins) can be recovered during or post-infection (Fig. 1.3). Besides the straightforward approach to express heterologous genes and collect large amounts of the protein to enable studies on the structure and function of it, viral vectors have also been developed (and optimized) for specific uses, and generate a new branch of research lines in medicine, called virotherapy e.g. human gene therapy, or cancer therapy but also used in vaccinology and immunotherapy (Abrahamian et al., 2020; Ura et al., 2014). Meanwhile, the attention for viruses (or elements thereof) as tool has also expanded to the viral structural proteins due to their ability to form Capsid/Virus-like particles (CLPs/VLPs) even in the absence of other non-structural viral proteins and the viral genome. VLPs mimic the organization and conformation of the authentic native viruses, and can be icosahedral, rod-shaped, membrane-bound, or even a combination (membrane-bound capsid-like particle). Currently, various VLPs are being developed/exploited in vaccination (VLP-based vaccines or as carriers of epitopes from other viruses), as drug delivery vehicles, and in nanotechnology (Hun et al., 2020).

Nowadays, numerous animal-, insect- and plant-cell based viral vector platforms are available and with the advancement in genetic engineering, many more are likely to be developed (Abrahamian et al., 2020; Ibrahim et al., 2019; Modric and Mergia, 2009). Among them, Semliki forest and Sindbis, Tobacco mosaic, Cowpea mosaic, Turnip yellow mosaic and Potato X viruses, baculovirus and bacteriophages (e.g. M13, Qβ, Lambda, T7 or T4) are exploited as viral expression vectors and/or VLPs production systems (Henry and Debarbieux, 2012). This allows, accordingly, to use different host systems (mammalian, insects, plants) for the production of recombinant proteins and biopharmaceuticals. New technological advancements are continuously being made to implement the usage of microorganisms (e.g. yeast, bacteria) for this purpose (Vogl et al., 2013). However, the usage of (certain) microorganisms is often hindered by their incompatibility in making post-translational modifications, such as and especially, *N*-glycosylations. N-glycans represent a critical quality attribute for the efficacy of biopharmaceuticals, conferring biological activity, stability and long half-life (Lalonde and Durocher, 2017). In this context, microalgae are considered a promising (and alternative) candidate for the production of therapeutic proteins (Dehghani et al., 2020; Rosales-Mendoza et al., 2020), as they are capable of performing N-glycosylations similar to mammalians (Mathieu-Rivet et al., 2020).

First attempts have already been made to produce monoclonal antibodies (mAbs) in microalgae, among other therapeutic proteins (e.g. human growth factors, cytokines) and several immunogens against human/animal viruses (Barrera and Mayfield, 2013; Chen et al., 2001; Geng et al., 2003; Vanier et al., 2018). Moreover, first examples of immunogen oral delivery using microalgae have been recently generated (Kiataramgul et al., 2020). Because one of the limitations up to date is the poor availability of efficient and strong gene expression tools in microalgae, establishing a viral-based platform can pave the road towards the establishment of novel and efficient biopharmaceutical production platforms. In this context, a very recent study provides a first example of transient expression of recombinant proteins using DNA viral vectors coming from a plant geminivirus (Malla et al., 2021). In this study, a geminiviral vector (pBYR2e) was modified to express SARS-CoV2 receptor binding domain (RBD) protein and the basic fibroblast growth factor (FGF), and transfected in two freshwater microalgal species, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*. Transfection of the vector was achieved through Agrobacterium-mediated transformation, during a co-cultivation. The system allowed the expression of the two recombinant proteins at 48 h post transformation. Although optimization is necessary to increase the product yields and understand the stability of the process, this study provides a first example of a virus-mediated technology in microalgae for the production of recombinant proteins (antigens for vaccine purpose).

The usage of viral vectors can avoid the difficulties faced in the genetic transformation of microalgae, such as low yields and genetic instability associated with gene silencing and genetic rearrangements, time-consuming processes for the transformation and selection of candidate clones. It can also allow to have efficient transient expression of transgene products that may result toxic for the cell. Moreover, viruses can be exploited on their natural capability to drive the expression of genes at different stage of the infection, by making use of different viral promoters (e.g. very early, early, late). Finally, depending on the scope and how the technology is developed, it is possible to directly purify the recombinant proteins after viral lysis. On the other hand, the viral vector will need to be engineered and optimized for the expression of transgenes and efficient protein recovery. Moreover, it needs to be delivered into the microalgal cell. In this context, several microalgae are already genetically accessible through different transformation methods and the research in this topic is advancing rapidly (Fajardo et al., 2020; Muñoz et al., 2018; Pralhad Rathod et al., 2017; Sproles et al., 2021). Obtaining knowledge on viral gene function and genome architecture is an important step to make rational design on which viral regulatory elements can be used to drive strong expression of desired transgenes, and to decide whether other viral gene(s) can be removed, to optimize the recovery of recombinant products. Depending on the type of virus, the expression of viral proteins that may induce host proteolytic activity, and the capacity for accommodating foreign DNA are current challenges that can slow down the development of viral vectors (Moleirinho et al., 2019; van der Loo and Wright, 2016).

5. Promising viruses infecting microalgae for biotechnological applications

While the research area in microalgae infecting viruses is growing and future prospects towards their exploitation in biotechnological applications is obvious, hardly any steps have yet been made in this direction. Among the isolated and cultured viruses there are a few that can be considered promising for two reasons: 1) they have not been reported to cause health problems in humans and 2) they can infect and sometimes have been used in microalgae which have a recognized status concerning industrial relevance. Table 2 summarizes the most promising

Table 2
Selected virus with high potential in microalgal biotechnology.

Virus name	Genome type	Genome size (kbp)	Host name	Explored application	Potential biotechnological application	References
CdebDNAV	ssDNA	ND	*Chaetoceros debilis*	heterologous gene expression tools	oral vaccine/biopharmaceutical/ biorefinery	(Tomaru et al., 2008)
ClorDNAV	ssDNA	5.8	*Chaetoceros lorenzianus*	heterologous gene expression tools	oral vaccine/biopharmaceutical/ biorefinery	(Tomaru et al., 2011)
PBCV-1	dsDNA	287–369	*Chlorella variabilis* NC64A	cell lysis	oral vaccine/biopharmaceutical/ biorefinery	(Jeanniard et al., 2013)
OSy-NE5	dsDNA	327	*Chlorella variabilis* Syngen 2–3	–	oral vaccine/biopharmaceutical/ biorefinery	(Quispe et al., 2017)
ATCV-1	dsDNA	288–327	*Chlorella heliozoae* SAG 3.83	–	oral vaccine/biopharmaceutical/ biorefinery	(Jeanniard et al., 2013)
TetV	dsDNA	668	*Tetraselmis* spp.	–	oral vaccine/biopharmaceutical/ biorefinery	(Schvarcz and Steward, 2018)
Tsv-N1	dsDNA	31	*Tetraselmis striata*	–	oral vaccine/biopharmaceutical/ biorefinery	(Pagarete et al., 2015)

viruses and emerging applications for this moment. However, we envision an expanding exploitation of many more among the already existing, and future novel isolated viruses infecting microalgae. Microalgae from the genus of *Chlorella* and *Tetraselmis* are already commercially exploited as animal and aquaculture feeds, and as human food supplements. As a logical consequence, viruses infecting those microalgae, can be exploited in biorefinery, as shown for the PBCV-1 infecting *Chlorella*, but also as tools for expressing functional proteins or immunogens for oral delivery. As earlier described, viral promoters from DIVs have shown to work well or even better in the pennate diatom *P. tricornutum* than current expression systems that rely on endogenous promoters. *P. tricornutum* is an industrially relevant microalga, used in aquaculture and as supplement of antioxidants pigments (i.e. fucoxanthin). Recent studies have shown that this microalga presents a competitive system for the production of monoclonal antibodies and high-value terpenoids with pharmaceutical potential (D'Adamo et al., 2018; Vanier et al., 2018). Moreover, and in general, besides their application as feed supplement and good lipid source (i.e. polyunsaturated fatty acids such as omega 3 and 6) both diatoms and coccolithophore microalgae (e.g. *Emiliana huxleyi*) are also attractive for their silica and calcite shells, respectively found in their external cell walls. These highly organized structures have been already considered for several biotechnological and biomedical applications, such as biosensor design and drug delivery systems, and also in cosmetic and cement industry as sustainable biomaterials (Grasso et al., 2020; Ragni et al., 2018; Skeffington and Scheffel, 2018). A controlled, virus-mediated lysis could potentially help in the, currently challenging, extraction procedure of these shells from the biomass. Moreover, it has been reported that *E. huxleyi* lytic virus EhV201EhV has the ability to downregulate host genes involved in de novo sphingolipid biosynthesis of the host, while the viral genes involved in the same pathway are upregulated (e.g., serine palmitoyltransferase), leading to the biosynthesis of unique sphingolipids (Ziv et al., 2016). Similarly, the genomes of PBCV-1 viruses infecting *Chlorella* spp. encode enzymes involved in making extracellular polysaccharides, such as the high value hyaluronan, and chitin (Van Etten et al., 2017). In this regard, more studies on virus–host interactions are needed to understand whether the metabolic engineering capability of these viruses can be used to enhance specific metabolites or molecules that can be exploited for other industrial applications. Finally, certain microalgae of the genera *Rhizosolenia, Heterosigma, Nitzschia, Skeletonema, Aureococcus, Heterocapsa, Chrysochromulina, Micratinium, Phaeocystis, Prymnesium, Pyramimonas, Aurantiochytrium, Gymnodinium, Stephanopyxis* are reported to form toxic blooms (Hallegraeff, 1992; Van Dolah et al., 2001). As future perspective, viruses that infect these micoralgae can be envisioned as a biotic control agents in environmental biotechnology. Similar considerations have been described in a recent review (Pal et al., 2020).

6. Conclusions and perspectives

Although we are just at the start of an era in which microalgae infecting viruses are being investigated from a scientific point of view, in time they will also become (and receive) growing interest to be explored and exploited in a versatile way for biotechnological applications using microalgae as production platform. Microalgae are a polyphyletic class of photosynthetic microorganisms that receive increasing industrial interest as next-generation resources with the potential to address urgent industrial and agricultural demands. The extensive biological diversity of microalgae can be leveraged to produce a wealth of valuable bioproducts (including antioxidants, carotenoids, proteins, polysaccharides, polyunsaturated fatty acids, sterols, and vitamins) either naturally or via genetic manipulation. Because they are relatively easy to be genetically amended, they can grow under containment (photobioreactors) and they are capable of performing post-translational modifications and glycosylations similar to mammalians, they are also attractive as new biopharmaceutical production platforms. To date, the establishment of a biotechnological chassis based on microalgae is still hindered by the high cultivation and refinery costs and the poor knowledge and tools for recombinant protein expression. In this context virus infecting microalgae may play a relevant role in emerging biotechnological applications. Virus-mediated technologies are opening the door to a faster, highly programmable genetic engineering of eukaryotic cells in many fields of research from basic science to biotechnology and medicine (Abrahamian et al., 2020; Lee et al., 2017; Roldão et al., 2019). Viruses are considered outstanding resources for recombinant protein expression, gene therapy, viral vaccine production, as well as nano-agents for drug delivery and nanomaterial technology. Their precise host recognition, entry and exit of the host cell through lytic activity can also be exploited for cell disruption, making easy the processing of the biomass. Viruses infecting microalgae have a recent history, and to date, only 63 viruses have been cultured, while metagenomic analyses have shown the presence of many more in the oceans. Here we have outlined the possible applications of these viruses and listed the currently known viruses that can infect industrially relevant species of microalgae and that may shape future biotechnological applications. While viruses in general have already proven their potential in areas of research and industry (e.g. pharma and animal health), there is still a road ahead. There are intrinsic challenges associated with the establishment of a viral technology, in general. Achieving high understanding of the infection mechanism, to be able to control and manipulate the process and to operate in safety, are a few examples (Moleirinho et al., 2019; van der Loo and Wright, 2016). It is important to highlight that the development of virus-mediated technologies will require the availability to access and isolate viruses. The need for culture collection of viruses isolated from aquatic environments has been very recently raised (Nissimov et al., 2020). In fact, the isolation of new

viruses involves screening of suitable susceptible cultured hosts with water samples thought to contain viruses. Afterwards, viruses should be propagated on a regular basis, unless long term viability is achievable, or ideally they can be cryopreserved to maintain the genetic integrity of the virus isolate. This will require skilled personnel, appropriate facilities, growth chambers and cultivation know-how, and specific biological knowledge of the host-virus system. If supported, this initiative will greatly facilitate the access to viruses and their exploitation for biotechnological applications.

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Declaration of Competing Interest

Authors declare that there is no conflict of interest.

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