

## Exploiting the potential of Cyanidiales as a valuable resource for biotechnological applications

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### ABSTRACT

The biotechnological uses of algae and cyanobacteria have been widely discussed in the context of climate change and consequent efforts to circularize economies, minimize carbon release and reuse waste streams. Their great potential in bioproduction and bioremediation has barely been exploited, particularly for the well-characterized red algae *Galdieria sulphuraria* and *Cyanidioschyzon merolae*. These and other Cyanidiales are excellent candidates for biotechnological enhancement and metabolic engineering for a broad spectrum of applications including the production of biofuels and thermostable colourants. In particular, extremophily, such as growth at thermophilic temperatures – up to 60°C – and at low pH and high salinity, make these algae unusually resistant to contamination and pathogens, and therefore potentially more commercially viable. We review existing applications of the Cyanidiales, as well as their available molecular tools. Their varied nutritional demands, from the broad heterotrophy of *G. sulphuraria* to strongly autotrophic *C. merolae*, along with their ability to grow to high densities, confer great potential as expression hosts. We also discuss the deficiencies that must be overcome to unlock further applications and ultimately to embed thermophilic red algae into a framework of circular and sustainable economic activity relying on bio-based sources.

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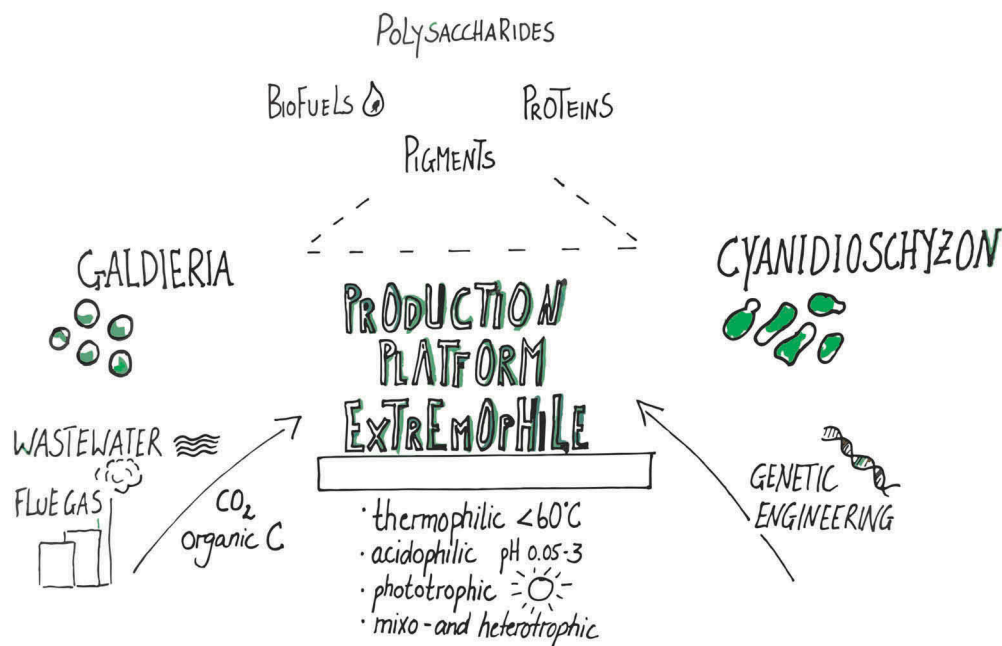
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### Introduction

In recent years, the interest in photosynthetic unicellular organisms as a sustainable resource for biotechnological applications has increased significantly (Doron, Segal, & Shapira, 2016; Eichler-Stahlberg et al., 2009; Guzmán-Zapata et al., 2016; Imbimbo et al., 2019; Varshney et al., 2015). Research and development efforts have gradually expanded the microalgal biotechnology repertoire, but also accentuated the limitations thereof. The main drawback remains the low cost effectiveness which impedes large-scale production of a wide array of substances (Barclay, Apt, & Dong, 2013; Chen et al., 2011; Ho et al., 2011; Lundquist et al., 2010; Razzak et al., 2013). Several key problems will have to be addressed in order to make microalgal technologies commercially competitive: (i) the biomass and target product yields need to be increased at least by a factor of two; (ii) the costs of cultivation systems (photobioreactors, open ponds) and contamination control will have to be reduced by at least a factor of five and (iii) costs of downstream processes, such as biomass harvesting and product

extraction will have to be lower (Clippinger & Davis, 2019; da Silva & Reis, 2015; Mallick et al., 2016; Milledge & Heaven, 2013). In this regard, Lundquist et al. (2010) calculated a 15–20% cost reduction of microalga-derived biofuels in 100–400 ha open pond facilities if the downstream processing cost was reduced by two-thirds. Furthermore, their estimate predicts a 20–25% increase in costs of biofuels if wastewater treatment was not included in the process.

To minimize the above-mentioned constraints, bioprospecting for suitable strains that exhibit the desired physiological properties is one way to make microalgal technologies more competitive (Barclay et al., 2013; Williams & Laurens, 2010). For example, substantial cost reductions were realized by exploitation of extremophilic algae that thrive under culture conditions that substantially inhibit the growth of predators and other algae (Patel et al., 2019; Varshney et al., 2015). These conditions could include high temperatures, high salt concentrations and extremes of pH (Varshney et al., 2015). Moreover, extremophiles contain unique genes and specialized, e.g., thermostable, enzymes that may prove useful for biotechnological and pharmaceutical



**Figure 1.** Graphic illustrating the biotechnological potential of *Galdieria* and *Cyanidioschyzon*. The remediation of waste streams (wastewater, flue gas, organic C) allows a valorization to products, such as pigments, lipids and polysaccharides. A toolbox for genetic engineering will render *Cyanidioschyzon* a heterologous expression platform.

applications (Lu et al., 2009; Varshney et al., 2015; Weber et al., 2007). The majority of extremophiles are bacteria and archaea; the few exceptions among eukaryotes are unique groups of green and red algae, in particular the rhodophyte lineage of Cyanidiales (Aguilera et al., 2007; Aguilera et al., 2010; Hirooka & Miyagishima, 2016; Weber et al., 2004). Despite their biotechnological potential, the number of published studies dealing with applied projects on Cyanidiales is relatively low. In a few patents, specialized production methods for cosmetics and pigments using strains of the order Cyanidiales are described (Bimonte et al., 2014; Cagnac, Richard, & Labro, 2017; Van der Maarel, Martinez-Garcia, & Sarian, 2016), but they are still far from commercial scale production.

In this review, we aim to provide a comprehensive picture of the current state of the art for two species of Cyanidiales, *Galdieria sulphuraria* and *Cyanidioschyzon merolae*. We highlight the commercial and research potential of microalgae to encourage scientists towards these less-studied algal groups and move microalgal technologies towards economic viability.

### Cyanidiales: an introduction

The order Cyanidiales represents a globally distributed group of unicellular rhodophytes (Archibald, Simpson, & Slamovits, 2017; Lee, 2009; Yoon et al., 2006), with

three known genera: *Cyanidium*, *Cyanidioschyzon* and *Galdieria*. So far, phylogenetic analyses have identified eight different species (Guiry & Guiry, 2017). All are well adapted to extreme conditions such as springs with highly acidic pH (from 0.05 to 3), fumaroles, mines and temperatures up to 60°C (Ciniglia et al., 2004; Gross et al., 2001; Schonknecht et al., 2013; Yoon et al., 2006). The cells of *Galdieria* and *Cyanidium* are spherical, surrounded by a mucilage cell wall, and they propagate through the formation of four endospores. Sexual reproduction has not been described (Guiry & Guiry, 2017), although it has been predicted to occur in *Cyanidioschyzon* based on the conservation of meiosis-specific genes (Guo & Yang, 2015). One unique feature of *Galdieria* is photo-organotrophic and heterotrophic growth, resulting in substantially higher biomass yields than under phototrophic growth conditions (Graverholt & Eriksen, 2007; Schmidt, Wiebe, & Eriksen, 2005). *Cyanidioschyzon* is club-shaped, lacking a vacuole and cell wall, and propagates through binary fission. It contains one nucleus, mitochondrion and plastid, and is considered one of the most primitive algae (Kuroiwa, 1998; Matsuzaki et al., 2004). Previously, *C. merolae* was thought to be an obligate phototroph, but, in a recent study, growth on organic carbon sources was reported (Moriyama, Mori, & Sato, 2015). So far, *C. merolae* is the only member of the Cyanidiales accessible for genetic modifications (Ohnuma et al., 2008; Table 1).

**Table 1.** Accessibility of *Galdieria* and *Cyanidioschyzon* for biotechnological applications.

	<i>Galdieria</i>	<i>Cyanidioschyzon</i>	References
Heterotrophic growth	Yes	Yes	Gross & Schnarrenberger, 1995; Oesterhelt et al., 1999; Schonknecht et al., 2013; Moriyama et al., 2015; Sloth et al., 2017
C-PC production	Yes	Yes	Schmidt et al., 2005; Sloth et al., 2006, Sloth et al., 2017; Graverholt & Eriksen, 2007; Wan et al., 2016; Carfagna et al., 2018; Wang et al., 2019; Rahman et al., 2020
C-PC extraction	Yes	Yes	Sørensen et al., 2013; Moon et al., 2014; Rahman et al., 2017; Imbimbo et al., 2019
Waste stream treatment	Yes	N.A.	Kurano et al., 1995; Selvaratnam et al., 2014; Minoda et al., 2015; Vítová et al., 2019
Human food and health	Yes	N.A.	Graziani et al., 2013; Carfagna et al., 2015; Bottone et al., 2018; Massa et al., 2019; Modeste et al., 2019
Floridoside	Yes	N.A.	Martinez-Garcia & van der Maarel, 2016
Genome sequences	Yes	Yes	Ohta et al., 1998; 2003; Matsuzaki et al., 2004; Barbier et al., 2005; Schonknecht et al., 2013; Rossoni et al., 2019a
Auxotrophic mutant strains	N.A.	Yes	Minoda et al., 2004
Nuclear transformation	N.A.	Yes	Ohnuma et al., 2008; Fujiwara et al., 2013
Selective markers	N.A.	Yes	Moriyama et al., 2015; Fujiwara et al., 2017; 2015; 2013
Expression vectors	N.A.	Yes	Ohnuma et al., 2008
Stable expression vectors	N.A.	N.A.	

## Bioresources

Most micro-organisms are thought to have a worldwide distribution. Extremophilic microalgae like Cyanidiales thrive in specific and rare polyextreme environments while elsewhere they will easily be outcompeted (Castenholz & McDermott, 2010). This habitat specificity, along with their prominent colour, enables focused sampling of Cyanidiales, even though their typical extreme habitats like acidic geothermal environments may be hard to access physically and legally. Environmental suitability seems to be the most important factor restricting the occurrence of Cyanidiales (Barcyte et al., 2018), which leads to further targeted sampling of, e.g., acidic heterotrophic or metal-contaminated areas. New habitats and localities for *Galdieria* were found by sampling anthropogenic localities that mimic the natural environment (Barcyte et al., 2018; Gross et al., 2002).

Cyanidiales species typically coexist in the field. However, they can be isolated separately by their specific growth conditions. After isolation, long-term maintenance in conditions mimicking the natural situation (acidic media, high temperature and salinity) suppresses growth of contaminants under autotrophic conditions. Therefore, phototrophic conditions are traditionally used for long-term maintenance of axenic and non-axenic Cyanidiales strains, while photo-organotrophic or heterotrophic conditions are only feasible for axenic holdings.

Bioprospection for potential production strains could be based on isolation of new strains, a rather time- and resource-consuming process. A much more effective and sustainable resource lies in the public and private culture collections acting as specialized biological resource centres.

Public algal culture collections (ACCs) in particular can provide a large number of well-characterized, pre-screened strains and valuable data in a legally compliant manner (Overmann & Smith, 2017). As centres of

competence for isolation, purification, identification, as well as characterization of newly established strains, they can underpin bioprospecting efforts. Furthermore, their expertise in cryopreservation of microbial strains eases the establishment of reliable cell banking protocols to enable long-term storage and preservation of genetic and physiological traits of production strains. Some ACCs act as International Depository Authorities (IDA) under the “Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedures and Regulations”.

Many laboratory strains of Cyanidiales have been archived since the first sustainable isolates, which were the basis for the description of the genus *Galdieria* by Merola (1981), including the transfer of *G. sulphuraria* (Galdieri) Merola. Public and private culture collections maintain more than 500 Cyanidiales strains, isolated globally from different habitats (A. Rossini, Düsseldorf University, pers. comm.). The ACUF Collection (Microalgal Collection at University of Naples Federico II) specializes in thermo-acidophiles and keeps about 400 Cyanidiales strains (Del Mondo et al., 2019). Further strains are kept in public and private ACCs worldwide. Despite the inter- and intra-specific variability shown (Albertano et al., 2000; Cho et al., 2019; Ciniglia et al., 2004; Davis et al., 2016; Eren et al., 2018) only a few strains of Cyanidiales have been studied in depth for their biotechnological potential.

## Cyanidiales as cell factories

In considering Cyanidiales strains for commercial scale production, it is important to evaluate their cellular properties, production parameters and the nature of the downstream processing. Thus, *Cyanidium* might not be the ideal candidate: it exclusively grows

phototrophically, has a cell wall, and has not yet been accessible to genetic modification. *Galdieria* reveals its key potential in its ability to grow heterotrophically on multiple organic carbon sources including glucose, glycerol, molasses, food wastes and cherry brine (Table 1; Schmidt et al., 2005; Sloth, Wiebe, & Eriksen, 2006; Sloth et al., 2017). Several studies have demonstrated that heterotrophically grown *G. sulphuraria* achieved a 20- to 50-fold increase in biomass productivity compared to phototrophic growth conditions, reaching up to 50 g dry biomass l<sup>-1</sup> d<sup>-1</sup> (Graverholt & Eriksen, 2007; Schmidt et al., 2005; Sloth et al., 2017; 2006). Heterotrophic growth rates of *C. merolae* are substantially lower than those of *Galdieria* (Moriyama et al., 2015). However, the genomic integration of a sugar transporter gene from *G. sulphuraria* resulted in higher growth rates of *C. merolae* (Fujiwara et al., 2019). Furthermore, *C. merolae* lacks a cell wall and thus allows for a simple and effective cell extraction by osmotic shock as demonstrated by Rahman and co-workers (Rahman et al., 2017).

There is growing interest in using microalgae as biocatalysts for converting waste streams into valuable products. For example, wastewater is an attractive resource for algal cultivation due to its high nutrient content and low price. Researchers have attempted to grow *G. sulphuraria* in wastewater and demonstrated satisfactory biomass productivities and moderate removal rates of nitrogen and phosphorus species (Selvaratnam et al., 2014; Tchinda et al., 2019). Furthermore, *Galdieria* was shown to be a valuable biocatalyst for the recovery of rare earth elements (Čížková, Vítová, & Zachleder, 2019; Minoda et al., 2015; Vítová, Čížková, & Zachleder, 2019). RuBisCo of Cyanidiales strains exhibits an unusually high affinity for CO<sub>2</sub>, mediating a high CO<sub>2</sub> tolerance of up to 100% (Kurano et al., 1995; Uemura, Anwaruzzaman, & Yokota, 1997). Coupled with the resistance to SO<sub>x</sub> and NO<sub>x</sub> species and very low pH, it makes them promising candidates for flue gas aeration in mass cultivation (Kurano et al., 1995; Varshney et al., 2015).

### Natural colourants – thermostability as an advantage

Natural colourants and antioxidants have received much attention from the food, cosmetics and pharmaceutical industries in recent years (Bux & Chisti, 2016; Packer, Harris, & Adams, 2016). Here, microalgae are considered a valuable source for these compounds. The commercial production of the blue accessory pigment phycocyanin (C-PC) is achieved by mass cultivation of cyanobacteria from the genus *Arthrospira* (*Spirulina*), now in its fourth decade. The current world market size

of C-PC is estimated to be 30 USD million, with *Arthrospira* being the sole source [<https://www.lpinformationdata.com/shop/2017-2022-global-top-countries-phyococyanin-market-report-2>]. Besides its role as a natural colourant in the food industry, C-PC also serves as a fluorescent probe in immunoassays, and exhibits antioxidant, anti-inflammatory and anti-cancer therapeutic properties (Bottone et al., 2018; Eriksen, 2008; Romay et al., 2003). With the market opening for natural blue colourants in the food sector, the demand for C-PC is expected to increase. We anticipate this development to also pave the way for alternative microalgal C-PC sources, such as *Cyanidioschyzon* and *Galdieria*.

The instability of C-PC, and its sensitivity towards light and air, is unfavourable for various biotechnological applications like thermal processing and extraction (Antelo, Costa, & Kalil, 2008; Chaiklahan, Chirasuwan, & Bunnag, 2012). The maximum temperature at which *Arthrospira*-derived C-PC retains its natural stability is 45°C and in a pH range of 5.5–6.0, for 30 min (Antelo et al., 2008; Chaiklahan et al., 2012; Patel et al., 2004). However, with preservatives, the thermostability of *Arthrospira*-derived C-PC for industrial applications can be increased to 60°C (Chaiklahan et al., 2012). There is therefore a significant market potential for a thermostable phycocyanin that does not depend on preservatives.

Consequently, extremophilic cyanobacteria and microalgae have been evaluated for their phycobiliproteins' (PBP) thermostability and chemical stability (Edwards et al., 1997; Edwards, MacColl, & Eisele, 1996; Eisele et al., 2000; Kao, Edwards, & Berns, 1975; Leu et al., 2013; Liang et al., 2018; Rahman et al., 2017). Of the three common Cyanidiales species studied in the literature, *C. merolae* harbours the C-PC with the highest thermostability at 75°C for 30 min (Rahman et al., 2017). C-PC isolated from *G. sulphuraria* and *C. caldarium* showed heat resistance up to 65°C and 55°C, for at least 30 min, respectively (Eisele et al., 2000; Moon, Mishra, & Kim, 2014). In contrast to the relatively scarce data on *C. merolae*, C-PC production in *G. sulphuraria* has been studied much more extensively (Carfagna et al., 2018; Eisele et al., 2000; Lee, 2009; Liang et al., 2018; Moon et al., 2014; Rahman, Sarian, & van der Maarel, 2020; Roth, Berns, & Chen, 1996; Sørensen, Hantke, & Eriksen, 2013; Varshney et al., 2015). In *G. sulphuraria*, phototrophic growth demonstrated similar C-PC contents compared to other well-studied cyanobacteria (Wang et al., 2020). Pigment productivities under photoorganotrophic and heterotrophic growth conditions revealed a complex interplay between carbon and nitrogen availability, as well as light intensity (Sloth et al., 2006; Van der Maarel et al., 2016). Even though, at 1–2% of dry

biomass, the cellular C-PC content was comparatively low, the high growth rate compensated for the low pigment content. The production rates were 20- to 30-fold higher than determined for *A. platensis* growing photo-trophically in open pond systems (Graverholt & Eriksen, 2007; Schmidt et al., 2005). Contrary to many other algae, some *G. sulphuraria* strains maintain the photosynthetic apparatus and associated pigments even when cultivated heterotrophically (Gross & Schnarrenberger, 1995; Carfagna et al., 2018; unpublished data, University of Applied Sciences Bremerhaven).

All studies published so far have revealed the challenges of establishing a standardized process to produce high-quality thermostable C-PC with *Galdieria*, irrespective of the nutritional mode. This highlights the need for detailed research on Cyanidiales to further advance the technology. As outlined before, there are many *Galdieria* strains available in public and private collections, but only a few are discussed in the literature. Consequently, a comprehensive screen for *Galdieria* strains exhibiting the physiological attributes that are necessary for high C-PC yields would be required. Another option is to generate a “high C-PC” strain through mutagenesis, as in the case of *Arthrospira* (Takeuchi & Roberts, 2016).

In conclusion, both *G. sulphuraria* and *C. merolae* represent natural sources of thermostable C-PC as an advantageous alternative to the use of preservatives for *Arthrospira*-derived C-PC. The development of a C-PC-overexpressing *C. merolae* strain represents one option in addition to photo-organotrophic or heterotrophic production in an enclosed system with *G. sulphuraria*. For either approach, further research and development is necessary to develop standardized operations and produce high-quality pigments.

### **Cyanidioschyzon merolae as a model for biofuel production**

In recent years, microalgae have received growing interest as a potential resource for biofuels. The advantage of microalgae over first- and second-generation biofuels is their high biomass production and ability to accumulate substantial amounts of oil (50–65% per unit dry weight) within the cells (Georgianna & Mayfield, 2012). Desired properties for suitable species include tolerance of changing environmental factors like light quality, temperature and water quality, as well as resistance to contamination and pathogens. *C. merolae* represents one such potential model organism. Its thermo-acidophilic nature enables cultivation in most kinds of culture systems including open, closed, terrestrial, marine,

wastewater and cultures supplemented with industrial exhaust gas (Sato et al., 2017). The small nuclear genome of 16.5 Mb, genetic tractability, availability of protein expression systems and homologous recombination tools further add to the attributes of *C. merolae* as a potential model for biofuel production (Fujiwara et al., 2013; Sato et al., 2017). In particular, its high lipid content and precise lipid composition facilitate processing of algal biomass and its utilization as biodiesel, jet fuel or other biofuels.

Based on comparative genomics, 121 genes in total have been predicted to play a role in *C. merolae* lipid metabolism (Mori et al., 2016). In combination with protein expression data, it was possible to generate a lipid metabolic map of *C. merolae*. In contrast to both eukaryotic and prokaryotic pathways, lipid biosynthesis in *C. merolae* occurs through a novel coupled pathway, in which monogalactosyl diacyl glycerol precursors, palmitic acid and linoleic acid, are supplied by the plastid and endoplasmic reticulum, respectively (Sato & Moriyama, 2007). The reduced *C. merolae* lipid profile is unique and contains C16–C18 saturated and unsaturated fatty acids (FAs), lacks highly unsaturated lipids, and has neither cardiolipin nor phosphatidylserine (Sato & Moriyama, 2007; Toyoshima et al., 2016). This rather simple lipid profile makes *C. merolae* an attractive candidate for biodiesel production.

Like other microalgae, *C. merolae* has been shown to accumulate triacyl glyceride (TAG) up to 20% of dry weight in response to nitrogen deprivation (Takusagawa et al., 2016). In contrast to other oleaginous algae, lipid and starch accumulation are independent of each other, representing a potentially valuable characteristic of *C. merolae* since both products could be used to produce high levels of biofuels (Sato et al., 2017). In order to identify rate-limiting steps in TAG accumulation, overexpression of two glycerol-3-phosphate acyltransferases, CmGPAT1 and CmGPAT2, was tested. The genetically modified *C. merolae* strains exhibited up to 59-fold greater TAG accumulation compared to the wild-type strain under normal cell growth conditions (Fukuda et al., 2018). Also, in other algae, TAG accumulation has been increased by metabolic engineering and is regarded as a promising solution in strain development for algae-based biodiesel production (Ajjawi et al., 2017; Iwai et al., 2014).

All of these data together support *C. merolae* as a promising model organism for biofuel production. However, growth of *C. merolae* is still too low and further attempts with regard to strain selection and biomass productivity are necessary in order for it to become a suitable production strain.

## Expression platform extremophile

Coupling photosynthesis with a homologous and/or heterologous implementation of enzymes or pathways to obtain natural products in microalgae offers great opportunities for green biotechnology. For several microalgae, genetic and metabolic engineering was demonstrated and has paved the way for the production of, e.g., bioactive compounds for pharmaceutical applications (Griesbeck, Kobl, & Heitzer, 2006; Hempel et al., 2011).

To have a maximally useful system for bioproduction, it is essential to have a broad set of molecular genetic tools. These include the means to introduce heterologous DNA into the host, genome editing tools such as CRISPR-Cas9, orthogonal inducible and repressible promoters that do not interfere with endogenous gene function, and well-characterized gene and metabolite expression pathways. Thus, a prerequisite for genetic engineering is the knowledge about the genome sequence of the respective microalga. The thermophilic red algae *C. merolae* and *G. sulphuraria* have been recognized as potentially valuable targets for green biotechnology due to their many advantageous growth properties.

The first complete algal genome sequence was that of *C. merolae* 10D, which became available in 2004, revealing a dramatically reduced set of 5331 genes on 20 chromosomes, and yielding unique insights into the evolution of land plants and eukaryotic cells in general (Matsuzaki et al., 2004). *C. merolae* has a genome size of around 16.5 million base pairs (bp), which is comparable to baker's yeast or fission yeast. Only 0.5% of all *C. merolae* genes contain a spliceosomal intron with a strict consensus sequence (Matsuzaki et al., 2004). Consistent with the paucity of introns, *C. merolae* has a dramatically reduced set of spliceosomal factors (Stark et al., 2015). Apart from the nuclear genome, mitochondrial and plastid genomic sequences of *C. merolae* are available (Ohta, Matsuzaki, & Misumi, 2003; Ohta, Sato, & Kuroiwa, 1998). The low frequency of introns, a haploid genome and the availability of organellar DNA sequences make *C. merolae* an excellent target for biotechnological applications involving genetic modification.

Initially, comparative genomics were conducted based on the *C. merolae* genome sequence and an EST set of *G. sulphuraria* (Barbier et al., 2005; Weber et al., 2004). Finally, the 13.7 million bp genome sequence of *G. sulphuraria* was published in 2013 (Schonknecht et al., 2013). To date, full genomes of 13 Cyanidiales strains have been reported, isolated from Italy, the Czech Republic, Spain, Azores, the USA, Taiwan and Indonesia, and including authentic strains derived from

the type material of *G. sulphuraria*, *G. phlegrea* and *C. merolae* (Rossoni et al., 2019a). Notably, the authors found a low frequency (1%) of horizontal gene transfer among the sequenced species and their finding may have implications for the entire eukaryotic lineage (Rossoni et al., 2019a). However, *G. sulphuraria* was shown to contain 5% of horizontally transferred genes, and with RNA-seq analyses these genes were identified as conferring polyextremophilic traits like cold tolerance and a concomitantly altered carbon metabolism, even affecting photosynthesis (Rossoni, Schönknecht, & Lee, 2019b). Obviously, the horizontal gene transfer to *G. sulphuraria* is responsible for the adaptation of this alga to extreme environments (Rossoni et al., 2019a).

Despite the available *G. sulphuraria* nuclear genome sequences, progress has only been made in genetic and metabolic engineering with *C. merolae* (Matsuzaki et al., 2004; Minoda et al., 2004; Fujiwara et al., 2013; Zienkiewicz et al., 2017a, 2017b) and these initial experiments offer great opportunities for homologous or heterologous protein expression.

The entire genomic and organellar DNA sequences of *C. merolae* are available and thus, the codon usage of an expressed heterologous target gene can be optimized, and sites of gene integration by homologous recombination are easily defined (Nozaki, 2007). Harboring a haploid genome, *C. merolae* is ideal for gene modification by homologous recombination – to replace a wild-type gene by a mutated version, alter a promoter, introduce heterologous genes or delete endogenous ones. Nuclear gene modification is well established (see below), and organellar gene modification has also been reported (Zienkiewicz et al., 2017a).

For *C. merolae*, a reliable PEG transformation protocol has been published (Ohnuma et al., 2009; 2008), optimized (Ohnuma et al., 2011) and is now used as the standard in the field. Several studies have documented successful PEG transformations, e.g., with B tubulin or tubulin (Ohnuma et al., 2009; 2008). One potentially useful method for rapidly selecting transformants is cell sorting. Flow cytometry has been reported in *C. merolae* to assess cell density and viability (Nikolova et al., 2017), but there have not yet been any demonstrations that *C. merolae* cells can be grown after cell sorting.

For a stable transformation, selectable markers are required. One potential drawback to growing microbes at acidic pH is the possibility that antibiotics or other small molecule drugs could be degraded in the medium. This has recently been reported for CDK inhibitors (Kobayashi & Tanaka, 2018) and may explain some of the apparent antibiotic resistance of *C. merolae*. Therefore, initially auxotrophic strains were characterized for their suitability for transformation protocols.

The endogenous URA5.3 gene has been used as a selectable marker for homologous recombination in the uracil auxotrophic strain M4 (Fujiwara et al., 2013), but integration at the target locus is competed by integration at the URA5.3 locus. When the *Galdieria* URA5.3 gene was used as the marker, this competition was markedly reduced (Imamura et al., 2010). More recently, a uracil auxotroph with a complete URA5.3 deletion has been reported that circumvents problems with integration at the URA5.3 locus (Taki et al., 2015). Finally, a second selection marker for *C. merolae* has been introduced – chloramphenicol acetyl transferase, or CAT, which can be stably integrated into the nuclear and also chloroplast genomes (Fujiwara et al., 2017; Zienkiewicz et al., 2017a). Using the CAT marker, the authors were able to determine that 200 bp of flanking homology is the minimum required for homologous recombination of DNA into the genome, but 500 bp substantially increases the recombination efficiency (Fujiwara et al., 2017). However, a stable vector for plasmid-based expression for *C. merolae* is not yet available and would greatly advance this red alga as an expression host.

With methods available to introduce and select for heterologous DNA, the next question is how to regulate expression of the transgene(s) by suitable promoters. Several target promoters have been identified. Watanabe et al. (2011) report successful implementation of the phycocyanin-associated rod linker protein promoter, APCCp. This promoter is strongly induced under illumination, and was used in their study to drive GFP expression and a CENH3-GFP fusion protein. Furthermore, Imamura et al. (2010) identified a nitrogen-responsive transcription factor, CmMyb1, that up-regulates nitrogen assimilation genes in response to nitrogen depletion (Imamura et al., 2010). CmMyb1 acts by binding several promoters, including NRp, NRTp and NiRp, which were subsequently shown to be effective regulators of heterologous expression (Fujiwara et al., 2015). Similarly, a 200 bp region containing the promoter from the CmHsp20 gene was found to reliably induce expression of two different genes in response to a shift to temperatures above 50° C (Sumiya et al., 2014).

Two methods for down-regulation of genes are to either express anti-sense RNA, complementary to the gene of interest, or simply to delete the gene by homologous integration of a selectable marker. As *C. merolae* lacks the RNAi machinery (Casas-Mollano et al., 2008), gene expression can be down-regulated by expression of an anti-sense RNA, as reported for the catalase gene (Ohnuma et al., 2009). Regarding homologous recombination, Imamura et al. (2010) report knocking out the

nitrite reductase gene by targeting a selectable marker to the appropriate locus. Both methods appear to be reliable for reducing gene expression, with transient expression of anti-sense RNA being substantially faster as transformed cells can be selected within 24 h.

For cells growing at elevated temperatures, an elaborate protein folding machinery, e.g., chaperones are required. Since *C. merolae* grows up to temperatures of 60°C, we expect the presence of specialized chaperones that aid protein solubility and stability and thus compensate for the elevated temperatures in the hot sulphur springs (Kobayashi et al., 2010). Nevertheless, stable expression of GFP required the use of the superfolder variant (Sumiya et al., 2014), and to date there are only a few reports of other heterologously expressed genes in *C. merolae*.

In summary, the basic tools for heterologous gene expression in *C. merolae* are available, but have so far only been used in the context of homologous recombination. There is still no suitable self-replicating – and thus stable – plasmid for *C. merolae* which would facilitate fast and reliable expression trials.




## The future for exploitation of Cyanidiales

Both *G. sulphuraria* and *C. merolae* have unique advantages for biotechnological applications – on one hand an efficient generation of biomass for *Galdieria* and on the other an advanced understanding of the genome and options for genetic manipulation in *C. merolae* (Table 1). It is evident that knowledge of these organisms could be combined, as shown by generating modified *C. merolae* strains with metabolite transporters from *G. sulphuraria* (Fujiwara et al., 2019). Lately, the potential of Cyanidiales as a biotechnological resource has increasingly been recognized. Cultivation procedures, extraction protocols and the validation of biomass extracts for applications in human health and food have been developed, however, only for a handful of strains. Public and private culture collections maintain more than 500 Cyanidiales strains, isolated globally from different habitats. These precious bioresources harbour great potential to further advance the selection for suitable strains for various applications. Activities related to cell banking and strain development have recently been initiated and are indispensable for the establishment of biotechnological processes.

## Disclosure statement

No potential conflict of interest was reported by the authors.

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