Protistology 15 (3), 107–126 (2021)

Protistology

New life for old discovery: amazing story about how bacterial predation on *Chlorella* resolved a paradox of dark cyanobacteria and gave the key to early history of oxygenic photosynthesis and aerobic respiration

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| Submitted August 13, 2021 | Accepted September 9, 2021 |

Summary

In late 1970s, Russian microbiologists B. Gromov and K. Mamkaeva discovered chlorophyll-less bacterium *Vampirovibrio chlorellavorus* attacking *Chlorella vulgaris* and described its morphology and life cycle, although misattributed it to the phylum Proteobacteria. Over four decades, freeze-dried samples of infected *Chlorella* have been stored in oblivion, until in the early 2010s "proteobacterial" predator was reattributed to the phylum Cyanobacteria. *V. chlorellavorus*, the type species of the order Vampirovibrionales within the class Vampirovibrionia became the first, and to date unique cultured "dark" (non-photosynthetic, chlorophyll-less) cyanobacterium in contrast to "light" (photosynthetic, chlorophyll-containing) members of the class Oxyphotobacteria that habitually encompassed the phylum Cyanobacteria. Thus, taxonomic reattribution of *V. chlorellavorus* confirmed the early suggestions that cyanobacteria (blue-green algae) were not only photosynthetic microorganisms. Consequent metagenomic studies have extended the described diversity of dark cyanobacteria: besides Vampirovibrionales, the class Vampirovibrionia was shown to contain the orders Gastranaerophilales, Obscuribacterales, and Caenarcanales embracing metabolically diverse species with different lifestyles from development in ground water to obligate symbiosis with microalgae and oxymonad protists. Metagenomic research of dark cyanobacteria over the past decade elicited three phyla sibling to Cyanobacteria – Blackallbacteria (former Sericytochromatia), Margulisbacteria, and Saganbacteria. Comparative analysis and annotation of their metagenome-assembled genomes (MAGs) revived the discussion on the origin of oxygenic photosynthesis and aerobic respiration, primarily focusing at dilemma "dark cyanobacteria: primordial or late". Thus, besides opening separate page in research of symbioses between protists and bacteria, and apart from looking deeper into diversity of cyanobacteria, the discovery of *V. chlorellavous* got a new life within evolutionary biology mainstream.

Key words: aerobic respiration, *Chlorella*, cyanobacteria, oxygenic photosynthesis, predatory bacteria, *Vampirovibrio chlorellavorus*, Melainabacteria/Vampirovibrionia

doi:10.21685/1680-0826-2021-15-3-2

Introduction

Although this story attains apotheosis in new evolutionary scenarios for oxygenic photosynthesis and aerobic respiration, it traces back to a sophisticated symbiosis between bacteria and protists (note: here protists are interpreted *sensu lato* as unicellular eukaryotes; see Adl et al., 2012). Despite the up-to-date taxonomic perception of protists relies on molecular phylogeny criteria (Adl et al., 2019), an overall "metabolic" classification is habitually used, and it distinguishes between protozoan phagotrophs, microalgal photosynthetizers including facultative heterotrophs, and microfungal obligate osmotrophs. Here, we report on some representatives of the second group.

Microalgae produce symbioses with various partners, interactions with bacteria ranging from mutually beneficial to parasitic and even predatory (predatory bacteria are those which hunt actively to kill prey microorganisms and consume low molecular products of their post-mortal degradation). Specific strategies of bacterial predation are: i) association, when predator does not attach to the prey, but collectively ("wolf pack" strategy) or individually ("fortuitous" strategy) secretes detrimental hydrolytic enzymes; ii) epibiosis, when predator attaches to the prey; iii) endobiosis, when predator penetrates periplasmic or cytoplasmic compartment of the prey. In particular, periplasmic predators *Bdellovibrio* spp. and *Bdellovibrio*-and-like-organisms (BALOs) that feed on gram-negative bacteria, are widespread and studied in detail (Pérez et al., 2016).

Bacterial predation on microalgae has been extensively explored (e.g., Furusawa et al., 2003; Mayali and Azam, 2004; Kim et al., 2009). Main predators were found among the genera *Alteromonas* and *Pseudoalteromonas* (the class Gammaproteobacteria), as well as *Cytophaga* and *Saprospira* (the phylum Bacteroidetes). In turn, main preys belonged to algal classes Bacillariophyceae, Chlorophyceae, Dinophyceae, Haptophyceae, Rhaphidophyceae, and Rhodophyceae. To note, most of research has been concentrated on monitoring of microbial loop and hazardous bloom biocontrol, while only rarely intimate details of algal-bacterial interaction were characterized. It was all the more surprising to find epibiotic predation in cyanobacteria – paradoxical phenomenon described by Gromov and Mamkaeva (1972). In fact, until the early 2010s, all cyanobacteria have been considered "light" (photosynthetic, chlorophyll-containing) microorganisms. Thus, early report on *C. vulgaris* attacked by vibrioid predator, which was later identified as cyanobacterium (Di Rienzi et al., 2013; Yarza et al., 2013), as well as current plethora of data on other "dark" (non-photosynthetic, chlorophyll-less) cyanobacteria are not only important from viewpoints of microbiology and protistology, but they also receive great response from symbiotic ecology and evolutionary biology.

Here, we revisit the discovery and properties of cyanobacterium attacking *Chlorella* spp. as well as describe the diversity of dark cyanobacteria. Taking into account that the analyses of such cyanobacteria and their genomes (Di Rienzi et al., 2013; Soo et al., 2014, 2017; Sánchez-Baracaldo and Cardona, 2020; Oliver et al., 2021) have dramatically challenged the concepts of evolutionary bioenergetics, we also dwell upon this issue in detail.

For obvious reason, we could not constrain our analysis by focusing on only predatory and other dark cyanobacteria, and thus commence the review with a momentary excursion into diversity of light cyanobacteria, their photosynthetic apparatuses, and their symbioses.

Light cyanobacteria: general prelude

For many years, phycologists have considered cyanobacteria botanical objects and named them blue-green algae (in search of consensus with bacteriology, the counterproductive term "cyanoprokaryotes" was coined; Komárek et al., 2014). However, as early as in the 1970s these microorganisms were reconsidered in light of prokaryotic concept (Stanier and Cohen-Bazire, 1977), and attributed to the phylum Cyanobacteria (Bonen et al., 1979; Castenholz, 2001). Notably, with the advent of standardized taxonomy supported by the Genome Taxonomy Database (GTDB; Parks et al., 2018), phyla were proposed to be denoted by the suffix –ota (Whitman et al., 2018). However, renaming Cyanobacteria into "Cyanobacteriota" would be premature because these bacteria have not been placed under coverage of the International Code of Nomenclature of Prokaryotes (ICNP; see Pinevich, 2015).

Within the evolutionary tree (Fig. 1), the phylum Cyanobacteria is represented by two principal groups. The first group termed "the crown group" (Shih et al., 2017) embraces non-archaic light cyanobacteria as well as chloroplasts of Chlorophyceae, Charophyceae, and green plants.

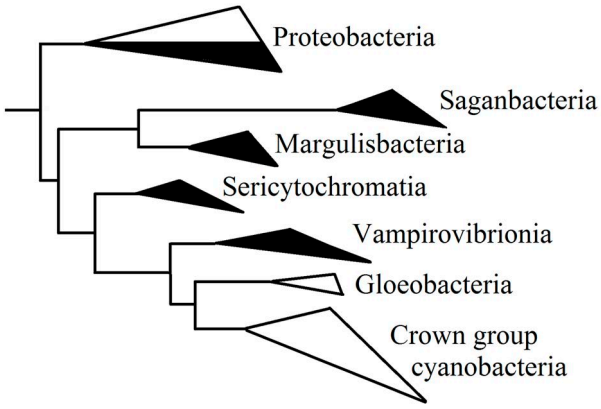

Fig. 1. Phylogenetic tree of cyanobacteria based on 16S rRNA sequences (modified from: Garcia-Pichel et al., 2020; Grettenberger et al., 2020). White and black triangles denote photosynthetic and non-photosynthetic representatives, respectively.

This type of chloroplasts is termed "simple" or "primary" due to their first-hand origin from deep branching cyanobacteria, with *Gloeomargarita lithophora* as present-day closest relative (Ponce-Toledo et al., 2016). The second group is archaic; it includes several genera of light cyanobacteria from the new class Gloeobacteria (Couradeau et al., 2012; Grettenberger et al., 2020; Rahmatpour et al., 2021), as well as dark cyanobacteria from the class Vampirovibrionia. The phyla sibling to Cyanobacteria are Sericytochromatia, Margulisbacteria, and Saganbacteria (see below).

Pigment moiety of cyanobacterial photosynthetic apparatus is distributed between two reaction centers (and corresponding core antennae) and light-harvesting complexes that are either membrane-anchored or membrane-embedded (Averina et al., 2018). Cyanobacteria produce different chlorophylls: *a, a*$_2$ (3,8-divinyl form of *a*), *b, b*$_2$ (3,8-divinyl form of *b*), *c, d*, and *f*. Chlorophylls *a, a*$_2$, *d* (and possibly *f*) are reaction center and core antenna pigments (sometimes also light-harvesting complex pigments), while chlorophylls *b, b*$_2$ or *c* participate only in light-harvesting complexes (Averina et al., 2019). In relation to chlorophyll subset, cyanobacteria can be subdivided into one-chlorophyll group, two-chlorophyll group, and multi-chlorophyll group (see below).

Symbioses of light cyanobacteria are extremely diverse, with the partners spanning from cyanophages (Gromov, 1983) to higher plants (Bergman et al., 1992) and vertebrates (Venn et al., 2008). In the case of protists that generally produce a variety of symbioses, the partnership with cyanobacteria is especially important (Gavelis and Gile, 2018). In fact, ancient merger of a protist with a cyanobacterium (that turned into chloroplast) resulted in the holistic assemblage of global significance.

One-chlorophyll (blue-green) cyanobacteria

One-chlorophyll cyanobacteria belong to the crown group. Here, chlorophyll *a* is part of reaction centers and core antennae. The light-harvesting complex is the so-called standard phycobilisome, the hemi-discoid ~50 nm assemblage of phycobiliproteins and linkers bound to the thylakoid membrane (rarely, to the plasmalemma). The content and ratio of phycobiliproteins vary in response to environmental stresses, as well as during complementary chromatic adaptation (Grossman et al., 1993) or far-red light photoacclimation (FaRLiP; Averina et al., 2018). Within the archaic group, one-chlorophyll cyanobacteria comprise the class Gloeobacteria with the species *Gloeobacter violaceus, G. kilaueensis, Anthocerotibacter panamensis*, and *Candidatus* Aurora vandensis (Rahmatpour et al., 2021). In this case, phycobilisome has non-standard cylindrical form and specific subunit array (Grettenberger et al., 2020). Depending on ratios of chlorophyll *a*, phycobiliproteins, and carotenoids cyanobacteria can substitute trivial blue-green pigmentation with blue, red, violet or yellow-brown color (Pinevich et al., 1994).

Two-chlorophyll (green) cyanobacteria

These crown group cyanobacteria are represented by fresh or brackish water species *Prochlorothrix hollandica* and *P. scandica* (Pinevich et al., 2012). Their reaction centers incorporate chlorophyll *a*; light-harvesting complex is embedded in thylakoid membrane and contains chlorophylls *a* and *b*. Due to the same chlorophylls with green algae, these species as well as other chlorophylls *a*/*b*-containing cyanobacteria are named "green cyanobacteria" (Pinevich et al., 2012). They were initially identified as the algal division Prochlorophyta and trivially named "prochlorophytes" (Lewin, 1981). However, the prefix pro- is used to designate ancestry, while a cyanobacterium cannot alone form endosymbiotic association (Pinevich, 2020b). Besides, two-chlorophyll cyanobacteria cannot be chloroplast

ancestors because their chlorophyll *a/b*-proteins are dissimilar from chloroplast counterparts (Bullerjahn et al., 1987). This implies an independent origin of crown group cyanobacteria and simple chloroplasts from common ancestor which possibly had the genes coding for chlorophyll *a/b*-proteins and the genes for phycobilisome biogenesis (Pinevich, 2020a).

Multi-chlorophyll cyanobacteria

These crown group cyanobacteria produce at least three chlorophylls. For instance, *Prochloron didemni*, an epibiont of colonial ascidians has chlorophylls *a, b,* and *c* (Larkum et al., 1994). Of note, the so-called chlorophyll *c* differs from chlorophylls proper because it combines the traits of porphyrins (double bond in C17–C18 position; 22 π electrons) and chlorophyllides (phytyl tail absent). In the case of *P. didemni*, chlorophyll *c* (~10% of total chlorophyll) is thought to be part of light-harvesting complex together with chlorophylls *a* and *b* (Larkum et al., 1994). Also, minor chlorophyll *c* was found in *Prochlorococcus marinus*, an ultra-small bacterium, which inhabits low latitude euphotic zone of oceans (Chisholm et al., 1988). In the case of *P. marinus*, chlorophylls *a* and *b* are replaced with a_2 and b_2 (some strains produce both forms of *b*; Goericke and Repeta, 1992). The function of chlorophyll *c* in *P. marinus* is obscure; most likely, this pigment associates with light-harvesting complex. Chlorophyll *c* was also found in *Acaryochloris marina* which, in analogy to *P. didemni*, is a symbiont of ascidians (Miyashita et al., 1997), inhabits white light depleted niches, and constitutively produces far-red light (700–750 nm) absorbing chlorophyll *d* which partially replaces chlorophyll *a* in reaction centers, and dominates in the light-harvesting complex (Averina et al., 2019). In analogy to chlorophyll *c* of *P. marinus*, chlorophyll *c* in *A. marina* is proposed to be light harvesting complex pigment. The strain RCC1774 isolated from foreshore near Roscoff (France) is related to *Acaryochloris* genus although it lacks chlorophyll *d* (Partensky et al., 2018). Instead, it possesses chlorophylls *a* and *b* at molar ratio of ~6 (close to that in *Prochloron* and *Prochlorothrix*). Based on phylogeny, ultrastructure, and pigment suite, it is described as a novel species *A. thomasi.*

Notably, in the abovementioned cases chlorophylls are constitutively produced in niches penetrated by white light (*P. didemni, P. marinus*) or enriched with far-red light (*A. marina*). Unlike them, some one-chlorophyll strains produce accessory chlorophylls if light maximum moves to infrared region, and thus the FaRLiP response is induced (Averina et al., 2018). Cyanobacteria that inducibly produce far-red light absorbing chlorophylls *d* and *f* comprise a small number of filamentous species (e.g., *Halomicronema hongdechloris*; Chen et al., 2012) or unicellular species (e.g., *Altericista variichlora*; Averina et al., 2021). They customarily form marine and freshwater biofilms, or biofilms in springs, bogged soil, and karst caves. Functions of inducible chlorophylls *d* and *f* are poorly understood, although energy coupling of chlorophyll *f* with chlorophyll *a* on the periphery of photosystems is most plausible (Itoh et al., 2015).

Dark cyanobacteria: prior to discovery

Evolutionary tree of bacteria is paradoxical in that genotypic relatedness is distinct from phenotypic resemblance matrix (this phenomenon is also observed in protists, but not in higher organisms). Thus, on the one hand, contrast phenotypes can be met within a separate lineage. On the other hand, similar phenotypes can occur within distant lineages. Photosynthesis is an especially striking example: at present, eight phyla – Acidobacteria, Chloroflexi, Fibrobacteres-Chlorobi-Bacteroidetes, Firmicutes, Gemmatimonadetes, and *Candidatus* Eremiobacterota (WPS-2) have been shown to contain both light and dark representatives (Cardona, 2015; Hug et al., 2016; Ward et al., 2019). At the same time, over a long time span the phylum Cyanobacteria has not been shown to include dark representatives although a reality of such microorganisms was suggested by some phycologists and bacteriologists.

Thus, Ferdinand Cohn, one from the cohort of great bacteriologists, pointed out that blue-green algae and morphologically similar chlorophyll-less microorganisms (non-photosynthetic bacteria, in current terminology) represented two related taxonomic groups (Cohn, 1875). He also concluded that the former group should be treated as the class Schizophyceae (S[c]hi.zo´phy.ce[a]e; Gr. v. *shizo* to split; Gr. n. *phycos* a seaweed; *-phyceae* ending to designate class in botany; Schizophyceae algae reproducing by binary fission). In turn, he concluded that the latter group should be treated as the class Schizomycetes (S[c]hi.zo´my.ce.tes; Gr. v. *shizo* to split; Gr. n. *mykes* a fungus; *-myceae* ending which at that time designated fungal class; Schizomyceae

fungi reproducing by binary fission). According to F. Cohn, both these classes should be placed together in the lower plant division Schizophyta (S[c]hi.zo´phy.ta; Gr. v. *shizo* to split; Gr. n. *phyton* a plant; -*phyta* ending to designate a division in botany; Schizophyta plants reproducing by binary fission). In treatises on plant taxonomy, e.g., by Russian botanist Christian Gobi (1916), the class Bacteriata (= Schizophyta Cohn) was subdivided in two subclasses: Bacteriomorpha Gobi, i.e. chlorophyll-less bacteria, and Cyanomorpha Gobi (= Schizophyceae Cohn), i.e. bacteria morphologically similar to blue-green algae. In particular, the subclass Cyanomorpha was suggested to include, besides chlorophyll-containing genera, also apochlorotic genera (L. prefix *apo*- a loss of; Gr. adj. *chloros* green; chlorophyll-less). Notably, a resemblance between filamentous blue-green algae and filamentous chlorophyll-less bacteria had been noticed just near to the end of the XIX century (hence, early proposals of their relatedness; see De Bary, 1885; Fischer, 1897). Moreover, such bacteria, e.g., *Beggiatoa* and *Leptothrix*, were thought to represent regressive Cyanomorpha (= Trichobacterineae; Fischer 1897).

Thus, over more than a century (see: Reichenbach, 1981), chlorophyll-less filamentous bacteria *Leucothrix, Saprospira, Vitreoscilla*, and similar heterotrophic genera have been considered apochlorotic analogues of colored cyanobacteria. However, with the invention and improvement of molecular phylogeny methods, these bacteria were shown to be unrelated to photosynthetic counterparts, e.g., filamentous cyanobacteria *Oscillatoria* spp. (Reichenbach et al., 1986). Deeper insight into 16S phylogeny attributed *Leptothrix* (Spring and Kämpfer, 2005), *Sphaerotilus* (Kämpfer and Spring, 2005), and *Vitreoscilla* (Strohl, 2005b) to the class Betaproteobacteria. In turn, *Beggiatoa* (Strohl, 2005a) and *Leucothrix* (Bland and Brock, 2005) were assigned to the class Gammaproteobacteria. Finally, *Lewinella* (former Herpetosiphon) (Sly and Fegan, 2011) and *Saprospira* (Lewin, 2011) were shown to belong to the phylum Bacteroidetes.

Nevertheless, no negative data could prove total "absence of presence" of dark cyanobacteria, especially those with another morphology and/or physiology than in *Beggiatoa, Leptothrix*, and other gliding filamentous bacteria. In particular, these hypothetical objects were inferred to associate with the most archaic branches of cyanobacterial tree (Pinevich, 1991). However, real advent of dark cyanobacteria has happened two decades later (Di Rienzi et al., 2013). This break-through took root in the finding of predatory bacterium initially assigned to the genus *Bdellovibrio* (see Williams et al., 2005) as the species *B. chlorellavorus* (Gromov and Mamkaeva, 1972), but later renamed *Vampirovibrio chlorellavorus* gen. and sp. nov. (Gromov and Mamkaeva, 1980a, 1980b). In other words, the discovery of rare predatory bacterium, followed after four decades by the elucidation of its true phylogeny, confirmed previous assumptions that dark cyanobacteria *de facto* exist.

The first known chlorophyll-less cyanobacterium *Vampirovibrio chlorellavorus*: its odyssey

In the beginning of the 1960s, occasional declines of *Chlorella* spp. in outdoor mass cultures monitored in Leningrad, Russia (see: Pinevich and Verzilin, 1963) gave rise for the attempts to find out an infectious agent, which provoked algal cell yellowing and death (Mamkaeva, 1966). Thus, laboratory studies on *C. vulgaris* cultures inoculated with samples from the aforementioned pilot plant (incorrect references to water reservoir in the Ukraine are habitual; see, e.g.: Coder and Starr, 1978; Hovde et al., 2020) demonstrated, after 5–7 days of dark incubation, dramatic changes of algal cells – clumping, bleaching from green to yellow-green to colorless, and formation of refractile bodies (Gromov and Mamkaeva, 1972). Attached vibrioid bacteria were named, in analogy to predatory BALOs, *Bdellovibrio chlorellavorus* (Bdel.lo.vi´bri.o. Gr. n. *bdella* a leech; L. m. n. *Vibrio* a generic name; L. n. *Bdellovibrio* a leech-like vibrio; *B. chlo.rel.la.vo´rus.* L. f. n. *Chlorella* a genus of algae; L. v. *voro* to devour; L. adj. *chlorellavorus Chlorella*-devouring). The first EM survey of attached bacteria viewed a pad (holdfast) produced by the predator to adhere to prey cell (Mamkaeva and Rybal'chenko, 1979; the detail later pinpointed by Park et al. 2018). In 1978, the "lysate" (i.e. lyophylized co-culture of *C. vulgaris* and *B. chlorellavorus*) supplied directly by Russian discoverers was deposited at three respected microbial culture collections under the indexes ICBP 3707, NCIMB 11383, and ATCC 29753 (Coder and Starr, 1978).

Contrary to the initial taxonomic diagnosis offered by Gromov and Mamkaeva (1972), it was concluded that chlorellavorous bacterium should not be ascribed to the genus *Bdellovibrio* mainly because of a sharp difference in predation strategy

– epibiotic rather than endobiotic (Coder and Starr, 1978). Soon thereafter, Gromov and Mamkaeva (1980a, 1980b) offered emended generic name for this bacterium – *Vampirovibrio* (Vam.pi.ro.vi´bri.o. Fr. n. *vampire* a vampire; L. m. n. *Vibrio* a generic name; L. n. *Vampirovibrio* vibrio [that sucks out the prey contents] as a vampire). However, initial higher-level taxonomic assignment was retained (order Bdellovibrionales, class Deltaproteobacteria, phylum Proteobactertia; see: Baer and Williams, 2005b). In fact, this bacterium was similar to the type genus *Bdellovibrio* in predatory ability, morphology (except an unsheathed flagellum), and in nearly the same mol% GC of the DNA.

Of note, the order Bdellovibrionales has been transferred to the new class Oligoflexia recently proposed for the phylum Proteobacteria (Hahn et al., 2017). Even more recently, based on the standardized classification network supported by the GTDB database (Parks et al., 2018), the new phylum Bdellovibrionota has been proposed (Waite et al., 2020).

Phenotype characters of *V. chlorellavorus* sufficed for a separate taxonomic treatment of this bacterium. First, it could not develop without its prey, and was feeding only on the prey's living cell. Second, it attacked preys within a narrow taxonomic spectrum: namely, only 31 strains of *Chlorella vulgaris, C. sorokiniana*, and *C. kessleri* (from the total number of 76 tested strains) became preys in contrast to only two strains of two other species, *C. saccarophila* and *C. luteoviridis* (Coder and Goff, 1986). The observed high host specificity could be due to similar cell surface signatures in attacked strains (Burczyk et al., 1979). Notably, *V. chlorellavorus* was initially considered the relative of another vibrioid bacterium, *Micavibrio admirandus* (Lambina et al., 1982; Baer and Williams, 2005a), which also had vampire lifestyle, although fed on a broad range of Gram-negative bacterial preys. However, *M. admirandus* has been shown to belong to the Alphaproteobacteria BALOs (a-BALOs) subgroup (Pasternak et al., 2013) while *V. chlorellavorus* unexpectedly turned out to be a representative of the phylum Cyanobacteria (see below).

The details of *V. chlorellavorus* predatory life cycle (Figs 2 and 3) were reconstructed based on morphological images (Gromov and Mamkaeva, 1972; Coder and Starr, 1978; Mamkaeva and Rybal'chenko, 1979) and genomic deduction (Soo et al., 2015). Main stages are as follows: i) prey location, attack, and adhesion promoted by

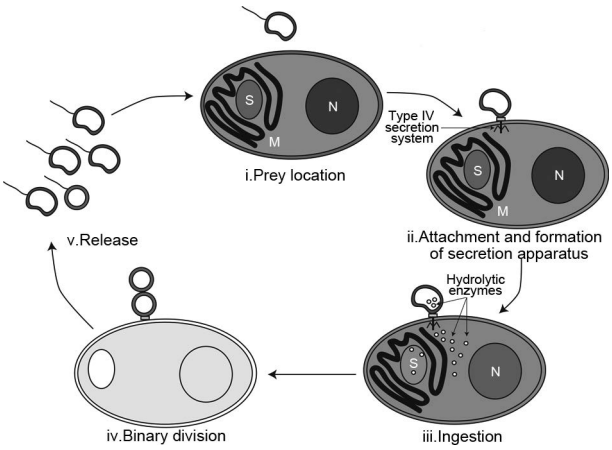

Fig. 2. Proposed predatory life cycle of *Vampirovibrio chlorellavorus* (modified from: Soo et al., 2015). See the text for explanation.

aerotaxis system, as well as by the flagellum and type-IV pili (TFP); ii) attachment, formation of conjugative type-IV secretion system (T4SS), and injection of the T-plasmid single-stranded DNA and hydrolytic enzymes into prey cell; iii) lysis of the prey contents, excretion of obtained products using efflux transporters encoded by the T-plasmid, and import of these products into predator cell using its ABC transporters; iv) binary fission of attached predator cell; v) escape of flagellated progeny, and their dispersal in hunting for a new prey. This scenario essentially differs from the endobiotic predation strategy of the FD111 a-BALO attacking the microalga *Nannochloropsis salina* (Lee et al., 2018).

Meanwhile, in the framework of Living Tree Project (Di Rienzi et al., 2013; Yarza et al., 2013), the American Type Culture Collection team sequenced the 16S rRNA gene of *V. chlorellavorus*. DNA template was extracted directly from freeze-dried sample ATCC 29753 (because of failed attempts to revive cyanobacteria from this state; see: Corbett and Parker, 1976). Nucleotide sequence (the GenBank database accession number HM038000) was compared with the Greengenes (McDonald et al., 2012) and SILVA (Quast et al., 2013) database matches. Unexpectedly, close relatedness was shown to the basal lineage SM1D11 of the phylum Cyanobacteria rather than to the Proteobacteria.

Several years later, template DNA directly extracted from sample 11383 of the NCIMB collection was used by Soo et al. (2015) to assemble a near-complete *V. chlorellavorus* genome (the GenBank database accession number GCA_001858525.1). The genome encoded full array of the EMP path-

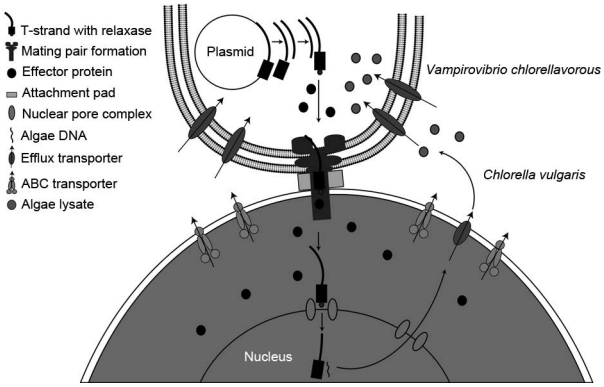

Fig. 3. Proposed conjugative mechanism in *Vampirovibrio chlorellavorus* (modified from: Soo et al., 2015). See the text for explanation.

way, oxidative pentose phosphate pathway, and the TCA cycle enzymes. Also, annotation data witnessed for the respiratory chain with Complexes I–IV, as well as for cbb_3-type cytochrome oxidase and *bd*-type quinol oxidase (both oxidases are involved in microaerobic respiration; see: Preisig et al., 1996). In addition, the genes to produce lactate via fermentation in low oxygen conditions were annotated. Additionally, *V. chlorellavorus* had the genes to produce nucleotides and coenzymes although it lacked the genes for synthesis of several amino acids.

Two *V. chlorellavorus* draft MAGs (metagenome-assembled genomes; accession numbers GCA_003149345.1 and GCA_003149375.1) were recovered from Vc_AZ_1 and Vc_AZ_2 isolates. These samples were obtained from algal cultivation ponds, Los Alamos National Laboratory, Tucson, AZ, USA, and repeatedly transferred on the culture of *C. sorokiniana* (Park et al., 2018; Hovde et al., 2020). The MAGs contained the genes responsible for predator cell cycle (see above). In particular, annotation data helped to identify pathogenicity proteins, virulence system (Vir) with the T4SS secretion apparatus, as well as motility and quorum sensing proteins. Notably, the MAG of Vc_AZ_1 isolate differed from the MAG of Vc_AZ_2 isolate in that it lacked orthologs of VirB gene with the sample NCIMB 11383 (Soo et al., 2015). Additionally, both Arizona isolates lacked plasmids. Thus, the availability of at least three divergent annotated MAGs of *V. chlorellavorus* showed that this genus was more genetically and functionally diverse than proposed at the beginning of its study.

In addition to annotated genomes of *V. chlorellavorus*, four non-annotated draft MAGs were deposited in 2020 at the GenBank database (accession numbers GCA_903839075.1, GCA_903835245.1, GCA_903866275.1, and GCA_903826385.1). They were all assembled from environmental DNA templates originating from a lake near Umeå city, Sweden.

The overall distribution of *V. chlorellavorus* (the type species of new order Vampirovibrionales) has also been evaluated. Besides two complete 16S rRNA gene sequences of collection samples ICBP 3707 and ATCC 29753 (accession numbers NR_104911.1 and HM038000.1 respectively), as well as of uncultured clone P36-3 (Ganuza et al., 2016; accession number KU570459.1), the GenBank database query yields more than a hundred 245–460 bp fragments annotated as "Uncultured *Vampirovibrio* sp." and originating from different locations and environments.

Taking into account comparatively broad distribution of *V. chlorellavorus*, this predator should be considered hazardous to *Chlorella* farming, hence the need of an effective in situ detection (Steichen and Brown, 2018), as well as of a search for host resistance mechanisms and protective measures during industrial *Chlorella* cultivation (Bagwell et al., 2016; Ganuza et al., 2016; Park et al., 2018).

To summarize, *V. chlorellavorus* is of great basic and applied importance because this species is: a) one of comparatively rare examples of bacterial predators attacking microalgae; b) the first known epibiotic predatory bacterium, which uses conjugative type-IV secretion system to deliver destructive genes and enzymes into prey cell; c) the first dark cyanobacterium found; d) the only one cultured representative of dark cyanobacteria *sensu lato* known to date.

Dark cyanobacteria: expanded diversity

The knowledge on phylogenetic confines of the phylum Cyanobacteria, especially of dark cyanobacteria, has been propelled by metagenomic studies. In particular they elicited new deep-branching lineages, such as ML635J-21, mle1-12, SM1D11, SM2F09, and YS2/4C0d-2 (in Greengenes and SILVA classification).

A survey of human gut microbiota revealed 16S rRNA gene sequences clustered in the lineage initially thought to be sister clade with Cyanobacteria (Ley et al., 2005). The origin of these tags from ingested plant-based foods was ruled out because of their high divergence from chloroplast rDNA

sequences. Anoxic water and sediment samples were also found to contain the 16S rRNA gene sequences of cyanobacteria deep branching from the crown group (Ley et al., 2005).

The first MAG of dark cyanobacteria was recovered from an anaerobic microbial consortium degrading lignocellulose-rich biomass (van der Lelie et al., 2012). Despite the MAG contained no genes for photosynthetic apparatus, the researchers did not comment on taxonomic aspects of their finding. However, very soon such proposal appeared based on the assembly of eight partial and complete MAGs of cyanobacteria that inhabited human gut and aquifer sediment and belonged to the YS2/4C02d-2 lineage (Di Rienzi et al., 2013). These uncultured cyanobacteria deeply branched from the crown group, that empowered to propose for them the candidate phylum Melainabacteria (Me.la.ina.bac.te´ria; *Melaina*, the Greek nymph of dark waters; Gr. adj. *melas* black; Gr. n. *bakterion* a small rod; suff. -*ia* ending to denote a class; L. neut. pl. n. *Melainabacteria* dark [or light-independent] bacteria).

Annotated MAGs of Melainabacteria did not contain the genes for photosystems, electron transport chain, and carbon dioxide fixation, that explained the inability of photosynthesis. At the same time, the presence of the EMP pathway, oxidative pentose phosphate pathway, and the TCA cycle enzyme genes indicated that these bacteria were obligate fermenters. Notably, the MAGs from aquifer sediment sample encoded the genes for nitrogen fixation distinct from those in the crown group cyanobacteria. Additionally, most of analyzed MAGs contained the genes to produce functional flagella.

The next step in the analysis of Melainabacteria was to assess whether this clade actually represented sister phylum with Cyanobacteria, and whether it consisted of only fermenters as was initially proposed by Di Rienzi et al. (2013) in their study on the lineage YS2/4C02d-2. Broader phylogenetic coverage was offered by six near-complete MAGs representing the lineages mle1-12 and SM2F09 (Soo et al., 2014). Environmental samples used in this culture-independent molecular survey included: a) feces from *Phascolarctos cinereus* koala bear; b) activated sludge from EBPR (enhanced biological phosphorus removal) lab-scale reactor; c) activated sludge from UASB (upflow anaerobic sludge blanket) bioreactor to treat waste water from food-processing factory. Based on the comparative analysis of a dataset including 81 reference cyanobacterial genomes, six newly assembled MAGs, and five most complete MAGs previously obtained by Di Rienzi et al. (2013), as well as by using phylogeny criteria previously formulated by Hugenholtz et al. (1998), it was concluded that Melainabacteria and Cyanobacteria are in fact not sister phyla, but rather two classes. Correspondingly, the first class became Melainabacteria *sensu novo* (later renamed Vampirovibrionia; see: Soo et al., 2019). The second class (the former phylum Cyanobacteria) was termed Oxyphotobacteria (Gr. adj. *oxus* acid; in combined words indicating oxygen; Gr. n. *phos* light; Gr. n. *bakterion* a small rod; suff. -*ia* ending to denote a class; L. neut. pl. n. *Oxyphotobacteria* oxygen [producing] light [dependent] bacteria; see: Gibbons and Murray, 1978). Both these classes are now within the phylum Cyanobacteria *sensu novo*.

Based on 16S rRNA phylotyping, the MAGs analyzed by Soo et al. (2014) belong to three distantly related lineages classified as three candidate orders. Together with the order Vampirovibrionales they are within the class Melainabacteria/Vampirovibrionia (Table 1).

The first candidate order named Gastranaerophilales was reported for feces of koala *P. cinereus*, and it was represented by the type species *Candidatus* Gastranaerophilus phascolarctosicola (Gas.tra.na.ero.phi´lus, Gr. n. *gaster* stomach; Gr. pref. *an-* not; Gr. masc. n. *aer* air; L. masc. adj. *philus* loving; *Gastranaerophilus* [a bacterium] that inhabits anaerobic gastric niche; phas.col.arc.to.si´co.la, L. *Phascolarctos* the name of koala; L. suff. -*cola* ending to denote association with a certain environmental niche; L. masc. n. *phascolarctosicola* [a bacterium] living within a body of koala). The Gastranaerophilales MAGs (1.8–2.3 Mb, a single exception is 2.7 Mb) recovered from *P. cinereus* feces were similar with those obtained from human feces by Di Rienzi et al. (2013), and they also belonged to fermenters possessing the EMP pathway enzymes.

Additional data on the distribution and phylogeny of Gastranaerophilales has been obtained in the study of MAGs from different termite species, as well as in the analysis of SAGs (single cell sorting/genome sequencing/assembled genomes) from the gut of higher termite *Termes propinquus* (Utami et al., 2018). In 45 out of 60 termite samples, as many as eighty 16S rRNA phylotypes related to Gastroanaerophilales were recovered. Annotated SAGs (~1.6 Mbp) for the phylotype Tpq-Mel-01 had many features in common with other Gastranaerophilales. In particular, the genes for photosynthesis, respiration, carbon dioxide fixation, and nitrogen fixation were absent. Incomplete set of

Table 1. Main characters of species in the class Melainabacteria/Vampirovibrionia.

Order and lineage	Species name	Genome size	Metabolism	Flagella	Habitat
Vampirovibrionales (SM1D11)	*Vampirovibrio chlorellavorus*	~3 Mb	Microaerobic respiration or fermentation	+	Predatory epibiosis on *Chlorella vulgaris, C. kessleri*, and *C. sorokiniana*
Gastranaerophilales (YS2)	*Gastranaerophilus phascolarctosicola*, *G. termiticola*	~2 Mb	Fermentation	±	The gut of mammals or termites
Obscuribacterales (mle1-12)	*Obscuribacter phosphatis*	~5 Mb	Fermentation or respiration (aerobic or nitrate); diazotrophy	–	Digestors; ground water
Caenarcaniphilales (ACD20)	*Caenarcanum bioreactoricola*	~2 Mb	Fermentation	–	Anaerobic bioreactors

the TCA cycle genes was counterbalanced with the EMP pathway genes indicating strictly fermentative metabolism. The SAGs also encoded the genes of flagella and type-IV pili involved in swimming and twitching motility, as well as chemotaxis genes. In addition, FISH microscopy with the oligonucleotide probe MelTpq-646 helped to visualize 1.0 × 0.5 μm rod-shaped cells. For this bacterium, the species name *Candidatus* Gastranaerophilus termiticola was proposed (ter.mi.ti´co.la, L. gen. n. *termitis*, termite; L. suff. -*cola* ending to denote life within a certain niche; *G. termiticola*, [a bacterium] from the genus *Gastranaerophilus* that lives within [a body of] termite).

The second candidate order named Obscuribacterales, represented by the type species *Candidatus* Obscuribacter phosphatis (Ob.scu.ri. bac´ter, L. adj. *obscurus* dark; Gr. n. *bakterion* a small rod; *Obscuribacter* a bacterium living in the dark; L. adj. phos.pha´tis *phosphate*; L. masc. n. accumulating phosphate), was found in industrial digestor and ground water. This dark cyanobacterium was suggested to use aerobic or nitrate respiration, as well as mixed-type fermentation. Comparatively large MAG (~5 Mb) inferred a broader adaptation span to dynamic environments than in other Melainabacteria. In particular, *O. phosphatis* possessed a complete array of enzymes for inorganic phosphate uptake and polyphosphate metabolism (orthophosphate transporter, polyphosphate kinase, polyphosphate glucokinase, polyphosphate: AMP phosphotransferase, as well as endo- and exopolyphosphatases).

The third candidate order named Caenarcaniphilales (sic!) (note: the order name correctly derived from the type species name should be Caenarcanales) is represented by the species *Candidatus* Caenarcanum bioreactoricola which was found in an anaerobic pilot plant (Caen.ar.ca´num, L. neut. n. *caenum* sludge; L. neut. n. *arcanum* secret; L. neut. n. *Caenarcanum* [a bacterium] hidden in sludge; bio.re.ac.to.ri´co.la, L. n. *bioreactor* a bioreactor; L. suff. -*cola* ending to denote association with certain habitat; L. masc. n. *bioreactoricola* [a bacterium living] within a bioreactor). Annotated MAG (~2 Mb) lacked the genes for the TCA cycle and respiration, indicating a fermentative metabolism. Remarkably, this species has the lowest GC content (27.7%) ever reported for cyanobacteria.

Expanded knowledge on dark cyanobacteria *sensu lato* is due to the discovery by Soo et al. (2017) of first phylum sibling to Cyanobacteria – Sericytochromatia (Se.ri.cy.to.chro.ma´tia, Gr. adv. *sero* late; Engl. n. *cytochrome*, from Greek n. *kytos* vessel [living cell in biology] and Greek n. *chroma* pigment; suff. -*ia* ending to denote a class; new Latin neut. pl. n. *Sericytochromatia* [bacteria] with cytochromes acquired late [in evolution]). Notably, this curious name denotes a richer, probably later in evolution acquired array of respiratory proteins than in other cyanobacterial lineages (see below). Three draft Sericytochromatia MAGs representing the lineage ML635J-21 were recovered from different environments such as coal well biofilm, lab-scale bioreactor, and subsurface groundwater (Soo et al., 2017). Additionally, the presence of this deep branching group has been demonstrated in ten temperate lakes of the European peri-Alpine region (Monchamp et al., 2019). Near-complete MAGs of the Sericytochromatia were obtained for the lineages S15B-MN24, LSPB_72, and GL2-53 (Soo et al., 2017). All to date reported Sericytochromatia MAGs did not contain the genes for photosynthesis and carbon dioxide fixation.

Recent metagenomic study on the prokaryotic community in erupted water of cold Crystal Geyser, Paradox Basin, Colorado Plateau, Utah, USA revealed 104 phylum-level clades including those

sibling to Cyanobacteria. They were named preferentially in honor of prominent contemporary researches (Probst et al., 2018). In particular, the clade overlapping with Sericytochromatia was named Blackallbacteria after Australian microbial ecologist Linda Blackall. Further, sister clades GWF2-35-9 and TG2/ZB3 were jointly named Margulisbacteria after American evolutionary biologist Lynn Margulis. This phylum overlapped with terabase-scale metagenomics (see: Hofmeyr et al., 2020) detected clade RIF30 – one of 47 new phyla found in sediment and ground water samples (Anantharaman et al., 2016). Finally, the clade WOR-1 firstly discovered among the lineages from White Oak River, NC, USA (Baker at al., 2015) was named Saganbacteria after American astrophysicist, exobiologist and science popularizer Carl Sagan.

Within the phylum Margulisbacteria, two subgroups are detected. One subgroup is represented by the clade GWF2-35-9 (so designated in the GTDB Taxonomy Database; see: Parks et al., 2018). It was named Riflemargulisbacteria after the draft MAG from an aquifer adjacent to the Colorado River, Rifle city environs, CO, USA (Anantharaman et al., 2016). The other subgroup is represented by the TG2/ZB3 clade from 16S rDNA library of the Zodletone spring, OK, USA (Elshabed et al., 2003). It was named Marinamargulisbacteria after SAGs obtained from several oceanic sites. Margulisbacteria and Saganbacteria represent sibling clades distantly related to the monophyletic assemblage encompassing Vampirovibrionia, Sericytochromatia/Blackallbacteria, Gloeobacteria, and the crown group cyanobacteria (Matheus Carnevali et al., 2019) (see Fig. 1).

According to MAGs annotation, sediment-associated Riflemargulisbacteria are fermenters that use different hydrogenases and fix nitrogen. In turn, water column-associated Marinamargulisbacteria depend on aerobic respiration. Finally, Saganbacteria that live in ground waters with variable oxygen content (Anantharaman et al., 2016) are either fermenting or aerobically respiring cyanobacteria with terminal oxidase of a new type.

Analysis of 16S rDNA amplicons from the TG2/ZB3 clade in combination with FISH microscopy helped to elicit cyanobacteria epibiontic on the spirochete *Treponema* sp. which itself is the epibiont of oxymonad protists within the gut of higher termites *Reticulitermes speratus* and *Neothermes koshunensis* (Utami et al., 2019). These cyanobacteria were assigned to four species of the genus *Candidatus* Thermititenax (ter.mi.ti.te´nax, L. m. n. *termes* termite, L. adj. *tenax* tenacious; L. m. n. *termititenax* [bacteria] clinging to termites) that represents a separate lineage of the Margulisbacteria. According to MAG annotation data, *Termithitenax* spp. have a complete set of enzymes to convert cellulose into acetate and H_2/CO_2 while *Treponema* sp. recycles acetate from H_2/CO_2. In other words, bacterial consortium exploits the symbiotic mechanism of "interspecies hydrogen transfer", and supplies partner protist with acetate.

Another type of symbiosis has been recently demonstrated for Marinamargulisbacteria that live within ventral epithelial cells of the marine placozoan *Trichoplax* sp. haplotype H2 (Gruber-Vodicka et al., 2019). Molecular data were obtained using 1.51 Mb metagenome assembly and annotation, as well as proteomic and morphologic analysis. These dark cyanobacteria were assigned to the species *Candidatus* Ruthmannia eludens (Ruth´man.nia, after August Ruthmann, a devoted researcher of placozoans; L. adj. *eludens* escaping or deceiving, [bacteria] that escaped [detection better than other intracellular symbionts in metazoans, and withstood interpretation of a large part of genome due to absence of characterized homologs]). Rod-shaped 1.2 × 0.5 μm bacterial cells are within host phagosomes, and their aerobic heterotrophic metabolism is based on the complete TCA cycle to degrade lipid material supplied by the host. Notably, symbiotic bacteria withstand digestion and possibly supply the host with essential amino acids.

To summarize, MAG and SAG data empowered to attribute the classes Melainabacteria/Vampirovibrionia and Oxyphotobacteria to the phylum Cyanobacteria, and showed that their metabolism is fermentative and/or respiratory (Soo et al., 2014). Among four orders within the class Melainabacteria/Vampirovibrionia, only Vampirovibrionales has been shown to include a cultivable species; among other orders, only Gastranaerophilales had a FISH-detected representative. Over the past decade, accelerated metagenomic research of dark cyanobacteria has revealed phylum-grade clades Blackallbacteria (former Sericytochromatia), Margulisbacteria, and Saganbacteria. At the same time, one should admit that most of knowledge was issued from a limited set of MAG and SAG data that provide indirect and comparatively meager insight into the metabolism and environmental role of sibling groups to light cyanobacteria.

Anyhow, the analysis of genomes in dark cyanobacteria helps to reconstruct the early history of bioenergetics, in particular, to solve the dilemma

"light cyanobacteria: primordial or later acquired", as well as to access the roots of aerobic respiration.

Dark cyanobacteria: primordial or later acquired

Dark cyanobacteria are not only interesting from an ecological viewpoint; their co-origin with light cyanobacteria is especially intriguing. In fact, the advent of oxygenic photosynthesis ensured the transition from completely anaerobic biosphere to the aerobic biosphere including higher organisms (Sánchez-Baracaldo and Cardona, 2020). Aerobes take the opportunity to live and flourish at the expense of water oxidation and primary productivity by free-living cyanobacteria and chloroplasts (Partensky et al., 1999).

Most of bacterial phyla diverged in the late Archaean (~3.25 Ga ago) due to massive gene loss (David and Alm, 2011). In particular, phototrophy, which is considered the primeval energy metabolism (Fox et al., 1980; Woese, 1987) was subsequently lost in the majority of bacterial lineages. Today, this great loss is echoed in coexistence of light and dark representatives within several branches of bacterial tree (Woese et al., 1985). No wonder that the phylum Proteobacteria, in which the largest number of phototrophs is found, initially bore trivial name "Purple bacteria and their relatives" (Stackebrandt et al., 1988).

The most conserved part of photosynthetic apparatus is type-1 and type-2 reaction centers. They are inferred to originate from a RCI-prototype via gene duplication, splitting, and horizontal transfer (Schubert et al., 1998; Baymann et al., 2001; Heathcote et al., 2002). As the result, they distribute among diverged lineages that inter alia contain anoxygenic bacteria. Thus, only RCI is present in some members of the phyla Acidobacteria, Fibrobacteres-Chlorobi-Bacteroidetes, and Firmicutes, whereas only RCII is present in some members of the phyla *Candidatus* Eremiobacterota (WPS-2), Gemmatimonadetes, Chloroflexi, and Proteobacteria.

Cyanobacteria are the only known phylum with co-occurring RCI and RCII (the latter reaction center acts as unique water dehydrogenase photoenzyme). To date, the acquisition of two reaction centers could be arbitrarily explained by three scenarios. The first scenario, or "the selective loss model", argues that RCI and RCII were concomitantly present in primordial anoxygenic ancestor, and its descendents vertically obtained one or both of them, while other lineages acquired one or the other reaction center by horizontal transmission (Olson and Pierson, 1987; Raymond et al., 2002). The second scenario called "the fusion model" implies that RCI and RCII were separately acquired by non-photosynthetic ancestor via horizontal gene transfers from two one-RC lineages (Mathis, 1990). The third scenario, or "the export model", postulates that ancestor two-RC cyanobacterium endowed other lineages with RC type-1 or RC type-2 (Mulkidjanian et al., 2006; Martin et al., 2018). Despite discrepancies among reaction center proteins-, chlorophyll synthesizing enzymes-, and 16S rRNA-phylogenies, current comparative genome and molecular clock data do not support "the fusion model". In other words, most plausible scenarios are either "the selective loss model" or "the export model" (both surmise two reaction centers in primordial anoxygenic bacterium; Cardona, 2015).

Approximate timing and proposed mechanism for the origin of oxygenic photosynthesis are debatable. In the one opinion (e.g., Crowe et al., 2013), it happed 3.5 Ga ago, i.e. before radiation of extant bacterial groups. In the other opinion (e.g., Soo et al., 2017), it happened 2.5 Ga ago, just before the Great Oxidation Event (the first rise of oxygen in atmosphere and oceans; see: Bekker et al., 2004; Lyons et al., 2014). Age estimates of PSI and PSII genes (Cardona, 2015; Cardona et al., 2015, 2019) are consistent with geochronology data on the presence of oxygen in Archaean (4–2.5 Ga ago) that implies the origin of oxygenic photosynthesis 3 Ga ago. Anyway, comparative analysis of 132 microbial proteomes showed that cyanobacteria represented a unique hub from obligate anaerobes to obligate aerobes (Harel et al., 2015).

The discovery of dark cyanobacteria gave new impetus to the discussion on how oxygenic photosynthesis appeared and evolved. Missing genes for light energy and carbon dioxide assimilation pose the dilemma of whether dark cyanobacteria are (or are not) firstborn. In other words, whether they originated via the loss of ancestral photosynthetic capacity (in analogy with non-purple Proteobacteria; see above) or whether they initially lacked such capacity. To date, three scenarios are considered (Fig. 4).

According to the first scenario (Fig. 4A), primordial cyanobacteria *sensu lato* were fermentative anaerobes (Matheus Carnevali et al., 2019). The Oxyphotobacteria lineage "turned light" (for a proposed mechanism, see above) after successive divergence

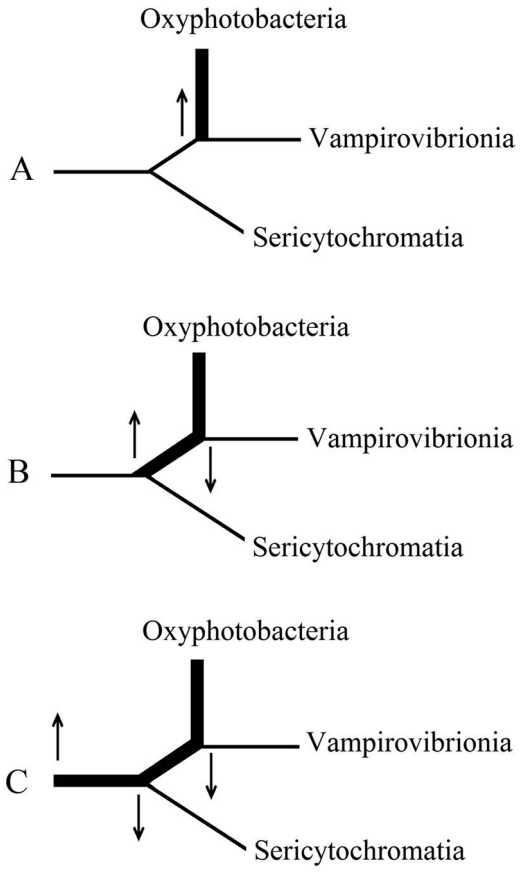

Fig. 4. Proposed scenarios for evolution of oxygenic photosynthesis. Photosynthetic lineage is in bold. Arrows pointing up denote acquisition, arrows pointing down – loss of oxygenic photosynthesis. For details, see the text.

of Sericytochromatia and Vampirovibrionia. It means that these classes were never photosynthetic (Soo et al., 2017; Shih et al., 2017). Molecular clock estimates for divergence of Oxyphotobacteria and Vampirovibrionia are 2.5 Ga ago (Shih et al., 2017) while the crown group cyanobacteria diverged 2.0 Ga ago. To note, ancestral absence of photosynthetic genes is also demonstrated in Margulisbacteria and Saganbacteria (Matheus Carnevali et al., 2019). The second scenario (Fig. 4B) surmises that common ancestor of Oxyphotobacteria and Vampirovibrionia acquired RCI and RCII after divergence from Sericytochromatia. In Vampirovibrionia, photosynthetic genes were lost after divergence of this lineage (Soo et al., 2019). According to the third scenario (Fig. 4C), common ancestor of cyanobacteria *sensu lato* was initially photosynthetic, while Sericytochromatia and Vampirovibrionia successively lost their photosynthetic capacity (Thiel et al., 2018). Taking into account the complete absence of photosynthetic genes in all known dark cyanobacteria, scenario "A" in which light cyanobacteria developed the photosynthetic capacity by themselves (or acquired it via horizontal gene transfer; see above) looks most plausible unless remnant representatives of photosynthetic genes are found in Sericytochromatia and Vampirovibrionia (Garcia-Pichel et al., 2020).

Then and now: aerobic respiration in cyanobacteria

It is generally accepted, at least to date, that photosynthesis was absent in common ancestor of cyanobacteria (Soo et al., 2017, 2019). A photosynthetic apparatus was acquired only during early evolution of the class Oxyphotobacteria (see Fig. 4A), and from that time on, little abiogenic oxygen became overbalanced by accumulated biogenic oxygen. With the rise of dissolved oxygen in oceans, electron transfer chain (ETC) in Oxyphotobacteria adapted fast to this terminal acceptor. Similar ability for aerobic respiration independently evolved in Vampirovibrionia and Sericytochromatia. In all three lineages evolutionary pressure has led to the establishment of highly effective energy metabolism, although respiratory chains evolved in separate directions. In other words, aerobic respiration originated in cyanobacteria several times, and cyanobacterial lineages independently acquired complexes III and IV (Soo et al., 2017).

In general, two dissimilar complexes III are known: i) cytochrome bc_1 complex with its b_6f variant, and ii) alternative ACIII complex. The first complex is widespread in respiring Bacteria and Archaea; in particular, the b_6f variant is typical for Oxyphotobacteria. The second complex (see: Vanyushin et al., 2005) is comparatively rare and present only in bacteria, Sericytochromatia being among the examples. In turn, complex IV is represented by heme-copper oxygen reductases (HCOs) or by cytochrome *bd* oxygen reductases. High oxygen adapted HCOs of "A" type are widespread, while low oxygen adapted HCOs of "B" or "C" type are less common. Another low oxygen adapted reductases, of the cytochrome *bd* type, appear to be widely distributed. Broad occurrence of the aforementioned complexes is possibly due to horizontal gene transfer; their structure-functional details (see: Pereira et al., 2001; Refojo et al., 2012) are omitted here.

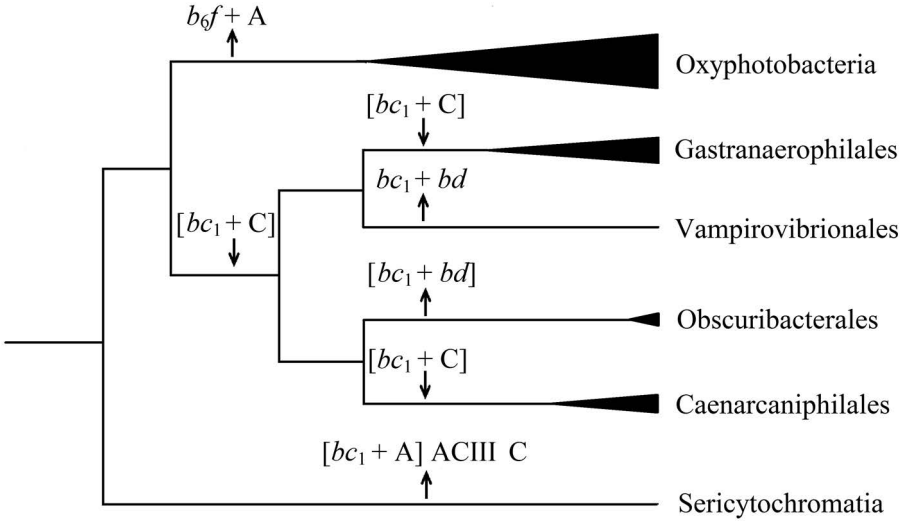

Fig. 5. Evolution of respiratory systems in Oxyphotobacteria, Vampirovibrionia, and Sericytochromatia (modified from: Soo et al., 2017, 2019). bc_1, b_6f, and ACIII – complexes III; A, C, and *bd* – complexes IV. Square brackets denote operon fusions. Arrows pointing up denote acquisition, arrows pointing down – loss of complex. For details, see the text.

According to phylogenetic analysis (Soo et al., 2017, 2019; Fig. 5), all Oxyphotobacteria have the b_6f-type complex III, and the A-type complex IV; both complexes were acquired soon after the divergence of this class. In ~10% of oxyphotobacterial genomes, the genes for additional A-type complex IV or, very rarely, the genes for additional C-type complex IV were found. They were acquired later in evolution of this lineage, and are adaptively used in low oxygen conditions (Schmetterer, 2016).

The class Vampirovibrionia is more diverse regarding the respiratory chain structure. Thus, upon the divergence, this lineage acquired the "fused operon" bc_1-complex III, as well as the C-type complex IV (Fig. 5). In the orders Gastranaerophilales and Caenarcaniphilales that mostly include fermenting cyanobacteria, respiratory chain was secondarily lost (in Ceanarcaniphilales, it is rarely retained). Later in their evolution, respiring Vampirovibrionales additionally acquired the bc_1-type complex III and the *bd*-type complex IV, while Obscuribacterales additionally acquired the "fused operon" bc_1-type complex III, and the *bd*-type complex IV. To note, the absence of A-type complex IV in these cyanobacteria demonstrates general adaptation of these cyanobacteria to low oxygen conditions.

The phylum Sericytochromatia is most diverse with respect of respiratory systems. Thus, several available MAGs of this lineage contain the genes for the bc_1-type, and the ACIII-type complexes III, as well as the genes for the A-type and the C-type complexes IV. All of them were independently acquired late in evolution of this lineage (hence, its figurative name); importantly, their structure is dissimilar from the corresponding complexes in other lineages.

Thus, oxygenic photosynthesis is thought to evolve comparatively late in evolution of the domain Bacteria, and therefore Oxyphotobacteria should not be considered "the earliest taxa" in contrast to the previous assumptions of their 3.5 Gyr age (Schopf, 2000). In particular, divergence of Oxyphotobacteria with Vampirovibrionia can be dated back to ~2.5 Gyr, that approximately coincides with geochemical data on the rise of oxygen (Garcia-Pichel et al., 2019).

Attempt at prediction: lithotrophic cyanobacteria

Primordial cyanobacteria are suggested to be fermentative anaerobes (Matheus Carnevali et al., 2019). Correspondingly, ETC and particularly complex III are proposed to appear in Oxyphotobacteria soon after the divergence of this class from Vampirovibrionia and Sericytochromatia. However, in our opinion, complex III could alternately be present in the common ancestor of

Cyanobacteria and its sibling phyla that had the respiratory rather than fermentative metabolism. General arguments for this speculation are as follows.

First, photosynthetic apparatus is known to include reaction center(s) and ETC (the redox active moiety) as well as light-harvesting complex (the excited state conducting moiety). It possibly originated via supplementing the ETC (which assimilates energy at membrane level) with pigment system (which converts light energy into chemical energy) (Xiong and Bauer, 2002). Second, ETC can channel two electron flows, respiratory and photosynthetic correspondingly; in both cases, energy metabolism is much more efficient compared with fermentation. Third, complex III is a champion in antiquity among other energy transducing systems; noteworthy, among the Archaea there are no photosynthetic representatives although these prokaryotes possess complex III closely similar to the bacterial counterpart (Schütz et al., 2000).

Based on these considerations, we hypothesize that common ancestor of Cyanobacteria could depend on respiration, and that the reductant for ETC could be either organic or inorganic. The second alternative seems more plausible taking into account that dependence on organic substrates developed comparatively late in cell evolution (Thauer, 2007). Noteworthy, certain strains of present cyanobacteria are able to use sulfide (Arieli et al., 1994).

Here, we propose that yet-to-be found lithotrophic cyanobacteria could inhabit (an)oxic freshwater or marine sediments. Whether these still cryptic objects additionally assimilate inorganic carbon (i.e. whether they are chemosynthetic bacteria) is also a matter of speculation. Future metagenomic analyses, together with attempts to isolate cultured strains, would confirm or reject these assumptions.

Concluding remark

By putting a full stop at the end of this story, we should eulogize early explorers less technically armed than those who benefit from their discovery, and stress the importance of hunting for cultured microbes, and of their laboratory observation. This old-fashioned task, recently supported by culturomics (Lagier et al., 2012) does not loose its relevance.

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