Título de la Tesis:

‘Uso del Nitrógeno en Algas: Desvelando Piezas del Rompecabezas de la Asimilación del Nitrógeno y su Regulación en el Alga Modelo *Chlamydomonas reinhardtii*’

‘Nitrogen Nutrition in Algae: Unraveling Pieces of the Nitrogen Assimilation Puzzle and its Regulation in the Model Alga *Chlamydomonas reinhardtii*’.

Programa de Doctorado: Biociencias y Ciencias Agroalimentarias

Directores: Aurora Galván Cejudo y Emilio Fernández Reyes

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TITULO: *Nitrogen Nutrition in Algae: Unraveling Pieces of the Nitrogen Assimilation Puzzle and its Regulation in the Model Alga Chlamydomonas reinhardtii*

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Informan que:

La presente Tesis Doctoral titulada "Uso del Nitrógeno en Algas: Desvelando Piezas del Rompecabezas de la Asimilación del Nitrógeno y su Regulación en el Alga Modelo *Chlamydomonas reinhardtii*". En inglés, "Nitrogen Nutrition in Algae: Unraveling Pieces of the Nitrogen Assimilation Puzzle and its Regulation in the Model Alga *Chlamydomonas reinhardtii*" realizada por Dª. María Victoria Calatrava Porras se ha desarrollado en los laboratorios del Departamento de Bioquímica y Biología Molecular de la Universidad de Córdoba bajo nuestra dirección.

Esta Tesis Doctoral reúne todas las condiciones exigidas según la legislación vigente. Su evolución y desarrollo ha seguido el plan de trabajo inicialmente previsto, alcanzándose los objetivos previamente marcados. Durante la elaboración de la Tesis, el doctorando ha realizado una labor altamente satisfactoria dentro del grupo de investigación.

Parte de los resultados obtenidos en esta tesis, han sido publicados en una revista internacional de reconocido prestigio. Otros resultados están actualmente en proceso de publicación.

Además, el doctorando ha realizado una estancia de cuatro meses en un grupo de investigación extranjero de reconocido prestigio en el área en la que se enmarca la tesis.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 25 de Octubre de 2018

Firma del/de los director/es

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Mediante la defensa de esta memoria se pretende optar a la obtención de la mención de 'Doctorado Internacional', habida cuenta que el doctorando reúne los requisitos exigidos para tal mención:

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El trabajo que engloba la presente Memoria se ha realizado durante el periodo de disfrute de un Contrato Predoctoral (Personal Investigador Predoctoral en Formación) de la Junta de Andalucía como parte del proyecto de investigación de excelencia de la convocatoria de 2012 (P-12-BIO-502), y el Plan Propio de Investigación de la Universidad de Córdoba (Programa de Fortalecimiento de las Capacidades en I+D+I, 2014).

A mis Padres y
a Juan Carlos

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ABSTRACT

Algae, lying on the basis of food webs in marine and freshwater ecosystems, are key for aquatic life. These photosynthetic organisms live under continuously fluctuating nutrients availability, showing a high level of adaptability to these dynamic environments. Although the essential nutrient Nitrogen (N) is usually used by algae in its inorganic form, some algal species can use organic N compounds, which may become especially abundant due to terrestrial leaking and runoff of highly fertilized areas. The model alga *Chlamydomonas reinhardtii (Chlamydomonas)* uptakes inorganic N sources (i.e. ammonium, nitrate and nitrite), as well as L-arginine and urea. Moreover, this alga presents an extracellular L-amino acid oxidase (LAO1) with a broad substrate specificity that scavenges N from L-amino acids. In this work we studied the signaling control that leads the preference for nitrate over organic N in *Chlamydomonas*, the key role of LAO1 in the use of amino acids and peptides, as well as the establishment of new mutualistic interactions with bacteria to facilitate growth on organic N.

Chapter 1

The transcription factor NIT2 is the key regulator of nitrate assimilation genes in *Chlamydomonas*. First, we compared the transcriptome of *Chlamydomonas* wild type (WT) and a *nit2* mutant in response to nitrate. We observed that nitrate and NIT2 down-regulated genes involved in organic N scavenging, including *LAO1*. By the use of *Chlamydomonas* mutant strains we demonstrated that both nitrate and nitrite negatively impact the use of amino acids by this alga.

Chapter 2

L-amino acid oxidase (LAAO) enzymes are widely present in nature and a major role as N scavenger has been proposed in fungal and algal LAAOs. By comparative genomic searches, we could not find any LAO1 ortholog in any green plant or plant, but we identified orthologs in 10 out of 27 other algal species, including Rhodophyta, Alveolata, Heterokonta, Haptophyta and Dinophyta algae. The construction of a LAAO phylogenetic tree revealed that algal protein sequences identified as LAO1 orthologs -named here as ALAAOs (Algal LAAOs)-, clustered on the same evolutionary branch. We observed that *Chlamydomonas LAO1* gene is clustered to a putative *RidA* gene *(LAO2),* which resulted to be closely related to cyanobacterial members. Our phylogenetic analysis favoured the idea that ALAAOs may have a common origin in the archaeplastidan ancestor, originated by a protist engulfing cyanobacteria. By the use of a *lao1* mutant, we showed that LAO1 was crucial for *Chlamydomonas* growth on 16 out of 20 proteinogenic amino acids, as well as for some di- and tripeptides.

Besides ammonium, LAAO produces keto acids and hydrogen peroxide. We have demonstrated that the spontaneous reaction of the LAO1-derived products generated by L-alanine deamination, pyruvic acid and hydrogen peroxide, generates acetic acid.

Although *Chlamydomonas* can grow on most amino acids and some di-/tri-peptides as the sole N sources, this growth is far less efficient than that on inorganic N, and yet, there are some amino acids and peptides that cannot be used by this alga. We serendipitously found a contaminating *Methylobacterium* sp. that allowed *Chlamydomonas* growth on a dipeptide that is not readily assimilated by this alga. *Methylobacterium* spp. are included in the PGPB group of bacteria (Plant Growth-Promoting Bacteria), which improve plant growth and fitness. After field sampling, isolation and identification of some bacteria, we found that some wild species, included in *Methylobacterium, Sphingomonas, Deinococcus,* and *Chitinophagaceae* genera, promoted *Chlamydomonas* growth on L-serine. Moreover, some *Methylobacterium* spp. allowed *Chlamydomonas* growth on amino acids and peptides that are not used by this alga. We have demonstrated a new mutualism based on carbon-nitrogen metabolic exchange between *Chlamydomonas* and *M. aquaticum*. Otherwise, some *Methylobacterium* spp. improved *Chlamydomonas* growth on assimilable amino acids. For this growth promotion, LAO1 was crucial for consortia growth with some *Methylobacterium* spp., including *M. extorquens, M. hispanicum* and *M. organophilum.*

The chemical cross-talk between interacting organisms mediates the beneficial and pathogenic symbiotic relationships. Within these inter-kingdom signal molecules IAA (Indole-3-Acetic Acid) is one of the best studied. We found that L-tryptophan-dependent indoles production in *Chlamydomonas*, observed here for the first time, was significantly reduced in the *lao1* mutant. Moreover, we observed that high levels of exogenously added IAA (> 30 μM) inhibits *Chlamydomonas* growth and that this inhibition may be relieved by the presence of *Methylobacterium* spp.

RESUMEN

Las algas, formando parte de la base de la cadena trófica de ecosistemas marinos y de agua dulce, son clave para la vida acuática. Estos organismos fotosintéticos, sometidos a fluctuaciones constantes de disponibilidad de nutrientes, muestran un alto nivel de adaptabilidad a estos ambientes dinámicos. Aunque el nitrógeno (N), nutriente esencial para la vida, es comúnmente usado por las algas en su forma inorgánica, algunas especies de algas pueden usar compuestos de N orgánico, los cuales pueden ser especialmente abundantes debido a la escorrentía y filtrado de áreas fertilizadas de forma intensiva. El alga modelo *Chlamydomonas reinhardtii* (*Chlamydomonas*) puede consumir fuentes de nitrógeno inorgánico (amonio, nitrato y nitrito), así como L-arginina y urea. Además, este alga presenta una L-aminoácido oxidasa extracelular (LAO1) que desamina un amplio rango de aminoácidos. En este trabajo hemos estudiado el control de la señalización que da lugar a la preferencia de nitrato sobre N orgánico en *Chlamydomonas,* el papel clave de LAO1 en el uso de aminoácidos y péptidos, así como el establecimiento de nuevas interacciones mutualistas con bacterias que promueven el crecimiento en N orgánico.

Capítulo 1

El factor de transcripción NIT2 es el regulador clave de los genes de la asimilación de nitrato en *Chlamydomonas.* En primer lugar, comparamos el transcriptoma de una estirpe silvestre y otra mutante *nit2* de *Chlamydomonas* en respuesta a nitrato. Observamos que nitrato y NIT2 reprimen los genes involucrados en el uso de fuentes de N orgánicas, incluyendo LAO1. Mediante el uso de mutantes de *Chlamydomonas* demostramos que tanto el nitrato como el nitrito afectan negativamente el uso de aminoácidos por este alga.

Capítulo 2

Las enzimas L-aminoácido oxidasa (LAAO, L-Amino Acid Oxidase) están ampliamente distribuidas en la naturaleza y se propone que su papel principal en hongos y algas es la captación de nitrógeno. Mediante búsquedas genómicas comparativas, no pudimos encontrar ningún ortólogo de LAO1 en ningún alga verde ni en plantas, pero identificamos ortólogos en 10 de otras 27 especies de algas, incluyendo Rhodophyta, Alveolata, Heterokonta, Haptophyta y Dinophyta. La construcción de un árbol filogenético de enzimas LAAO reveló que las secuencias identificadas como ortólogas de LAO1 -denominadas aquí como ALAAOs (Algal LAAOs)-, se agrupaban en la misma rama evolutiva. Observamos que en *Chlamydomonas* el gen *LAO1* está situado adyacente a un gen que codifica una putativa proteína RidA, que resultó estar evolutivamente cercana a la de cianobacterias. Nuestro análisis filogenético apoya la idea de que las proteínas ALAAOs pueden tener un origen en el ancestro común de las algas, el cual se originó por la endosimbiosis de una cianobacteria por un protista. Mediante el uso de un mutante *lao1* hemos mostrado que LAO1 era

crucial para el crecimiento de *Chlamydomonas* en 16 de 20 aminoácidos proteinogénicos, así como para algunos di-/tri-péptidos.

Además de amonio, las enzimas LAAO producen el correspondiente cetoácido y peróxido de hidrógeno. Hemos demostrado que la reacción espontánea de los productos derivados de la desaminación por LAO1 de L-alanina -ácido pirúvico y peróxido de hidrógeno- genera ácido acético.

Capítulo 3

Aunque *Chlamydomonas* puede crecer en la mayoría de los L-aminoácidos y en algunos di-/tri-péptidos como únicas fuentes de N, este crecimiento es mucho menos eficiente que en fuentes de N inorgánicas, y además, algunos aminoácidos y péptidos no pueden ser usados por este alga. De forma fortuita descubrimos una contaminación *Methylobacterium* sp. que permitió el crecimiento de *Chlamydomonas* en un di-péptido que no puede asimilar. Las especies de *Methylobacterium* están incluidas en el grupo de bacterias promotoras del crecimiento de plantas (PGPB, del inglés *Plant Growth-Promoting Bacteria),* las cuales mejoran el crecimiento de las plantas. Tras el muestreo en campo, aislamiento e identificación de bacterias, encontramos que algunas especies salvajes, incluidas en los géneros *Methylobacterium, Sphingomonas, Deinococcus* y *Chitinophagaceae*, mejoran el crecimiento de *Chlamydomonas* en L-serina. Además, algunas especies de *Methylobacterium* permitieron el crecimiento de *Chlamydomonas* en algunos aminoácidos y péptidos que este alga no puede usar. Hemos demostrado un nuevo mutualismo basado en un intercambio metabólico de carbono y nitrógeno entre *Chlamydomonas* y *M. aquaticum*. Por otro lado, algunas especies de *Methylobacterium* mejoraron el crecimiento de *Chlamydomonas* en aminoácidos asimilables. Para esta mejora, la enzima LAO1 fue esencial para el crecimiento del consorcio con algunas estirpes de *Methylobacterium*, incluyendo *M. extorquens, M. hispanicum* y *M. organophilum.*

La comunicación química en la interacción entre organismos diferentes media las relaciones simbióticas. Entre estas moléculas de señalización, el ácido indolacético es una de las más estudiadas. Descubrimos que la producción de índoles dependiente de L-triptófano por *Chlamydomonas*, observada aquí por primera vez, disminuyó significativamente en el mutante *lao1*. Además, observamos que altas concentraciones de ácido indolacético (> 30 μM) inhibe el crecimiento de *Chlamydomonas* y que esta inhibición se puede reducir por la presencia de especies de *Methylobacterium.*

ABBREVIATIONS

- **-N**, without nitrogen
- **A**, absorbance
- **ACC**, 1-aminoyclopropane-1-carboxylic acid
- **ATP**, adenosine triphosphate
- **C,** carbon
- **cDNA**, complementary DNA
- **Cre,** *Chlamydomonas reinhardtii*
- **DHPS**, 2,3-dihydropropane-1-sulfonate
- **DIN**, dissolved inorganic nitrogen
- **DMS**, dimethylsulfide
- **DMSP**, dimethylsulfoniopropionate
- **DNA**, deoxyribonucleic acid
- **DNase**, deoxyribonuclease
- **DOM**, dissolved organic matter
- **DON**, dissolved organic nitrogen
- **EDTA**, ethylenediaminetetraacetic acid
- **FAD**, flavine adenine dinucleotide
- **FC**, fold change
- **gDNA**, genomic DNA
- **GSNO**, nitrosoglutathione
- **HPLC**, high-performance liquid chromatography
- **IAA**, Indole-3-acetic acid
- **ID**, identification number
- **Maqu,** *Methylobacterium aquaticum*
- **MIN**, minimal
- **MSX**, methionine sulfoximine
- **MW**, molecular weight
- **N**, Nitrogen
- **NADH**, nicotinamide adenine dinucleotide
- **NADPH**, nicotinamide adenine dinucleotide phosphate
- **NO**, nitric oxide
- **PCR**, polymerase chain reaction
- **pDNA**, plasmid DNA
- **PGPB**, Plant Growth-Promoting Bacteria
- **PPFM**, Pink-Pigmented Facultative Methylotrophs
- **PyA**, pyruvic acid
- **qPCR**, quantitative polymerase chain reaction
- **QS**, quorum sensing
- **rDNA**, ribosomal DNA
- **RNA**, ribonucleic acid
- **RNase**, ribonculease
- **ROS**, reactive oxygen species
- **SDS**, sodium dodecyl sulfate
- **TAP**, tris-acetate-phosphate
- **WT**, wild type

GENES, PROTEINS

- **AAAP**, amino acid/auxin permease family of transporters
- **ACD**, 1-aminocyclopropane-1-carboxylic acid deaminase
- **ADI**, arginine deiminase
- **AH**, allophanate hydrolase
- **ALAAO**, Algal L-Amino Acid Oxidases
- **AMT1.1-8**, *C. reinhardtii* ammonium transporters
- **AO**, amine oxidase
- **AOC1-6**, *C. reinhardtii* AAAP family proteins
- **AOF2**, amino acid oxidase family 2
- **AOT1-7**, *C. reinhardtii* APC family proteins
- **APC**, Amino acid Polyamine organoCation family of transporters
- **CNX**, calnexin
- **CYG**, guanylate cyclase
- **CYP**, cytochrome P450
- **DAAO**, D, amino acid oxidase
- **DUR1**, urea carboxylase
- **DUR2**, allophanate hydrolase
- **DUR3A-B**, *C. reinhardtii* urea transporters
- **Fd**, ferredoxin
- **GDH**, glutamate dehydrogenase
- ***GLB1*** (**PII**), nitrogen regulatory protein
- ***GLN*** **(GS),** glutamine synthetase
- **GOGAT**, glutamate synthase
- **GOGAT**, glutamate synthase
- **GS**, glutamine syntethase
- **HAAT**, high affinity ammonium transporter
- **HANNiT**, high affinity nitrate/nitrite Transporters
- **LAAT**, low affinity ammonium transporter
- **LAO1**, *C. reinhardtii* extracelular L-amino acid oxidase 1
- **LAO2/RidA**, L-amino acid oxidase 2
- **LAO3**, intracelular L-amino acid oxidase 3
- **LASPO**, L-aspartate oxidase
- **LodA**, (L-Lysine ε-oxidase)
- **MOT**, Molybdenum transporter
- **MYB**, transcription factor
- **NAGK**, N-acetyl-L-glutamate kinase
- **NAR1-2,** Nitrate assimilation related component
- ***NIA1*** **(NR**), nitrate reductase
- ***NII1*** **(NiR),** nitrite reductase
- **NIT2**, nitrate assimilaton regulatory protein
- **NOFNIR,** nitric oxide forming-nitrite reductase
- **NOS**, nitric oxide synthase
- **NPF**, nitrate transporter 1/peptide transporter family
- **NRR1**, nitrogen response regulator 1
- **NRT1**, nitrate transporter 1
- **NRT2**, nitrate transporter 2 family
- **RidA**, Reactive intermediate/imine deaminase A family
- **RuBisCo**, ribulose-1,5-biphosphate carboxylase/oxygenase
- **sGC**, soluble guanylate cyclase
- **SLC14**, Solute carrier family 14
- **SLC7A1-3,** human cationic amino acid transporters
- **SSF**, Sodium:solute Symporter Family of transport proteins
- **THB**, truncated hemoglobin
- **UALase**, ATP-Urea amidolyase
- **UAPA**, xanthine/uracil/vitamin C permease-like
- **UC**, urea carboxylase

CONTENTS

FIGURES

TABLES

INTRODUCTION

I.1 Algae: Ecological Impact and Biotechnological Potential.

Algae, covering about 70% of the Earth surface, provide approximately 50% of the oxygen we breath (Chapman, 2013). A primary endosymbiotic event, in which a protist engulfed a cyanobacterium, gave rise to the Archaeplastida (De Clerck et al., 2012). From this common ancestor, different algal lineages emerged: Glaucophytes, Rhodophytes -red algae-, and Chlorophytes -green algae- (Figure I.1.). This latter group led to the origin of land plants. Moreover, further events of endosymbiosis in algae originated other algal groups including Cryptophyta, Haptophytes and Heterokontophyta (De Clerck et al., 2012). **Due the high level of algal diversity, algae are interesting targets for ecological, evolutionary and physiological research.** These eukaryotic and mostly photosynthetic organisms cover multiple branches of the tree of life and inhabit different niches from marine and freshwater ecosystems to desert crusts. Both red and green algal lineages present unicellular and complex multicellular members. Algae show high genomic plasticity and can evolve complex phenotypic resilience (Schlüter et al., 2016). They can undergo sexual cycles with a range of sexual systems from isogamous -e.g. the unicellular alga *Chlamydomonas reinhardtii* with two gametes of plus and minus mating types- to oogamous -e.g. the multicellular alga *Volvox carteri* with males that produce sperm and females that produce eggs-. Although most algal species are photosynthetic and can grow under strictly autotrophic conditions, some algal species have evolved to strictly heterotrophic growth like *Plasmodium*, and many can grow mixotrophically, like *Chlamydomonas reinhardtii*.

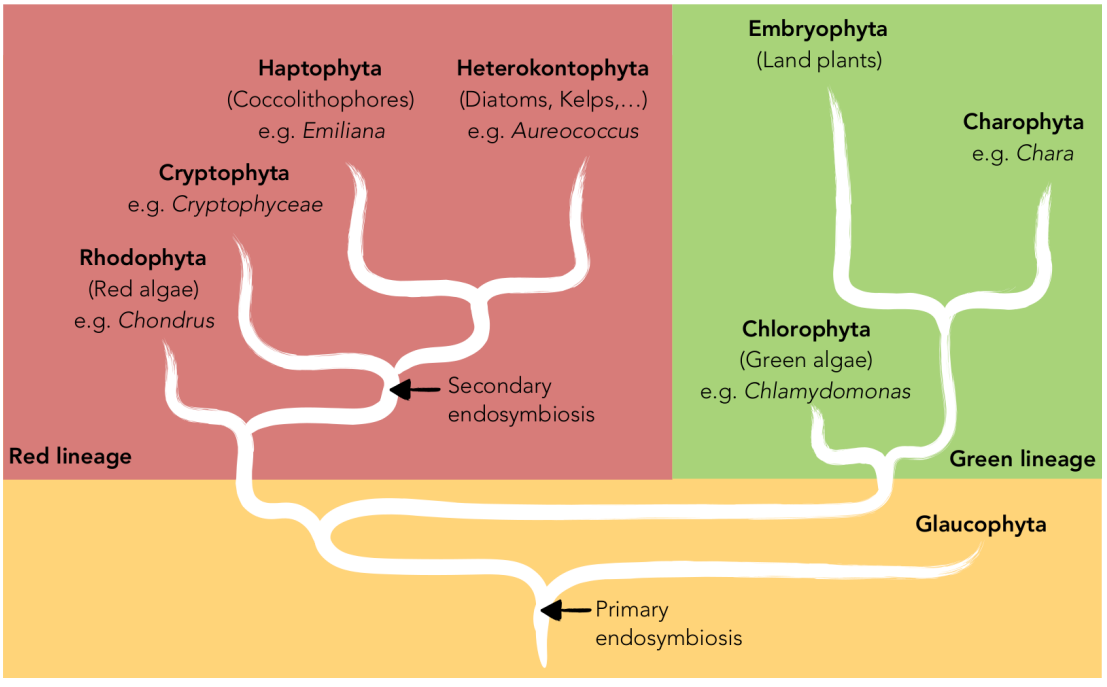


Figure I.1 Algal phylogeny. In the primary endosymbiosis event, a protist engulfed a cyanobacterium, which persisted as a stable endosymbiont and evolved to form the plastid. From this common ancestor, different algal groups diverged: Glaucophyta, Rhodophyta and Chlorophyta. The last group led to the origin of land plants. Moreover, secondary symbiosis events generated more complex algal groups, like Cryptophyta, Haptophyta and Heterokontophyta.

As primary producers, algae are key for aquatic life. However, some algal proliferation can lead to harmful algal blooms that may disrupt the ecosystem by toxins release and/or blocking the sunlight avoiding photosynthesis in the deeper layers (Figure I.2). This low photosynthetic activity, together with a high respiration rate by bacteria that rapidly grow feeding on the algal decay, results in local hypoxia and migration or death of animal life. These phenomena, occurring with high regularity, affect marine and freshwater ecosystems representing a significant socio-economic impact that affects different sectors such as human health, commercial fisheries and tourism (Sanseverino et al., 2016). Moreover, some algae can alter the climate since they produce the sulfur compound dimethylsulfoniopropionate (DMSP), which is ultimately converted by bacteria into dimethylsulfide (DMS), a volatile gas and a major precursor of cloud condensation nuclei (Archer et al., 2001).

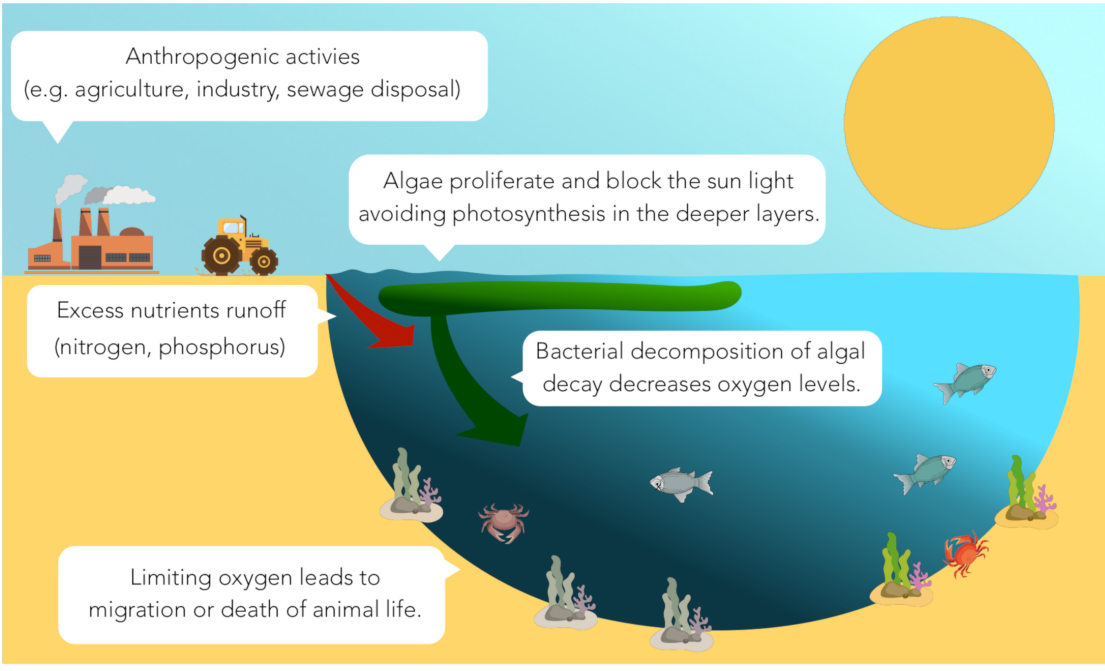


Figure I.2. Harmful algal bloom. Anthropogenic activities such as agriculture, industry and sewage disposal may generate a runoff loaded with high concentration of nutrients (mainly nitrogen and phosphorous) that promote algal growth. Algae rapidly grow forming an algal bloom. This phenomenon can harm the water quality by releasing toxins that are toxic for animal life and by blocking the sunlight, preventing photosynthesis of vegetal life in the deeper areas. As the bloom evolves, algal decay falls into the shallower areas and feeds bacteria that respire the oxygen. This oxygen consumption by bacteria, together with the impeded photosynthesis, generates hypoxia. This condition leads to migration or death of mollusks, fishes and other aquatic animals.

The recent technological advances in genomics and transcriptomics, with more than thirty algal genomes now available, have facilitated the opportunities to exploit algal metabolism diversity for biotechnological applications (Keeling et al., 2014). These applications include: biofuel production by many microalgae that highly accumulate triacylglycerides (Ahmed et al., 2017); biohydrogen production by the green microalga *Chlamydomonas reinhardtii* (Jurado-Oller et al., 2015); nutraceuticals production, such as pigments, vitamins and

antioxidants; as well as different applications such as bioremediation, aquaculture and human foods (Brodie et al., 2017; Mobin, 2017; Sathasivam et al., 2017).

I.2 Algae and the Nitrogen Cycle

Nitrogen (N) is a key component of biomolecules such as nucleic acids and proteins and therefore, it is essential for all forms of life. Although N is one of the most abundant elements on Earth, it is mostly present as N_2, which can be only used by prokaryotic organisms but is not readily available for eukaryotes. Otherwise, inorganic and organic nitrogen molecules can be assimilated by eukaryotic organisms. Within aquatic ecosystems, N -together with phosphorous- is usually considered as the main limiting nutrient for aquatic algal production since its supply is usually reduced compared to its requirement for cell growth. Inorganic N is often the most abundant form of N in marine and freshwater systems. However, in many aquatic systems, terrestrial leaking and runoff are important inputs of organic N. Sewage and other anthropogenic activities lead to significant infusions of organic N into rivers that are eventually transported to estuarine and coastal waters. The organic inputs into water systems have dramatically increased over the last century due to industrial growth and global warming, and have a great impact on the biodiversity of associated ecosystems (Berman and Bronk, 2003; Seitzinger and Sanders, 1997, 1999; Vaquer-Sunyer et al., 2015). Thus, organic N sources, mainly urea and free amino acids can regularly change spatially or temporarily reaching high levels while inorganic N concentrations became limited. Although bacteria have long been considered to be the only consumers of this organic N, **recent studies have shown than many phytoplankton species are also able to use organic N sources** and are especially evident during algal blooms (Berman and Bronk, 2003; Fan and Glibert, 2005; Ramûnas et al., 2002; Seitzinger and Sanders, 1999). For instance, the bloom-forming alga *Aureococcus anophagefferens* causes the 'brown tide' in the United States, South Africa and China (Dong et al., 2014; Gobler et al., 2011; Mulholland et al., 2002; Probyn et al., 2001). The input of inorganic nitrogen from extensively fertilized areas runoff originates a first bloom by diatom species (Figure I.3). Then, when most inorganic N is consumed and its concentration is limiting, these species die off increasing the concentration of organic matter rich in organic N. The ability to use this organic N by *A. anophagefferens* allows this alga to outcompete with other species and rapidly proliferate causing the brown tide. Thus, **the degree to which primary producers can use organic N may be decisive to the species proliferation over other competitors**, which should be factored into models of ecosystem balance.

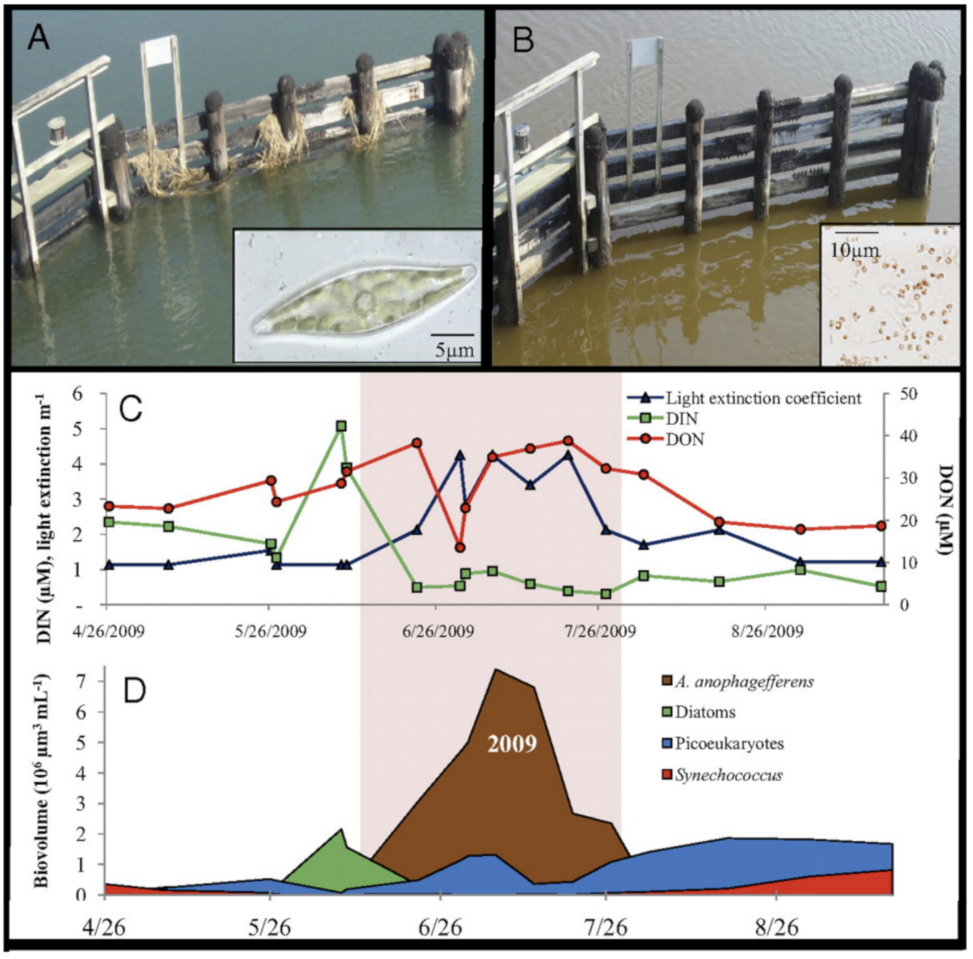


Figure I.3. Nitrogen sources and algal species evolution in a brown tide (Adapted from Glober et al., 2011) Field observations from Quantuck Bay, NY. (*A*) Macro- and microscopic images (*Inset*) of an estuary (Quantuck Bay, NY) under normal conditions on June 9, 2009 before a brown tide (note the diatom in *Inset* micrograph image). (*B*) Similar macro- and microscopic images (*Inset*) taken July 6, 2009 during a harmful brown tide bloom caused by *A. anophagefferens* (note the dominance of *A. anophagefferens* in *Inset* micrograph). (*C*) The dynamics of dissolved inorganic nitrogen (DIN) and dissolved organic nitrogen (DON) and the extinction coefficient of light within seawater during the spring and summer of 2009 in Quantuck Bay. (*D*) The dynamics of phytoplankton during the spring and summer of 2009, a year when *A. anophagefferens* bloomed almost to the exclusion of other phytoplankton, including picoeukaryotes, which are often dominated by *Ostreococcus* sp. in estuaries that host brown tides, and *Thalassiosira* and *Phaeodactylum*, genera that are found in this system. The shaded regions in *C* and *D* indicate the period when *A. anophagefferens* blooms, highlighting that *A. anophagefferens* blooms when levels of DIN and light levels are low and DON levels are high and also highlighting that *A. anophagefferens* blooms can persist for more than 1 month during the summer when this species dominates phytoplankton biomass inventories.

I.3 *Chlamydomonas* as a Model Organism

Phytoplankton -cyanobacteria and microalgae- are the primary producers of aquatic ecosystems and therefore comprise the base of the food chain in these niches. Within algal members, **the green microalga *Chlamydomonas reinhardtii* (*Chlamydomonas*) has served as a model organism for physiological studies for more than fifty years** (Harris, 2001). The development of molecular and genetic tools, relying on efficient transformation system, generation of vectors and the sequencing of its three genomes -nuclear, mitochondrial and chloroplast- (Jinkerson and Jonikas, 2015; Kindle, 1998; León-Bañares et al., 2004; Li et al., 2016; Merchant et al., 2007), have been useful for many diverse studies such as photosynthesis, chloroplast evolution, flagella structure, and function and metabolic responses to stress conditions (Harris, 2009). Nitrogen metabolism in this alga has been one of the most extensively studied of any alga (Sanz-Luque et al., 2015a) and findings have been extrapolated to other algae and mostly to land plants leading to a relevant progress (Bittner, 2014; Krapp et al., 2014; Xu et al., 2012).

I.4 Inorganic Nitrogen Nutrition in Algae

I.4.1 Uptake:

Ammonium is the preferred N source for algae due to a lower energetic cost for its assimilation than that for other oxidized forms (Ruiz-Marin et al., 2010). In most organisms, two systems of ammonium transporters have been identified: low affinity (LAATS), related to passive diffusion K^+ channels (Crawford and Forde, 2002; Ullrich et al., 1984; Wang et al., 1993); and high affinity (HAATS), related to active transport activity coupled to H^+ antiport (Howitt and Udvardi, 2000; von Wirén et al., 2000).

The HAATs, present in bacteria, plants, fungi and animals, constitute the superfamily AMT/MEP/Rh. On one hand, the AMT/MEP family includes ammonium transporters in plants, algae, fungi and bacteria (Crawford and Forde, 2002; Loque and von Wiren, 2004; Marini et al., 1997; Thomas et al., 2000; von Wirén et al., 2000). On the other hand, the Rh family of glycoproteins is mostly present in animals. *Chlamydomonas* presents both families. **Rh proteins are low affinity ammonium transporters** and can act as bidirectional channels of CO_2 (Soupene et al., 2002, 2004). This alga bears the largest family of **High Affinity Ammonium Transporters (HAATs)**, coded by **AMT1 genes**, consisting of eight members (**AMT1.1-8**) (González-Ballester et al., 2004) (Figure I.4). These genes show differential expression patters in response to nitrogen availability (González-Ballester et al., 2004) and light (Ermilova et al., 2010). It is interesting that this unicellular organism shows a numerous set of transporters for ammonium, showing a great adaptability to changing environments to complement different affinity and activity requirements.

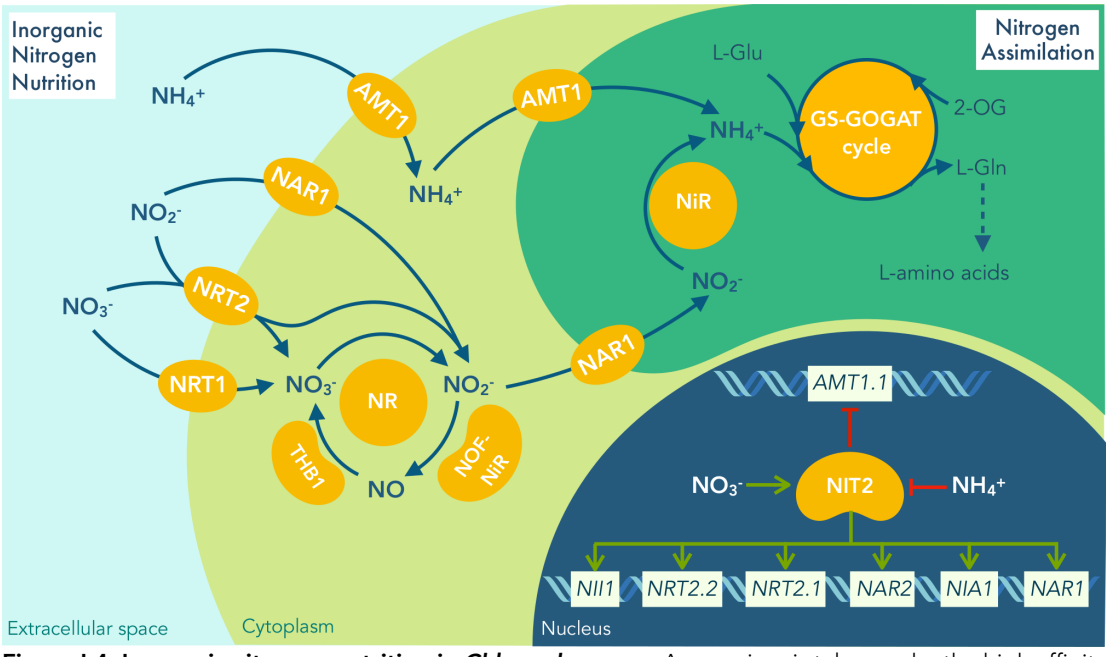


Figure I.4. Inorganic nitrogen nutrition in *Chlamydomonas*. Ammonium is taken up by the high affinity ammonium transporters AMT1. Nitrite can enter into the cell by NAR1 (Nitrate Assimilation-Related component 1) and by the high affinity nitrate/nitrite NRT2 uptake systems. Nitrate is taken up by NRT2 and might also be transported by NRT1. Once inside the cell, nitrate can be reduced to nitrite by Nitrate Reductase (NR) -coded by the gene *NIA1*- and nitrite is transported into the chloroplast by NAR1. Nitrite is then reduced to ammonium by nitrite reductase (NiR), coded by the gene *NII1*. In the chloroplast, ammonium is then assimilated into carbon skeletons by the glutamine synthase-glutamate synthetase (GS-GOGAT) enzymes. Moreover, nitrite can be reduced to nitric oxide (NO) by the NR/NOFNiR (NO-Forming Nitrite Reductase) complex. NO can be re-oxidized to nitrate by the NR/THB1 (Truncated Hemoglobin 1) complex. The master regulatory protein NIT2, activated by nitrate and inhibited by ammonium, is involved in the induction of the expression of the nitrate assimilation genes (*NII1, NRT2.2, NRT2.1, NAR2, NIA1* and *NAR1),* and in the repression of the ammonium uptake transporter *AMT1.1*.

Nitrate is not only a nutrient but also a signal molecule that regulates an important number of processes in microalgae (Crawford, 1995; Krapp et al., 2014). Nitrate and nitrite are transported into the cell by different transport systems: NRT1, NRT2 and NAR1 protein families (Crawford and Glass, 1998; Fernandez and Galvan, 2007, 2008; Forde, 2000; Forde and Cole, 2003; Galván and Fernández, 2001).

NPF transporters, previously known as NRT1 (Léran et al., 2014), are widely represented in plants, but often found in low numbers if any in algae. In plants, most NPF transporters are **low-affinity nitrate transporters** and some of them have been as well reported to be related to peptides and auxin transport (Gojon et al., 2011; Ho et al., 2009; Tsay et al., 2007). *Chlamydomonas* has a unique *NRT1* gene, like most red and green algal genomes. However, *NRT1* seems to be absent in the green microalga *Micromonas pusilla* and the Glaucophyta *Cyanophora paradoxa* (Sanz-Luque et al., 2015a).

NRT2 transporters are present in prokaryotes and eukaryotes (Forde, 2000). This family includes most significant **high affinity nitrate transporters** in fungi, algae and plants (Crawford and Forde, 2002; Galván and Fernández, 2001; Siverio, 2002). Some of them require a **membrane-anchored partner protein NAR2** to be functional (Quesada et al., 1994). In *Chlamydomonas*, two out of six NRT2 members require NAR2, the bispecific high affinity nitrate/nitrite transporter NRT2.1 and the high affinity nitrate transporter NRT2.2 (Fernandez and Galvan, 2007; Quesada et al., 1996). Also, the accessory protein NAR2 gene is present in other Chlorophytes and plants, but absent in Rhodophytes and Glaucophytes.

NAR1 family of transporters, Nitrate Assimilation-Related component 1, present in some eukaryotes like fungi, algae and protozoa but absent in plants, is constituted by FNT (Formate Nitrite Transporters) (Rexach et al., 2000). Similar to NAR2, NAR1 genes are present in Chlorophytes, but absent in Rhodophytes and Glaucophytes. *Chlamydomonas*, with the highest number of members so far, presents six *NAR1* genes. NAR1.1 is involved in **nitrite uptake to the chloroplast** and NAR1.2 (LCIA) transports **nitrite and bicarbonate**, involved in the CO_2-concentration mechanism that makes photosynthesis efficient in aquatic microalgae by accumulating CO_2 close to RuBisCO (Mariscal et al., 2006; Rexach et al., 2000; Wang and Spalding, 2014; Yamano et al., 2015).

Akin to AMT genes, the high number of NRT2 and NAR1 genes present in *Chlamydomonas* genome suggests a high optimization of nutrient utilization in different environmental conditions.

I.4.2 Reduction and Assimilation

Nitrate is reduced to nitrite by the enzyme nitrate reductase (NR) in the cytosol (Figure I.4). In eukaryotes, NR is a homodimeric protein containing three prosthetic groups in each subunit: FAD, b_{557} heme and Molybdenum Cofactor (MoCo) (Zhou and Kleinhofs, 1996). Like in most algae, *Chlamydomonas* NR is coded by a single gene (*NIA1*). In this alga, additional activities have been demonstrated for NR such as nitrite reduction to nitric oxide with a partner protein ARC, recently renamed as NOFNiR (Chamizo-Ampudia et al., 2016); and oxidation of nitric oxide to nitrate with another partner protein, a truncated hemoglobin named THB1 (Sanz-Luque et al., 2015b). Thus, NR participates in maintaining the intracellular homeostasis of NO.

Nitrite is reduced to ammonium by the enzyme Fd-nitrite reductase (NiR) in the chloroplast. Additionally, nitrite can be reduced in the absence of light with electrons from NAD(P)H by Fd-$NADP^+$ oxidoreductase (Jin et al., 1998). In algae, cyanobacteria and plants, NiR is a monomer containing a [4Fe-4S] grouping and a siroheme as prosthetic groups. In *Chlamydomonas*, NiR is encoded by a single gene (*NII1*) clustered with other essential genes for nitrate/nitrite assimilation (Guerrero et al., 1981; López-Ruiz et al., 1991; Quesada et al., 1993).

Finally, **ammonium is incorporated into carbon skeletons by means of the glutamine synthetase-glutamate synthase (GS-GOGAT) cycle** in the chloroplast

(Howitt and Udvardi, 2000; von Wirén et al., 2000). Also, the ammonium generated in the mitochondria by photorespiration can be exported to the cytosol for subsequent assimilation (Britto et al., 2001; Glass, 2003; von Wirén et al., 2000). Additionally, under specific stress conditions, glutamate dehydrogenase (GDH) activity can incorporate ammonium to alpha-ketoglutarate to yield glutamate (Muñoz-Blanco and Cárdenas, 1989). GS genes are numerous, from 3-4, in Volvocales algae, including *Chlamydomonas* and *Volvox carteri, and Coccomyxa subsellipsoidea*. However, functional and location studies have only been performed in *Chlamydomonas* (Chen and Silflow, 1996; Vallon and Spalding, 2009; Vega, 1983). This alga presents two *GS* genes encoding cytosolic GS of type 1 (*GLN1*, *GLN4*) and two pastidic GS of type 2 (*GLN2, GLN3*). However, *Ostreococcus* spp. and *Cyanidioschyzon merolae* show a single *GS* gene more related to bacterial *GLN* than to plant-like *GLN* genes present in other algae. In Chlorophyta genomes, genes for the plastidic NADH-GOGAT and Fd-GOGAT are present, in contrast to Mamiellales, Rhodophytes and Glaucophytes, which show only Fd-GOGAT (Sanz-Luque et al., 2015a).

I.4.3 Regulation of Nitrate Assimilation.

Nitrate and ammonium are usually positive and negative signals, respectively, for nitrate assimilation genes. Thus, **NR gene expression is induced by nitrate and repressed by ammonium** in different algae (Cannons and Shiflett, 2001; Imamura et al., 2010; Llamas et al., 2002; Loppes et al., 1999). In *Chlamydomonas*, a finely tuned regulation mediated by the nitrate/ammonium balance, driven by a complex network of signal molecules and effectors, has been proposed (de Montaigu et al., 2011; Llamas et al., 2002). In *Chlamydomonas*, it is well known that ammonium represses nitrate assimilation genes at both transcriptional and posttranscriptional levels (Fernandez and Galvan, 2008).

A master regulatory gene for nitrate assimilation, NIT2, is necessary for *Chlamydomonas* nitrate assimilation (Camargo et al., 2007). This RWP-RK domain-containing transcription factor is needed for fully upregulation of the main genes involved in nitrate assimilation: nitrate/nitrite transport *NRT2.1, NRT2.2, NAR2, NAR1.1, NAR1.6,* nitrate reductase *NIA1* and nitrite reductase genes *NII1* (Mariscal et al., 2006; Quesada et al., 1993, 1994) (Figure I.4). This gene is also downregulated by ammonium and upregulated by nitrate, suggesting other unknown unidentified regulatory members working upstream to NIT2 (Camargo et al., 2007). Also, this transcription factor is involved in the repression of genes downregulated by nitrate like the ammonium transporter *AMT1.1* (González-Ballester et al., 2004). Additionally, other regulatory protein NZF1 (Nitrate Zinc Finger 1) is involved in nitrate positive regulation of *NIT2* expression (Higuera et al., 2014).

The mechanism by which NIT2 senses nitrate to bind gene promoters remains unknown. However, a NES domain present in the transcription factors NIRA and NLP7, involved in nitrate induction in *Aspergillus* and *Arabidopsis*, respectively, is involved in its exportation to the cytosol in the absence of nitrate (Bernreiter et al., 2007; Marchive et al., 2013). Although transcription factors upregulating nitrate assimilation genes in

green algae other than *Chlamydomonas* remain unknown, in the Rodophyta *Cyanidioschyzon merolae* a R2R3-type MYB transcription factor, *MYB1,* has a key role similar to *Chlamydomonas* NIT2 in the nitrate upregulation of the main N assimilation genes (*NIA2, NII1, NRT2, AMT* and *GLN).* This transcription factor is as well upregulated by nitrate and N starvation (Imamura et al., 2009, 2010).

On the other hand, **nitrate reductase and nitrate transporter genes are downregulated by ammonium**. A soluble guanylate cyclase (sGC), CYG56, is involved in this repression (de Montaigu et al., 2010; Iyer et al., 2003). Moreover, it has been recently revealed that arginine is involved in this signaling cascade (González-Ballester et al., 2018) . This protein, activated by NO, generates cGMP from GTP, and represses *NIA1, NRT2.1, AMT1.1* and *AMT1.2* genes expression (de Montaigu et al., 2010). Canonical sGC genes are well represented in *Chlamydomonas* and *Volvox carteri* genomes. However, very few members are found in *Coccomyxa subellipsoidea, Micromonas* spp. and *Ostreococcus* spp., and none is found in plants. Nevertheless, non-canonical GC could have similar roles in algae and plants lacking canonical sGC domains.

Regarding posttranslational regulation by ammonium, reports are scarce in algae and it is starting to be elucidated in *Chlamydomonas.* **NR and high affinity nitrate/nitrite uptake activities are partially inhibited by nitric oxide (NO)** in a reversible way (Sanz-Luque et al., 2013). The cytosolic truncated hemoglobin THB1 catalyzes the conversion of NO to nitrate by taking up electrons from the FAD group of NR enzyme acting as a sink of reducing power and thus limiting NR activity. In other algae the NR gene has been found to be fused to a truncated hemoglobin (Stewart and Coyne, 2011). This fusion was proposed to be advantageous to use the bloom-induced NO as N source. However, NiR does not seem to be regulated by NO. Under low photosynthetic activity, NiR activity is reduced (Jin et al., 1998), leading to an uncoupled of NR-NiR activity that results in higher nitrite production than reduction. Under this condition, *Chlamydomonas* excretes the overproduced nitrite to avoid toxicity (Navarro et al., 2000). As a compensatory mechanism for N and reducing power loss, NR can synthesize NO from accumulated nitrite to inhibit nitrate uptake and reduction till both NR and NiR activities are coupled again.

Although glutamine is an important signal of N status in bacteria, its sensing in photosynthetic organisms is yet poorly understood. In *Chlamydomonas, CreAMT1.1* repression and nitrite uptake inhibition by ammonium are partially released by methionine sulfoximine (MSX), a specific glutamine synthetase inhibitor (Galván et al., 1991; González-Ballester et al., 2004). In nearly all bacteria and chloroplasts of algae and plants, intracellular level of glutamine is sensed by the PII protein (Chellamuthu et al., 2013). Within green algae, PII proteins have been reported in *Chlamydomonas, Chlorella* and *Micromonas* (Ermilova et al., 2013; McDonald et al., 2010). Notwithstanding, PII ortholog genes seem to be absent in red algal and diatom genomes (Uhrig et al., 2009). In *Chlamydomonas,* PII protein (GLB1) binds to glutamine and activates a N-acetyl-L-glutamate kinase (NAGK) in the chloroplast. However, if 2-oxoglutarate levels reach high concentrations, the complex is inhibited. Thus, when

N/C balance in the cell is shifted to N, arginine biosynthesis is promoted. Moreover, ammonium repression of *AMT1.1* and *NIA1* has been recently reported to be arginine-dependent by means of the NO-inducible CYG56 in *Chlamydomonas* (González-Ballester et al., 2018)

While NO is being extensively studied and many different roles have been attributed to this molecule, knowledge about how it is synthesized and metabolized in photosynthetic organisms is still limited. **NO can be synthesized by the oxidative pathway, from arginine, polyamines, or hydroxylamines; and by the reductive pathway from nitrite** (Gupta et al., 2011a; Moreau et al., 2010; Wilson et al., 2008). The last one has been described as the main pathway for NO synthesis in photosynthetic organisms using NAD(P)H as electron donor (Mallick et al., 2000; Rockel et al., 2002; Sakihama et al., 2002; Yamasaki and Sakihama, 2000). In this pathway, NR is involved in plants and algae (Hao et al., 2010; Horchani et al., 2011; Lozano-Juste and Leon, 2010; Sun et al., 2015; Wei et al., 2014; Zhao et al., 2009). In *Chlamydomonas*, a NR-NOFNiR (Nitric Oxide-Forming Nitrite Reductase) dual system has been recently reported to produce NO in the presence of nitrate, a condition where NR alone cannot do it (Chamizo-Ampudia et al., 2016) (Figure I.4). Besides NR-dependent NO production, an additional reductive pathway has been recently reported in *Chlamydomonas* under nitrogen starvation for 20 hours (Wei et al., 2014). Further alternative pathways have been described in plants, presumably mediated by Mo-enzymes like xanthine oxidoreductase (del Río et al., 2004; Gupta et al., 2011a), aldehyde oxidase (Li et al., 2009) or sulfite oxidase enzymes (Wang and Spalding, 2014), under anaerobic conditions, similar to that described in animals (Bethke et al., 2004). Additionally, nitrite can be chemically reduced to NO in the apoplast at acidic pH.

Oxidative production of NO from arginine is catalyzed by NOS in animals and bacteria. Nevertheless, arginine-dependent NO production has been physiologically described (Corpas et al., 2006, 2009; Gupta et al., 2011a) and arginine involvement in NO-mediated repression by CYG56 in *Chlamydomonas* further suggests that this amino acid may be a source for NO production (González-Ballester et al., 2018). However, no NOS homolog has been yet found in any plant genome and are rarely found in algae. Only some algae, as two *Ostreococcus* spp. and *Bathycoccus prasinos,* show animal-type NOS orthologs in their genomes (Foresi et al., 2010, 2015; Kumar et al., 2015). The unusual presence of this gene in these primitive algae may be explained by their high horizontal gene transfer events in their genome (Moreau et al., 2012).

In *Chlamydomonas,* while NO-induced inhibition of nitrogen assimilation genes under high nitrite levels can be mediated by NR, the inhibition by ammonium is arginine-dependent (González-Ballester et al., 2018). On the other hand, while NO is involved in inhibition of inorganic nitrogen assimilation under inorganic N-replete conditions, it has also been reported that in the absence of N, NO induces genes for alternative N sources use, such as LAO1, an extracellular L-amino acid oxidase involved in N scavenging from amino acids (Wei et al., 2014).

Finally, NO must be rapidly removed to prevent toxicity. Thus, Hemoglobins (Hb) are one of the major NO scavengers, reported in plants (Gupta et al., 2011b;

Perazzolli et al., 2004) and algae. In *Chlamydomonas*, two truncated hemoglobins *THB1* and *THB2* are differentially expressed by the N source and regulated by NIT2 (Johnson et al., 2014; Sanz-Luque et al., 2015b). *THB1* and *THB2* are upregulated and downregulated, respectively, by NO. Also, as mentioned before, THB1 uses the reducing power from NAD(P)H working with NR. Moreover, other truncated hemoglobin, THB8, is crucial for survival under hypoxia in a process involving NO (Hemschemeier et al., 2013). Truncated hemoglobins in other algae have also been identified, such as in other *Chlamydomonas* spp., *Volvox carteri, Chlorella* sp. NC64A and *Micromonas pusilla* (Hemschemeier et al., 2013; Vázquez-Limón et al., 2012). Although less studied in algae, other NO scavenging pathways include spontaneous oxidation to nitrite, reaction with glutathione to produce nitrosoglutathione (GSNO) -a main reservoir of NO in photosynthetic organisms (Wang et al., 2006)-, reaction with other ROS like superoxide anion to produce peroxynitrite, and with metals as iron of heme groups (Gupta et al., 2011a; Moreau et al., 2010; Mur et al., 2013).

I.5 Organic Nitrogen Nutrition in Algae

While inorganic N can be limiting in some natural habitats, organic N sources may be locally or temporarily otherwise available (Berman and Bronk, 2003). These organic molecules, mainly urea and amino acids, but also amines, nucleic acids and other nitrogenous compounds, may be alternative N sources that can contribute substantially to algal N demand (Tyler et al., 2003, 2005). Thus, the ability to exploit these molecules by some species may result advantageous over other competitors. Although the use of organic N sources by algae is of eco-physiological importance, these studies are still scarce. Here, we review the algal use of the two major organic N molecules found in aquatic ecosystems: urea and amino acids.

I.5.1 Urea uptake and catabolism

Even though urea concentration in aquatic ecosystems is often lower than that for inorganic N molecules, it can reach greater concentrations, especially due to runoff from fertilized areas (Glibert et al.; Kudela et al., 2008; Solomon et al., 2010). Urea input in aquatic ecosystems can also come from natural sources such as excretion by zooplankton, regeneration by heterotrophic bacteria, release by phytoplankton and some animals (Berman and Bronk, 2003; Miller and Roman, 2008; Painter et al., 2008; Pedersen et al., 1993).

Urea can be readily used as N source by many different algae including the Phaeophyceae macroalga *Chordaria flagelliformis* (Probyn and Chapman, 1982), the Rhodophyta *Gracilaria vermiculophylla*, the Chlorophyta *Ulva lactuca*, *Chlamydomonas reinhardtii* and *Volvox carteri* (Kirk and Kirk, 1978b, 1978c, 1978a; Tyler et al., 2005), and the diatom *Phaeodactylum* (Rees and Syrett, 1979). Urea uptake rates by algae can be higher than that for inorganic N (Berman and Bronk, 2003; Berman and Chava, 1999; Kudela and Cochlan, 2000; McCarthy, 1972). This uptake can be driven by DUR3 proteins, high-affinity active transporters included in the sodium:solute symporter family (SSSF) of transport proteins present in mosses, higher plants and fungi (Wang et al., 2008). These urea transporters are widely distributed in algal genomes, found in Chlorophyta, Rhodophyta, Heterokonta, Alveolata and

Haptophyta genomes (Solomon et al., 2010). *Chlamydomonas* can readily assimilate urea by an active and energy-dependent system (Williams and Hodson, 1977) (Figure I.5). Two urea transporters are found in *Chlamydomonas* genome *DUR3A* and *DUR3B*. Besides the high-affinity transport system DUR3, urea transport can be mediated by urea/amide channels, aquaporins and low-affinity transporters of the solute carrier family 14 (SLC14) (Raunser et al., 2009). Within the red lineage, the Haptophyta *Emiliana huxleyi* and the diatoms *Thalassiosira pseudonana* and *Phaeodactylum tricornutum* bear *SLC14A* urea transporters in their genomes (Solomon et al., 2010). In higher plants, urea uptake is driven by DUR3A but low-affinity transport systems such as aquaporins can become more important when urea concentration reaches low levels (Wang et al., 2008).

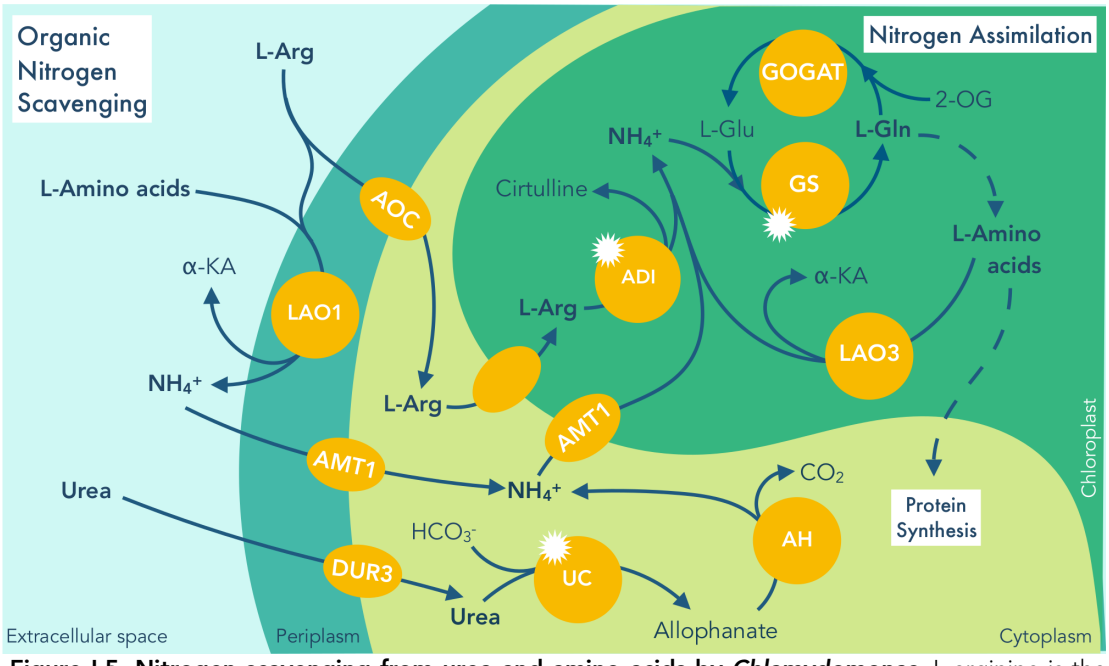


Figure I.5. Nitrogen scavenging from urea and amino acids by *Chlamydomonas*. L-arginine is the only amino acid actively transported by a high affinity transporter (AOC, included in the APC -Amino acid Polyamine organoCation- family of transporters). Once inside the cell, arginine is deaminated by the arginine deiminase (ADI) generating ammonium and citrulline. Most amino acids can be extracellularly deaminated by the L-amino acid oxidase LAO1, generating ammonium and the corresponding α-keto acid (α-KA). Ammonium is taken up efficiently by the high affinity ammonium transporters AMT1. Otherwise, the keto acid remains in the media. An intracellular LAO1 homolog is predicted to be present in the chloroplast. Urea is taken up by specific transporters (DUR3). Then, urea is catabolyzed by the urea carboxylase (UC) and the allophanate hydrolase (AH), producing ammonium and CO_2. Ammonium is assimilated into carbon skeletons in the chloroplast by the glutamine synthase and glutamate synthetase (GS-GOGAT) enzymes. White spiked circles indicate ATP-dependent enzymatic activity.

Once inside the cell, **urea can be hydrolyzed intracellularly** by two different enzymes that catalyze the degradation of urea to ammonia and CO_2: **urease** (EC 3.5.1.5), which catalyze the hydrolytic cleavage of urea; or by **ATP-urea amidolyase** (UALase). This last enzyme carries out two catalytic activities: urea carboxylase (EC 6.3.4.6) that catalyzes the ATP-dependent carboxylation of urea to allophanate; and allophanate hydrolase (EC 3.5.1.13). Like in cyanobacteria and higher plants, urease genes have been found in some marine algae within the red lineage including *Thalassiosira pseudonana*, *Phaeodactylum tricornutum, Emiliana huxleyi,* and *Aureococcus anophagefferens*, and in some marine green algae such as *Ostreococcus* and *Micromonas* (Solomon et al., 2010). Alternatively, UALase genes can be arranged as a single gene or as two genes coding two independent proteins catalyzing the two different activities. In *Chlamydomonas* genome, two separated genes, *DUR1* and *DUR2*, coding for urea carboxylase (UC) and allophanate hydrolase (AH) enzymes, respectively, are found to form the UALase complex (Hodson et al., 1975; Whitney and Cooper, 1972) (Figure I.5). Both genes *DUR1* and *DUR2* are found adjacent and tail-to-tail oriented, and clustered with the urea transport genes *DUR3A* and *DUR3B*. UALase activity has been found different in Chlorophyta including *Chlamydomonas reinhardtii, Dunaliella terticolecta, Nanochloris coccoides, Scenedesmus brasiliensis, Schizomeris liebleinii, Ulothrix* and *Stichococcus* spp. (Al-Houty and Syrett, 1984; Leftley and Syrett, 1973).

I.5.2 Amino acids utilization

Amino acids in aquatic ecosystems may come from different sources such as phytoplankton exudation, zooplankton excretion, viral lysis and detritus particles from biomass decay (Rosenstock and Simon, 2001; Sarmento et al., 2013). Even though, bacteria have long been thought to be the major consumers, some phytoplankton species are reported to use amino acids and **can contribute as well to bulk amino acids uptake** (Flynn and Butler, 1986; Mulholland et al., 2002; Zhang et al., 2015).

The use of amino acids as N source for growth by different algal species has long been demonstrated (Algéus, 1949; Cho and Komor, 1985; Ietswaart et al., 1994; Kirk and Kirk, 1978a; Palenik et al., 1988; Palenik and Morel, 1990a). However, information about how algae use amino acids as a N source is still poorly understood.

Amino acids can be utilized as N sources by two pathways: direct uptake and subsequent intracellular catabolism; or extracellular deamination and further uptake of the generated ammonium.

I.5.2.1. Amino acids uptake and intracellular catabolism

***Chlamydomonas* can use most amino acids as N source, however only L-arginine is efficiently taken up by a specific transport system** (Kirk and Kirk, 1978b) (Figure I.5). Likewise, other green microalgae show efficient uptake for only one amino acid, L-arginine in *Volvox* (Kirk and Kirk, 1978c) and L-histidine in *Dunaliella* (Eaton-Rye et al., 2017). However, the identity of those transporters has not been determined experimentally. *Chlamydomonas*

genome shows a vast number of genes coding putative amino acid transporters (Vallon and Spalding, 2009). These are included in two families: the **AAAP (Amino Acid/Auxin Permease)** and the **APC (Amino acid Polyamine organo-Cation) families of transporters**. Seven genes are included in the first one, named *AOT1-7*, and six in the second one, known as *AOC1-6*. ***AOC5* and *AOC6* have been proposed to be responsible of L-arginine uptake in *Chlamydomonas*** (Vallon and Spalding, 2009). These transporters are the most closely related to plant Cationic Amino Acid Transporters and to human SLC7A1-3, which transport arginine and lysine. On the other hand, the specificity of *AOC1-4* genes cannot be deduced by their sequence and some of them could be involved in intracellular amino acid transport (Vallon and Spalding, 2009). Otherwise, within the *AAAP* transport genes, *AOT1-4* are more closely related to plant homologues, while *AOT5-7* are more similar to animal and fungal members. More than one carrier system exist in the green algae *Scenedesmus* and *Ankistrodesmus,* one for arginine, and another one or more for non-basic amino acids (Kirk and Kirk, 1978a). In contrast to the Chlorophyceae algae *Chlamydomonas, Volvox,* and *Dunaliella* that show uptake for only one or two amino acids, the Trebouxophyceae alga *Chlorella vulgaris* shows surprisingly complex and diverse amino acid uptake systems. At least seven different uptake systems have been described: system I, for most neutral and acidic amino acids; system II, for short-chain neutral amino acids including proline; system III, for basic amino acids including histidine; system IV, for acidic amino acids; and specific systems for L-methionine (system V), L-threonine (system VI) and L-glutamine (system VII) (Cho and Komor, 1985). Interestingly, amino acids supply to *Chlorella* by symbiotic hosts have been reported (Kato and Imamura, 2008; McAuley, 1991). Thus, the development of such a broad number of amino acids uptake systems may have been driven by nutritional advantages during symbiotic relationships. In contrast, a limited number of amino acid uptake systems seems to be a common trait within green algae (Kirk and Kirk, 1978a; Murphree et al., 2017).

I.5.2.2 Extracellular deamination of amino acids

In addition to uptake, extracellular oxidation is an effective mechanism to scavenge N from amino acids carried out by some bacteria and different phytoplankton species. Indeed, amino acid oxidase enzymes are proposed to significantly contribute to N supply in the oceans (Palenik and Morel, 1990a). **L-Amino Acid Oxidase (LAAO) enzymes are flavin adenine dinucleotide (FAD)-containing proteins that catalyze the oxidative deamination of L-amino acids to α-keto acids and ammonia** (EC 1.4.3.2.). These enzymes are widely distributed in nature and are present in diverse organisms including bacteria (Faust et al., 2007), fungi (Aurich et al., 1972; Nuutinen et al., 2012), algae (Fujisawa et al., 1982; Rees and Allison, 2006; Vallon et al., 1993), fishes (Kitani et al., 2007), the sea hare (Yang et al., 2005), mammals (Nagaoka et al., 2009; Sun et al., 2002), and in snake venom (Bordon et al., 2015; Tõnismägi et al.,

2006). LAAOs catalyze a similar reaction in different organisms with similar structures that may have derived from an old evolutionary origin but evolved to cope with different physiological functions in distinct groups (Campillo-Brocal et al., 2015; Macheroux et al., 2001). While the major role of some LAAO enzymes has been suggested to be a defense against pathogens by H_2O_2 generation (Kasai et al., 2015), it has been shown that **fungal and algal LAAOs help to scavenge N from amino acids** (Nuutinen and Timonen, 2008; Piedras et al., 1992). Consistent with this function in algae and fungi, **LAAO enzymes show a broad substrate specificity and are induced under N-limiting conditions** (Davis et al., 2005; Sikora and Marzluf, 1982; Vallon et al., 1993). LAAOs may provide an ecological advantage in instances where inorganic N is limiting, and organic N is otherwise available.

Chlamydomonas bears a periplasmic L-amino acid oxidase encoded by the gene *LAO1* (*LAO1*). The LAO1 enzyme deaminates a wide range of L-amino acids (Vallon et al., 1993), releasing ammonium that can be efficiently taken up by the high-affinity ammonium transporters AMT1 (Figure I.5). However, the α-keto acids are not further metabolized and remain extracellular (Muñoz-Blanco et al., 1990). This enzyme was found in two highly glycosylated different forms with similar enzymatic properties and high molecular weight: a lighter form containing LAO1 -previously named as Mα or catalytic subunit-; and a heavier form containing an additional subunit coded by the gene *LAO2* -previously named as Mβ or non-catalytic subunit- (Vallon et al., 1993). Moreover, a *LAO1* homolog can be found in *Chlamydomonas* genome, encoded by the gene *LAO3* (Vallon and Spalding, 2009). Although its location requires further experimental demonstration, LAO3 is predicted to be intracellular and possibly located in the chloroplast.

Surprisingly, no LAO1 homologs have been found in any sequenced green algae or plant (Vallon and Spalding, 2009). Nevertheless, extracellular amino acid oxidation has been reported in different algae including the Rhodophyta *Gymnogongrus flabelliformis* and *Amphiora crassissima* (Fujisawa et al., 1982; Ito et al., 1987), the Alveolata *Amphiridium carterae* and *A. operculatum* (Palenik and Morel, 1990b), the Heterokonta *Phaeodactylum tricornutum* and *Aureococcus anophagefferens* (Mulholland et al., 2002; Rees and Allison, 2006), and the Haptophyta *Pleurochrysis* spp. and *Prymnesium parvum* (Palenik and Morel, 1990b). However, other algal species were reported to lack this activity (Algéus, 1949; Ietswaart et al., 1994; Murphree et al., 2017; Palenik and Morel, 1990b). Therefore, LAAO presence in algae seems to be an adaptive trait probably to cope with different nutritional availability.

The use of peptides as N source by algae has been scarcely studied. Vallon et al. (1993) reported that the purified *Chlamydomonas* LAO1 enzyme is not active on dipeptides, although the dipeptides tested were not specified. Besides, a high-throughput screening based on cellular respiration of this alga in response to a large array of metabolites has shown that *Chlamydomonas* is

metabolically active on a number of di- and tri-peptides (108 out of 267 di-peptides, and 3 out of 14 tri-peptides) (Chaiboonchoe et al., 2014). However, whether those peptides can support *Chlamydomonas* growth remains unknown.

Moreover, other organic nitrogenous compounds such as acetamide and purines like adenine, guanine and hypoxanthine can be used as N sources by *Chlamydomonas* (reviewed by Vallon and Spalding, 2009).

I.5.3 Regulation of organic nitrogen utilization

Organic N compounds are usually considered 'poor' sources since they support much slower growth rates than inorganic N sources. However, it may be due to a fine-tuned regulatory network that prioritizes nutrient retention over fast growth to avoid N loss, as previously proposed for bacteria (Wang et al., 2016b). In eukaryotic microorganisms, genes clustering is often found for genes related to the same metabolic pathway to facilitate a coordinated regulation (Osbourn and Field, 2009). For instance, genes related to nitrate assimilation are found clustered in filamentous fungi, yeasts and algae (Galván and Fernández, 2001; Johnstone et al., 1990; Pérez et al., 1997). Similarly, genes related to organic N utilization can be found in *Chlamydomonas* genome where *UC, AH, DUR3A, DUR3B* and *ADI* genes, related to urea and arginine assimilation, are found physically clustered in Chromosome VIII. Urea uptake is induced by N deficiency, urea and arginine in *Chlamydomonas* and Volvox (Kirk and Kirk, 1978b, 1978c). Urea uptake is as well stimulated by N starvation in *Chlorella ellipsoidea* (Hattori, 1960) and in *Phaeodactylum* (Rees and Syrett, 1979), and otherwise repressed by the presence of inorganic N in *Chlamydomonas, Volvox, Scenedesmus, Ulva, Gracilaria* and *Chordaria* species (Healey, 1977; Kirk and Kirk, 1978b, 1978c; Probyn and Chapman, 1982; Tyler et al., 2005). In *Chlamydomonas, Chlorella, Scenedesmus, Phaeodactylum* urea uptake is induced by light and/or depressed by energy uncouplers or dark (Healey, 1977; Hodson and Thompson, 1969; Rees and Syrett, 1979; Williams and Hodson, 1977). Uptake in the green alga *Ulva lactuca* and the red alga *Gracialaria vermiculophylla*, a similar pattern for urea uptake has been reported: a fast initial uptake phase and a lower sustained phase, suggesting feedback inhibition by fulfilled intracellular pools (Tyler et al., 2005). Moreover, UALase is inducible by N deficiency in *Chlamydomonas* and *Chlorella* (Hodson et al., 1975). Also, a regulatory link between urea and acetamide utilization by *Chlamydomonas* has been proposed (Hodson and Gresshoff, 1987).

Similarly to urea, arginine uptake is induced by N-deficiency and the presence of urea or arginine, and repressed by inorganic N in *Chlamydomonas* and *Volvox* (Kirk and Kirk, 1978b, 1978c; Strijkert et al., 1971). However, alanine uptake in *Ulva lactuca* is not repressed by ammonium (Tyler et al., 2005). Amino acid transport systems in *Chlorella* are induced by glucose (Cho and Komor, 1985).

LAAO activity in *Chlamydomonas*, considered a hallmark for nitrogen deprivation, is highly induced by N starvation and strongly repressed by high levels of ammonium (Vallon et al., 1993). Also, it is induced by supplementation of acetate, an assimilable carbon source for *Chlamydomonas* (Muñoz-Blanco et al., 1990). Additionally, *Chlamydomonas* can use adenine or guanine as the sole nitrogen source for growth by means of an inducible system that is repressed by ammonia (Lisa et al., 1995).

In summary, although with some exceptions, a common regulation seems to be present for the assimilation of organic N sources by algae, driven by nitrogen, carbon and light/energy availability. Thus, the presence of **inorganic N is a negative signal for the assimilation of alternative N sources.** This preference might be explained

by the lower energetic cost for assimilation of inorganic forms. Otherwise, **available carbon sources**, such as glucose for *Chlorella* or acetate for *Chlamydomonas*, **are positive signals for organic N assimilation.** In this case, the requirement of carbon skeletons to assimilate N by the GS/GOGAT cycle and/or the requirement of metabolic energy may be limiting the capacity of N scavenging from alternative sources. Lastly, **the presence of light activates N scavenging from alternative N sources**. This regulation seems to be related to the energy demand by the assimilation of these sources, since energy uncouplers but not photosynthesis inhibitors inhibited assimilation. In this way, a fine-tuned regulatory network may make organic N forms 'poor' sources in a deliberated way, supporting slow growth, likely to couple N scavenge with N assimilation capacity in terms of C/N and energy availability to avoid N loss, which might result advantageous for competitors (Wang et al., 2016b). Nevertheless, the molecular basis of the nitrogen nutrition preference in algae requires further investigation.

I.6 Algal Metabolic Complementation by Bacteria

I.6.1 The Phycosphere

Phytoplankton account for approximately 50% of the total global carbon fixation (Field et al., 1998). Heterotrophic bacteria, comprising almost 25% of the total biomass in the euphotic zone of the oceans (Pomeroy et al., 2007), are fed on up to 50% of the carbon fixed by phytoplankton (Fuhrman and Azam, 1982). Thus, these two linked groups have a great impact on the global carbon and nutrient cycling, and strongly shape the ecology of aquatic systems. Interactions between phytoplankton and bacteria have long been reported and can be from cooperative to competitive associations (Amin et al., 2012). These interactions are based on the exchange of metabolites and infochemicals, such as nutrients or vitamins, and the complexity of these partnerships is only starting to be uncovered (Hom et al., 2015). The zone immediately surrounding phytoplankton cells is the physical interface where these chemicals are exchanged (Cole, 1982). This microenvironment, known as the **phycosphere -a planktonic analogous of the rhizosphere in land plants-,** is considered the key meeting place for phytoplankton-bacterial interactions (Seymour et al., 2017) (Figure I.6).

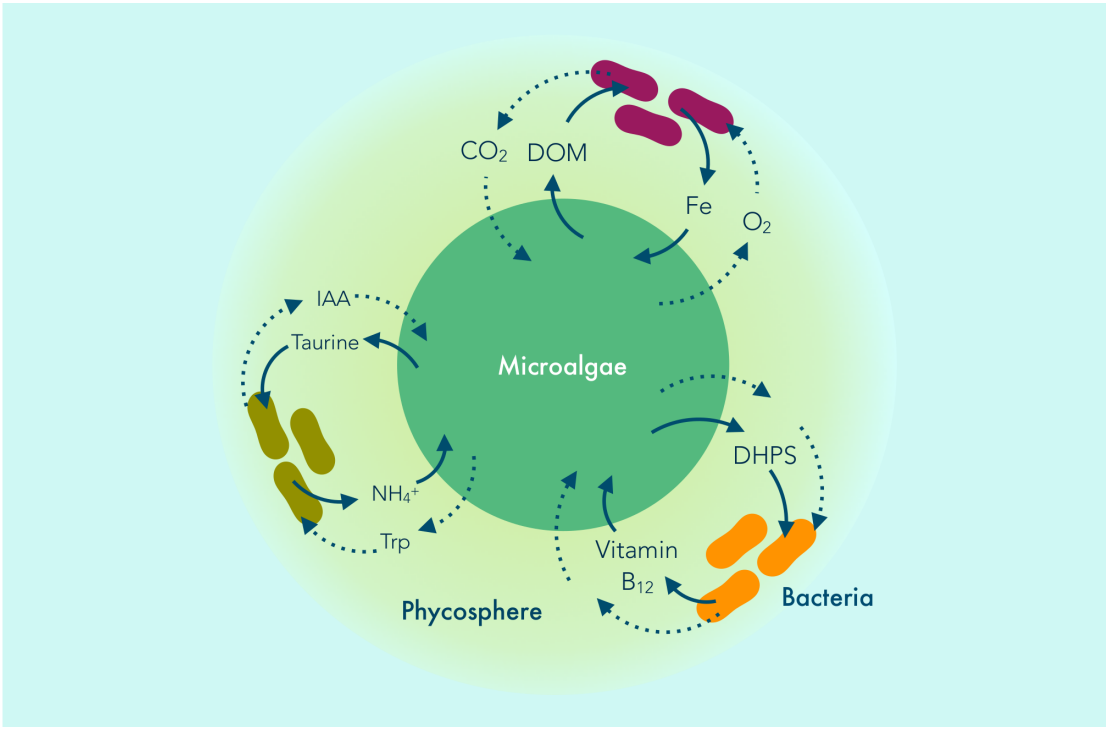


Figure I.6. The phycosphere. The zone immediately surrounding phytoplankton cells is the physical interface where nutrients and infochemicals are exchanged with interacting bacteria. This microenvironment, known as the phycosphere -a planktonic analogous of the rhizosphere in land plants-, is considered the key meeting place for phytoplankton-bacterial interaction. Examples of mutualistic bacteria provide microalgae with soluble iron (Fe), vitamin B_{12}, ammonium and phytohormones (IAA). In turn, microalgae can supply bacteria dissolved organic matter (DOM), 2,3-dihydropropane-1-sulfonate (DHPS), taurine, tryptophan (Trp) and oxygen.

The growing evidences for only particular bacterial taxa co-existing with phytoplankton species make clear that these interactions have been selected throughout the evolution (Culham et al., 1993; Krohn-Molt et al., 2017; Ramanan et al., 2015). For instance, the coccolithophores *Emiliana huxleyi* and *Coccolithus pelagicus* cultures are dominated by *Marinobacter* and *Marivita* spp. (Green et al., 2015), which have also been found in association with dinoflagellates and diatoms (Green et al., 2004, 2010; Hatton et al., 2012; Hwang et al., 2009; Seibold et al., 2001). Moreover, the major bacterial phyla found in the phycosphere of different green algae -mainly Bacteroidetes and α-Proteobacteria- are found as well in symbiosis with plants, which strongly suggests a possible **co-evolution occurring between algae and their bacterial symbionts** (Ramanan et al., 2015, 2016).

Different associations can be classified mainly as: mutualism, when both partners get benefit; commensalism, in which only one partner is benefitted; and parasitism, in which a partner is negatively affected by the interaction. Nevertheless, microbial interactions are dynamic and represent a continuum of benefiting and competitive interactions that can simultaneously be influencing the association. The specific conditions will determine if the interactions shift from

beneficial to harmful, showing the so-called 'Jekyll and Hyde' lifestyle as previously reported for some algal-bacterial interactions (Aminov et al., 2016; Wang et al., 2014).

Here, we will focus exclusively on algal-bacterial mutualistic interactions.

I.6.2 Microalgae Growth-Promoting Bacteria

Mutualistic interactions between algae and bacteria are widespread in nature. Many symbiotic associations are based on nutrients exchange. Likewise some land plants get benefited by fixed N from symbiotic diazotrophic bacteria, some marine diatoms establish symbiotic relationships with N_2-fixing cyanobacteria that metabolically complement these alga by providing them with bioavailable N (Thompson et al., 2012). Also, Haptophytes can feed on cyanobacterial fixed N in exchange of algal fixed carbon (Foster et al., 2011).

In corals reef ecosystems, symbiotic algae provide high concentrations of oxygen to bacteria, promoting growth of both the animal host and the prokaryote, and avoiding infectious organisms (Kuhl et al., 1995). Furthermore, it has been suggested that symbiotic microbial community may increase heat tolerance of corals protecting them from bleaching (Ziegler et al., 2017). Likewise, mutualistic vitamin B_{12}-producing bacteria enhance thermal tolerance in *Chlamydomonas* (Xie et al., 2013). In another example, the diatom *Thalassiosira pseudonana* gets benefited as well by bacterial-produced vitamin B_{12} in exchange of the organosulfur molecule DHPS (2,3-dihydropropane-1-sulfonate). Interestingly, only a limited groups of bacteria can catabolize this molecule, leading to a strong selectivity of bacterial species by diatoms (Durham et al., 2015).

Other examples of benefiting actions by bacteria are those in which **bacteria metabolically complement algal nutrition by turning into bioavailable nutrients that are not readily assimilable by algae**, such as N mineralization or iron solubilization. Bacterial mineralization of amino acids can improve algal growth by the coccolithophore *Emiliana huxleyi* and the diatom *Thalassiosira pseudonana* (Ietswaart et al., 1994). Some *Marinobacter* species produce vibrioferrin, a siderophore that binds iron with high affinity and, in the presence of light, releases a more soluble form of iron that is rapidly taken up by both bacteria and algal hosts. In turn, the algal partner feed the prokaryote with organic carbon (Amin et al., 2009; Yarimizu et al., 2018).

These diverse algal-bacterial interactions can be exploited for biotechnological purposes that include: microalgal harvesting by bacterial induced aggregation (Powell and Hill, 2013), algal cells disruption by bacterial induced autolysis (Demuez et al., 2015), enhanced algal biohydrogen production (Ban et al., 2018), wastewater treatment (de-Bashan et al., 2004; Higgins et al., 2018), bioremediation (Boivin et al., 2007; Tang et al., 2010), bloom control (Lee et al., 2018) and sustainable aquaculture (Toi et al., 2013).

I.6.3 Microalgae-bacteria inter-kingdom signaling

Similar to the regulatory machinery present in single organisms driven by signaling molecules, linked metabolisms of different organisms is association show a fine-tuned regulatory network. These inter-kingdom signaling pathways by which algae and bacteria communicate are only starting to be elucidated (Zhou et al., 2016).

Bacteria communicate between each other by means of released chemical signal molecules. By quorum sensing (QS), individual cells can sense the population density by detecting released autoinducers and activating specific genes expression when the concentration threshold is reached. In this way, bacteria work coordinately as a community rather than as individual cells to trigger different responses, such as biofilm formation and antibiotic production (Evans et al., 2018; Li and Tian, 2012). **Algae have developed the ability to detect and respond to bacterial QS signals**. For instance, the Rhodophyta *Delisea pulchra* releases QS analogs that interfere with the expression of bacterial genes related to virulence and antibiotic production (Givskov et al., 1996; Manefield et al., 2000, 2001). Likewise, *Chlamydomonas* releases molecules that mimic QS molecules to disrupt bacterial communication (Teplitski et al., 2004). Moreover, it has been reported that *Chlamydomonas* excretes a molecule that can induce the synthesis of a particular bacterial protein (Stegeman and Hoober, 1975), showing its ability to impact bacterial physiology.

Some Plant Growth-Promoting Bacteria (PGPB) have developed the ability to generate the auxin indole-3-acetic acid (IAA). **IAA is a phytohormone that promotes growth in land plants and algae** (Kiseleva et al., 2012; Labeeuw et al., 2016; Mazur et al., 2001; Tarakhovskaya et al., 2007). Moreover, this molecule is involved in the signaling communication in symbiotic interactions (Spaepen and Vanderleyden, 2011). Plant-exuded L-tryptophan is used by indole-producing bacteria to synthesize and release IAA, which is taken up by the plant. This bacterial IAA, together with the endogenous IAA, produces in the plant a set of physiological responses such as cell proliferation or elongation and the biosynthesis of 1-aminocyclopropane-1-carboxylic acid (ACC), the precursor of ethylene. This ACC is exuded and utilized by ACC deaminase-producing bacteria, using it as a N and carbon source. Thus, bacteria act as a sink for this molecule preventing ACC accumulation in the plant, as well as the formation of ethylene, a plant growth inhibitor. In this way, **a synergistic interaction between bacterial ACC deaminase and both plant and bacterial IAA has been proposed to be key for optimal function of PGPB** (Glick, 2014). A similar inter-kingdom signaling pathway has been described in the interaction between the bloom-forming coccolithopohore *Emiliana huxleyi* and a Roseobacter bacteria (Segev et al., 2016). Similarly, **bacterial IAA has been found to promote algal growth** during a mutualistic interaction based on nutrients exchange between the diatom *Pseudo-nitzschia multiseries* and a

Sulfitobacter species (Amin et al., 2015). Thus, although further studies are necessary, these recent findings suggest that this signaling pathway dominating symbiotic interactions within the rhizosphere might be as well present within the phycosphere.

A better understanding on algal-bacterial interactions will provide new insights into the evolution of these associations that may be included in ecology principles and applied for different biotechnological applications. To that end, the use of model organisms can be extremely useful.

OBJECTIVES

1. To study the Nitrate signal and the role of NIT2 on organic nitrogen assimilation (amino acids) in *Chlamydomonas*.
2. To investigate the role of the *Chlamydomonas* extracellular L-amino acid oxidase (LAO1) as a nitrogen scavenger from amino acids and peptides, and the study of its evolution in algae.
3. To elucidate possible algal-bacterial mutualistic interactions for facilitating algal growth on amino acids and peptides.

RESULTS AND DISCUSSION

Chapter 1:

Nitrate and NIT2 are Negative Effectors for Organic Nitrogen Assimilation in *Chlamydomonas*.

1.1 Nitrate and NIT2 as signaling effectors regulating genes expression

To better understand the nitrate signaling involved in regulating different metabolic processes as well as the role of the key regulator of nitrate assimilation gene, NIT2, we have studied the transcriptome of *Chlamydomonas* wild type (WT) and a nit2 mutant in response to nitrate. Two conditions were evaluated: A) **mixotrophic conditions** -supplemented with acetate (TAP medium)-; and B) **autotrophic conditions** -without acetate but in the presence of CO_2 (minimal medium bubbled with 4% CO_2). After 1 hour of incubation, samples were processed for transcriptome analysis (Figure 1.1). This time was chosen because the major nitrate assimilation genes are fully induced at this point. Thus, we found interesting to compare the transcriptomes between strains able to sense and assimilate nitrate (WT) and that unable to assimilate nitrate (*nit2* mutant).

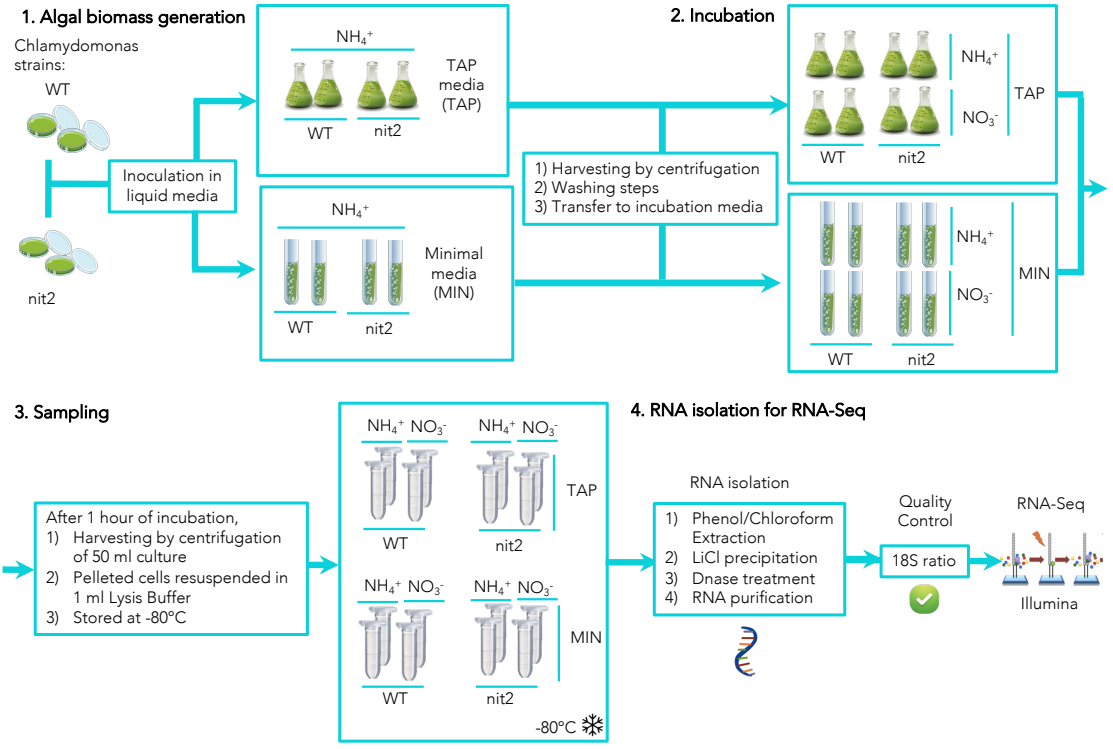


Figure 1.1. Workflow for transcriptomic analysis. For further details, see Materials and Methods section.

The results showed that 416 genes were differentially regulated (fold change >2) in response to nitrate under mixotrophic conditions in the WT: 342 up-regulated and 74 down-regulated. However, under autotrophic conditions the number of genes differentially regulated was 3,840, significantly higher to mixotrophic conditions. Within these, 1759 genes were up-regulated and 2081 down-regulated (Figure 1.2). Fold changes were greater for up-regulated genes (up to log_2FC = 9.80 and log_2FC = 11.00 on TAP and minimal media, respectively) than for down-regulated genes (up to log_2FC = -3.05 and log_2FC = -5.4 on TAP and minimal media, respectively).

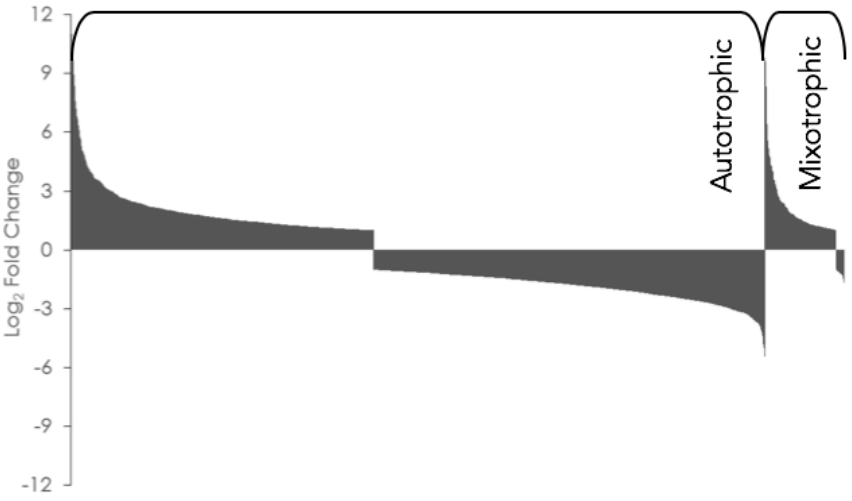


Figure 1.2. Distribution of transcripts differentially accumulated on nitrate by Chlamydomonas WT. Bars represent each transcript differentially expressed in the WT.

The comparison between both mixotrophic and autotrophic conditions -TAP and minimal media, respectively- helps to highlight common genes differentially expressed due to the presence of the specific inorganic nitrogen molecule –ammonium or nitrate-, independently of the carbon source being used by this alga. When compared, only 230 genes were shared under both mixotrophic and autotrophic conditions in the WT (core) (Figure 1.3).

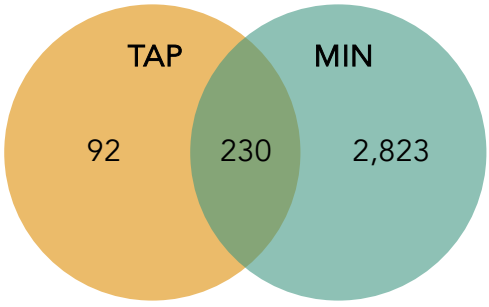
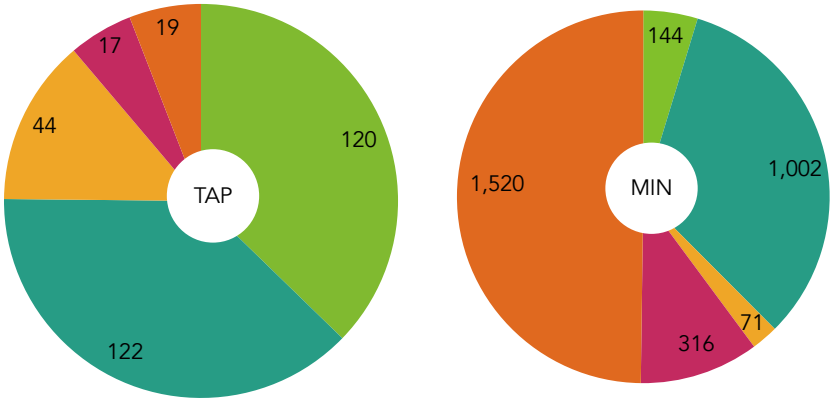


Figure 1.3. Venn Diagram showing the distribution of transcripts differentially expressed. Included transcripts showed a fold change above 2 in the wild-type, comparing nitrate with ammonium; the *nit2* mutant, comparing nitrate with ammonium; and/or nitrate, comparing the wild-type with the *nit2* mutant. TAP, under mixotrophic conditions (with acetate). MIN, under strictly autotrophic conditions (without acetate, but bubbled with 5% of CO_2).

When WT and *nit2* mutant strains are compared, different and interesting expression patterns can be observed. We divided them into five clusters, and within each cluster shared genes between mixotrophic and autotrophic conditions were analyzed (core genes) (Figure 1.4 and 5):



	WT	*nit2*	NO_3^-
Cluster A	$NO_3^- > NH_4^+$	$NO_3^- \geq NH_4^+$	WT > *nit2*
Cluster B	$NO_3^- > NH_4^+$	$NO_3^- > NH_4^+$	WT = *nit2*
Cluster C	$NO_3^- < NH_4^+$	$NO_3^- < NH_4^+$	WT < *nit2*
Cluster D	$NO_3^- < NH_4^+$	$NO_3^- < NH_4^+$	WT = *nit2*
Cluster E	$NO_3^- > NH_4^+$	$NO_3^- = NH_4^+$	WT < *nit2*

Figure 1.4. Pie charts showing the distribution of differentially expressed genes corresponding to each cluster (A-E) on TAP and minimal (MIN) media. The table refers to the differential abundance of transcripts on ammonium and nitrate, and in the wild-type (WT, 704 strain) and the mutant (*nit2*, 89.87 strain).

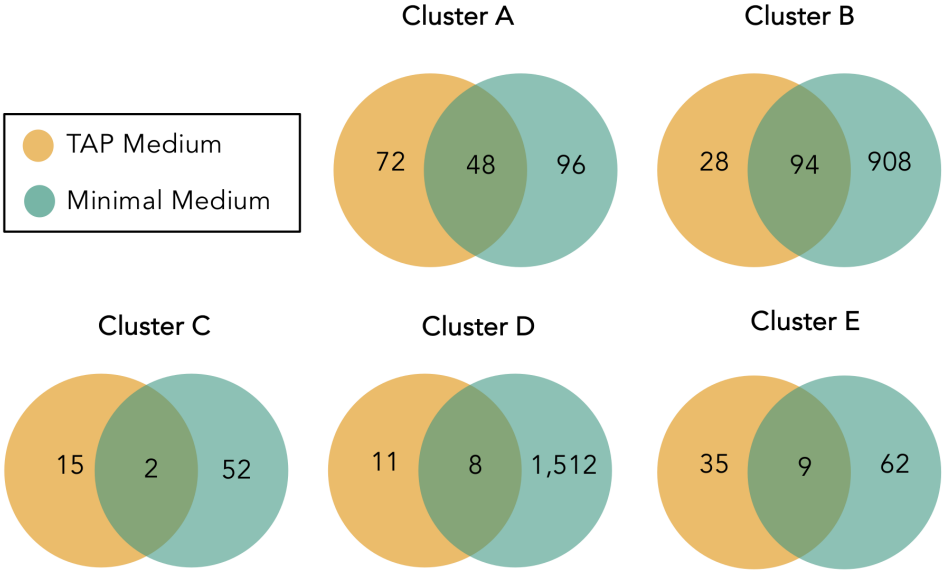


Figure 1.5. Venn diagrams showing the distribution of differentially expressed genes considered in Figure 1.1., in each divided cluster. Numbers refers to the number of transcripts differentially accumulated (fold change > 2) according to each expression pattern or cluster.

Cluster A: up-regulated and NIT2-dependent.

C**luster A** represents those genes that were overexpressed on nitrate, with higher expression in the WT than in the *nit2* mutant. This cluster showed 120 and 144 transcripts in TAP and minimal media, respectively, with 48 genes in common (core). Since these genes were under-expressed in the mutant, this pattern suggests that these genes are **up-regulated by nitrate in the presence of NIT2.** The core of this cluster contained transcripts corresponding to key genes for nitrate assimilation such as such nitrate reductase (*NIA1),* high affinity nitrate/nitrite transporters (*NRT2*.1, *NRT2*.2, *NRT2.3, NAR2*), nitrite reductase (*NII1*), nitrite transporter (*NAR1*.1), as well as genes related to molybdenum-cofactor biosynthesis, essential for the activity of proteins involved in nitrate assimilation, such as *MOT1*, *CNX2*, CNX5, *CNX6* and *CNX7*. Also, genes involved in nitric oxide metabolism were included in this core such as the truncated hemoglobins (*THB1* and *THB2*).

Cluster B: up-regulated and NIT2-independent.

This cluster included genes that were overexpressed on nitrate, showing similar levels in the WT to the *nit2* mutant. The number of genes included in this cluster was 122 genes in TAP medium, in contrast to 1,002 in minimal medium, and 94 genes were found in both conditions. This expression pattern suggests that these genes are **up-regulated by nitrate** –or downregulated by ammonium- but, in contrast to cluster A, by means of a **NIT2-independent signaling pathway**, since both WT and *nit2* mutant showed similar transcript levels. The core of this cluster included the nitrogen response regulator 1 (*NRR1*) and glutamine synthetase (*GLN1*) transcripts, both genes known to be induced in response to nitrogen starvation (Schmollinger et al., 2014)[a].

Cluster C: down-regulated and NIT2-dependent.

This cluster included genes that were down-regulated in the WT when transferred to nitrate, but not differentially represented when comparing nitrate and ammonium in the nit2 mutant. This pattern suggests that these genes are **down-regulated by nitrate by means of NIT2**. This cluster showed 15 and 52 transcripts in TAP and minimal media, respectively, and interestingly, only two genes in common (core).

Cluster D: down-regulated and NIT2-independent.

This cluster included transcripts whose levels did not differ between the WT and the *nit2* mutant on nitrate and were lower on nitrate than on ammonium in both strains, WT and *nit2* mutant. In this case, this pattern suggests that these genes are **down-regulated by nitrate in a NIT2-independent signaling pathway.** The number of genes included in cluster D corresponded to 19 in TAP and 1,520 in minimal medium, 8 of them were present in both situations.

[a]Schmollinger, S., Mühlhaus, T., Boyle, N. R., Blaby, I. K., Casero, D., Mettler, T., et al. (2014). Nitrogen-Sparing Mechanisms in *Chlamydomonas* Affect the Transcriptome, the Proteome, and Photosynthetic Metabolism. *Plant Cell*. doi:10.1105/tpc.113.122523.

Cluster E: overexpressed in nitrate, with negative effect of NIT2

This cluster represents transcripts that were over-expressed on nitrate, in both the WT and nit2 strains. However, this cluster represents a special case since paradoxically, transcript levels on nitrate were higher in the *nit2* mutant that in the WT. This pattern suggests that these genes could be **down-regulated by NIT2 in the presence of nitrate**. To explain this, we propose two possible explanations: either (1) ammonium is a stronger repressor than nitrate for these genes, or (2) the switch from ammonium during pre-culturing to nitrate generates a lag for nitrate signaling leading to a transient situation of nitrogen starvation. The number of genes included within this cluster was 44 and 71 in mixotrophic and auxotrophic conditions, respectively. Nine of them where shared in both conditions (Table 1.1). These genes are related to organic nitrogen scavenging –e.g. the extracellular L-amino acid oxidase (*LAO1*) and transporters like the Xanthine/uracil/vitamin C permease-like (*UAPA1* and *UAPA6)*. Given that these genes are known to be upregulated by nitrogen starvation, we consider the second explanation as the most plausible one. See Figure 1.6 for a comprehensive view on the expression patterns corresponding to the genes from the different clusters).

Table 1.1. Differentially accumulated transcripts included in core of cluster E.

Phytozome ID	Gene	Functional Annotation
Cre12.g551352	*LAO1* (partial)	Extracellular L-Amino Acid Oxidase (LAAO)
Cre12.g551353	*LAO1* (partial)	Extracellular L-Amino Acid Oxidase (LAAO)
Cre03.g159254	*AMT1.1*	Ammonium Transporter (AMT)
Cre10.g442800	*UAPA1 (XUV6)*	Xanthine/uracil/vitamin C permease-like
Cre06.g260700	*UAPA6 (XUV1)*	Xanthine/uracil/vitamin C permease-like
Cre12.g510350	*BLZ17*	Transcription Factor
Cre01.g012700	*KCN6*	Ion transport protein/Ankyrin repeats
Cre09.g389050	-	-
Cre01.g008250	-	-

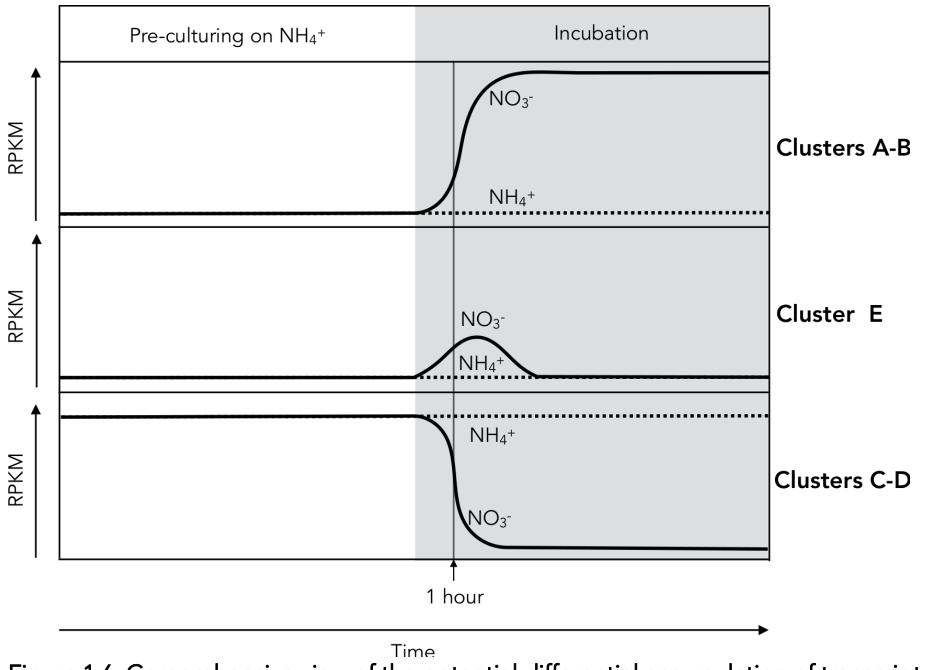


Figure 1.6. Comprehensive view of the potential differential accumulation of transcripts by the time after the switch from ammonium (pre-culture), to nitrate (solid line) or ammonium (dotted line). 1 hour indicates the time of sampling.

1.2 The assimilation of extracellular amino acids is down-regulated by nitrate and NIT2

From here on, in this thesis we focus on genes included in the core of cluster E, especially on *LAO1*, coding an **extracellular L-amino acid oxidase**. This protein can deaminate most proteinogenic amino acids generating ammonium, which is efficiently assimilated by *Chlamydomonas* (Piedras et al., 1992; Vallon et al., 1993)[a,b]. The generated ammonium can be taken up by this alga by **high affinity ammonium transporters** (AMT family). *Chlamydomonas* contains eight *AMT* genes (***AMT1.1-8***) and among them, *AMT1.1, 1.2, 1.4* and *1.5* have been reported to be up-regulated under nitrogen starvation (González-Ballester et al., 2004)[c]. We analyzed the expression pattern of these transporters in the present transcriptomic analysis (Table 1.2). As a result, *AMT1.2, 1.4, 1.5* and *1.8* genes were up-regulated when cells were transferred from ammonium to nitrate, both in the WT and the nit2 mutant strains, and under both autotrophic and mixotrophic conditions (core of cluster B). *AMT1.7* was down-regulated in a NIT2-indepentent fashion (cluster D), but only under strictly autotrophic conditions. *AMT1.6* also was down-regulated in a NIT2-indepentent fashion and included in core of cluster D. *AMT1.3* was the only AMT gene that showed no regulation under

[a]Piedras, P., Pineda, M., Muñoz, J., and Cárdenas, J. (1992). Purification and characterization of an L-amino-acid oxidase from *Chlamydomonas reinhardtii*. *Planta* 188, 13–18.

[b]Vallon, O., Bulté, L., Kuras, R., Olive, J., and Wollman, F.-A. (1993). Extensive accumulation of an extracellular L-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 215, 351–360. doi:10.1111/j.1432-1033.1993.tb18041.x.

[c]González-Ballester, D., Camargo, A., and Fernández, E. (2004). Ammonium transporter genes in *Chlamydomonas*: The nitrate-specific regulatory gene *Nit2* is involved in *Amt1;1* expression. *Plant Mol. Biol.* 56, 863–878. doi:10.1007/s11103-004-5292-7.

neither autotrophic or mixotrophic conditions. Finally, the expression pattern of *AMT1.1* was included in the special cluster E, together with *LAO1* gene, and thus showing a co-regulation. This fact suggests that AMT1.1 may be the ammonium transporter coordinated with LAO1 for nitrogen scavenging from amino acids.

Further experiments were performed to confirm the relationship between *LAO1* and *AMT1.1* genes, as well as the impact of nitrate and NIT2 on the expression of these genes and the use of amino acids for growth.

Table 1.2. Differential expression of AMT family genes in response to nitrate on TAP (mixotrophic conditions).

Gene	Cluster	Locus ID	WT NH_4^+/NO_3^-		nit2 NH_4^+/NO_3^-		NO_3^- WT/nit2	
			log_2FC	pvalue	log_2FC	pvalue	log_2FC	pvalue
AMT1.1	E	*Cre03.g159254*	2.43	0.00	5.70	0.00	-4.08	0.00
AMT1.2	B	*Cre14.g629920*	8.77	0.00	8.89	0.00	0.98	0.00
AMT1.3	N.R.	*Cre06.g293051*	0.30	0.02	0.39	0.02	0.08	0.51
AMT1.4	B	*Cre13.g569850*	8.34	0.00	7.66	0.00	0.30	0.00
AMT1.5	B	*Cre09.g400750*	4.52	0.00	2.27	0.00	-0.28	0.01
AMT1.6	D	*Cre07.g355650*	-1.11	0.00	-1.64	0.00	0.31	0.18
AMT1.7	N.R.	*Cre02.g111050*	-0.89	0.00	-1.25	0.00	0.05	0.68
AMT1.8	B	*Cre12.g531000*	2.71	0.00	2.47	0.00	0.49	0.02

N.R. refers to no differential regulation (fold change > 2) under the considered conditions.

First, we performed quantitative-PCR (qPCR) to study the gene expression of *LAO1* and the ammonium transporters *AMT1.1-2* and *1.4-5* from WT and *nit2* mutant in response to ammonium, nitrate and nitrogen starvation (Figure 1.7). We observed a co-regulation of both *LAO1* and *AMT1.*1 in terms that ammonium, but also nitrate, clearly inhibited gene expression of *LAO1* and *AMT1.1* in the WT strain. Moreover, on nitrate, *NIT2* mutation led to higher levels of relative expression of both genes compared to that in the WT.

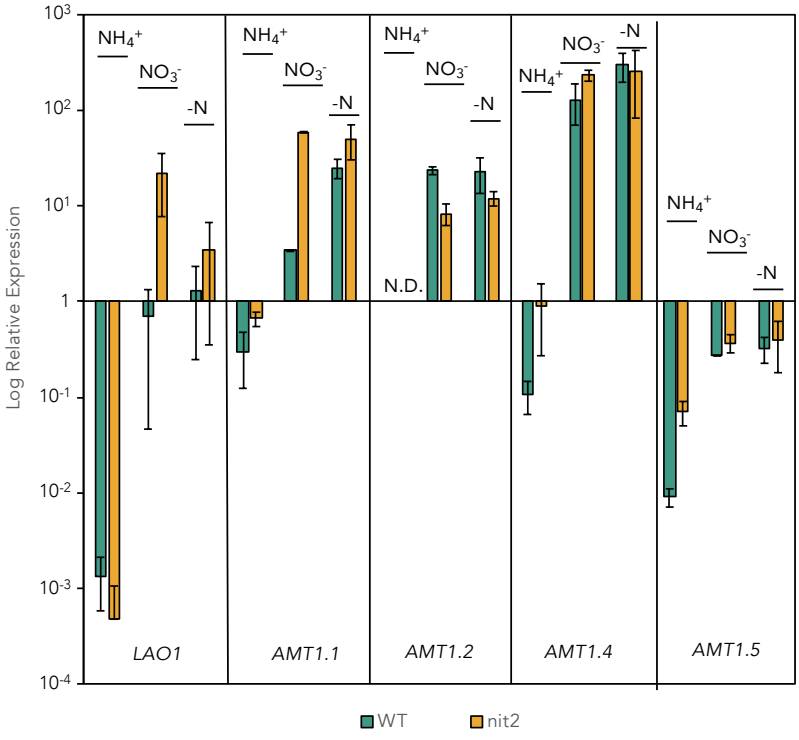


Figure 1.7. Relative expression of LAO1 and AMT genes in WT and *nit2* mutant. The impact of ammonium and nitrate in both wild-type (704 strain) and *nit2* (89.87 strain) mutant strains are shown. Transcripts (*LAO1, AMT1.1, 1.2, 1.4* and *1.5*) were quantified after 1 hour of incubation in TAP medium supplemented with 4 mM of ammonium (NH_4^+), nitrate (NO_3^-) or without any nitrogen source (-N).

Further, the expression of these genes was quantified along the time when cells were transferred from ammonium to nitrate and nitrogen starvation (Figures 1.8 and 1.9). Here, four strains were compared: the WT, the *nit2* mutant, the complemented *nit2* mutant with *NIT2*, and a *nit1* mutant. In fact, the results showed that both *LAO1* and *AMT1.1* were up-regulated in response to nitrogen starvation and that the overexpression in nitrate is consequence of the absence of a functional *NIT2*. Moreover, a *nit1* mutant strain (305) lacking nitrate reductase was also analyzed. This strain has a functional *NIT2* but because it is not able to reduce nitrate, there is a persistent nitrate signal in the cell. Results showed that nitrate *per se* repressed both *LAO1* and *AMT1.1* expression as well as in the *nit1* mutant.

In summary, the transcriptional co-regulation of **both LAO1 and AMT1.1 suggests that both proteins are coordinated for nitrogen scavenging from amino acids**. In addition, we hypothesize that nitrate, through a NIT2-dependent signaling pathway, is negatively regulating amino acids assimilation in *Chlamydomonas*. This is an interesting aspect because NIT2 could determine a preference for nitrate (inorganic nitrogen) over amino acids (organic nitrogen), which in addition has implications on natural environments.

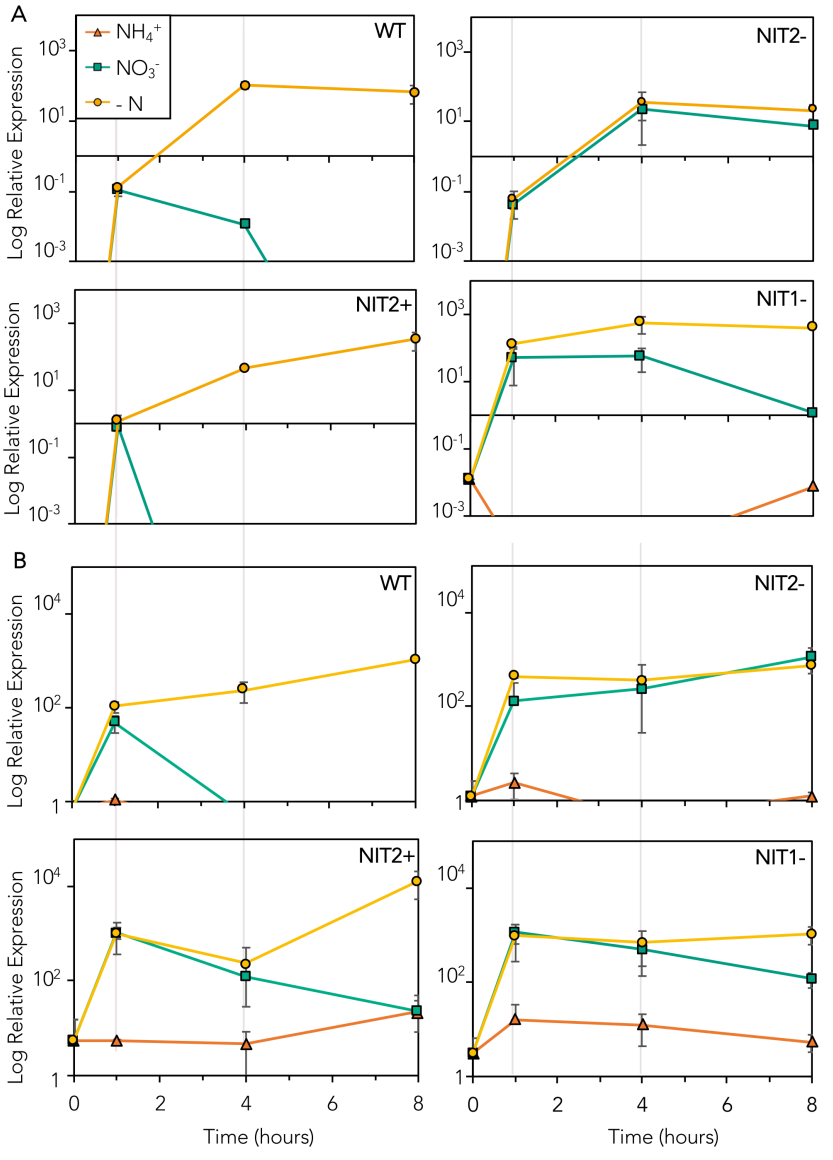


Figure 1.8. LAO1 (A) and AMT1.1 (B) gene expression. Cells, grown in ammonium, were transferred to fresh media containing 4 mM of ammonium (NH_4^+), nitrate (NO_3^-) or without any nitrogen source (-N). Different strains were used: the wild-type strain 704 (WT), the *nit2* mutant 89.87 strain (NIT2-), the mutant complemented with NIT2 p68.8 strain (NIT2+), and the *nit1* mutant 305 (NIT1-). Gene expression is represented relative the expression of the housekeeping gene ubiquitin ligase in each condition, which has a value of 1.

1.3 *NIT2* mutation enhances growth on amino acids.

Chlamydomonas growth was studied on the following amino acids as the sole nitrogen source: L-arginine, L-lysine, L-leucine, L-alanine, L-phenylalanine and L-cysteine (Figure 1.9). Two different *nit2* mutants and their corresponding parental NIT2$^+$ strains were tested: 89.87 (*NIT2*$^-$) and 704 (NIT2$^+$); and 203 (NIT2$^-$) and 6145c (NIT2$^+$). Strains 89.87 and 704 were cell wall deficient (cw-). As a result, both *nit2* mutants showed a greater growth on L-arginine, L-lysine, L-leucine, L-alanine and L-phenylalanine than that of their respective parental wild type strains. To further confirm such effect by *NIT2* mutation, mutants complemented with *NIT2* gene (p68.6, p68.8 and p68.11) were grown on different amino acids, which resulted in the reversion of the phenotype, then similar to that in the WT. However, growth on L-cysteine was not affected by *NIT2* mutation. Additionally, a negative effect for growth on amino acids by cell wall-deficient strains 704 and 89.87 was observed, possibly due to the diffusion of the enzyme from the periplasm to the media (Vallon et al., 1993).

These results suggest that NIT2 may have a negative impact on the use of amino acids as the sole N source. All tested amino acids except for L-cysteine are specific substrates for LAO1 activity (Vallon et al., 1993)[a]. Thus, we found a correlation between the impact of *NIT2* mutation and LAO1, on different amino acids. This phenotype further supported the idea that NIT2 is inhibiting the use of L-amino acids by means of LAO1.

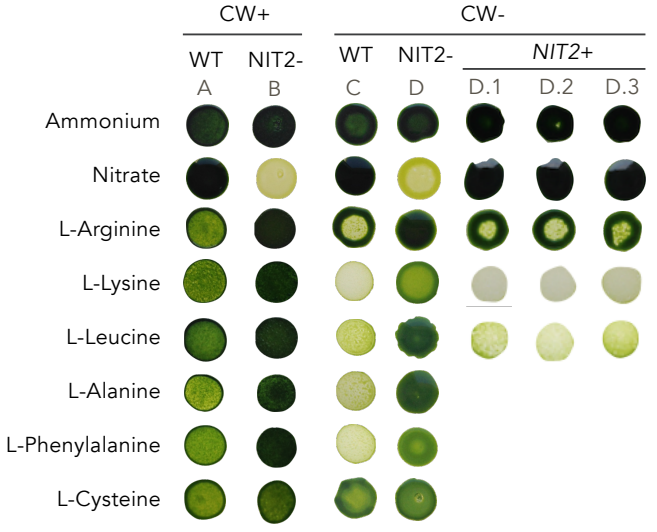


Figure 1.9. Impact of NIT2 mutation in *Chlamydomonas* growth on different L-amino acids. Each amino acid was supplemented at 4 mM concentration medium as the sole nitrogen source in solid TAP, for 3 days (ammonium, nitrate and L-arginine) or 7 days (the rest of amino acids). A, wild-type strain for nitrate assimilation (6145c strain). B, *nit2* mutant (203 strain). C, wild-type strain for nitrate assimilation (704 strain). D, *nit2* mutant (89.87 strain). D.1-3, different *NIT2*-complemented strains of the *nit2* mutant 89.87 (p68.6, p68.8, and p68.11). CW+ refers to intact cell wall, and CW- to cell wall mutant strains.

[a]Vallon, O., Bulté, L., Kuras, R., Olive, J., and Wollman, F.-A. (1993). Extensive accumulation of an extracellular l-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 215, 351–360. doi:10.1111/j.1432-1033.1993.tb18041.x

1.4 Nitrate *per se* negatively impacts the use of amino acids by *Chlamydomonas*

To confirm that nitrate by means of NIT2 has a negative impact on the use of amino acids in *Chlamydomonas*, we studied algal growth on different amino acids in the presence of nitrate. Given that in WT genetic backgrounds nitrate is rapidly converted to nitrite by NR activity and further assimilated to ammonium, in order not to exhaust intracellular nitrate signaling, we used a nitrate reductase mutant (*nit1*) for these growth tests. As a result, the supplementation of 100 µM nitrate inhibited algal growth on L-arginine, L-lysine and L-leucine (Figure 1.10). This effect was not observed in a double mutant *nit1nit2* lacking both NR and NIT2, which showed increased growth on all amino acids, independently of nitrate presence. Growth on L-cysteine was not affected by either nitrate or the *NIT2* presence.

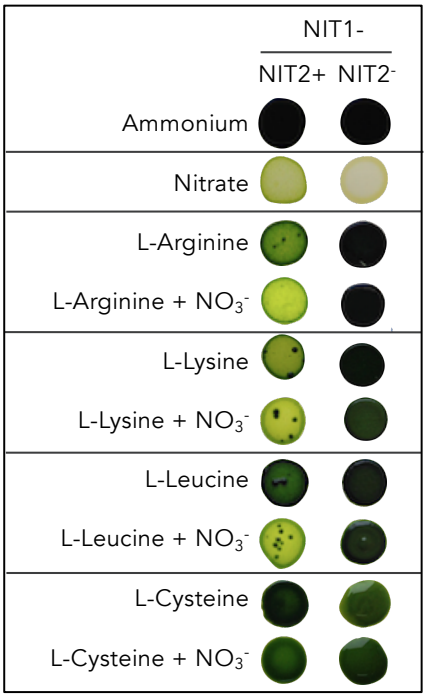


Figure 1.10. The impact of nitrate in *Chlamydomonas* growth nit1 mutant on different L-amino acids. Both strains lacked NIT1, but contained or lacked the nitrate sensing regulator NIT2, 305 (NIT1-NIT2+) and 137c (NIT1-NIT2-) strains, respectively. Cell drops containing $4{\cdot}10^4$ cells were placed on agar plates with 4 mM of ammonium, nitrate or the amino acid, and where indicated, further supplemented with 100 µM of nitrate (+ NO_3^-). Plates were imaged after incubation under continuous light for 4 days (on ammonium and arginine) or for 7 days (on nitrate, lysine, leucine and cystein

These results support the hypothesis that nitrate is an effective signal by which NIT2 inhibits *LAO1* and thus, the use of amino acids. Given that it has been proposed that L-cysteine is not deaminated by LAO1 but taken up by a passive diffusion component, this lack of phenotype may be explained by the existence of an alternative pathway for L-cysteine use (Zuo et al., 2012)[a].

In order to evaluate the inhibitory effect by nitrate in the *nit1* mutant, amino acid dependent growth was quantified along the time in liquid media (Figure 1.10). In fact, a strong inhibition by nitrate was observed when the alga was growing on L-lysine and L-arginine. To further confirm that nitrate *per se* is inhibiting the use of L-arginine, which relies on both

[a]Zuo, Z., Rong, Q., Chen, K., Yang, L., Chen, Z., Peng, K., et al. (2012). Study of amino acids as nitrogen source in *Chlamydomonas reinhardtii*. *Phycol. Res.* 60, 161–168. doi:10.1111/j.1440-1835.2012.00646.x.

specific transport and deamination by LAO1, *nit1* mutant cells were incubated under nitrate and nitrogen starvation conditions. After 10 hours, cells were changed to fresh media containing 1 mM of L-arginine, and its concentration was tracked in the media along 8 hours (Figure 1.11). As a result, L-arginine was consumed efficiently from cells that were induced under nitrogen starvation, but the presence of nitrate inhibited its use.

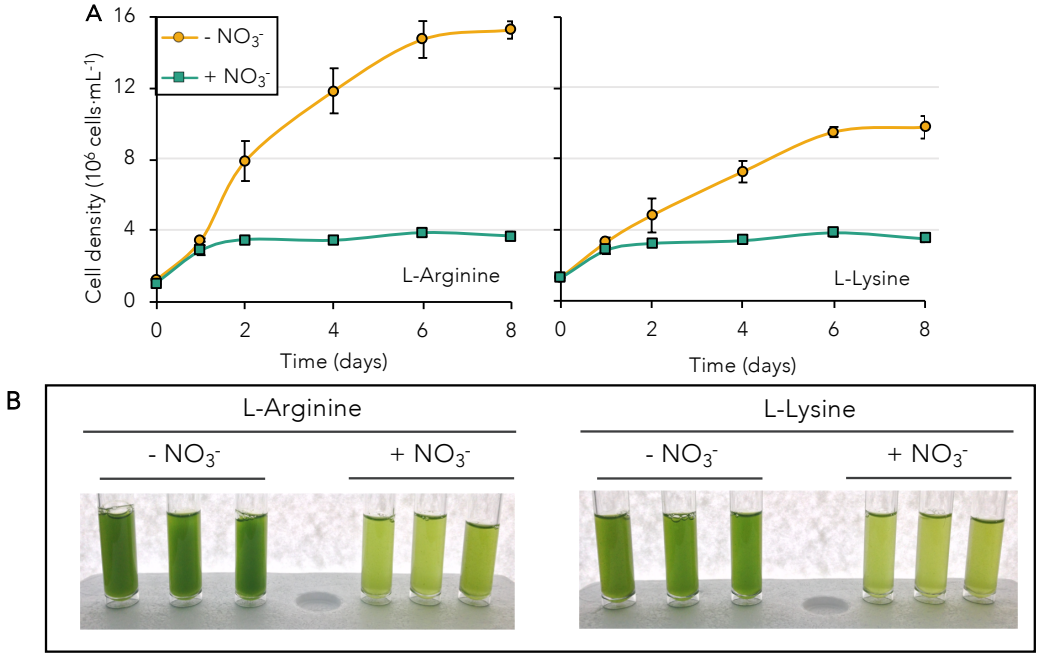


Figure 1.11. Impact of nitrate presence in *Chlamydomonas nit1* mutant growth on L-arginine and L-lysine. The *nit1* mutant strain (305) cells were incubated on TAP media supplemented with 4 mM of the amino acid with 100 μM of nitrate where indicated (+ nitrate). **A**, cell growth on L-arginine (left) and L-lysine (right). **B**, liquid cultures imaged after 8 days of incubation under continuous light.

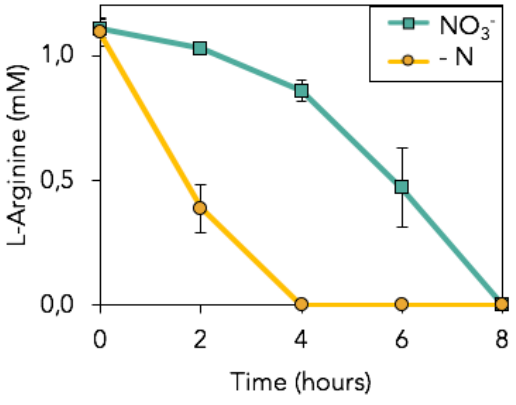


Figure 1.12. Impact of nitrate in L-arginine uptake by a *Chlamydomonas nit1* mutant. 305 strain cells were incubated for 10 hours on TAP media supplemented with 100 μM of nitrate (+ Nitrate) or without any nitrogen source (- N). Then, 1 mM of L-arginine was added and its concentration measured in the media along the time

Table 1.3. Phenotype ratios of the resulting segregants from the genetic cross between Chlamydomonas wild-type (CC-1690 strain) and a double mutant in nitrate reductase and NIT2 genes (nit1; nit2) (CC-5325 strain).

Growth	NH_4^+	NO_3^-	NO_2^-
Positive	100	30	55
Negative	0	70	45
Total	100	100	100

Table 1.4. Phenotypes and assigned genotypes of the selected segregants resulting from genetic cross in Table 1.3.

Strains	Growth			Phenotype	Assigned Genotype
	NH_4^+	NO_3^-	NO_2^-		
CC-1690	+	+	+	nit+ nii+	WT
CC-5325	+	-	-	nit- nii-	nit1nit2
2J1-6	+	+	+	nit+ nii+	WT
2J1-8	+	+	+	nit+ nii+	WT
2J1-45	+	-	+	nit- nii+	nit1
2J1-2	+	-	+	nit- nii+	nit1
2J1-14	+	-	-	nit- nii-	nit2
2J1-35	+	-	-	nit- nii-	nit2

nit: growth on nitrate; nii: growth on nitrite. (+) positive growth; (-) negative growth

The impact of *NIT1 (NIA1)* and/or *NIT2* mutations in *Chlamydomonas* use of amino acids was also analyzed by a genetic cross between a WT strain and a double mutant lacking *NIT1* and *NIT2* functional genes. A total of one hundred segregants resulting from this cross were streaked on nitrate and nitrite plates to test nit (growth on nitrate) and nii (growth on nitrite) phenotypes (Table 1.3). Since growth on nitrate relies on both NIT1 and NIT2 presence, and growth on nitrite relies only on NIT2 but not NIT1, the expected segregation ratios were 25% for nit+ and 50% for nii+. Thus, 30% and 55% of segregants did grow on nitrate (nit+), and nitrite (nii+), respectively. To study *NIT1* and *NIT2* mutations effect on amino acids growth, we selected two segregants of each of the following phenotypes: nit+/nii+ (NIT1+ NIT2+, i.e. WT), nit-/nii+ (NIT1- NIT2+) and nit-/nii- (NIT1- NIT2- or NIT1+ NIT2-) (Table 1.4). As a result, growth on amino acids L-alanine and L-serine was enhanced for NIT2- segregants, highlighted into a blue square (Figure 1.13). In contrast, the single NIT1- segregants were defective for growth on these amino acids (red square). From data in Figure 1.13, it should be noted that not only genetic background affects the growth on amino acids, but also the presence or absence of acetate in the media (TAP and minimal media, respectively). Understanding such differences requires further experiments but will be discussed belo

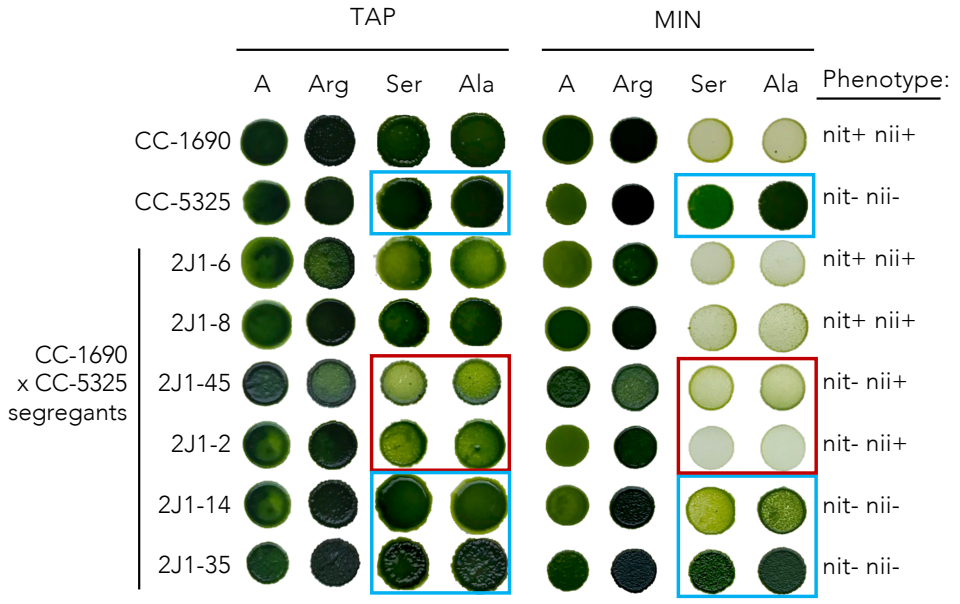


Figure 1.13. Impact of NIT1 and NIT2 mutations for the use of amino acids as nitrogen source. Both parental strains, the wild-type (CC-1690) and the *nit1nit2* mutant (CC-5325), and six selected segregants resulting from this genetic cross were cultured on TAP and minimal media (MIN) agar plates supplemented with 8 mM of the indicated nitrogen source: ammonium (A), L-arginine (Arg), L-serine (Ser) or L-alanine (Ala) as the sole nitrogen source. Plates were imaged after 7 days of incubation under continuous light. Phenotypes for nitrate and nitrite assimilation observed in Table 1.4 are indicated on the right. Observable differential growth is highlighted in blue and red boxes.

1.5 Nitrite reductase mutants and the use of amino acids.

In this section we analyzed the impact of nitrite reductase mutation (*nii1* mutants) in *Chlamydomonas* growth on amino acids (i.e., L-alanine and L-lysine). Two *nii1* mutants were used, the M3 and M4 strains (Navarro et al., 2000; Wei et al., 2014)[a,b]. The difference between these two strains is the presence (M3) or the absence (M4) of the High Affinity Nitrate/Nitrite Transporters (HANNiT) System I, encoded by *NRT2*.1 and *NAR2* genes. This transport system allows the efficient entrance into the cell of both nitrate and nitrite (Quesada et al., 1996; Rexach et al., 1999)[c,d]. Once inside the cell, nitrate is reduced to nitrite, but it cannot be

[a]Navarro, M. T., Guerra, E., Fernández, E., and Galván, A. (2000). Nitrite Reductase Mutants as an Approach to Understanding Nitrate Assimilation in *Chlamydomonas reinhardtii*. *PLANT Physiol.* 122, 283–290. doi:10.1104/pp.122.1.283.

[b]Wei, L., Derrien, B., Gautier, A., Houille-Vernes, L., Boulouis, A., Saint-Marcoux, D., et al. (2014). Nitric Oxide-Triggered Remodeling of Chloroplast Bioenergetics and Thylakoid Proteins upon Nitrogen Starvation in *Chlamydomonas* reinhardtii. *Plant Cell* 26, 353–372. doi:10.1105/tpc.113.120121

[c]Quesada, A., Quesada, A., and Fernández, E. (1996). Nitrate and Nitrite Are Transported by Different Specific Transport Systems and by a Bispecific Transporter in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* 271, 2088–2092. doi:10.1074/jbc.271.4.2088.

[d] Rexach, J., Montero, B., Fernández, E., and Galván, A. (1999). Differential regulation of the high affinity nitrite transport systems III and IV in *Chlamydomonas reinhardtii*. *J. Biol. Chem.* 274, 27801–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10488125

subsequently reduced in *nii1* mutants, and it is stoichiometrically excreted to the media (Navarro et al., 2000)[a] (see scheme in Figure 1.14-A). As shown on Figure 1.14-B, **nitrite as well as nitrate inhibited M3 strain growth on L-arginine and L-lysine** but not in the strain lacking the HANNiT (M4 strain). Also, the evolution of L-arginine media during M3 strain growth on this amino acid in the presence of nitrate or nitrite was measured (Figure 1.14-C). **In this mutant, the use of L-arginine was inhibited by the presence of either nitrate or nitrite.** However, in contrast to the observed inhibition by nitrate in the *nit1* mutant (Figure 1.10), in this case the inhibition was significantly relieved after 3 days on nitrate and after 5 days on nitrite. Thus, we observed a **transient inhibition** that was slightly longer by the presence of nitrite than nitrate. Then, we quantified both nitrite and nitrate in the media. As expected, nitrate disappeared from the media after 1 day (Figure 1.14-E) and nitrite was excreted almost at equimolar concentration (Fig 1.14-F). Interestingly, even though NII1 is not functional in this mutant, nitrite concentration decreased in the media after 3-7 days, being especially evident on nitrate. This effect, observed here for the first time, could be explained by the action of the $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow NO_3^-$ cycle, played by NR, NOFNiR and THB1 enzymes (Calatrava et al., 2017; Sanz-Luque et al., 2015b, 2015a)[b,c,d] (see scheme in Figure 1.14-A). Thus, nitrite, initially inhibiting growth on this amino acid, may be along the time converted into nitric oxide (NO) by the NR/NOFNiR complex. Besides inhibition release by nitrite conversion, NO induction of LAO1 may also promote *Chlamydomonas* growth on this amino acid (Wei et al., 2014)[e]. Also, NO may be further oxidized to nitrate by the NR/THB1 complex (Sanz-Luque et al., 2015b) or reduced to nitrous oxide by the CYP55 (Plouviez et al., 2017)[f], both gaseous molecules that can be dissipated. Given that the NO oxidation or reduction involves genes that are up-regulated by nitrate such as *NIA1*, *THB1* and *CYP55*, the mentioned pathway could explain the observation of faster disappearance of nitrite when nitrate was present. However, nitrite reduction by an alternative reductase cannot be discarded.

[a] Navarro, M. T., Guerra, E., Fernández, E., and Galván, A. (2000). Nitrite Reductase Mutants as an Approach to Understanding Nitrate Assimilation in *Chlamydomonas reinhardtii*. *PLANT Physiol.* 122, 283–290. doi:10.1104/pp.122.1.283.

[b] Calatrava, V., Chamizo-Ampudia, A., Sanz-Luque, E., Ocaña-Calahorro, F., Llamas, A., Fernandez, E., et al. (2017). How *Chlamydomonas* handles nitrate and the nitric oxide cycle. *J. Exp. Bot.* 68, 2593–2602. doi:10.1093/jxb/erw507.

[c] Sanz-Luque, E., Ocaña-Calahorro, F., de Montaigu, A., Chamizo-Ampudia, A., Llamas, Á., Galván, A., et al. (2015b). THB1, a truncated hemoglobin, modulates nitric oxide levels and nitrate reductase activity. *Plant J.* 81, 467–479. doi:10.1111/tpj.12744.

[d] Sanz-Luque, E., Chamizo-Ampudia, A., Llamas, A., Galvan, A., and Fernandez, E. (2015a). Understanding nitrate assimilation and its regulation in microalgae. *Front. Plant Sci.* 6, 899. doi:10.3389/fpls.2015.00899.

[e] Wei, L., Derrien, B., Gautier, A., Houille-Vernes, L., Boulouis, A., Saint-Marcoux, D., et al. (2014). Nitric Oxide-Triggered Remodeling of Chloroplast Bioenergetics and Thylakoid Proteins upon Nitrogen Starvation in *Chlamydomonas* reinhardtii. *Plant Cell* 26, 353–372. doi:10.1105/tpc.113.120121.

[f] Plouviez, M., Wheeler, D., Shilton, A., Packer, M. A., McLenachan, P. A., Sanz-Luque, E., et al. (2017). The biosynthesis of nitrous oxide in the green alga Chlamydomonas reinhardtii. *Plant J.* 91, 45–56. doi:10.1111/tpj.13544.

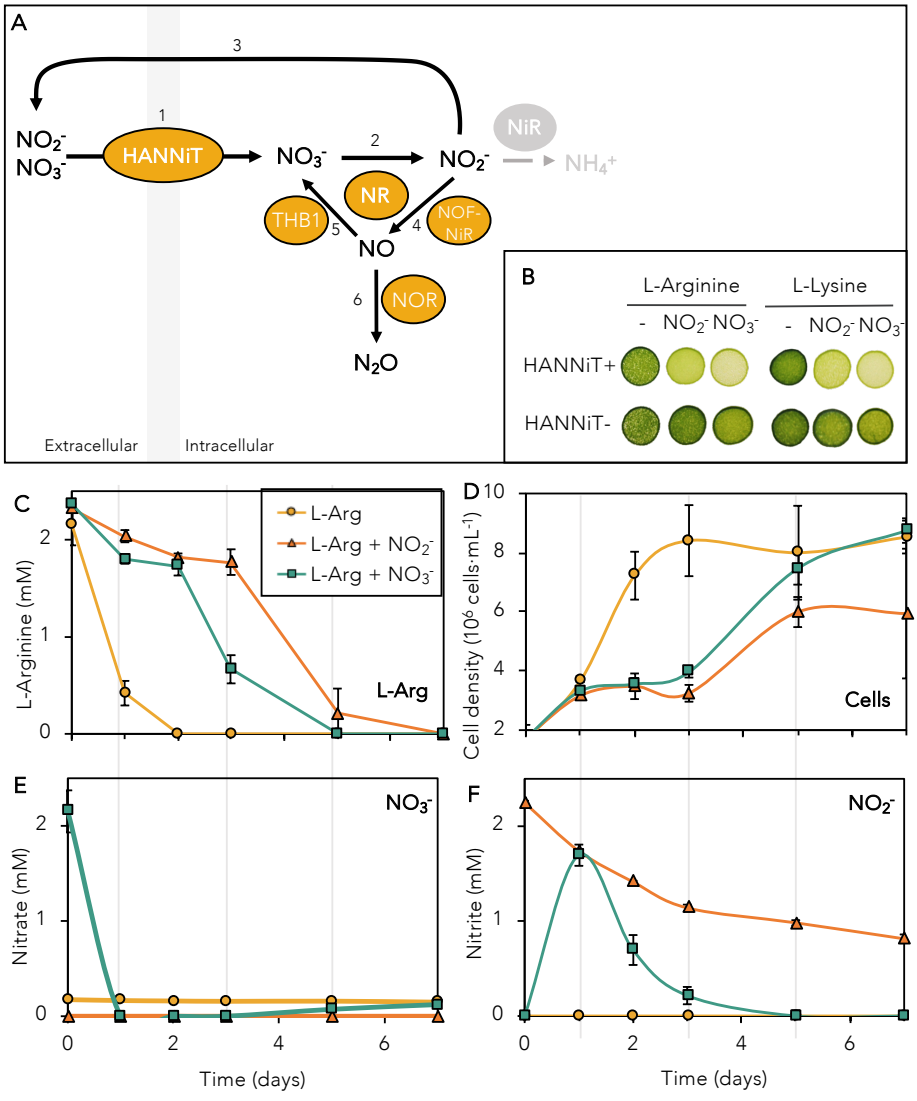


Figure 1.14. Impact of nitrate and nitrite on the use of amino acids by nitrite reductase mutants. A, representation of the possible pathways for nitrate and nitrite in *Chlamydomonas nii1* mutants. 1: Nitrate/nitrite uptake by High Affinity Nitrate/Nitrite transporters (HANNiT); 2: nitrate reduction to nitrite by nitrate reductase (NR); 3, nitrite excretion to avoid toxicity by high intracellular concentration due to nitrite reductase (NiR) inactivity in the *nii1* mutant; 4, nitrite reduction to nitric oxide by NR/NOFNiR complex; 5, nitric oxide oxidation to nitrate by the NR/THB1 complex; 6, nitric oxide reduction to nitrous oxide by the nitric oxide reductase (NOR), encoded by the gene CYP55. **B**, *nii1* mutants M3 and M4, containing and lacking HANNiT (NRT2.1/NAR2), respectively, grown on solid TAP media containing 4 mM of L-arginine or L-lysine, supplemented with 100 µM of nitrite (NO_2^-) or nitrate (NO_3^-) for 4 days. In C-F, M3 cells were grown on liquid TAP media containing 4 mM L-arginine, with and without 100 µM of nitrite (NO_2^-) or nitrate (NO_3^-). During this growth on liquid media, cell density was quantified (**C**), as well as L-arginine (**D**), nitrate (**E**) and nitrite (**F**) in the supernatant of each culture.

In summary, we have shown that the presence of the inorganic nitrogen form **nitrate is a negative signal for *Chlamydomonas* use of amino acids**. Otherwise, **the lack of *NIT2* resulted in an enhanced use of amino acids,** even in the presence of nitrate. Also, we have observed that **nitrate, by means of NIT2, down-regulates gene expression of *LAO1* and the high-affinity ammonium transporter AMT1.1.** Finally, we have shown that **nitrite is as well a negative signal for this algal use of amino acids**.

These results suggest that *Chlamydomonas* is regulating nitrogen assimilation pathways that lead to a preference for inorganic nitrogen (i.e. ammonium, nitrate and nitrite) over amino acids. The preference of inorganic nitrogen over organic nitrogen in algae can determine the dynamics of ecological niches where both inorganic and organic nitrogen sources are present. How important is LAO1 for nitrogen scavenging from amino acids in *Chlamydomonas* and how *LAO1* gene is distributed in algal genomes was an objective for the following chapter.

Chapter 2:

The Role of *Chlamydomonas* Extracellular L-Amino Acid Oxidase LAO1 in Nitrogen Scavenging and the Study of its Evolution

2.1 *In silico* analysis of *Chlamydomonas LAAO* genes

L-Amino Acid Oxidases (LAAOs) have been associated with amino acid catabolism and cellular defense. In natural waters where algae are present, LAAOs are proposed to significantly alter amino acids distributions (Palenik and Morel, 1990b)[a]. In *Chlamydomonas reinhardtii* genome, two *LAAO* genes are present: *LAO1*, located on chromosome XII, encoding a periplasmic LAAO (NCBI protein ID: AAB97101.1); *LAO3*, located on chromosome V, enconding a putative intracellular LAAO (NCBI protein ID: XP_001700756.1). LAO1 enzyme has been more extensively characterized and generates ammonium that *Chlamydomonas* uses for growth with a corresponding α-keto acid byproduct that is not assimilated (Muñoz-Blanco et al., 1990)[b]. The enzyme was purified and showed its specific activity for a broad range of proteinogenic amino acids (Piedras et al., 1992; Vallon et al., 1993)[c,d], and the cDNA was verified by cloning (Vallon and Wollman, 1997)[e]. The purified enzyme appeared in two high-molecular weight forms. The lighter form (900-1,000 kDa) containing only the protein product of *LAO1* -a flavoprotein previously named as subunit Mα or catalytic subunit of protein M, coded by the gene *LAO1*-, while the heavier form (1,300 kDa) contained an additional subunit of 135 kDa, previously named as subunit Mβ or non-catalytic subunit of protein M, coded by *LAO2* gene. This last accessory protein -LAO2- was not necessary for LAAO activity, and both forms were found to be glycosylated, as most cell wall components in this alga. The basic structure of the so-called protein M was proposed to be an oligomer with approximately 16 copies of the catalytic subunit (LAO1) to which 2-3 copies of the accessory subunit (LAO2) can be associated (Vallon et al., 1993)[f]. Both subunits are present in *Chlamydomonas* during nitrogen starvation, becoming LAO1 one of the major proteins in gametes deprived of nitrogen for more than 50 hours. Thus, LAO1 expression has been considered a hallmark cell response to nitrogen deprivation (Bulté and Wollman, 1992; Vallon et al., 1993)[g].

Even though LAO2 protein product is not necessary for deamination activity of LAO1, and given that both proteins are found to be interacting and co-regulated during nitrogen starvation, it seems likely that this accessory protein may have a role together with LAO1. *LAO2* gene (*Cre12.g551350*, Phytozome ID) is found in the adjacent upstream region of *LAO1*, tail

[a] Palenik, B., and Morel, F. M. M. (1990b). Comparison of cell-surface L-amino acid oxidases from several marine phytoplankton. *Mar. Ecol. Prog. Ser.* 59, 95–201. doi:10.3354/meps059195.

[b] Muñoz-Blanco, J., Hidalgo-Martínez, J., and Cárdenas, J. (1990). Extracellular deamination of amino acids by *Chlamydomonas*. *Planta* 182, 194–198

[c] Piedras, P., Pineda, M., Muñoz, J., and Cárdenas, J. (1992). Purification and characterization of an L-amino-acid oxidase from *Chlamydomonas reinhardtii*. *Planta* 188, 13–18

[d] Vallon, O., Bulté, L., Kuras, R., Olive, J., and Wollman, F.-A. (1993). Extensive accumulation of an extracellular l-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 215, 351–360. doi:10.1111/j.1432-1033.1993.tb18041.x.

[e] Vallon, O., and Wollman, F.-A. (1997). cDNA sequence of M(**α**), The catalytic subunit of the *Chlamydomonas reinhardtii* L-amino acid oxidase (Accession No. U78797): A new sequence motif shared by a wide variety of flavoproteins. (PGR97-171). *PLANT Physiol.*, 1729.

[f] Vallon, O., Bulté, L., Kuras, R., Olive, J., and Wollman, F.-A. (1993). Extensive accumulation of an extracellular l-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 215, 351–360. doi:10.1111/j.1432-1033.1993.tb18041.x.

[g] Bulté, L., and Wollman, F. a (1992). Evidence for a selective destabilization of an integral membrane protein, the cytochrome b_6/f complex, during gametogenesis in *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 204, 327–336. doi:10.1111/j.1432-1033.1992.tb16641.x

to tail orientated, coding a putative YjgF protein (Figure 2.1-A). Recently, certain members of the YjgF/YER057c/UK114 family, renamed as RidA (reactive intermediate/imine deaminase A) have been shown to hydrolyze the reactive imines generated by PLP-dependent enzymes or FAD-dependent amine oxidases, thus avoiding metabolite damage by the intermediate imine (Niehaus et al., 2014)[a]. Interestingly, *RidA* genes have been found to frequently cluster with FAD-dependent amine oxidase family genes, which produce an imine intermediate that spontaneously hydrolyzes to an α-ketoacid (Lambrecht et al., 2012)[b]. Thus, LAO2 (referred herein as LAO2/RidA) could accelerate the hydrolysis of the imines generated by LAO1 activity, similarly to what RidA proteins do for imines produced by threonine dehydratase (Niehaus et al., 2015)[c] (Figure 2.1-B). Indeed, within the intergenic region of both genes, the LAAO-coding gene *LAO1* and the putative RidA-coding gene *LAO2*, we found two G-box regions (CACGTG) that suggests that a bidirectional transcription factor may be co-regulating both genes expression to facilitate a potentially coordinated activity between both proteins (Figure 2.1).

According to *LAO2/RidA* gene sequence, the calculated molecular weight of the coded protein is 120 kDa, which fits with the approximately 135 kDa of the purified protein previously reported. This protein shows six YjgF/RidA domains. This protein sequence can be divided into two halves showing 98% of identity to each other, with three RidA domains each one. Thus, LAO2/RidA must be included in the previously reported subset of RidA proteins termed as 3x-RidA that contain three RidA domains fused in tandem (Niehaus et al., 2015)[c]. Apparently, LAO2/RidA consists of two 3x-*RidA* almost identical fused genes.

[a] Niehaus, T. D., Nguyen, T. N. D., Gidda, S. K., ElBadawi-Sidhu, M., Lambrecht, J. A., McCarty, D. R., et al. (2014). Arabidopsis and maize RidA proteins preempt reactive enamine/imine damage to branched-chain amino acid biosynthesis in plastids. *Plant Cell* 26, 3010–22. doi:10.1105/tpc.114.126854

[b] Lambrecht, J. A., Flynn, J. M., and Downs, D. M. (2012). Conserved YjgF protein family deaminates reactive enamine/imine intermediates of pyridoxal 5'-phosphate (PLP)-dependent enzyme reactions. *J. Biol. Chem.* 287, 3454–61. doi:10.1074/jbc.M111.304477.

[c] Niehaus, T. D., Gerdes, S., Hodge-Hanson, K., Zhukov, A., Cooper, A. J., ElBadawi-Sidhu, M., et al. (2015). Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. *BMC Genomics* 16. doi:10.1186/s12864-015-1584-3.

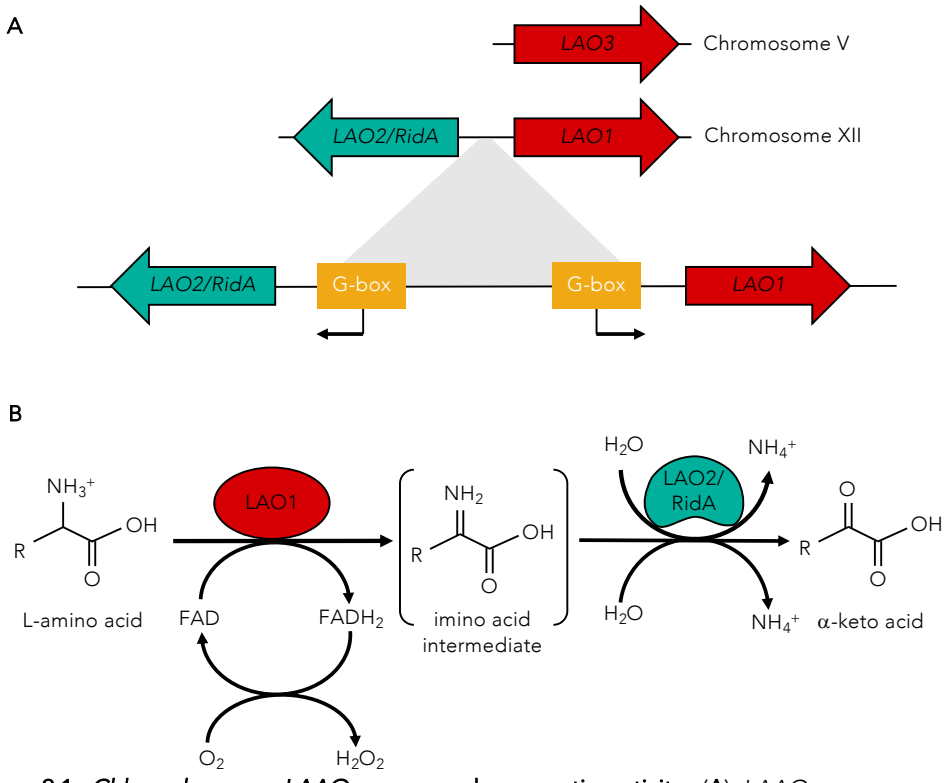


Figure 2.1. *Chlamydomonas LAAO* genes and enzymatic activity. (**A**) *LAAO* genes arrangement in *Chlamydomonas* genome. Red boxes represent *LAAO* genes *LAO1* and *LAO3*) and blue boxes *RidA* gene (*LAO2*/*RidA*). G-box sequence: 5'-CACGTG-3'. (**B**) LAAO activity reaction by LAO1 and putative LAO2/RidA activity.

The putative intracellular LAAO, coded by the gene *LAO3*, shows a 59% of identity to LAO1 and does not seem to be however regulated by nitrogen (Schmollinger et al., 2014)[a].

Intringuingly, even though *Chlamydomonas* genome presents two *LAAO* genes (*LAO1* and *LAO3*), other sequenced genomes of closely related green algae such as *Volvox* and *Ostreococcus* do not contain LAAO genes (Vallon and Spalding, 2009)[b].

2.2 *LAAO* genes occurrence in algal genomes

In order to clarify the so-far uniqueness of *LAAO* genes in *Chlamydomonas reinhardtii* within the green lineage, we queried several genome databases (see 'Material and Methods' section) with the LAO1 protein sequence using reciprocal BLASTp to identify potential orthologs following these criteria: (a) translated protein size of 400-600 amino acid residues; (b) sequence identity greater than 20%; and (c) sequence coverage greater than 60%. The resulting protein hits where subsequently used in tBLASTn queries against *C. reinhardtii* genome to identify reciprocal best-hits and confirm LAO1 orthology. Our data confirmed that **no LAO1 orthologs**

[a] Schmollinger, S., Mühlhaus, T., Boyle, N. R., Blaby, I. K., Casero, D., Mettler, T., et al. (2014). Nitrogen-Sparing Mechanisms in Chlamydomonas Affect the Transcriptome, the Proteome, and Photosynthetic Metabolism. *Plant Cell*. doi:10.1105/tpc.113.122523.

[b] Vallon, O., and Spalding, M. H. (2009). "Amino acid Metabolism," in *The Chlamydomonas Sourcebook*, 115–158

are found in other Viridiplantae members, including other closely related green algae like *Dunaliella* and *Coccomyxa*, and land plants. Also, LAO1 orthologs seem to be absent among five currently available *Chlamydomonas* spp. genomes (*C. eustigma, C. sphaeroides, C. debaryana, C. asymmetrica and C. applanata*).

In contrast, **we identified LAO1 orthologs in 10 out of 27 algal species:** *Porphyra umbilicalis, Gracilariopsis lemaneiformis* and *Chondrus crispus* (**Rhodophyta**), *Vitrella brassicaformis* (**Alveolata**), *Aureococcus anophagefferens* and *Pelagophyceae sp*. CCMP2097 (**Heterokonta**), *Pavlovales* sp. CCMP2436, *Emiliana huxleyi* and *Chrysochromulina* sp. CCMP291 (**Haptophyta**), and *Symbiodinium microadriaticum* (**Dinophyta**). Most of these algae seem to have a single *LAO1* ortholog gene, except for the diatom *Emiliana huxleyi* that seems to contain two and the red alga *Porphyra umbilicalis* four genes.

To understand the evolutionary history of these algal LAO1 orthologs, we constructed a phylogenetic tree along with other LAAO proteins previously analyzed in other work (Campillo-Brocal et al., 2015)[a]. Fungal, gastropod and vertebrate LAAO members, as well as other distantly related amino oxidases were included: D-amino acid oxidases (DAAOs), LodA (L-Lysine ε-oxidase)-like proteins, L-aspartate oxidases (LASPOs) and other enzymes with experimentally reported amine oxidase activity (Campillo-Brocal et al., 2015)[a]. Our analysis revealed that **algal protein sequences identified as LAO1 orthologs cluster on the same evolutionary branch** that we named as **ALAAOs (Algal LAAOs** in Figure 2.2). This ALAAO branch was distinct in lineage from fungal, gastropods and vertebrates LAAOs. In addition, two bacterial LAAOs from *Oceanobacter kriegii* and *Aquabacterium* sp. NJ1 were included in the phylogenetic analysis since they were found to be LAO1 orthologs. However, these two LAAOs did not cluster on the same branch as ALAAOs, suggesting that ALAAOs may have evolved separately rather than acquiring the genes from a recent lateral gene transfer event (Figure 2.2).

Within this new ALAAO branch, the sequence identity between LAO1 and other ALAAOs was low (from 23 to 31% of identity), but fits with previously reported data in other LAAO proteins within the same group (Nuutinen et al., 2012)[b]. Nevertheless, conserved motives in the substrate- and FAD- binding domains are present (Figure 2.3). All the LAAO members from the algal branch contain a $GGRX_2$[S/T] motif as found in fungal, gastropod and vertebrate LAAOs, but absent in DAAOs, LASPOs and LodA-like proteins. Sequences within the FAD-binding domain, particularly the dinucleotide binding domain $GXGX_2G$ and WAEXS[L/V] motives, which are highly conserved across LAAOs, were as well present in all ALAAOs. Within the substrate-binding domain, a conserved sequence [D/E]hGAYR in algal members is present, 'h' representing a hydrophobic amino acid residue. In algal members, an aromatic tyrosine is conserved in this position, whereas in bacteria, fungi and animals, a methionine residue is conserved at this position.

[a] Campillo-Brocal, J. C., Lucas-Elió, P., and Sanchez-Amat, A. (2015). Distribution in different organisms of amino acid oxidases with fad or a quinone as cofactor and their role as antimicrobial proteins in marine bacteria. *Mar. Drugs* 13, 7403–7418. doi:10.3390/md13127073.

[b] Nuutinen, J. T., Marttinen, E., Soliymani, R., Hilden, K., and Timonen, A. S. (2012). L-Amino acid oxidase of the fungus Hebeloma cylindrosporum displays substrate preference towards glutamate. *Microbiology* 158, 272–283. doi:10.1099/mic.0.054486-0

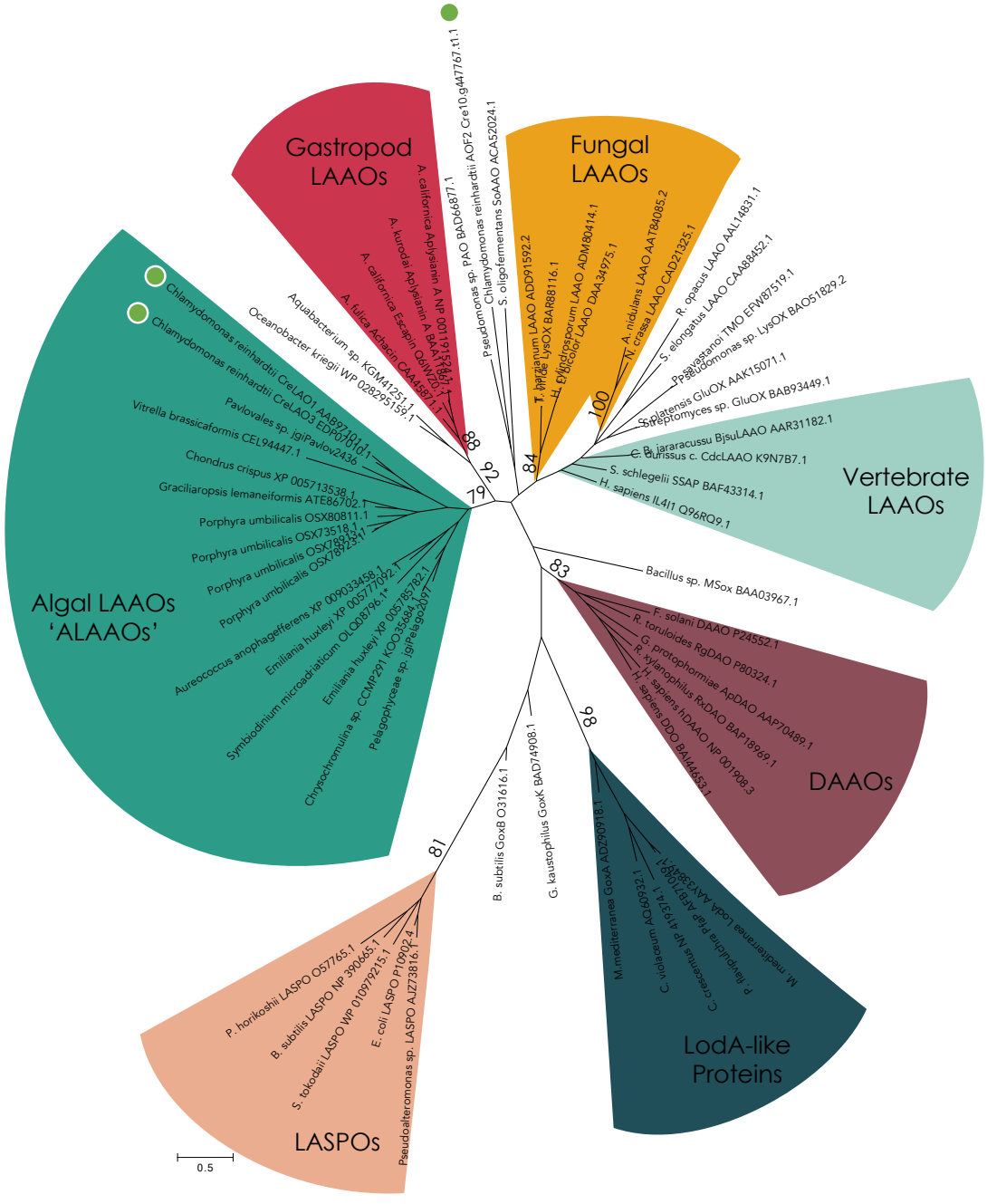


Figure 2.2. Evolutionary relationships of LAAOs and other enzymes with amine oxidase activity. The tree was obtained using the Maximum Likelihood method integrated in the software MEGA7 (Kumar et al., 2016). The alignment was performed using Clustal method, and the evolutionary distances were computed using the Poisson correction method. The distances are the units of the number of amino acid substitutions per site. Numbers in branches show bootstrap values in percentage. Green circles correspond to *C. reinhardtii* protein sequences.

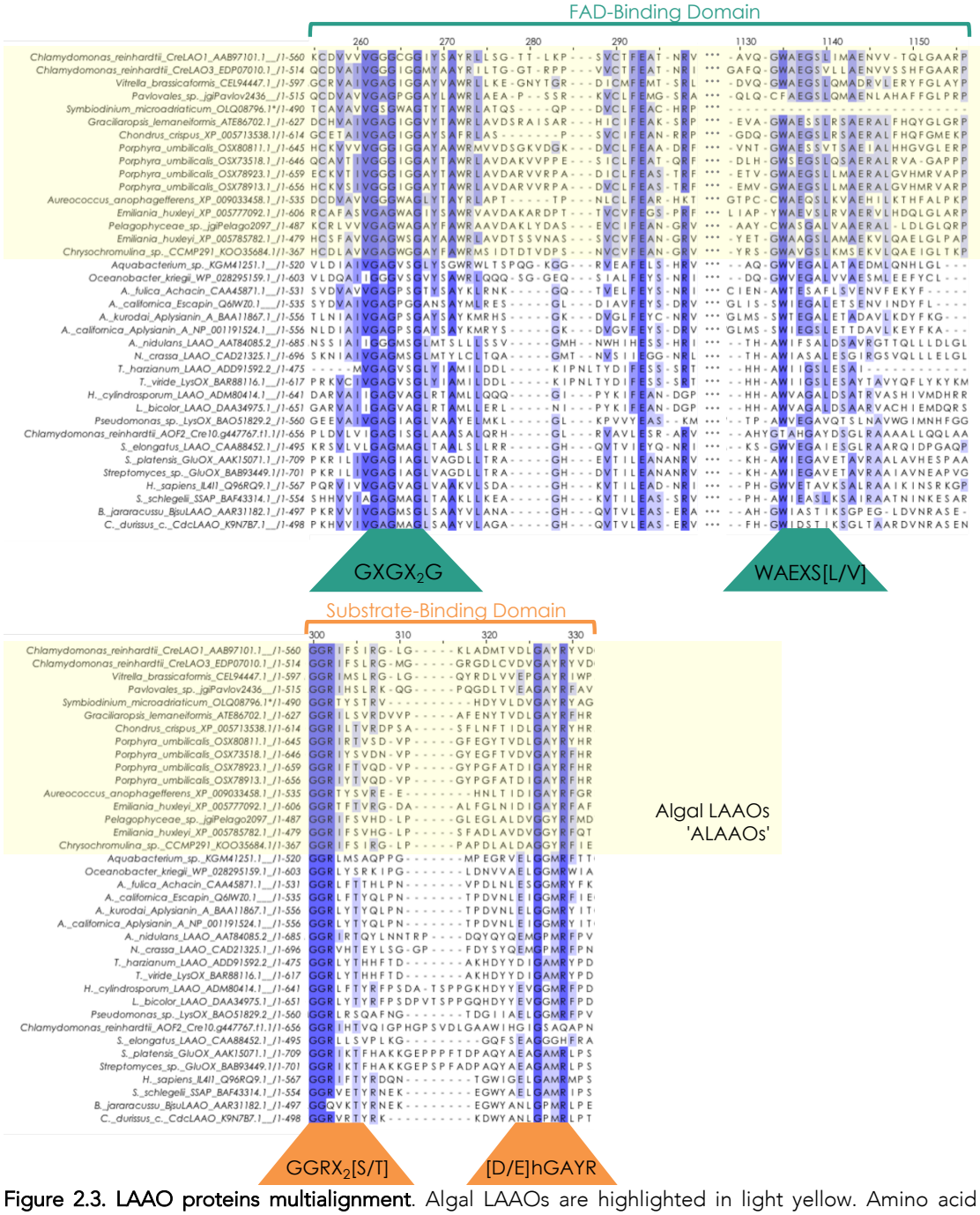


Figure 2.3. LAAO proteins multialignment. Algal LAAOs are highlighted in light yellow. Amino acid residues are highlighted in blue, according to percentage of identity. **Symbiodinium microadriaticum* LAAO protein sequence is partial. Alignment performed with Clustal Omega program.

2.3 Putative algal LAAO gene origins

Although our data support LAO1 and LAO3 being grouped together within ALAAOs, their origin is puzzling since we could find no other orthologs within the green algal lineage. In the hope of shedding light into the possible evolutionary history of *Chlamydomonas ALAAO* genes, we analysed the immediately adjacent gene to *LAO1*, *LAO2/RidA*. When this gene was queried, the most closely related gene was found in cyanobacteria and interestingly, adjacent to a putative amine oxidase gene, as previously reported for other bacteria (Niehaus et al., 2015)[a]. However, this cyanobacterial amine oxidase showed low sequence identity to LAO1, but higher to other putative amine oxidases in *Chlamydomonas*, AOF2 (*Cre10.g447767.t1.1*) (Figure 2.4). In contrast to *LAO1* and *LAO3*, *AOF2* shows orthologs in most genomes within the green and red algal lineages, as well as in cyanobacteria (indicated as AOs in Figure 2.5) and did not cluster in the same ALAAO branch in the Maximum Likelihood analysis (Figure 2.2), suggesting that the AOF/AO family may have given rise to ALAAOs. However, since no ortholog to ***LAO2/RidA*** was found in any other algal genome, this gene **may have been prior acquired in the archaeplastidian ancestor and later lost in most algal lineages but uniquely retained in *Chlamydomonas*, or either *Chlamydomonas* may have acquired it by lateral gene transfer.** Thus, we propose two possible explanations for the presence of *ALAAO* genes in the red algal lineage and the thus far unique co-ocurrence of *ALAAO-RidA* cluster in *C. reinhardtii* (Figure 2.6): In the ancestral archaeplastidan, prior to the divergence of red and green lineages, (1) an *in situ* duplication of the putative archaetypal *LAAO* occurred with further divergence of this copy towards *ALAAO*, followed by *AO* and *ALAAO* genes separating; or (2) a duplication of *LAAO-RidA* cluster with *LAAO* divergence to *ALAAO* with a subsequent loss of the *RidA* gene copy adjacent to archaetypal *LAAO*. For both, subsequent loss of the *RidA* gene in the red algae and loss of the *ALAAO-RidA* cluster in the green algae (except for *Chlamydomonas*), could explain the genome arrangements observed today. Interestingly, transcriptomics and functional data have demostrated that genes acquired as a result of endosymbiotic gene transfer events (e.g. from the choloroplast to the nucleus) are usually involved in tolerance to oxidative stress and nitrogen limitation (Méheust et al., 2016)[b]. Moreover, the fact that the LAO1 homolog, LAO3, is predicted to be located in the chloroplast, further suggests that thes genes might have been acquired by an endosymbiotic gene transfer event from the chloroplast to the nucleus (McFadden, 2014)[c]. Alternatively, the *ALAAO-RidA* cluster from a microbe related to the ancestral archaeplastidan could have been laterally gene transferred to *Chlamydomonas*.

[a] Niehaus, T. D., Gerdes, S., Hodge-Hanson, K., Zhukov, A., Cooper, A. J., ElBadawi-Sidhu, M., et al. (2015). Genomic and experimental evidence for multiple metabolic functions in the RidA/YjgF/YER057c/UK114 (Rid) protein family. *BMC Genomics* 16. doi:10.1186/s12864-015-1584-3

[b] Méheust, R., Zelzion, E., Bhattacharya, D., Lopez, P., and Bapteste, E. (2016). Protein networks identify novel symbiogenetic genes resulting from plastid endosymbiosis. *Proc. Natl. Acad. Sci. U. S. A.* 113, 3579–84. doi:10.1073/pnas.1517551113.

[c] McFadden, G. I. (2014). Origin and evolution of plastids and photosynthesis in eukaryotes. *Cold Spring Harb. Perspect. Biol.* 6, a016105.

Thus, given the current available genomes and genome assemblies, **our phylogenetic analysis favors the idea that ALAAOs may have a common origin in an archaeplastidan ancestor** that was the result of a primary endosymbiosis of a protist engulfing a free cyanobacterium.

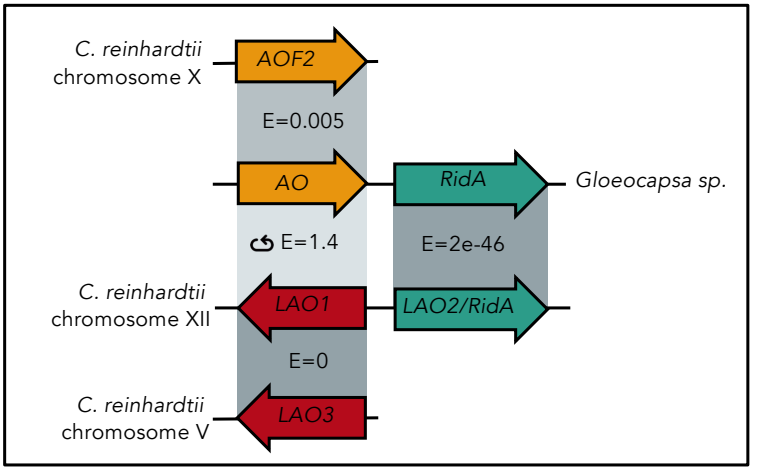


Figure 2.4. Amine oxidase-*RidA* sinteny analysis between the green alga *C. reinhardtii* and the cyanobacterium *Gloeocapsa* sp. PCC7428. E, e-value.

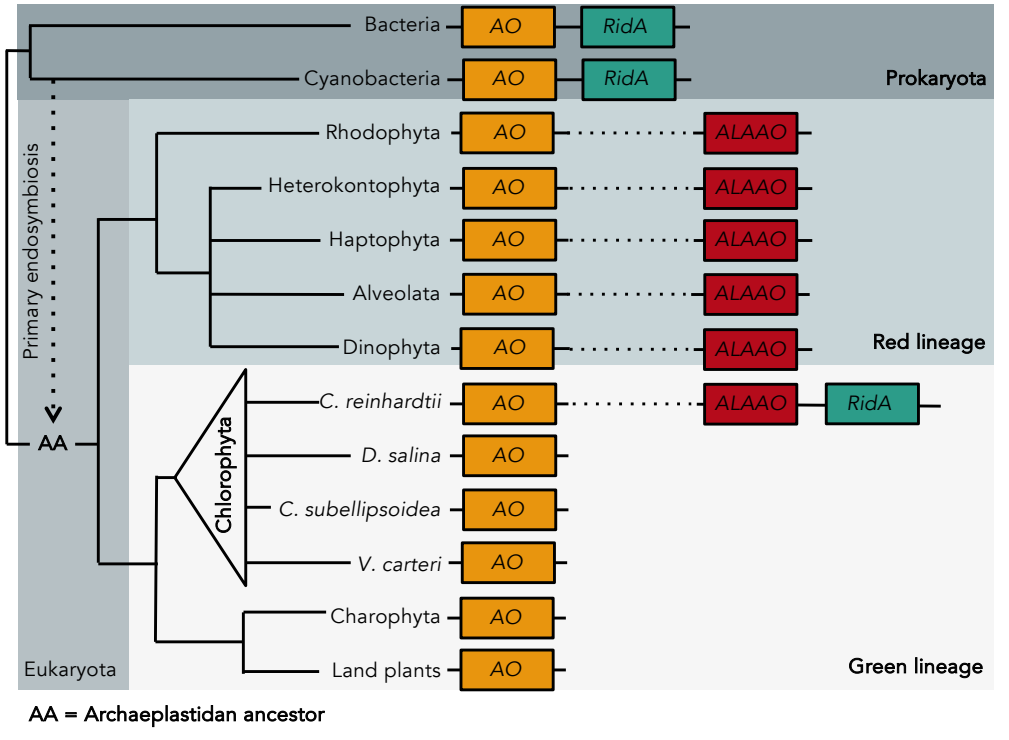


Figure 2.5. Comparative genomic analysis of algal *LAAOs*. *AO* refers to putative *AOF2* family ortholog genes; *ALAAO*, Algal L-Amino Acid Oxidase genes; *RidA*, Reactive intermediate/ imine deaminase A family genes.

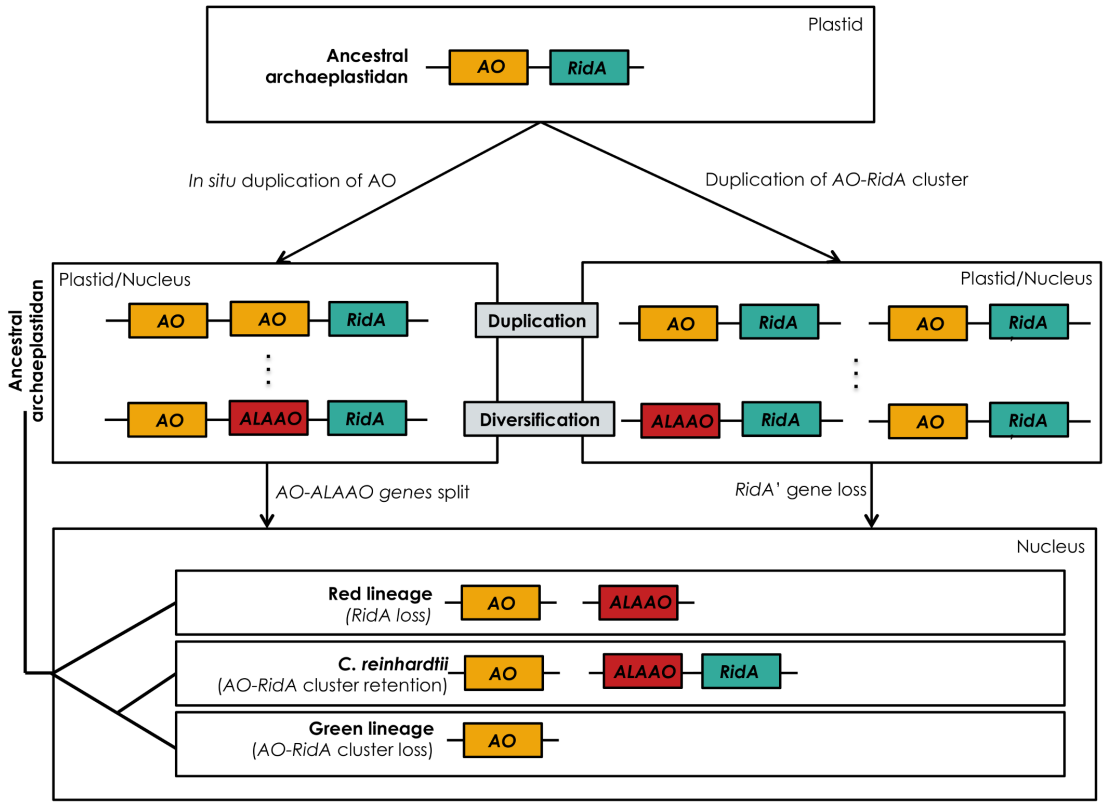


Figure 2.6. Putative algal *LAAO* (*ALAAO*) genes origin. Red boxes represent algal L-amino acid oxidase genes (*ALAAO*); orange boxes refer to putative *AOF2* family ortholog genes (*AO*); blue boxes indicate Reactive intermediate/imine deaminase A family genes (*RidA*).

2.4 The impact of LAO1 mutation on the use of amino acids and peptides as nitrogen sources.

Genetic tractability in *Chlamydomonas* has allowed the creation of a continuously-growing strains collection[a] that make available a large number of mutants for the scientific community. To better understand LAO1 role in *Chlamydomonas*, we obtained a *lao1* mutant (*LMJ.RY0402.044073*) from the *Chlamydomonas Resource Center*[a]. According to supplier indications, this mutant presents an insertion in the seventh exon of the gene that we further confirmed by PCR (Figure 2.7).

[a] *https://www.chlamycollection.org/strains/*

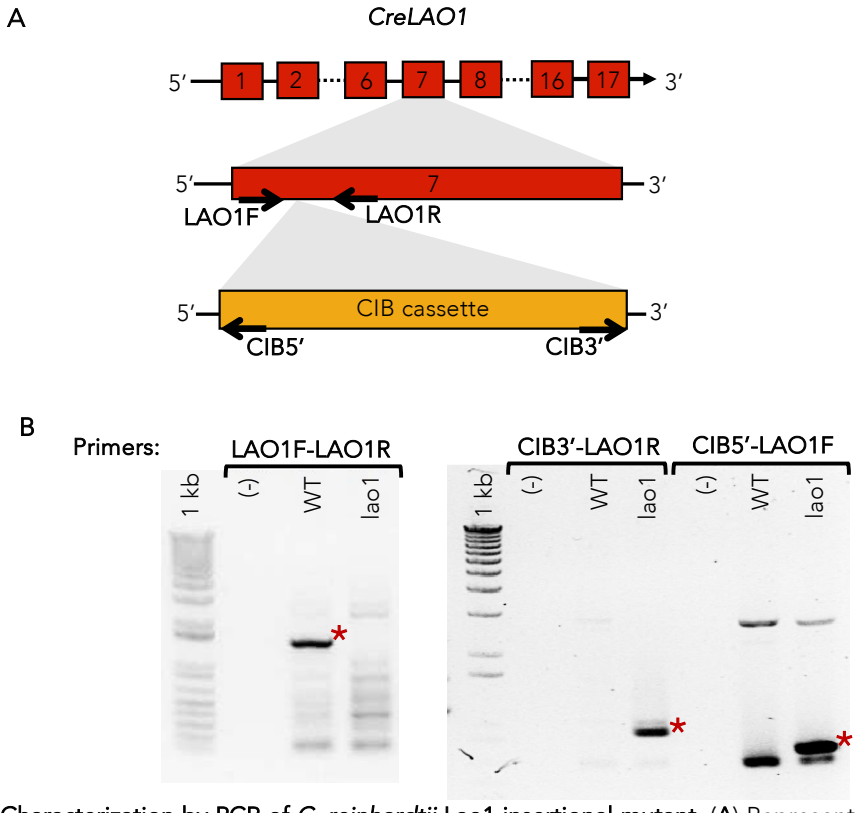


Figure 2.7. Characterization by PCR of *C. reinhardtii* Lao1 insertional mutant. (**A**) Representation of CIB cassette insertion localization in *LAO1* gene in the *lao1* mutant and primers localization. (**B**) PCR products for *lao1* mutant confirmation. Wild-type strain (WT) corresponds to CC-5325 and *lao1* mutant (*lao1*) to LMJ.RY0402.044073. (-) PCR negative control without DNA. 1 kb, DNA molecular weight marker. DNA from expected bands (*) were purified and sequenced for further confirmation. See *Materials and Methods* section for further details.

To explore LAO1 role as nitrogen scavenger from amino acids, we evaluated the effect of *LAO1* knock-out mutation in *Chlamydomonas* growth on each amino acid as the sole nitrogen source. These growth tests were performed in TAP media (with acetate), since it has been reported to enhance LAO1 activity (Vallon et al., 1993)[a]; after short- and long-term periods (4 and 12 days, respectively). First, to relate efficient growth on specific amino acids with LAO1 amino acid-specific activity, we quantified cell density after a short-term period to ensure exponential growth phase (Figure 2.8-A). Then, 8 days later, the cultures were imaged to further evaluate if growth was positive or negative after a long-term period (12 days total) (Figure 2.8-B, C). We compared the growth of the parental WT strain (CC-5325) and the *lao1* mutant strain on twenty proteinogenic amino acids as well as several di- and tri-peptides (Figure 2.8). We grouped the observed growth phenotypes into 3 classes: (i) LAO1-independent, (ii) LAO1-dependent and (iii) no growth.

[a] Vallon, O., Bulté, L., Kuras, R., Olive, J., and Wollman, F.-A. (1993). Extensive accumulation of an extracellular L-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem*. 215, 351–360. doi:10.1111/j.1432-1033.1993.tb18041.x

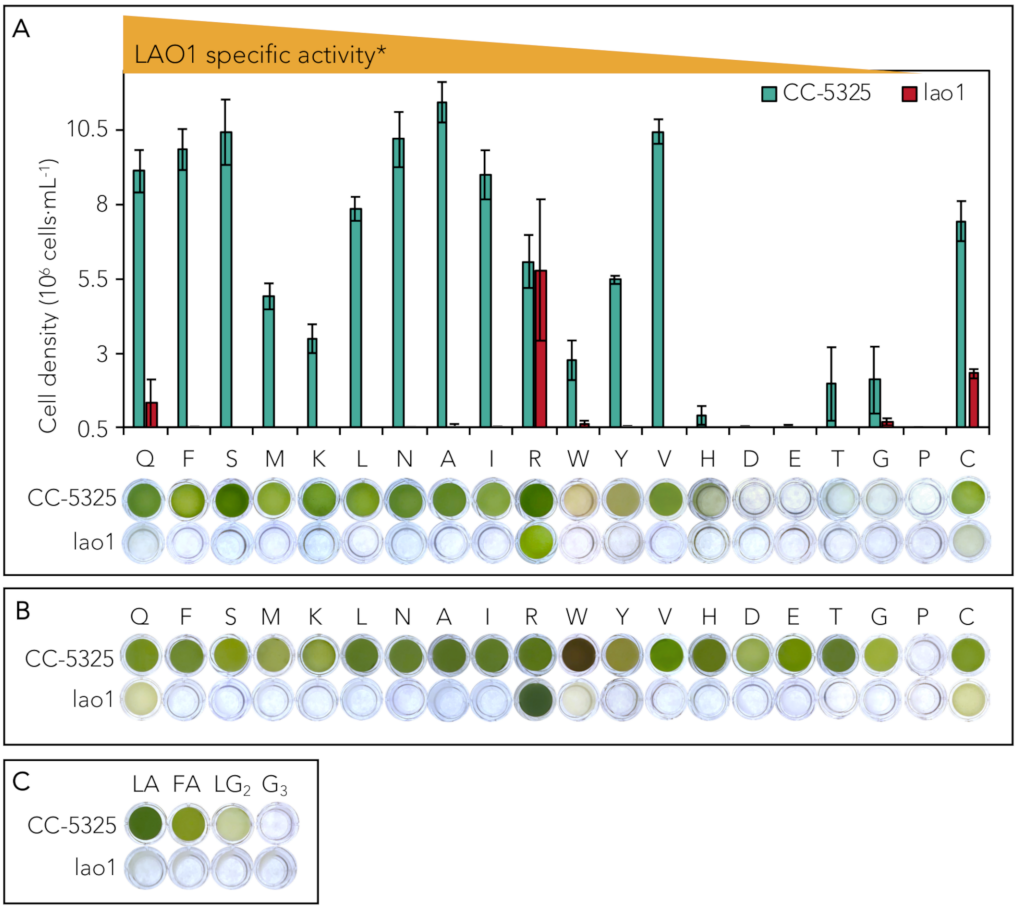


Figure 2.8. *Chlamydomonas* growth on amino acids and peptides as sole nitrogen source. (**A**) Short-term growth on L-amino acids (4 days). (*) From left to right, amino acids are arranged according to the specific activity, from higher to lower, reported for LAO1 (Vallon et al., 1993). Long-term growth on L-amino acids **(B)** and di-/tri-peptides (**C**) (12 days). Parental strain CC-5325 and *lao1* mutant strains were grown on each amino acid or peptide as the sole nitrogen (*Continued*) source (8 mM, except for L-Tyr: 2 mM) in TAP liquid media (with acetate). Q: L-glutamine; F: L-phenylalanine; S: L-serine; M: L-methionine; K: L-lysine; L: L-leucine; N: L-asparagine; A: L-alanine; I: L-isoleucine; R: L-arginine; W: L-tryptophan; Y: L-tyrosine; V: L-valine; H: L-histidine; D: L-aspartic acid; E: L-glutamic acid; T: L-threonine; G: glycine; P: L-proline; C: L-cysteine; LA: L-leucyl-alanine; FA: L-phenylalanyl-alanine; LG_2: L-leucyl-glycil-glycine; G_3: glycil-glycil-glycine. Error bars represent standard deviation of at least three biological replicates.

(i) Amino acids supporting LAO1-independent growth

Growth on L-gutamine, L-arginine and L-cysteine occured via a LAO1-independent pathway (Figure 2.8-B). In *Chlamydomonas*, L-arginine is the only reported amino acid that is taken up efficiently by a specific transporter (Kirk and Kirk, 1978b)[a], what is also true for the evolutionarily related green alga *Volvox* (Kirk and Kirk, 1978c)[b]. The identification of the corresponding arginine transporter has not yet been determined experimentally,

[a] Kirk, D. L., and Kirk, M. M. (1978b). Carrier-mediated Uptake of Arginine and Urea by *Chlamydomonas reinhardtii*. *Plant Physiol.*, 556–560.

[b] Kirk, M. M., and Kirk, D. L. (1978c). Carrier-mediated Uptake of Arginine and Urea by *Volvox carteri f. nagariensis*. *PLANT Physiol.* 61, 549–555. doi:10.1104/pp.61.4.549

however. The *Chlamydomonas* genome encodes six APC (Amino acid Polyamine organoCation) transporters and among them *AOC5* has been suggested as a candidate for specific arginine uptake (Vallon and Spalding, 2009)[a]. While LAO1 shows a high specific activity with L-glutamine as a substrate (Vallon et al., 1993)[b], we observed efficient growth of *lao1* mutants on this amino acid, suggesting for the first time that it may also be assimilated in a LAO1-independent manner, although with far less efficiency (Figure 2.8-B), via an unknown mechanism. It is possible that L-glutamine may just spontaneously oxidizes to form pyroglutamate and ammonium that could support *Chlamydomonas* growth, as it has been reported for *Dunaliella* spp. (Murphree et al., 2017)[c]. Similarly, we observed growth on L-cysteine by the *lao1* mutant. In this case, although LAO1 does not deaminate L-cysteine, a mechanism of passive diffusion into the cell has been proposed to allow *Chlamydomonas* growth on this amino acid (Zuo et al., 2012)[d], which could account for this growth in the *lao1* mutant. However, we observed a clear utilization defect compared to that in the WT, suggesting that LAO1 is involved in L-cysteine-dependent growth (Figure 2.8-A, B). Besides a passive uptake, we hypothesize that L-cysteine oxidizes in aqueous solution to form the dimer cystine (Kendall and Nord, 1926)[e], which is then efficiently deaminated by LAO1 (Vallon et al., 1993)[f] and could account for the observed enhanced growth in the WT. Nevertheless, growth on both L-glutamine and L-cysteine was significantly enhanced by LAO1 presence.

(ii) Amino acids supporting LAO1-dependent growth

LAO1-dependent growth occured with 16 amino acids (L-phenylalanine, L-serine, L-methionine, L-lysine, L-leucine, L-asparagine, L-alanine, L-isoleucine, L-tryptophan, L-tyrosine, L-valine, L-histidine, L-aspartate, L-glutamate, L-threonine and glycine), all of which have been shown to be enzymatic substrates for the LAO1 protein (Figure 1A) (Vallon et al., 1993)[a]. Consistent with the lower specific activity of LAO1 protein for L-histidine, L-aspartic acid, L-glutamic acid, L-threonine and glycine, growth on these amino acids was significantly low or even absent after 4 days, but it was evident after 12 days. Note that the oxidation of L-tryptophan turned the media orange, as previously noticed (Murphree et al., 2017)[g]. Intringuinly, even though it has been previously reported that

[a] Vallon, O., and Spalding, M. H. (2009). "Amino acid Metabolism," in *The Chlamydomonas Sourcebook*, 115–158

[b] Vallon, O., Bulté, L., Kuras, R., Olive, J., and Wollman, F.-A. (1993). Extensive accumulation of an extracellular l-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 215, 351–360. doi:10.1111/j.1432-1033.1993.tb18041.x

[c] Murphree, C. A., Dums, J. T., Jain, S. K., Zhao, C., Young, D. Y., Khoshnoodi, N., et al. (2017). Amino Acids Are an Ineffective Fertilizer for *Dunaliella* spp. Growth. *Front. Plant Sci.* 8, 847. doi:10.3389/fpls.2017.00847.

[d] Zuo, Z., Rong, Q., Chen, K., Yang, L., Chen, Z., Peng, K., et al. (2012). Study of amino acids as nitrogen source in *Chlamydomonas reinhardtii*. *Phycol. Res.* 60, 161–168. doi:10.1111/j.1440-1835.2012.00646.x

[e] Edward Kendall, B. C., and Nord, F. (1926). Reversible oxidation-reduction systems of cysteine-cystine and reduced and oxidized glutathione. *J. Biol. Chem.* 69, 295–337. Available at: http://www.jbc.org/ [Accessed July 20, 2017].

[f] Vallon, O., Bulté, L., Kuras, R., Olive, J., and Wollman, F.-A. (1993). Extensive accumulation of an extracellular l-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 215, 351–360. doi:10.1111/j.1432-1033.1993.tb18041.x

[g] Murphree, C. A., Dums, J. T., Jain, S. K., Zhao, C., Young, D. Y., Khoshnoodi, N., et al. (2017). Amino Acids Are an Ineffective Fertilizer for *Dunaliella* spp. Growth. *Front. Plant Sci.* 8, 847. doi:10.3389/fpls.2017.00847

Chlamydomonas is not able to grow and dies rapidly on L-histidine as nitrogen source with acetate as a carbon source (Hellio et al., 2004)[a], we observed efficient growth.

(iii) No growth

Chlamydomonas did not grow on L-proline, not even after long-term period, according to the absent substrate activity of LAO1 to this imino acid (Vallon et al., 1993)[b].

The use of peptides (di- and tri-peptides) as nitrogen source by *Chlamydomonas* has not been extensively studied. It was previously reported that the purified LAO1 enzyme is not active on dipeptides, although the dipeptides tested were not specified (Vallon et al., 1993)[c]. Recently, a high-throughput screening based on cellular respiration of *Chlamydomonas* in response to a large array of metabolites has shown that this alga is metabolically active on a number of di- and tri-peptides (108 out 267 di-peptides, and 3 out of 14 tri-peptides) (Chaiboonchoe et al., 2014)[d]. However, whether those peptides are nitrogen sources for *Chlamydomonas* growth is unknown. Here, we tested three respiration-positive (L-Leu-Ala, L-Phe-Ala and L-Leu-Gly-Gly) and one respiration-negative (Gly-Gly-Gly) peptides (Chaiboonchoe et al., 2014)[b]. We found that peptides that showed respiration-positive phenotype supported growth of *Chlamydomonas* WT cells, and the one that showed respiration-negative Gly-Gly-Gly tri-peptide did not (Figure 2.8-C). We also observed that the *lao1* mutant was unable to grow on any of the four tested peptides, suggesting that LAO1 may have a role in peptides assimilation as well, either deaminating the peptide directly or via deamination of free amino acids generated through the activity of some extracellular peptidase.

Thus, LAO1 seems to have a key role for the use of most of the proteinogenic amino acids and di-peptides as sole nitrogen source by this alga.

2. 5 The putative role of keto acids generated by LAO1 activity.

Besides nitrogen sources, amino acids might theoretically be used as well as carbon sources. However, it was previously reported that *Chlamydomonas* does not use the generated ketoacids and remained in the media (Muñoz-Blanco et al., 1990)[e]. This paradoxically waste of carbon made us wonder if keto acids, resulting from amino acids deamination, might have any additional role other than carbon sources for algae. It has been reported that keto acids may spontaneously react with hydrogen peroxide to be decarboxylated generating the

[a] Hellio, C., Veron, B., and Le Gal, Y. (2004). Amino acid utilization by *Chlamydomonas reinhardtii*: specific study of histidine. *Plant Physiol. Biochem.* 42, 257–64. doi:10.1016/j.plaphy.2003.12.005

[b] Vallon, O., Bulté, L., Kuras, R., Olive, J., and Wollman, F.-A. (1993). Extensive accumulation of an extracellular l-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 215, 351–360. doi:10.1111/j.1432-1033.1993.tb18041.x

[c] Vallon, O., Bulté, L., Kuras, R., Olive, J., and Wollman, F.-A. (1993). Extensive accumulation of an extracellular l-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 215, 351–360. doi:10.1111/j.1432-1033.1993.tb18041.x

[d] Chaiboonchoe, A., Dohai, B. S., Cai, H., Nelson, D. R., Jijakli, K., and Salehi-Ashtiani, K. (2014). Microalgal Metabolic Network Model Refinement through High-Throughput Functional Metabolic Profiling. *Front. Bioeng. Biotechnol.* 2, 1–12. doi:10.3389/fbioe.2014.00068

[e] Muñoz-Blanco, J., Hidalgo-Martínez, J., and Cárdenas, J. (1990). Extracellular deamination of amino acids by Chlamydomonas. *Planta* 182, 194–198

corresponding carboxylic acid and carbon dioxide (Bayliak et al., 2016)[a]. Thus, keto acid byproducts may act as hydrogen peroxide scavengers protecting from oxidative stress generated by the LAAOs deamination activity. To investigate if such decarboxylation was occurring in our algal cultures, we used the ketoacid pyruvic acid, which can not be assimilated by *Chlamydomonas*, in the presence of hydrogen peroxide, which would lead to the carboxylic acid acetic acid, one of the few organic carbon sources efficiently used by *Chlamydomonas*. Thus, we evaluated the impact of hydrogen peroxide in *Chlamydomonas* growth in the presence of pyruvic acid (Figure 2.9-A). Ammonium, pyruvic acid and hydrogen peroxide were added to the minimal media (without supplemented acetate) at equimolar concentrations to mimic LAAO byproducts generation. As expected, **hydrogen peroxide inhibited *Chlamydomonas* growth** (Figure 2.9 B). **However, pyruvic acid protected from such inhibition, according to its role as a hydrogen peroxide scavenger**. Moreover, **hydrogen peroxide addition led to a greater growth on pyruvic acid**, especially evident at 8 mM concentration. This enhanced growth might be due to the generation of an organic carbon source in the form of acetate, resulting from pyruvic acid decarboxylation promoted by hydrogen peroxide. Indeed, when this experiment was carried out **in the absence of light** to avoid CO_2 fixation**, hydrogen peroxide supplementation led to a significantly improved algal growth with pyruvic acid as the sole carbon source** (Figure 2.9-C), further suggesting the generation of acetic acid, which supports *Chlamydomonas* heterotrophic growth. Then, to further understand if such non-enzymatic decarboxylation was taking place after LAO1 activity, we quantified organic acids in the media of *Chlamydomonas* cultures grown on L-alanine as the sole nitrogen and carbon source (minimal media) (Figure 2.10). In fact, **we detected both pyruvic and acetic acid in the media subsequently to L-alanine dissapearence** (Figure 2.10-A). However, the *lao1* mutant strain, which did not use the amino acid, did not generate either pyruvic or acetic acid (Figure 2.10-B), further suggesting that both organic acids are a result of LAO1 activity in *Chlamydomonas*.

[a] Bayliak, M. M., Lylyk, M. P., Vytvytska, O. M., and Lushchak, V. I. (2016). Assessment of antioxidant properties of alpha-keto acids *in vitro* and *in vivo*. *Eur. Food Res. Technol.* 242, 179–188. doi:10.1007/s00217-015-2529-4

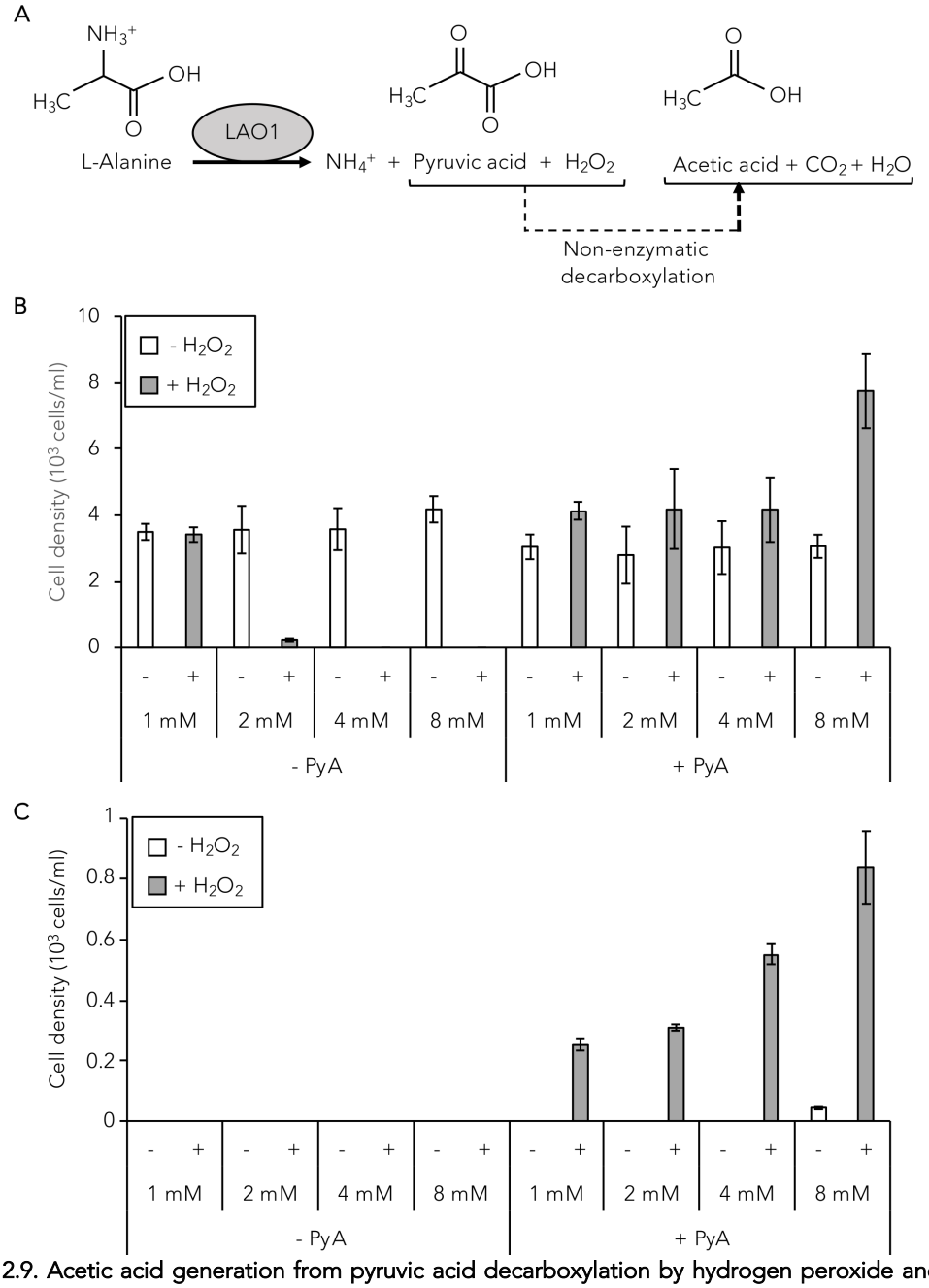


Figure 2.9. Acetic acid generation from pyruvic acid decarboxylation by hydrogen peroxide and algal growth. (**A**) L-Alanine deamination by LAO1 generates ammonium, pyruvic acid (PyA) and H_2O_2. The last two compounds can react to generate acetic acid, an assimilable carbon source for *Chlamydomonas*. (**B**) Pyruvic acid as H_2O_2-scanvenger in *Chlamydomonas* cultures after 3 days growing on ammonium under continuous light; – PyA: without pyruvic acid; + PyA: with pyruvic acid; (-) without H_2O_2; (+) with H_2O_2. 1, 2, 4 and 8 mM concentrations refer to equimolar concentrations of ammonium, pyruvic acid and/or H_2O_2, if present. (**C**) Similar experiment conditions as on (B) but carried out under continuous darkness. Error bars represent standard deviation of three biological replicates.

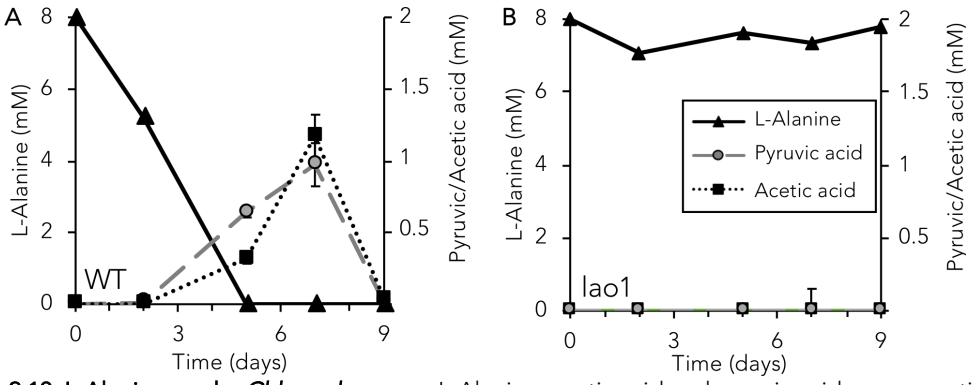


Figure 2.10. L-Alanine use by *Chlamydomonas*. L-Alanine, acetic acid and pyruvic acid were quantified in the media of cells grown on minimal media (without acetate) supplemented with 8 mM of L-Alanine as the sole nitrogen source under continuous light. (**A**) Wild-type cells (CC-5325). (**B**) *lao1* mutant cells. Error bars represent standard deviation of three biological replicates.

Thus, **keto acids, produced by LAO1 activity, may have a role as hydrogen peroxide scavengers to prevent oxidative stress due to deamination of amino acids by this enzyme in *Chlamydomonas***. In addition, ***Chlamydomonas* may use L-alanine not only as a nitrogen source, but also as a carbon source by means of the extracellular LAAO activity**. Moreover, if this spontaneous reaction is also occurring in other L-amino acids after deamination, further implications may rise up. For example, deamination of L-tryptophan may lead to the generation the carboxylic acid indole-acetic acid, a phytohormone that promotes algal growth (Park et al., 2013)[a]. Also, LAO2/RidA activity may have a role, not only avoiding reactive imine accumulation, but also for the rapid conversion to keto acids to immediately cope with the hydrogen peroxide generated by LAO1, pre-empting oxidative damage as well.

Then, LAAO in *Chlamydomonas* provides a wide-range substrate tool to scavenge nitrogen from amino acids and peptides when inorganic nitrogen is scarce, with a fine mechanism to avoid possible oxidative stress. However, since this enzyme is not a common trait within all algal species, it seems to represent an evolutionary adaptive attribute. Thus, different algae may have evolved different mechanisms to cope with different necessities and nitrogen sources availability in their natural niches.

Additionally, LAO1-generated keto acids roles may be involved in other roles such as iron solubilization as previously proposed in bacteria (Drechsel et al., 1993).

[a] Park, W.-K., Yoo, G., Moon, M., Kim, C. W., Choi, Y.-E., and Yang, J.-W. (2013). Phytohormone Supplementation Significantly Increases Growth of *Chlamydomonas reinhardtii* Cultivated for Biodiesel Production. *Appl. Biochem. Biotechnol*. 171, 1128–1142

2.6 How do algae scavenge nitrogen from amino acids?

From an ecological perspective, even though *LAAO* genes are not widely distributed within all algal genomes, it may however represent an advantage over other competitors to use alternative nitrogen sources when inorganic nitrogen is limiting. To that end, the extracellular localization of ALAAOs could be strategic for its role in nitrogen scavenging from exogenous amino acids and peptides, as proposed for LAO1 in *Chlamydomonas*. To get light whether this is a generalized fact for the other ALAAOs, we checked the cellular localization by *in silico* analysis. Although experimental confirmation is required, our data show that **out of 10 algal species containing LAAO, five of them were predicted to contain putative extracellular LAAOs** (Table 2.1). In addition, *Porphyra umbilicalis* was predicted to have three extracellular LAAOs. Thus, our data suggest that, like in *Chlamydomonas*, extracellular ALAAOs may have an impact on algal species growth on amino acids and peptides as nitrogen sources.

Even though the use of amino acids by algae is of eco-physiological importance, these studies are still scarce. We reviewed the currently available information in the literature. To that end, a broad search was performed combining reports of algal amino acid uptake, extracellular deamination activity and growth on amino acids. Although further research is necessary, the resulting data allow to propose different nitrogen scavenging patterns.

In the halophitic green alga *Dunaliella*, four different species were recently reported to be unable to use most proteinogenic amino acids as a sole nitrogen source, except L-histidine, which can be transported into the cell (Murphree et al., 2017)[a]. In *Volvox carteri*, a multicellular close relative of *Chlamydomonas*, only L-arginine is transported, although it is not catabolized and therefore not used as nitrogen source (Kirk and Kirk, 1978c)[b]. None of these algal genomes contains ALAAO (Murphree et al., 2017)[a]. This fact, together with the deficiency of transport systems could explain why these algae do not generally grow on most amino acids.

In the unicellular green alga *Chlorella*, amino acid uptake activities, as well as growth on many amino acids, have been reported (Algéus, 1949; Cho and Komor, 1985; Kirk and Kirk, 1978a; Zhang et al., 2015)[c,d,e,f]. Even though *Chlorella vulgaris* genome lacks ALAAO, the presence of amino acid transporters could explain the effective growth on amino acids.

[a] Murphree, C. A., Dums, J. T., Jain, S. K., Zhao, C., Young, D. Y., Khoshnoodi, N., et al. (2017). Amino Acids Are an Ineffective Fertilizer for *Dunaliella* spp. Growth. *Front. Plant Sci.* 8, 847. doi:10.3389/fpls.2017.00847

[b] Kirk, M. M., and Kirk, D. L. (1978c). Carrier-mediated Uptake of Arginine and Urea by Volvox carteri f. nagariensis. *Plant Physiol.* 61, 549–555. doi:10.1104/pp.61.4.549

[c] Algéus, S. (1949). Alanine as a Source of Nitrogen for Green Algae. *Physiol. Plant.* 2, 266–271. doi:10.1111/j.1399-3054.1949.tb07485.x

[d] Cho, B.-H., and Komor, E. (1985). The amino acid transport systems of the autotrophically grown green alga *Chlorella*. *Biochim. Biophys. Acta - Biomembr.* 821, 384–392. doi:10.1016/0005-2736(85)90042-2

[e] Kirk, D. L., and Kirk, M. M. (1978a). Amino Acid and Urea Uptake in Ten Species of Chlorophyta. *J. Phycol.* 14, 198–203. doi:10.1111/j.1529-8817.1978.tb02449.x

[f] Zhang, W., Zhang, Z., and Yan, S. (2015). Effects of various amino acids as organic nitrogen sources on the growth and biochemical composition of *Chlorella pyrenoidosa*. *Bioresour. Technol.* 197, 458–464. doi:10.1016/j.biortech.2015.08.100

The Algal LAAO enzymes and their putative cellular locations.

Phylum	Species	Natural habitat	Protein ID	DB	Putative cellular location (score)
Chlorophyta	*Chlamydomonas reinhardtii*	Freshwater/ soil microalga	AAB97101.1 (LAO1)	NCBI	SP (1.64)
			EDP07010.1 (LAO3)	NCBI	O
Rhodophyta	*Porphyra umbilicalis*	Marine macroalgae	OSX78923.1	NCBI	SP (0.70)
			OSX73518.1	NCBI	SP (1.16)
			OSX78913.1	NCBI	Chl (1.85)
			OSX80811.1	NCBI	SP (1.41)
	Graciliaropsis lemaneiformis		ATE86702.1	NCBI	SP (0.21)
	Chondrus crispus		XP_005713538.1	NCBI	SP (1.27)
Heterokonta	*Pelagophyceae* sp. CCMP2097	Marine microalgae	jgiPelago2097	JGI	O
	Aureococcus anophagefferens		XP_009033458.1	NCBI	SP (2.02)
Haptophyta	*Pavlovales sp.* CCMP2436		jgiPavlov2436	JGI	SP (1.51)
	Emiliania huxleyi		XP_005785782.1	NCBI	Mit (0.50)
			XP_005777092.1	NCBI	O
	Chrysochromulina sp. CCMP291		KOO35684.1	NCBI	O
Alveolata	*Vitrella brassicaformis*		CEL94447.1	NCBI	O
Dinophyta	*Symbiodinium microadriaticum*		OLQ08796.1*	NCBI	O

Putative cellular location was obtained using the multicellular location prediction tool dedicated to algae, PredAlgo program, which computes a score for three cellular compartments: the mitochondrion (Mit), the chloroplast (Chl), and the secretory pathway (SP). When the three scores were below the following cutoff, the target is indicated as 'other' (O): 0.42 for the mitochondrion, 0.41 for the chloroplast, and 0.14 or the secretion pathway (Tardif et al., 2012)[a]. Databases: NCBI (https://www.ncbi.nlm.nih.gov/), JGI (https://jgi.doe.gov/), and Phytozome (https://phytozome.jgi.doe.gov/). *Partial protein sequence.

[a] Tardif, M., Atteia, A., Specht, M., Cogne, G., Rolland, N., Brugière, S., et al. (2012). PredAlgo: A New Subcellular Localization Prediction Tool Dedicated to Green Algae. *Mol. Biol. Evol.* 29, 3625–3639. doi:10.1093/molbev/mss178

In Heterokonta algae with available genome sequences, different situations can be proposed. The harmful algal bloom-forming *Aureococcus anophagefferens* possesses similar abilities as *Chlamydomonas* to grow on amino acids, and show amino acids uptake as well as extracellular deaminase activities (Mulholland et al., 2002)[a]. According to the last mentioned activity, a putative extracellular ALAAO was found in its genome (Table 2.1). The pennate diatom *Phaeodactylum tricornutum* grows on a wide range of amino acids, it shows amino acid uptake activity for L-arginine and L-lysine, and extracellular deamination activity for several amino acids (Hayward, 1965; Rees and Allison, 2006)[b,c]. However, we did not find any LAO1 ortholog in the current assembly of *P. tricornutum* genome. Whether the reported extracellular deaminase activity is due to another amine oxidase enzyme should be resolved. Finally, the centric diatom *Thalassiosira pseudonana* is unable to grow on amino acids as sole nitrogen source (Ietswaart et al., 1994; Palenik and Morel, 1990b) [d, e] and lacks ALAAO gene. Nevertheless, the defective growth on amino acids or peptides by some algae may be complemented by interactions with bacteria that can mineralize this nitrogen (Ietswaart et al., 1994)[a]. How algae can be benefited by some bacteria that promote algal growth on amino acids and peptides, and what are the physiological basis that allow the success of these interactions, are the main questions that will be adressed in the following and final chapter.

[a] Mulholland, M. R., Gobler, C. J., and Lee, C. (2002). Peptide hydrolysis, amino acid oxidation, and nitrogen uptake in communities seasonally dominated by *Aureococcus anophagefferens*. *Limnol. Oceanogr.* 47, 1094–1108. doi:10.4319/lo.2002.47.4.1094

[b] Hayward, J. (1965). Studies on the Growth of *Phaeodactylum tricornutum* (Bohlin) I. The Effect of Certain Organic Nitrogenous Substances on Growth. *Physiol. Plant.* 18, 201–207. doi:10.1111/j.1399-3054.1965.tb06883.x

[c] Rees T.A.V.; Allison, V. J. (2006). Evidence for an extracellular l-amino acid oxidase in nitrogen-deprived *Phaeodactylum tricornutum* (bacillariophyceae) and inhibition of enzyme activity by dissolved inorganic carbon. *Phycologia* 45, 337–342. doi:http://dx.doi.org/10.2216/04-92.1

[d] Ietswaart, T., Schneider, P. J., and Prins, R. A. (1994). Utilization of organic nitrogen sources by two phytoplankton species and a bacterial isolate in pure and mixed cultures. *Appl. Environ. Microbiol.* 60, 1554–1560

[e] Palenik, B., and Morel, F. M. M. (1990b). Comparison of cell-surface L-amino acid oxidases from several marine phytoplankton. *Mar. Ecol. Prog. Ser.* 59, 95–201. doi:10.3354/meps05919

Chapter 3:

Alga-Bacteria Interactions Facilitate *Chlamydomonas* use of Amino Acids and Peptides as Nitrogen Sources

3.1 Contaminating bacteria can promote *Chlamydomonas* growth

Although *Chlamydomonas* can grow on most amino acids and some di-/tri-peptides as the sole nitrogen sources, this growth is far less efficient than that on inorganic nitrogen, and yet, there are some amino acids and peptides that cannot be used by this alga. Throughout the analysis of *Chlamydomonas* growth on these nitrogen compounds that cannot be used, we observed that a spontaneous pink-colored contamination allowed this alga to grow efficiently on the di-peptide L-alanyl-alanine (Figure 3.1-A). This contamination was isolated from this algal culture by repeated colony re-streaking and identified by the sequencing of a PCR-amplified product of a region of 16S rDNA from single colonies (Figure 3.1-B, C) (see *Material and Methods* section for further details). This sequence revealed a 99% of identity with *Methylobacterium* spp. (*M. marchantiae* and *M. bullatum)* and was deposited in NCBI database (accession number: MG287145).

Methylobacterium spp. are known as Pink-Pigmented Facultative Methylotrophs (PPFM) included in the PGPB group of bacteria (Plant Growth-Promoting Bacteria), which improve plant growth and fitness (Omer et al., 2004)[a]. Thus, it was not entirely surprising that this contaminating *Methylobacterium* sp. might as well benefit *Chlamydomonas*, a green alga ancestor to land plants.

Prompted by the ecological importance of this finding we got further insight to identify bacteria supporting *Chlamydomonas* cell growth and also to understand the mechanisms for metabolic complementation in synthetic ecosystems alga-bacteria.

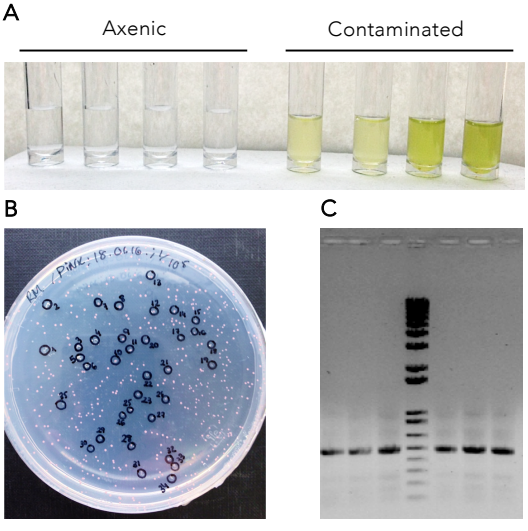


Figure 3.1. Isolation and identification of bacteria promoting *Chlamydomonas* growth. (**A**) *Chlamydomonas* liquid cultures (TAP) supplemented with L-Ala-Ala as the sole nitrogen source after 9 days. (**B**) Single colonies of the contaminant used for identification by PCR. (**C**) Amplification result of 16S rDNA region PCR of six different colonies. The middle lane corresponds to a 1kb MW marker. Intense bands observed in the gel were sequenced by Sanger sequencing.

We proceeded to isolate different bacteria from natural environments where algae usually live -such as water ponds and wet rocks-, and where *Methylobacterium* spp. are reported to be found -such as the surface of plant leaves and tap water- (Figure 3.2-A, B). We isolated and identified 12 bacterial colonies that were tested in co-culture with

[a] Omer, Z. S., Tombolini, R., Gerhardson, B., Green, P. N., Corpe, W. A., Corpe, W. A., et al. (2004). Plant colonization by pink-pigmented facultative methylotrophic bacteria (PPFMs). *FEMS Microbiol. Ecol.* 47, 319–26. doi:10.1016/S0168-6496(04)00003-0

Chlamydomonas to observe if they could promote algal growth on L-serine. As a result, 10 of them enhanced algal growth (Figure 3.2-C). Among them, different species of *Sphingomonas*, *Methylobacterium*, *Chitinophagacea* and *Deinococcus* genera were benefitting *Chlamydomonas* growth. Thus, **algal growth promotion on amino acids seem to be a relatively widespread ability of different bacteria that share natural niches with algae.**

A

B

C

Co-cultures

Isolate	Source	Identification	Id. (%)	Isolate	Source	Identification	Id. (%)
FS5	UMFS	*Chitinophagaceae bacterium*	98	TW1	Tap Water	*Sphingomonas parapaucimobili*	99
FS6	UMFS	*Sphingomonas panni*	99	TW3	Tap Water	*Methylobacterium extorquens*	98
FS8	UMFS	*Sphingomonas panni*	99	TW5	Tap water	*Methylobacterium extorquens*	100
FS9	UMFS	*Sphingomonas panni*	99	TW6	Tap water	*Sphingomonas yantingensis*	99
FS12	UMFS	*Sphingomonas panni*	99	M017	Cre-culture	*Methylobacterium marchantiae*	99
FS13	UMFS	*Deinococcus aquaticus*	99	CL4	Cre-culture	*Shingomonas paucimobilis*	100

Figure 3.2. Isolation and identification of *Chlamydomonas* growth-promoting bacteria. (**A**) Water pond covered with algae in the Field Station of the University of Mississippi (Oxford, MS) where some samples were taken. (**B**) Agar plates with isolates from different environments: green rocks (left, up and down) on BB and KS media; water pond (middle, up), tap water (middle, down) and plant leaves surface (right, up and down) on CYM:MH media. (**C**) Growth promotion test on minimal media (without any added carbon source) supplemented with 8 mM of L-serine as the sole nitrogen source. Liquid cultures were grown in 96 wells-plates for 5 days under continuous light, centrifuged and the pelleted cells in the wells were pictured from below. Yellow arrow indicates the *Chlamydomonas* (wild-type strain CC-1690) mono-culture control. FS isolates were obtained from the Field Station of the University of Mississippi (UMFS), TW isolates were obtained from tap water; and M017 and CL4 were isolated from *Chlamydomonas* cultures. Id., is the sequence identity found to the indicated species obtained by BLAST program using a region of 16S rDNA region amplified by PCR (see *Materials* and *Methods* for further details).

3.2 *Methylobacterium* allows *Chlamydomonas* growth on non-assimilable amino acids and peptides by the alga.

Given the high biotechnological potential of *Methylobacterium* spp., wide availability of sequenced genomes and strains, as well as their reported mutualistic relationship with plants (Dourado et al., 2015)[a], we decided to focus on this genus in the following work.

First, we studied the ability of these bacteria to allow *Chlamydomonas* growth on different amino acids and peptides that cannot be used by this alga. These results are included in the following publication (full text included in Supplementary Material):

OK, thanks! A new mutualism between *Chlamydomonas* and methylobacteria facilitates growth on amino acids and peptides.

Victoria Calatrava, Erik F Y Hom, Ángel Llamas, Emilio Fernández, Aurora Galván
FEMS Microbiology Letters, Volume 365, Issue 7, 1 April 2018, fny02,
DOI: 10.1093/femsle/fny021
Published: 29 January 2018

Abstract: Nitrogen is a key nutrient for land plants and phytoplankton in terrestrial and aquatic ecosystems. The model alga *Chlamydomonas reinhardtii* can grow efficiently on several inorganic nitrogen sources (e.g., ammonium, nitrate, nitrite) as well as many amino acids. In this study, we show that *Chlamydomonas* is unable to use proline, hydroxyproline, and peptides that contain these amino acids. However, we discovered that algal growth on these substrates is supported in association with *Methylobacterium* spp., and that a mutualistic carbon-nitrogen metabolic exchange between *Chlamydomonas* and *Methylobacterium* spp. is established. Specifically, the mineralization of these amino acids and peptides by *Methylobacterium* spp. produces ammonium that can be assimilated by *Chlamydomonas*, and CO_2 photosynthetically fixed by *Chlamydomonas* yields glycerol that can be assimilated by *Methylobacterium*. As *Chlamydomonas* is an algal ancestor to land plants and *Methylobacterium* is a plant growth-promoting bacterium (PGPB), this new model mutualism may facilitate insights into the ecology and evolution of plant-bacterial interactions and design principles of synthetic ecology.

[a] Dourado, M. N., Camargo Neves, A. A., Santos, D. S., and Araújo, W. L. (2015). Biotechnological and Agronomic Potential of Endophytic Pink-Pigmented Methylotrophic *Methylobacterium* spp. *Biomed Res. Int.*, 1–19

3.3 *Methylobacterium* spp. promoting *Chlamydomonas* growth on algal assimilable amino acids

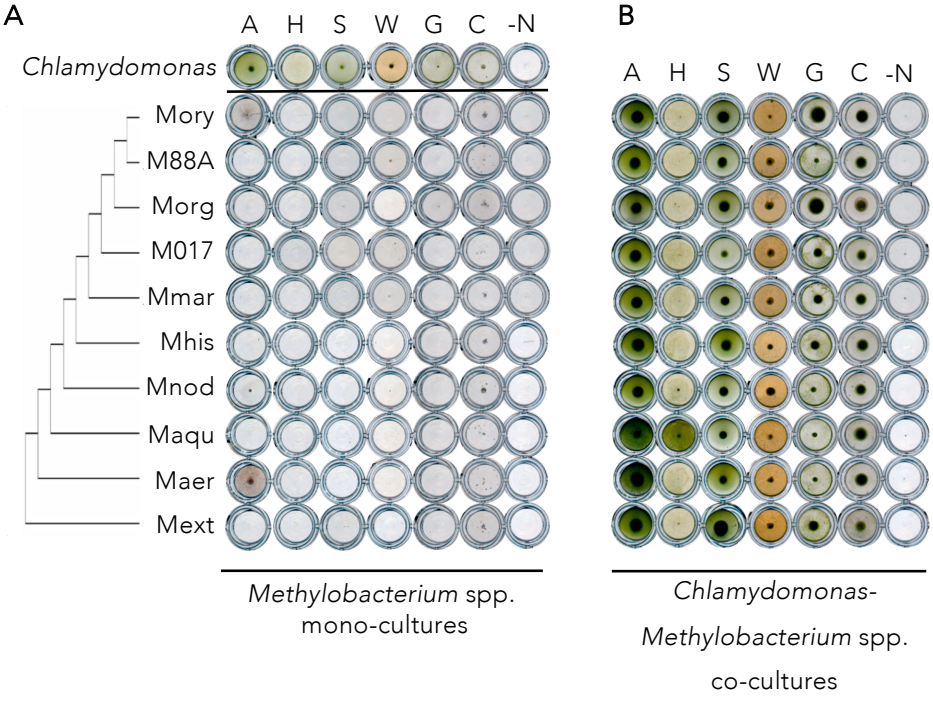
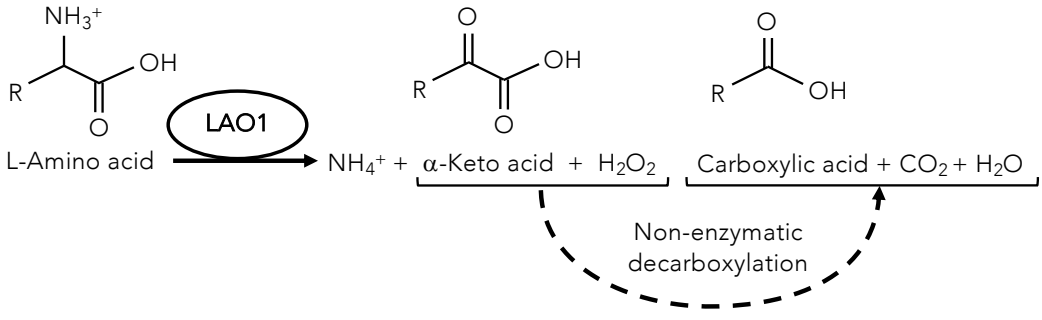


Figure 3.3. *Chlamydomonas* growth promotion by *Methylobacterium* spp. on amino acids as sole nitrogen source. (**A**) Mono-cultures and (**B**) co-cultures of *Chlamydomonas* WT strain CC-1690 and *Methylobacterium* spp. on liquid minimal media (without any added carbon source) supplemented with 8 mM of L-alanine (A), L-histidine (H), L-serine (S), L-tryptophan (W), glycine (G), L-cysteine (C) and without any nitrogen source (-N), for 3 days on the first three and for 5 days on the last three conditions. 96-wells plates were centrifuged, and the pelleted cells were imaged from below. Images are representative of three biological replicates. *Mory*, *M. oryzae*; *Methylobacterium* sp. 88A; *Morg, M. organophilum; M017, Methylobacterium* sp. M017; *Mmar, M. marchantiae; Mhis, M. hispanicum; Mnod, M. nodulans; Maqu, M. aquaticum; Maer, M. aerolatum; Mext, M. extorquens.*

Although *Chlamydomonas* can use most amino acids as nitrogen sources by means of the extracellular LAAO (LAO1), we observed that this growth was often enhanced by the presence of *Methylobacterium* spp. (Figure 3.3). As shown in Figure 3.3, **all the *Methylobacterium* spp. tested improved the algal growth in L-alanine, L-serine, L-glycine, L-tryptophan and L-cysteine.** However, **algal growth on L-histidine showed a species-specific metabolic complementation by *M. aquaticum*.** We hypothesized that such algal growth promotion by methylobacteria on L-amino acids could be driven by a nitrogen-carbon exchange, similarly to what we observed for L-proline. Thus, bacterial mineralization of amino acids provides the alga with ammonium. In this case however, given that these amino acids are LAO1 substrates, *Chlamydomonas* could also provide in turn α-keto acids as extra carbon sources to the bacteria. Given that LAO1 activity produces different α-keto acids -and presumably carboxylic acids- from each specific amino acid (Figure 3.4), these compounds could determine the specificity of *Chlamydomonas-Methylobacterium* mutualistic interactions.

Thus, we hypothesized that LAO1-producing α-keto acids, a waste product for the alga, could provide bioavailable carbon sources to bacteria. If this hypothesis were right, the extracellular LAO1 would have a key role in this *Chlamydomonas-Methylobacterium* interaction. To investigate this possibility, we studied the impact of *LAO1* mutation in the co-cultures on three amino acids that are deaminated by LAO1 (L-alanine, L-serine and L-glutamic acid) (Figure 3.5). As we observed in Chapter 2, and further confirmed here, *Chlamydomonas lao1* mutant did not grow on these amino acids under axenic conditions (mono-culture). During co-culture however, specific *Methylobacterium* spp. led to growth complementation on specific amino acids. On one hand, we observed that some *Methylobacterium* spp. did not complement *Chlamydomonas lao1* mutant growth on some amino acids, if any (Figures 3.3 and 3.4). For instance, *M. extorquens*, *M organophilum* and *M. hispanicum* did not complement *lao1* mutant growth on none of the tested amino acids. On the other hand, *M. oryzae* complemented algal growth on the three amino acids. According to the obtained results, we divided the interactions into two different phenotypes: (a) LAO1-dependent mutualism, for those that showed growth complementation by *Methylobacterium* spp. and was not observed in the *lao1* mutant; and (b) LAO1-independent mutualism, when bacteria were able to complement *lao1* mutant growth (Figure 3.5).

A



B

L-Amino acid	α-Keto acid	Carboxylic acid
L-Alanine	Pyruvic acid	Acetic acid
L-Histidine	Urocanic acid	4-Imidazoleacetic acid
L-Serine	3-Hydroxypyruvic acid	Glycolic acid
L-Tryptophan	Indole-3-pyruvic acid	Indole-3-acetic acid
Glycine	Glyoxylic acid	Formic acid
L-Cysteine	3-Mercaptopyruvic acid	Thioglycolic acid

Figure 3.4. Organic acids generated by LAO1 activity. (**A**) *Chlamydomonas* LAO1 can deaminate most proteinogenic amino acids generating ammonium, the corresponding α-keto acid and hydrogen peroxide. Subsequently, a non-enzymatic decarboxylation of the keto acid may occur by the action of hydrogen peroxide, generating the corresponding carboxylic acid, carbon dioxide and water. (**B**) Corresponding α-keto acids and carboxylic acids generated by LAO1 deamination of amino acids.

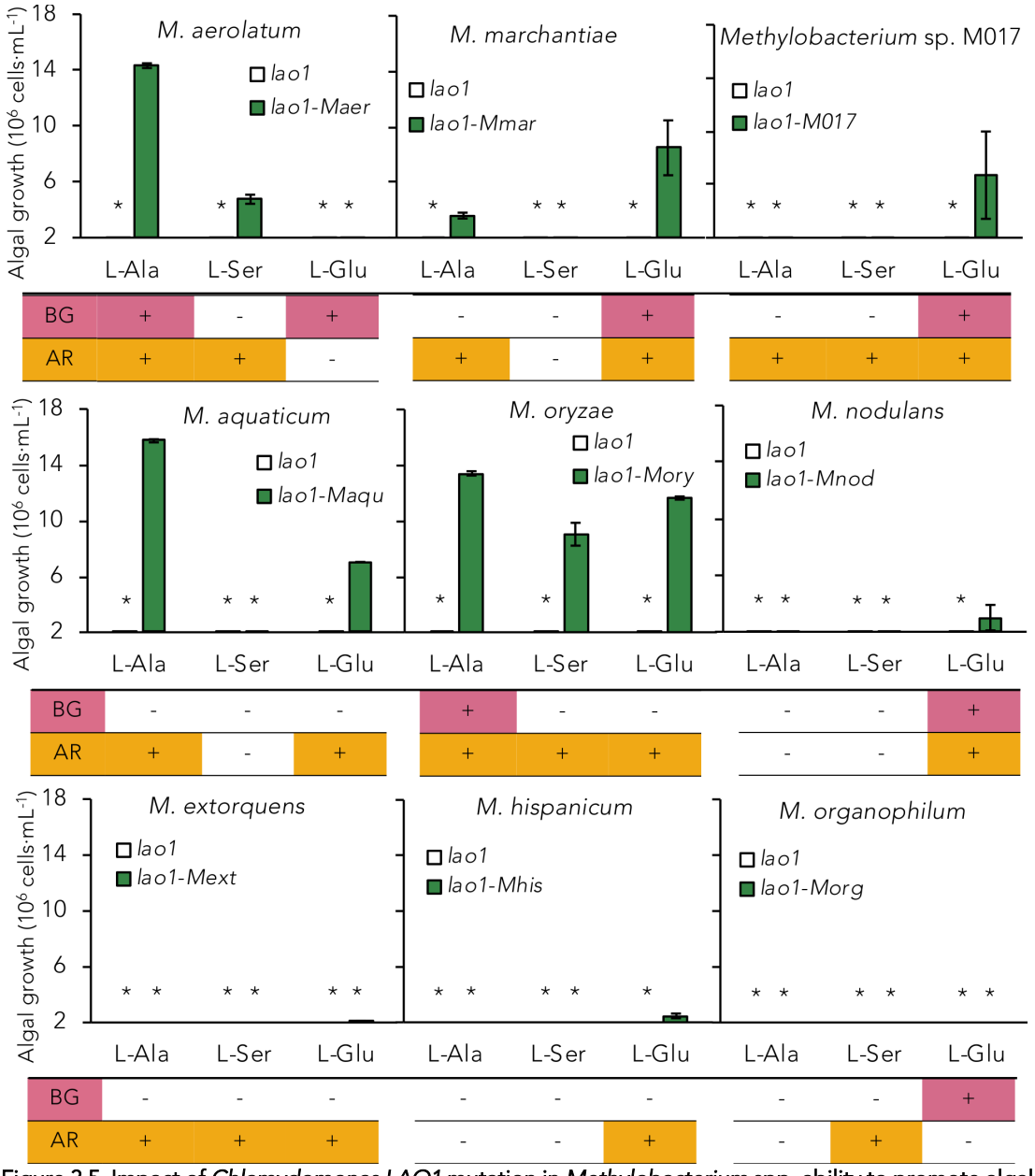


Figure 3.5. Impact of *Chlamydomonas LAO1* mutation in *Methylobacterium* spp. ability to promote algal growth on amino acids. Three amino acids that showed LAO1-dependent growth for *Chlamydomonas* (L-alanine, L-Serine and L-Glutamic acid) were tested during mono- and co-culture of *lao1* mutant with nine different *Methylobacterium* species. Algal cell density was quantified after 1 week incubated on each amino acid (8 mM) in minimal media (without any other added carbon source) under continuous light. Error bars represent standard deviation of two biological replicates. Mono-culture bacterial growth (BG) was determined, after 1 week under the same conditions, by measuring absorbance at 600 nm (A_{600}). Positive growth (+) was considered as A_{600}>0.05. Ammonium release (AR) was determined after 1 week in the supernatant of the same bacterial mono-cultures by a colorimetric method using Nessler's reagent. Positive ammonium release (+) was considered as A_{410}>0.1. (*), Not detected *Chlamydomonas* cell density (lower than $2 \cdot 10^6$ cells·mL^{-1}).

Additionally, to know how efficiently these bacterial species were assimilating these amino acids in the absence of *Chlamydomonas*, we tested bacterial growth and ammonium release under axenic conditions (Figure 3.5). We assumed that a positive ammonium release by the bacteria suggests an unbalanced C/N from the amino acid. In addition, the negative or positive growth might indicate how strong is this unbalance. For instance, L-alanine seems to be a better C/N source for *M. aerolatum* than for *M. aquaticum*.

(a) LAO1-dependent mutualism

Under mono-culture, we observed that *M. extorquens* released ammonium on L-alanine, L-serine and L-glutamate but these amino acids did not support bacterial growth. Although, this species promoted *Chlamydomonas* WT growth on these amino acids, it did not complement *lao1* mutant suggesting that **LAO1 activity may be crucial for this interaction with *M. extorquens***. The hypothesis of keto acids as the key carbon molecule exchanged during these consortia was further demonstrated in our lab by Ureña (2018)[a]. In his work, Ureña also shows that during bacterial mono-culture, *M. extorquens* was not able to grow on L-alanine as the sole nitrogen and carbon source, and that ammonium was released into the media. During *Chlamydomonas* WT-*M. extorquens* co-culture, the growth of both microbes was enhanced, demonstrating a mutualistic interaction. However, when the co-culture was performed with *Chlamydomonas lao1* mutant instead, none of the organisms were able to grow, which suggests that the keto acid generated by LAO1 activity (i.e., pyruvic acid) was key. In fact, when pyruvic acid was added exogenously to the bacterial mono-culture, *M. extorquens* growth was enhanced on ammonium and on L-alanine. In summary, it is proposed that alga-produced pyruvic acid, by feeding the bacterial TCA cycle, results in the balance of the C/N ratio allowing an efficient N assimilation and therefore bacterial growth on this amino acid. This effect might be applied to any LAO1 substrate, as long as the specific bacterial species are able to use the keto acid as an extra carbon source to properly assimilate the amino acid-derived N. In turn, *Chlamydomonas* may get benefited by the bacterial-produced ammonium. In the model proposed for LAO1-dependent mutualistic interaction (Figure 3.6), the different metabolites produced by both microbes may cross-regulate each other's activity, what may be essential for the success of the consortia growth. On one hand, ammonium can be generated from amino acids by either the bacteria or the alga (by LAO1 activity) but the bacteria is inefficient to use it for growth unless α-keto acids are available. Moreover, LAO1 activity generates hydrogen peroxide that could inhibit algal photosynthesis and growth (Baldry, 1983; Foyer, 2018; Pokora et al., 2018)[b,c,d]. Since

[a] Ureña, J. (2018) Caracterización fisiológico-molecular de la interacción mutualista alga-bacteria.Trabajo fin de Grado de Biología, Universidad de Córdoba. Córdoba, Spain

[b] Baldry, M. G. (1983). The bactericidal, fungicidal and sporicidal properties of hydrogen peroxide and peracetic acid. *J. Appl. Bacteriol.* 54, 417–23. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6409877 [Accessed September 5, 2018]

[c] Foyer, C. H. (2018). Reactive oxygen species, oxidative signaling and the regulation of photosynthesis. *Environ. Exp. Bot.* 154, 134–142. doi:10.1016/J.ENVEXPBOT.2018.05.003

[d] Pokora, W., Aksmann, A., Baścik-Remisiewicz, A., Dettlaff-Pokora, A., and Tukaj, Z. (2018). Exogenously applied hydrogen peroxide modifies the course of the *Chlamydomonas reinhardtii* cell cycle. *J. Plant Physiol.* 230, 61–72. doi:10.1016/j.jplph.2018.07.015

by releasing ammonium, bacteria could low down LAO1 activity (Vallon et al., 1993)[a] and hydrogen peroxide levels and thus, both organisms may get benefited by avoiding oxidative stress. Interestingly, all *Methylobacterium* spp. are reported to show catalase activity (Patt et al., 1976)[b], which might be a specific trait that favors the interaction with *Chlamydomonas*. Finally, other molecules can be exchanged between both partners. For instance, the CO_2 released by bacteria as a waste product feed algal photosynthesis. Otherwise, this algal photosynthetic activity releases O_2 as a waste product, which facilitates growth of *Methylobacterium* species, strictly aerobic organisms (Trotsenko et al., 2001)[c].

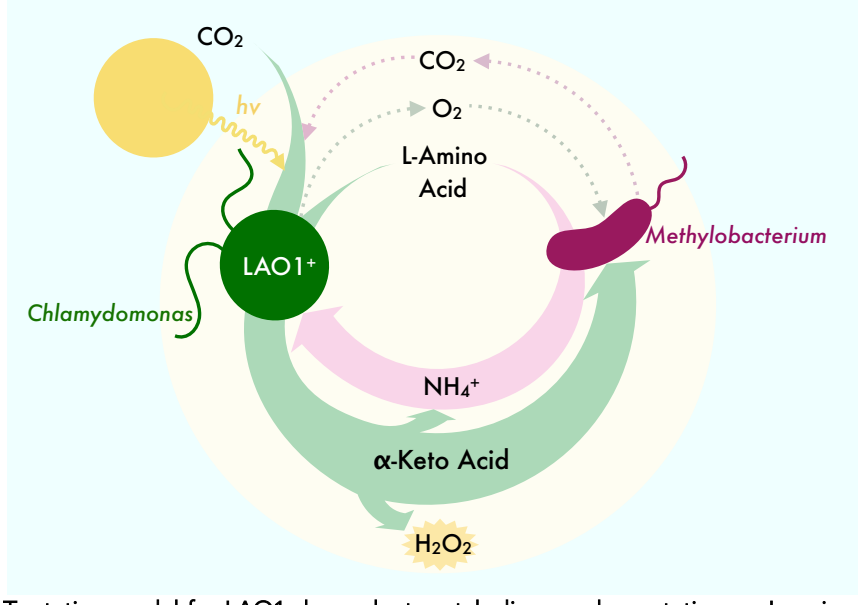


Figure 3.6. Tentative model for LAO1-dependent metabolic complementation on L-amino acids. Most proteinogenic amino acids are extracellularly deaminated by Chlamydomonas LAO1 generating ammonium and the corresponding α-keto acid. Then, ammonium can be efficiently assimilated. However, the α-keto acids cannot be used by this alga and remain in the media. Otherwise, some Methylobacterium spp., although they are able to catabolize amino acids, these compounds do not support efficient bacterial growth and lead to ammonium release to the media. This effect may be due to a C/N unbalance that requires an additional carbon input to properly assimilate ammonium. Then, bacterial-produced ammonium is efficiently taken up by Chlamydomonas facilitating algal growth. Otherwise, the algal-produced α-keto acids can be assimilated by these bacteria which provides extra carbon skeletons to properly assimilate ammonium and thus, promote bacterial growth. Moreover, LAO1 activity generates hydrogen peroxide, which inhibits both microbial growth and algal photosynthesis. Additionally, the CO_2 released by bacteria as a waste product, enhances algal photosynthesis. Otherwise, this algal photosynthetic activity releases O_2 as a waste product, which facilitates growth of Methylobacterium, which are strictly aerobic organisms.

[a] Vallon, O., Bulté, L., Kuras, R., Olive, J., and Wollman, F.-A. (1993). Extensive accumulation of an extracellular l-amino-acid oxidase during gametogenesis of *Chlamydomonas reinhardtii*. *Eur. J. Biochem.* 215, 351–360. doi:10.1111/j.1432-1033.1993.tb18041.x

[b] Patt, T. E., Cole, G. C., and Hanson, R. S. (1976). Methylobacterium, a New Genus of Facultatively Methylotrophic Bacteria. *Int. J. Syst. Evol. Microbiol.* 26, 226–229. doi:10.1099/00207713-26-2-226.

[c] Trotsenko, Y. A., Ivanova, E. G., and Doronina, N. V (2001). Aerobic methylotrophic bacteria as phytosymbionts. *Microbiology* 70, 623–632. doi:10.1023/A:1013167612105

(ii) LAO1-independent mutualism

Otherwise, ***Chlamydomonas*** **LAO1 presence was not crucial for algal-bacterial mutualism on some amino acids with other *Methylobacterium* spp.** For instance, *M. aquaticum, M. aerolatum and M. oryzae* allowed *Chlamydomonas lao1* mutant growth on L-alanine (Figure 3.5), on L-serine, *M. aerolatum and M. oryzae*; and on L-glutamic acid, *M. marchantiae, M. oryzae and M. aquaticum*. Taking in mind that those bacteria were unable to grow but released ammonium to the media under axenic condition, it could be suggested that these amino acids are also unbalanced C/N sources and thus, extra carbon input is necessary for bacterial growth. However, given that these bacteria were able to complement *lao1* mutant growth, this result suggests that, in this case, the carbon source provided by the alga is not only LAO1-produced. Thus, additional carbon compounds provided by *Chlamydomonas*, other than the α-keto acids generated by LAO1, must be involved in this mutualism (Figure 3.7). Given that these growth tests were carried out under autotrophic conditions, *Chlamydomonas* may release photosynthates, such as glycerol and glycolic acid, into the media (León and Galván, 1994; Wilson and Tolbert, 1991)[a,b]. In fact, we observed that growth promotion by *Methylobacterium* was light-dependent (Figure 3.8) even under mixotrophic conditions where an organic carbon was available for the alga (TAP media). Otherwise, ethanol, acetate, pyruvate, lactate and formate excretion has also been reported in *Chlamydomonas* (Bradley et al., 2017; Salem et al., 1973)[c,d]. However, these molecules are reported to be released under anaerobic conditions. Also, different sugars and amino acids have been reported to be exuded to the media (Vogel et al., 1978)[e], which could potentially feed bacteria.

[a] León, R., and Galván, F. (1994). Halotolerance studies *on Chlamydomonas reinhardtii*: glycerol excretion by free and immobilized cells. *J. Appl. Phycol.* 6, 13–20. doi:10.1007/BF02185898

[b] Wilson, B. J., and Tolbert, N. E. (1991). The transport of glycolic acid by *Chlamydomonas* reinhardtii. *FEBS Lett.* 279, 313–315. doi:10.1016/0014-5793(91)80176-

[c] Bradley, A. S., Swanson, P. K., Muller, E. E. L., Bringel, F., Caroll, S. M., Pearson, A., et al. (2017). Hopanoid-free *Methylobacterium extorquens* DM4 overproduces carotenoids and has widespread growth impairment. *PLoS One* 12, e0173323. doi:10.1371/journal.pone.0173323.

[d] Salem, A. R., Wagner, C., Hacking, A. J., and Quayle, J. R. (1973). The Metabolism of Lactate and Pyruvate by *Pseudomonas* AM1. *J. Gen. Microbiol.* 76, 375–388. doi:10.1099/00221287-76-2-375

[e] Vogel, S. L., Frisch, H. L., and Gotham, I. J. (1978). Qualitative assay of dissolved amino acids and sugars excreted by *Chlamydomonas reinhardtii* (Chlorophyceae) and *Euglena Gracilis* (Euglenophyceae). *J. Phycol.* 14, 403–406. doi:10.1111/j.1529-8817.1978.tb02459.x

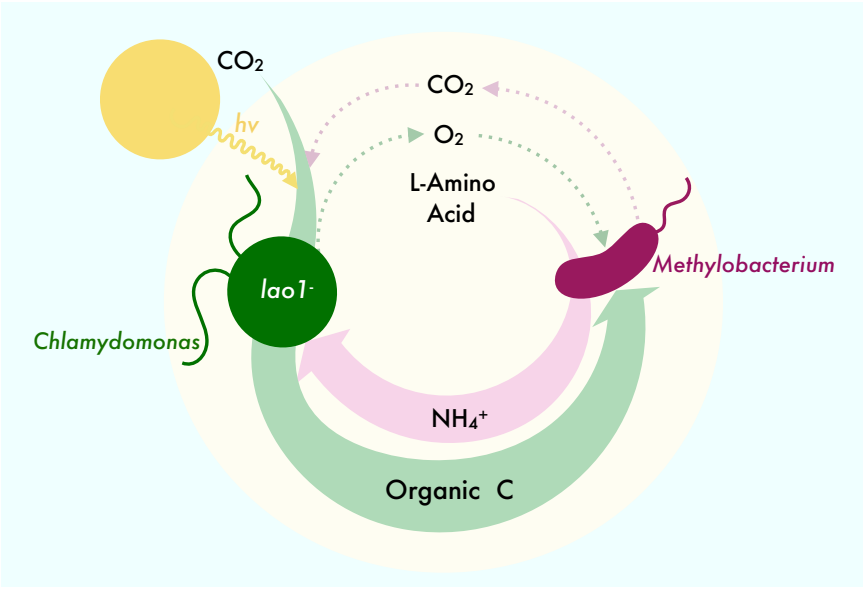


Figure 3.7. Tentative model of LAO1-independent metabolic complementation on L-amino acids. Some *Methylobacterium* spp. can complement growth of LAO1-lacking *Chlamydomonas*. Since these bacteria do not grow efficiently on these amino acids but release ammonium to the media, an additional carbon input may be required to support bacterial growth. In this case, since LAO1 activity is absent, additional organic carbon source(s) must be released by the alga that allows bacterial growth. *Chlamydomonas* has been reported to release different organic compounds such as glycerol, glycolic acid, sugars and amino acids, which could potentially facilitate bacterial growth. In this way, the alga feeds on the bacterial-released ammonium and bacteria feed on algal-produced organic carbon compound(s).

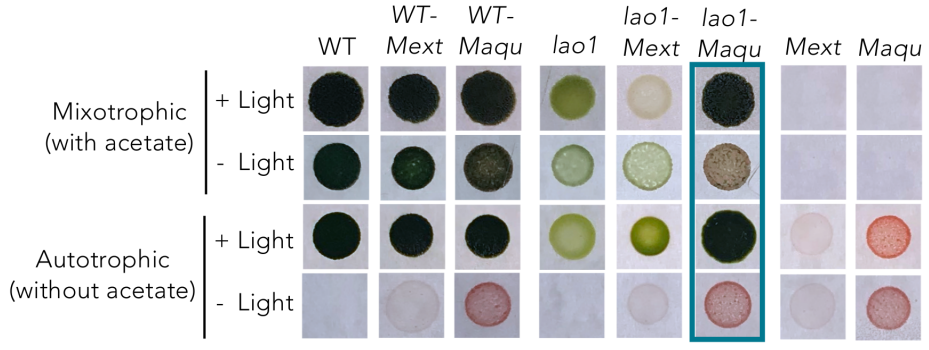


Figure 3.8. Light-dependent mutualism on L-Alanine. *Chlamydomonas* WT- and *lao1-Methylobacterium* spp. growth after 10 days on solid media containing 8 mM of L-alanine as the sole nitrogen source. TAP and minimal media were used for mixotrophic and autotrophic conditions, respectively, under continuous light (+ light) or darkness (- light). *Mext, M. extorquens; Maqu, M. aquaticum.*

3.4 Is IAA involved in the *Chlamydomonas-Methylobacterium* inter-kingdom signaling?

The chemical cross-talk between interacting organisms mediates the symbiotic and pathogenic relationships. Within these inter-kingdom signaling molecules that eukaryotes can produce, the auxin IAA (Indole-3-Acetic Acid) might be one of the best studied. This phytohormone regulates many different processes of plant development (Spaepen and Vanderleyden, 2011)[a]. However, IAA biosynthesis is not only present in plants, but it is proposed to evolve independently also in algae, bacteria and fungi (Fu et al., 2015; Labeeuw et al., 2016; Meza et al., 2015; Romani, 2017; Yue et al., 2014)[b],[c],[d],[e],[f]. This molecule plays also a role in inter-kingdom relationships (Lee et al., 2015), and has been proposed to be key for plant-PGPB mutualistic interactions (Glick, 2014). Although IAA has been detected in some algal species (Kiseleva et al., 2012; Mazur et al., 2001), its metabolism and sensing pathways in algae are still unclear (Lau et al., 2009; Romani, 2017; Wang et al., 2016a). Otherwise, evolutionary studies support that plant response machinery and transport mechanisms for auxin were at least partially present in green algae, though alternative pathways might be present (De Smet et al., 2011; Romani, 2017). Although IAA has not been detected before in *Chlamydomonas*, given the wide distribution of IAA biosynthesis, it seems likely that *Chlamydomonas* may synthesize it as well. Thus, this raises the questions: (1) if *Chlamydomonas* may synthesize IAA, what metabolic pathways may be involved? and (2), can *Chlamydomonas* sense IAA? and if so, is it involved in *Chlamydomonas-Methylobacterium* mutualism like plant-PGPB interactions?

3.4.1 Can *Chlamydomonas* biosynthesize IAA?

In *Chlamydomonas* genome, the plant-like auxin biosynthetic pathway seems to be absent and the presence of alternative mechanisms have been proposed (De Smet et al., 2011)[g]. Otherwise, the analysis of its genome shows that a bacterial-like biosynthetic pathway involving a putative tryptophan 2-monooxygenase and a putative indole-3-acetamide amidase. Moreover, an L-amino acid oxidase gene cluster has been recently reported to be involved in L-tryptophan-dependent IAA biosynthesis in bacteria

[a] Spaepen, S., and Vanderleyden, J. (2011). Auxin and plant-microbe interactions. *Cold Spring Harb. Perspect. Biol.* 3, 1–13. doi:10.1101/cshperspect.a001438.

[b] Fu, S.-F., Wei, J.-Y., Chen, H.-W., Liu, Y.-Y., Lu, H.-Y., and Chou, J.-Y. (2015). Indole-3-acetic acid: A widespread physiological code in interactions of fungi with other organisms. *Plant Signal. Behav.* 10, e1048052. doi:10.1080/15592324.2015.1048052

[c] Labeeuw, L., Khey, J., Bramucci, A. R., Atwal, H., de la Mata, A. P., Harynuk, J., et al. (2016). Indole-3-Acetic Acid Is Produced by Emiliania huxleyi Coccolith-Bearing Cells and Triggers a Physiological Response in Bald Cells. *Front. Microbiol.* 7, 828. doi:10.3389/fmicb.2016.00828

[d] Meza, B., de-Bashan, L. E., and Bashan, Y. (2015). Involvement of indole-3-acetic acid produced by Azospirillum brasilense in accumulating intracellular ammonium in Chlorella vulgaris. *Res. Microbiol.* 166, 72–83. doi:10.1016/j.resmic.2014.12.010

[e] Romani, F. (2017). Origin of TAA Genes in Charophytes: New Insights into the Controversy over the Origin of Auxin Biosynthesis. *Front. Plant Sci.* 8, 1616. doi:10.3389/fpls.2017.01616

[f] Yue, J., Hu, X., and Huang, J. (2014). Origin of plant auxin biosynthesis. *Trends Plant Sci.* 19, 764–770. doi:10.1016/j.tplants.2014.07.004

[g] De Smet, I., Voss, U., Lau, S., Wilson, M., Shao, N., Timme, R. E., et al. (2011). Unraveling the Evolution of Auxin Signaling. *PLANT Physiol.* 155, 209–221. doi:10.1104/pp.110.168161.

(Rodrigues et al., 2016)[a]. Interestingly, this gene cluster included genes coding a L-amino acid oxidase and a RidA protein, which resembles the physically clustered *LAO1-LAO2/RidA* genes in *Chlamydomonas,* reported in the previous chapter of this work. Thus, we wondered if LAO1 might have a role in extracellular production of IAA and therefore, in the inter-kingdom signaling during *Chlamydomonas-Methylobacterium* interactions. According to this hypothesis, LAO1 deaminating activity, which generates indole-pyruvic acid, that then would be decarboxylated to generate IAA, or either by the non-enzymatic decarboxylation in the presence of hydrogen peroxide previously suggested here (Chapter 2), or additionally by an enzymatic activity (Sardar and Kempken, 2018)[b]. As a first approximation, we studied the indoles production by *Chlamydomonas* in the presence of L-tryptophan and the effect of *lao1* mutation. As a result, **we observed that indoles production in *Chlamydomonas,*** observed here for the first time, **was significantly reduced in the *lao1* mutant** (Figure 3.9). However, this experimental determination was not specific for IAA, but other indoles such as indole-pyruvic acid and indole-lactic acid may interfere and different attempts to separate these molecules by HPLC were not successful. Thus, further analysis such as GCxGC-TOF (Labeeuw et al., 2016)[c], will confirm if IAA is produced by *Chlamydomonas*, and thus understand if LAO1 is mediating IAA production as previously reported in a plant-symbiotic bacteria (Rodrigues et al., 2016)[d].

[a] Rodrigues, E. P., Soares, C. de P., Galvao, P. G., Imada, E. L., Simoes-Araújo, J. L., Rouws, L. F. M., et al. (2016). Identification of genes involved in indole-3-acetic acid biosynthesis by *Gluconacetobacter diazotrophicus* PAL5 strain using transposon mutagenesis. *Front. Microbiol.* 7. doi:10.3389/fmicb.2016.01572.

[b] Sardar, P., and Kempken, F. (2018). Characterization of indole-3-pyruvic acid pathway-mediated biosynthesis of auxin in *Neurospora crassa*. *PLoS One* 13, e0192293. doi:10.1371/journal.pone.0192293.

[c] Labeeuw, L., Khey, J., Bramucci, A. R., Atwal, H., De La Mata, A. P., Harynuk, J., et al. (2016). Indole-3-acetic acid is produced by *Emiliania huxleyi* coccolith-bearing cells and triggers a physiological response in bald cells. *Front. Microbiol.* 7, 828. doi:10.3389/fmicb.2016.00828.

[d] Rodrigues, E. P., Soares, C. de P., Galvao, P. G., Imada, E. L., Simoes-Araújo, J. L., Rouws, L. F. M., et al. (2016). Identification of genes involved in indole-3-acetic acid biosynthesis by *Gluconacetobacter diazotrophicus* PAL5 strain using transposon mutagenesis. *Front. Microbiol.* 7. doi:10.3389/fmicb.2016.01572.

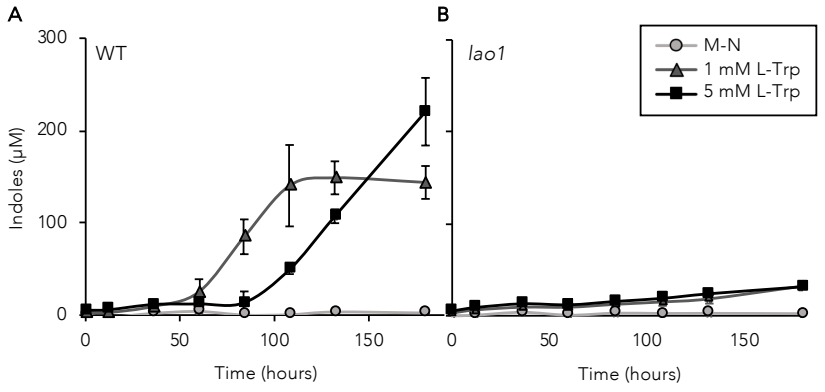


Figure 3.9. Impact of lao1 mutation on indoles production by *Chlamydomonas*. Chlamydomonas WT and lao1 mutant cells were grown on TAP medium supplemented with ammonium for three days and then washed and transferred to minimal medium without any nitrogen source (M-N) for 8 hours under continuous light. After incubation, L-tryptophan was added to obtain 1 mM or 5 mM of final concentration on the media. A control without any addition was included (M-N). Then, cells were incubated under continuous light and agitation for 1 week. Indoles concentration in the supernatant were determined by a colorimetric assay using Salkowski's reagent (see 'Material and Methods' section for further details). Error bars represent standard deviation of three biological replicates.

3.4.2 Is IAA involved in *Chlamydomonas-Methylobacterium* interactions?

IAA can promote plant growth under low concentrations but inhibit it when accumulated (Evans et al., 1994)[a]. During plant-bacteria interactions, a synergistic interaction between ACD (1-aminocyclopropane-1-carboxylate deaminase) enzyme and both plant and bacterial-produced IAA, has been proposed to be key for optimal function of PGPB (Glick, 2014)[b]. These bacteria can produce IAA from the L-tryptophan exuded by the roots. This auxin, together with the endogenous IAA, induces in the plant the expression of the ACC synthase that catalyzes the formation of ACC (1-aminocyclopropane-1-carboxylate), which is the precursor of ethylene, a growth inhibitory hormone. ACD-producing bacteria act as a sink of ACC, lowering the IAA-induced ACC accumulation, thus promoting plant growth.

Although ACC synthesis genetic pathway is well conserved in red and green algae (Ju et al., 2015)[c] and ethylene production has been previously reported in *Chlamydomonas* (Yordanova et al., 2010)[d], ACC synthesis in this alga has not been experimentally reported yet and further studies are needed to support this.

[a] Evans, M., Ishikawa, H., and Estelle, M. (1994). Responses of *Arabidopsis* roots to auxin studied with high temporal resolution: Comparison of wild type and auxin-response mutants. *Planta* 194, 215–222. doi:10.1007/BF00196390.

[b] Glick, B. R. (2014). Bacteria with ACC deaminase can promote plant growth and help to feed the world. *Microbiol. Res.* 169, 30–39. doi:10.1016/j.micres.2013.09.009

[c] Ju, C., de Poel, B., Cooper, E. D., Thierer, J. H., Gibbons, T. R., Delwiche, C. F., et al. (2015). Conservation of ethylene as a plant hormone over 450 million years of evolution. *Nat. Plants* 1, 1–7

[d] Yordanova, Z. P., Iakimova, E. T., Cristescu, S. M., Harren, F. J. M., Kapchina Toteva, V. M., and Woltering, E. J. (2010). Involvement of ethylene and nitric oxide in cell death in mastoparan-treated unicellular alga *Chlamydomonas reinhardtii*. *Cell Biol. Int.* 34, 301–308. doi:10.1042/CBI20090138

Here, we tested the effect of IAA on *Chlamydomonas* growth and how different species of the PGPB genus *Methylobacterium* affected this growth. As a result, **we observed growth inhibition of *Chlamydomonas* under high concentrations of IAA** (higher than 30 μM, Figure 3.10) under axenic conditions. IAA has been reported to promote growth at low concentrations in *Chlamydomonas* (Park et al., 2013)[a], but its inhibitory effect has never been reported yet in this alga. Otherwise, our results show that **during co-culture with ACD-producing *Methylobacterium* spp. we observed that this IAA-induced inhibition was relieved**. However, this effect was weaker in co-culture with the ACD-lacking bacterium *M. extorquens*. These results suggest that a similar signaling pathway might be regulating *Chlamydomonas-Methylobacterium* interactions (Figure 3.10). Nevertheless, if LAO1 is involved in this IAA-mediated interaction, a new mechanism in algae alternative to land plants may arise.

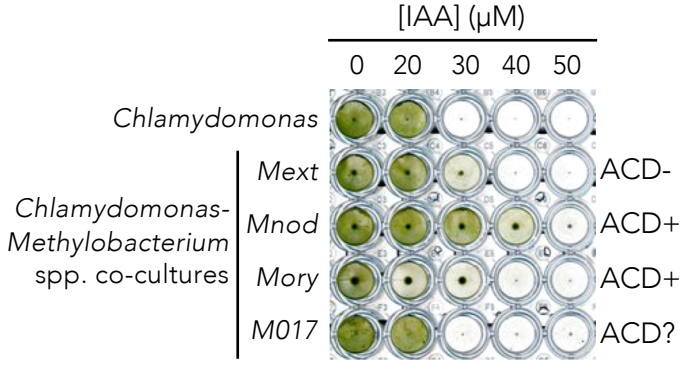


Figure 3.10. *Chlamydomonas* IAA-induced growth inhibition is released by some *Methylobacterium* spp. Liquid cultures in minimal media containing 8 mM of nitrate and increasing concentrations of IAA (indole-3-acetic acid). *Mext, M. extorquens*; *Mnod, M. nodulans; Mory, M. oryzae*; *M017, Methylobacterium* sp. M017. ACD-, ACC deaminase-lacking species. ACD+, ACC deaminase-producing species. ACD?, genome information not available.

Although further experiments are required to confirm this hypothesis, these preliminary results suggest that *Chlamydomonas-Methylobacterium* consortium may be a suitable model system for algae/plant-bacteria interactions to investigate possible co-evolutionary aspects of these interactions and better understand the inter-kingdom signaling pathways that modulate them.

[a] Park, W.-K., Yoo, G., Moon, M., Kim, C. W., Choi, Y.-E., and Yang, J.-W. (2013). Phytohormone Supplementation Significantly Increases Growth of *Chlamydomonas reinhardtii* Cultivated for Biodiesel Production. *Appl. Biochem. Biotechnol.* 171, 1128–1142

GENERAL DISCUSSION

As primary producers, algae are key for aquatic life. However, some algal proliferation can lead to harmful algal blooms that may disrupt the ecosystems, representing a significant socio-economical impact. Nitrogen (N) is a key nutrient essential for all forms of life. Although inorganic N is often the most abundant form of N in marine and freshwater systems, terrestrial leaking and runoff are important inputs of organic N -mainly urea and free amino acids- that can regularly change spatially or temporarily reaching high levels while inorganic N concentrations became limited. Although bacteria have long been considered to be major consumers of this organic N, several studies have shown than many phytoplankton species are also able to use organic N sources, what is especially evident during algal blooms (Berman and Bronk, 2003; Fan and Glibert, 2005; Gobler et al., 2011; Ramûnas et al., 2002; Seitzinger and Sanders, 1999). Thus, the degree to which primary producers can use organic N may be decisive to the species proliferation over other competitors, which should be factored into models of ecosystem balance.

The green microalga *Chlamydomonas reinhardtii* (*Chlamydomonas*) has served as a model organism for physiological studies for more than fifty years (Harris, 2001) and the N metabolism in this alga has been one of the most extensively studied of any alga. *Chlamydomonas* can use both inorganic and some organic N forms as N sources. Ammonium, is the preferred N source for this alga and inhibits the utilization of other N forms, including nitrate and organic N (Sanz-Luque et al., 2015a). Nitrate is not only a nutrient but also a signal molecule that regulates many processes (Crawford, 1995; Krapp et al., 2014). Nitrate and ammonium are usually positive and negative signals, respectively, for nitrate assimilation genes. A master regulatory gene for nitrate assimilation, *NIT2*, is necessary for fully upregulation of the main genes involved in nitrate assimilation in *Chlamydomonas* (Camargo et al., 2007; Mariscal et al., 2006; Quesada et al., 1993, 1994).

To better understand the nitrate signaling pathways involved in regulating different metabolic processes as well as the role of the key regulator of nitrate assimilation gene, *NIT2*, we studied the transcriptome of *Chlamydomonas* wild type (WT) and a *nit2* mutant in response to nitrate. We observed that nitrate and NIT2 up-regulated key genes for nitrate assimilation, molybdenum-cofactor biosynthesis and nitric oxide metabolism, as previously reported (Fernandez and Galvan, 2008; Sanz-Luque et al., 2015b). Moreover, **nitrate and NIT2 down-regulated genes related to organic N scavenging -e.g. the extracellular L-amino acid oxidase (*LAO1*) and the xanthine/uracil/vitamin C permease-like genes (*UAPA1* and *UAPA6*)**. This observation suggested that NIT2 might be involved in the N nutrition preference control in *Chlamydomonas*, activating nitrate assimilation and inactivating the scavenging of organic N sources. In fact, we have shown that, in a nitrate reductase mutant, nitrate persistently prevented *Chlamydomonas* growth on amino acids, and that *NIT2* mutation enhanced this growth.

Also, nitrite inhibited the use of amino acids in nitrite reductase mutants. However, this signal was not persistent, and this inhibition was only transient due to nitrite conversion. **The nature of this regulation is still unknown, but could be driven by the $NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow NO_3^-$ cycle, played by NR, NOFNiR and THB1 enzymes** (Calatrava et al., 2017; Sanz-Luque et al., 2015b, 2015a). Besides inhibition relieve by nitrate/nitrite decrease, NO production may induce *LAO1* and growth on amino acids (Wei et al., 2014). Also, NO may be further oxidized

to nitrate by the NR/THB1 complex (Sanz-Luque et al., 2015b) or reduced to nitrous oxide by the CYP55 (Plouviez et al., 2017), and both gaseous molecules that can be dissipated. However, nitrite reduction by an alternative reductase cannot be discarded. These results suggested that **NIT2 is involved in the control of N assimilation pathways leading to a preference for inorganic N (i.e. ammonium, nitrate and nitrite) over amino acids in *Chlamydomonas* (Figure 4.1). The preference of inorganic N over organic N in algae can determine the dynamics of ecological niches where both inorganic and organic N sources are present.**

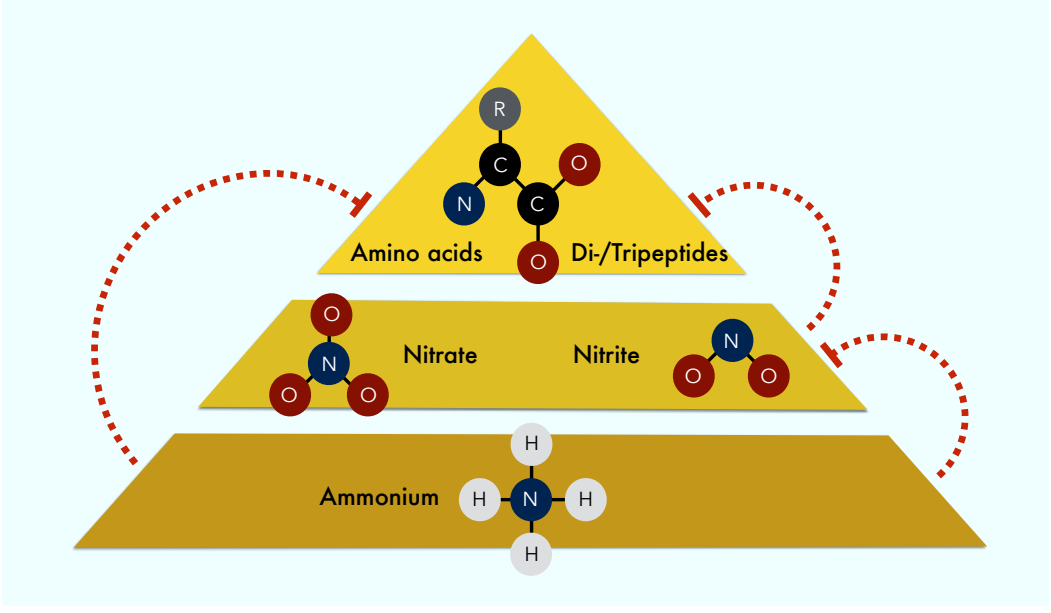


Figure 4.1. *Chlamydomonas* Nitrogen Pyramid. The preferential use of nitrogen sources in *Chlamydomonas* is driven by a fine-tuned regulation that prevents the use of nitrate when ammonium is present, and the use of amino acids when ammonium, nitrate or nitrite are available.

Although the use of amino acids as N source for growth by different algal species has long been demonstrated, how algae use amino acids as a N source is still poorly understood. Amino acids can be utilized as N sources by two different pathways: direct uptake and subsequent intracellular catabolism; or extracellular deamination and further uptake of the generated ammonium. In *Chlamydomonas*, only L-arginine is efficiently taken up by a specific transport system (Kirk and Kirk, 1978b). Alternatively, N can be scavenged by broad substrate specific extracellular L-amino acid deaminases (LAAO). *Chlamydomonas* bears a broad-substrate specific periplasmic LAAO encoded by the gene *LAO1* (*LAO1*). Intriguingly, we could not find any LAO1 orthologs in other available genomes of any green alga or land plant, but we identified LAO1 orthologs in 10 out of 27 algal species including Rhodophyta, Alveolata, Heterokonta, Haptophyta and Dinophyta algae. The construction of **a LAAO phylogenetic tree revealed that algal protein sequences identified as LAO1 orthologs clustered on the same evolutionary branch that we named as ALAAOs (Algal LAAOs)**. This ALAAO branch was distinct in lineage from fungal, gastropods and vertebrates LAAOs. Although our data support LAO1 and LAO3 being grouped together within ALAAOs, their origin was puzzling since we could

find no other orthologs within the green algal lineage. We observed that the gene clustered to *LAO1*, *LAO2/RidA*, was closely related to cyanobacterial members. Given the current available genomes and genome assemblies, our phylogenetic analysis favored the idea that ALAAOs may have a common origin in the archaeplastidan ancestor.

By the use of a *lao1* mutant, we showed that lao1 was crucial for *Chlamydomonas* growth on 16 out of 20 proteinogenic amino acids. Otherwise, *Chlamydomonas* did not grow on L-proline, according to the absent substrate activity of lao1 to this imino acid (Vallon et al., 1993). Additionally, some di-/tripeptides supported *Chlamydomonas* growth. However, the *lao1* mutant was unable to grow on any of these peptides, suggesting that LAO1 may have a role in peptides assimilation as well, either deaminating the peptide directly or via deamination of free amino acids generated through the activity of some extracellular peptidase. Thus, **LAO1 seems to have a key role for the use of most of the proteinogenic amino acids and di-peptides as sole N source by this alga**.

Besides ammonium, LAO1 produces keto acids and H_2O_2. The α-keto acids are paradoxically not used as a carbon source by *Chlamydomonas*. However, since α-keto acids are reported to be H_2O_2 scavengers, we hypothesized that these compounds may then have a role as H_2O_2 scavengers preventing the extracellular oxidative stress generated by LAO1 activity. We observed that subsequent to deamination of L-alanine by *Chlamydomonas* WT, the α-keto acid pyruvic acid and the carboxylic acid acetic acid were accumulated in the media, which suggests **that LAO1-produced H_2O_2 is decarboxylating the α-keto acid and generating the corresponding carboxylic acid**. Given that acetic acid is used by *Chlamydomonas,* this alga may use L-alanine not only as a N source, but also as a carbon source by means of the extracellular LAAO activity. Moreover, if this spontaneous chemical reaction is also occurring after the LAO1-mediated deamination of other L-amino acids, further implications may rise up. For instance, LAO1 deamination of L-tryptophan may lead to the generation of the carboxylic acid indole-acetic acid, a phytohormone that promotes algal growth (Park et al., 2013). Also, LAO2/RidA activity may have a role, not only avoiding reactive imine accumulation, but also for the rapid conversion to keto acids to immediately cope with the H_2O_2 generated by LAO1, pre-empting oxidative damage as well.

From an ecological perspective, LAAO presence in algae may represent an advantage over other competitors to use alternative N sources when inorganic N is limiting. To that end, the extracellular localization of ALAAOs could be strategic for its role in N scavenging from exogenous amino acids and peptides, as proposed for LAO1 in *Chlamydomonas*. Although experimental confirmation is required, our data showed that out of 10 algal species containing LAAO, five of them were predicted to contain putative extracellular LAAOs. In addition, *Porphyra umbilicalis* was predicted to have three extracellular LAAOs. Thus, our data suggested that, like in *Chlamydomonas*, extracellular ALAAOs may have an impact on algal species growth on amino acids and peptides as N sources. Although further research is necessary, a broad search concerning algal use of amino acids allowed us to propose different N scavenging patterns within the high diversity present in algae concerning algal mechanisms to scavenge N from amino acids (Figure 4.2). We have demonstrated that *Chlamydomonas* can grow on most proteinogenic amino acids by means of its extracellular LAAO enzyme, and also, that growth on arginine was efficient in the absence of this enzyme by means of a putative

specific transporter. The harmful algal bloom-forming *Aureococcus anophagefferens* possesses similar abilities as *Chlamydomonas* to grow on amino acids, and show amino acids uptake as well as extracellular deaminase activities (Mulholland et al., 2002). According to the last mentioned activity, we found a putative extracellular ALAAO in its genome. The pennate diatom *Phaeodactylum tricornutum* grows on a wide range of amino acids, shows amino acid uptake activity for L-arginine and L-lysine, and extracellular deamination activity for several amino acids (Hayward, 1965; Rees and Allison, 2006). However, we could not find any LAO1 ortholog in the current assembly of *P. tricornutum* genome. Whether the reported extracellular deaminase activity is due to another amine oxidase enzyme should be resolved.

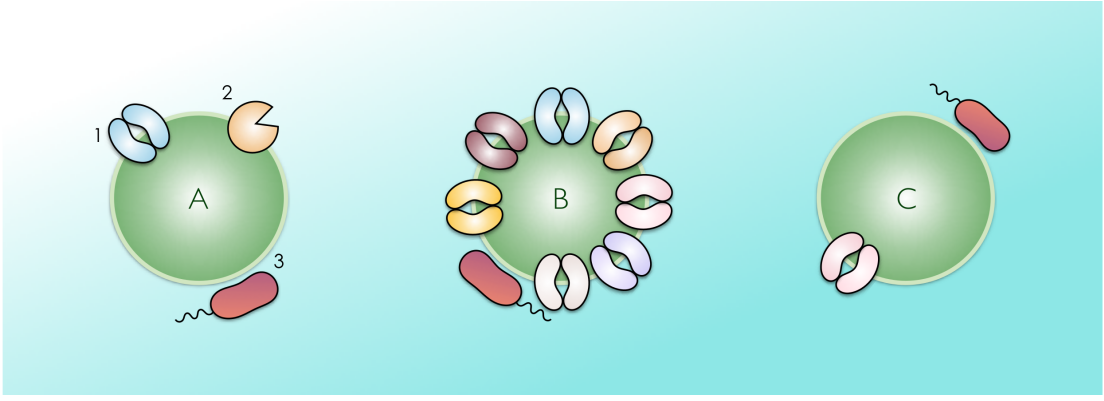


Figure 4.2. Algal Biodiversity in Nitrogen Scavenging from Amino Acids. A, *Chlamydomonas*-like model, containing extracellular deaminase activity by an ALAAO (Algal L-Amino Acid Oxidase) and a specific uptake system. B, *Chlorella*-like model, lacking ALAAO but containing several amino acid uptake systems. C, *Dunaliella*-like model, lacking ALAAO and containing a single amino acid transporter. Presumably, in all three systems, metabolic complementation by nitrogen mineralizing bacteria may support algal growth.

Otherwise, like *Chlamydomonas,* some green algae including *Dunaliella* and *Volvox* species, present only one specific transporter for amino acids uptake. In contrast to *Chlamydomonas*, these algae lack extracellular amino acid deaminase acitivity resulting in an inefficient ability to use amino acids as N sources. In contrast, the extracellular LAAO-lacking green algae *Chlorella* has developed a vast number of different specific transporters that allow this alga to efficiently grow on amino acids (Algéus, 1949; Cho and Komor, 1985; Kirk and Kirk, 1978a; Zhang et al., 2015). Interstingly, amino acids supplied to *Chlorella* by symbiotic hosts have been reported (Kato and Imamura, 2008; McAuley, 1991), which may explain this high dregee of especialization. Finally, the centric diatom *Thalassiosira pseudonana* is unable to grow on amino acids as sole N source (Ietswaart et al., 1994; Palenik and Morel, 1990b) and lacks ALAAO genes. Nevertheless, the defective growth on amino acids or peptides by some algae may be complemented by interactions with bacteria than can mineralize this N (Ietswaart et al., 1994).

Although *Chlamydomonas* can grow on most amino acids and some di-/tri-peptides as the sole N sources, this growth is far less efficient than that on inorganic N, and yet, there are some amino acids and peptides that cannot be used by this alga. We serendipitously found a spontaneous contaminating *Methylobacterium* spp. that allowed *Chlamydomonas* growth on

a dipeptide that is not readily assimilated by this alga. *Methylobacterium* spp. are included in the PGPB group of bacteria (Plant Growth-Promoting Bacteria), which improve plant growth and fitness. Thus, prompted by the ecological importance of this finding, we isolated different bacteria from natural environments and found that, out of 12 species that we isolated and identified, 10 enhanced *Chlamydomonas* growth on L-serine as the sole N source. This result suggested that **algal growth promotion on amino acids is a relatively widespread ability of different bacteria that share natural niches with algae**. Among the *Chlamydomonas* growth-promoting bacteria we found different species of *Sphingomonas*, *Methylobacterium*, *Chitinophagacea* and *Deinococcus* genera. Interestingly, bacterial species from *Methylobacterium* and *Sphingomonadaceae* families have been recently found as well in the phycospere biofilm of the green microalga *Chlorella saccharophila* (Krohn-Molt et al., 2017). This co-occurrence supports the idea that algal interactions with particular bacterial taxa have been selected throughout the evolution (Culham et al., 1993; Krohn-Molt et al., 2017; Ramanan et al., 2015). We observed that some *Methylobacterium* spp. complemented *Chlamydomonas* growth on amino acids and peptides that were not readily available for this alga.

We have discovered a species-specific mutualistic interaction between *Chlamydomonas* and *M. aquaticum* on L-proline (Calatrava et al., 2018). This amino acid is not assimilated by *Chlamydomonas* and did not support efficient bacterial growth leading to ammonium release, suggesting a C/N imbalance due to low carbon skeletons availability that prevented bacterial N assimilation. However, the co-culture facilitated the growth of both microbes. **This algal-bacterial interaction resulted in a metabolic complementation based on a carbon-N exchange: bacterial mineralization of the amino acid fed the alga with ammonium, and the algal photosynthetic CO_2 fixation yielded glycerol that can be used by *M. aquaticum***. Moreover, algal growth on amino acids that can be used by *Chlamydomonas* LAO1 activity was enhanced by the presence of some *Methylobacterium* spp. The use of a *Chlamydomonas lao1* mutant allowed us to define **a new role for LAO1 as a key enzyme for mutualistic growth with some *Methylobacterium* spp. on specific amino acids.** Thus, the organic carbon released by LAO1 activity may be used by bacteria to compensate the C/N imbalance to properly assimilate N from amino acids. This hypothesis was further confirmed for *Chlamydomonas-M. extorquens* co-culture on L-alanine (J Ureña 2018) and might be applied to any LAO1 substrate, as long as the specific bacterial species are able to use the α-keto acid. In turn, *Chlamydomonas* may get benefited by the bacterial-produced ammonium. In the model proposed for LAO1-dependent mutualistic interaction (Figure 4.3), the different metabolites produced by both microbes may cross-regulate each other's activity, what may be essential for the success of the consortia growth. On one hand, ammonium can be generated from amino acids by both algae -by LAO1 activity- and bacteria, but bacteria are inefficient to use it for growth unless LAO1-produced α-keto acids are available. α-Keto acids facilitate bacterial growth and prevent ammonium release (J Ureña 2018). Moreover, LAO1 activity generates H_2O_2 that could inhibit algal photosynthesis and growth (Baldry, 1983; Foyer, 2018; Pokora et al., 2018). Since by releasing ammonium, bacteria may low down LAO1 activity (Vallon et al., 1993) -and therefore H_2O_2 levels-, they may prevent oxidative stress and release *Chlamydomonas* from H_2O_2-driven photosynthesis inhibition.

Interestingly, all *Methylobacterium* spp. are reported to show catalase activity (Patt et al., 1976), which might be a specific trait that favors the interaction with *Chlamydomonas*. However, some *Methylobacterium* spp. did not complement algal growth with the *lao1* mutant. In this case, since LAO1 activity was not essential for the mutualistic growth, **additional algal-produced organic carbon compounds, other than those LAO1-produced, may promote bacterial growth** (Figure 4.4). Given that *Chlamydomonas* was under autotrophic conditions, photosynthates, such as glycerol or glycolic acid, may be released to the media (León and Galván, 1994; Wilson and Tolbert, 1991), and may in principle feed bacteria. Also, amino acids and sugars are also exuded by *Chlamydomonas* and could potentially be used by bacteria.

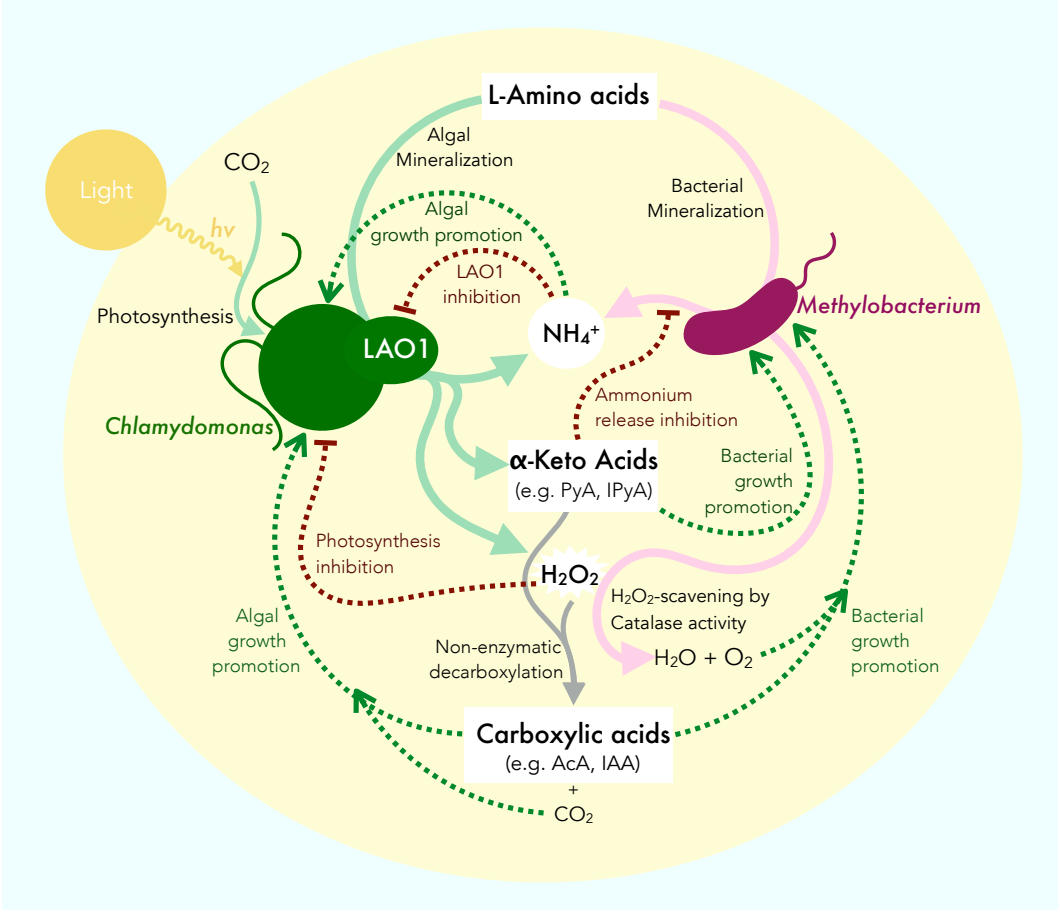


Figure 4.3. Tentative model for *Chlamydomonas* LAO1 role in algal-bacterial signaling. During *Chlamydomonas-Methylobacterium* mutualistic growth on amino acids, the different metabolites produced by both microbes may cross-regulate each other's activity, what may be essential for the success of the consortium.

The chemical cross-talk between interacting organisms mediates the symbiotic and pathogenic relationships. Within these inter-kingdom signaling molecules that eukaryotes can produce, the auxin IAA (Indole-3-Acetic Acid) is one of the best studied. Its biosynthesis is widespread in nature and regulates many different processes of plant development (Spaepen

and Vanderleyden, 2011). Although the plant-like auxin biosynthetic pathway seems to be absent in *Chlamydomonas* genome, a bacterial-like biosynthetic pathway can be found in its genome. Moreover, an L-amino acid oxidase gene cluster has been recently reported to be involved in L-tryptophan-dependent IAA biosynthesis in bacteria (Rodrigues et al., 2016). Interestingly, this gene cluster included genes coding an L-amino acid oxidase and a RidA protein, which resembles the physically clustered *LAO1-LAO2/RidA* genes in *Chlamydomonas*. We found that indoles production in *Chlamydomonas*, observed here for the first time, was significantly reduced in the *lao1* mutant. However, this experimental determination was not specific for IAA, and further experiments are needed to confirm if IAA is produced by *Chlamydomonas*, and thus to understand if LAO1 is involved in this production. Although IAA can promote plant growth under low concentrations, it inhibits it when accumulated (Evans et al., 1994). During plant-bacteria interactions, a synergistic interaction between ACC (1-aminocyclopropane-1-carboxylate) deaminase enzyme and both plant and bacterial-produced IAA, has been proposed to be key for optimal function of PGPB (Glick, 2014). We observed *Chlamydomonas* growth inhibition by IAA under axenic conditions. Otherwise, during co-culture with ACD-producing *Methylobacterium* spp. we observed that this IAA-induced inhibition was released. However, this effect was weaker in co-culture with the ACD-lacking bacterium *M. extorquens*, which suggested that a similar signaling pathway might be regulating *Chlamydomonas-Methylobacterium* interactions. However, further investigations are necessary to confirm this hypothesis. Nevertheless, if LAO1 involved in this IAA-mediated interaction, a new mechanism in algae alternative to land plants may arise. Although further experiments are required to confirm this hypothesis, these preliminary results suggest that *Chlamydomonas-Methylobacterium* consortium may be a suitable model system for algae/plant-bacteria interactions to investigate possible co-evolutionary aspects of these interactions and better understanding the inter-kingdom signaling pathways that modulate them.

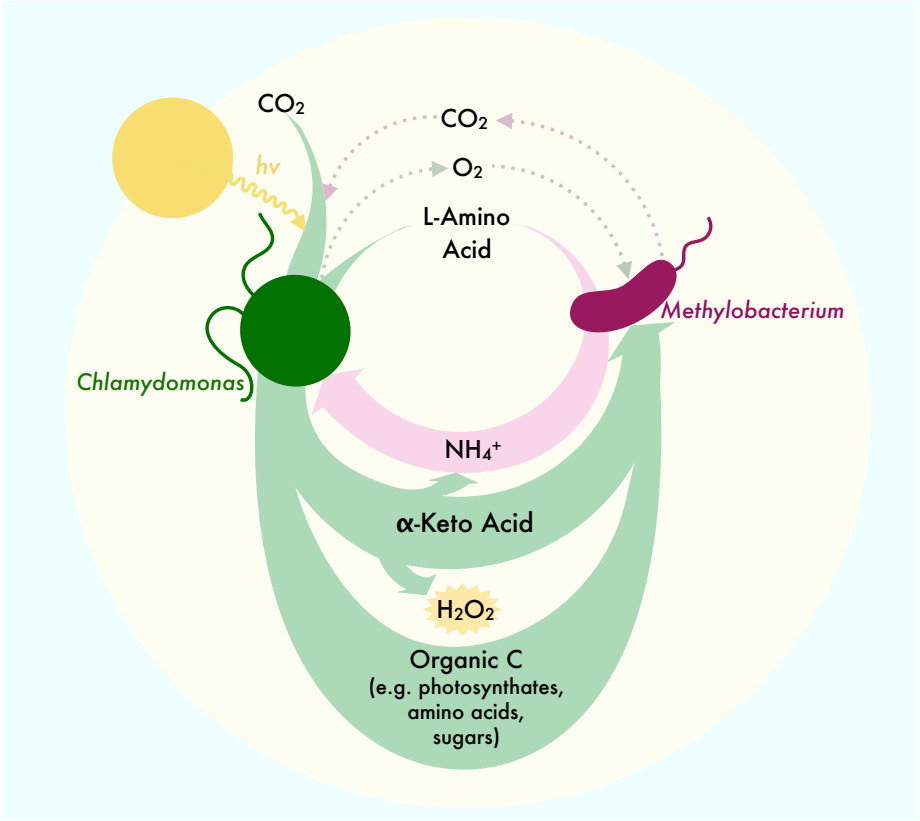


Figure 4.4. Metabolic complementation during *Chlamydomonas-Methylobacterium* mutualistic growth on L-amino acids. *Chlamydomonas* LAO1 deaminates L-amino acids generating ammonium, which is assimilated, and the corresponding keto acid, that is not used by this alga. Otherwise, some *Methylobacterium* spp., however, can catabolize amino acids, but these compounds do not support efficient bacterial growth and lead to ammonium release to the media. Then, bacterial-produced ammonium is efficiently taken up by *Chlamydomonas* facilitating algal growth. Otherwise, bacteria can feed on the algal-produced keto acids. Moreover, LAO1 activity generates hydrogen peroxide, which inhibits microbial growth and algal photosynthesis. Additionally, the CO_2 released by bacteria as a waste product may enhance algal photosynthesis. Otherwise, this algal photosynthetic activity releases O_2 as a waste product, which may facilitate the growth of the strictly aerobic *Methylobacterium* specie

CONCLUSIONS

1. Nitrate, by means of NIT2, down-regulates genes involved in organic nitrogen (N) scavenging in *Chlamydomonas*, including *LAO1*, *UAPA1* and *UAPA6* genes.
2. NIA1 mutation and nitrate supplementation avoid *Chlamydomonas* growth on amino acids as sole N source, and NIT2 mutations enhance this growth.
3. Nitrite prevents the use of amino acids in a *nii1* mutant.
4. No LAO1 orthologs can be found in any Viridiplantae member. Otherwise, LAO1 orthologs are present in other algal members within the red lineage, named here as ALAAOs (Algal LAAOs).
5. *Chlamydomonas LAO1* gene is clustered to a putative *RidA* gene (*LAO2*) gene cluster, what may be a result of an ancient gene transfer event from the chloroplast in the archaeplastidan ancestor.
6. *Chlamydomonas* can grow on all proteinogenic amino acids as the sole N source, except for L-Proline. It can grow as well on the peptides L-leucyl-alanine, L-phenylalanyl-alanine and L-leucyl-glycyl-glycine but do not on glycyl-glycyl-glycine.
7. *Chlamydomonas* efficient growth on amino acids and peptides relies on a functional LAO1, except for L-arginine.
8. The spontaneous reaction of LAO1-derived products generated by L-alanine deamination, pyruvic acid and hydrogen peroxide, generates acetic acid.
9. Co-cultures with different wild bacteria, including *Methylobacterium*, *Sphingomonas*, *Deinococcus*, and *Chitinophagaceae* spp., promoted *Chlamydomonas* growth on L-serine.
10. Some *Methylobacterium* spp. allow *Chlamydomonas* growth on amino acids and peptides that are not used by this alga. A mutualistic carbon-nitrogen metabolic exchange between *Chlamydomonas* and *M. aquaticum* improves the growth of both cell types on L-proline.
11. LAO1 is crucial for consortia growth on amino acids with some *Methylobacterium*, including *M. extorquens*, *M. hispanicum* and *M. organophilum*. Otherwise, this enzyme is not essential for algal growth complementation by other Methylobacterium spp., including *M. aquaticum, M. oryzae* and *M. aerolatum*.
12. In the presence of exogenously added L-tryptophan, *Chlamydomonas* can synthesize indoles. This biosynthesis is LAO1-dependent.
13. High levels of exogenously added indole-3-acetic acid (> 30 μM) inhibit *Chlamydomonas* growth. This inhibition is relieved by the presence of *Methylobacterium spp.*

MATERIAL AND METHODS

M.1 Biological Material.

M.1.1 *Chlamydomonas* strains

Chlamydomonas cells were maintained on TA 1.6% agar (w/v) slants (M.2.1.2.1) and refreshed every six months.

Table M.1. *Chlamydomonas* strains used in this work.

Strain	Genotype for nitrogen assimilation	Phenotype for nitrogen assimilation	Ref.
704	mt+ *cw15 ARG7- pNIA1:ARS+* WT	WT	1
89.87	mt+ *cw15 ARG7+ pNIA1:ARS+ nit2-*	Nit-	2
p68.6, p68.8, p68.11	mt+ *cw15 ARG7+ pNIA1:ARS+ nit2-pMN68*	Nit+	3
6145c	mt+ WT	WT	4
203	mt- *nit2*	Nit-	4
305	mt- *nia1*	Nit-	4
CC-125 (137c^{+})	mt+ *nia1 nit2*	Nit-	5
CC-1690 (21gr)	mt+ WT	WT	5
CC-5325	mt- *cw15 nia1 nit2*	Nit-Nii-	6
2J1-6	CC-1690 x CC-5325 segregants	Nit+Nii+	7
2J1-8		Nit+Nii+	
2J1-45		Nit-Nii+	
2J1-2		Nit-Nii+	
2J1-14		Nit-Nii-	
2J1-35		Nit-Nii-	
M3	*Δ(NII1 NRT2;2 NRT2;1 NAR2 NIT1)::NIT1 :: (NRT2;2 NRT2;1)::NAR2*	Nit-Nii-	8
M4	*Δ(NII1 NRT2;2 NRT2;1 NAR2 NIT1)::NIT1*	Nit-Nii-	8
LMJ.RY0402.044073	*lao1*	Nit- (Par^{R})	6

Nit, growth on nitrate; Nii, growth on nitrite; ParR, resistant to paromomycin (25 µg·mL-1). 1, (Loppes et al., 1999); 2, (Camargo et al., 2007); 3, (Álvarez-Gómez F, 2012).; 4, (Fernández and Matagne, 1986); 5, (Pröschold et al., 2005); 6, (Li et al., 2016); 7, this work; 8, (Schroda, 2006).

M.1.2 Methylobacterium strains.

Methylobacterium spp. strains were maintained in 40% glycerol (v/v) at -80°C in 0.5 mL-aliquots. Each aliquot was used for each experiment to avoid freezing-thawing cycles and potential contaminations.

Table M.2. *Methylobacterium* spp. strains used in this work.

Methylobacterium spp.	Strain	ID	Source	Ref.
M. oryzae	CBMB20	18207	DSMZ	1
Methylobacterium sp.	88A	-	*(1)	-
M. organophilum	XX	ATCC 27886	BBCM/LMG	2
Methylobacterium sp.	M017	-	This work	3
M. marchantiae	JT1	21328	DSMZ	4
M. hispanicum	GP34	16372	CECT	5
M. nodulans	-	21967	BBCM/LMG	6
M. aquaticum	GR16T	16371	DSMZ	5
M. aerolatum	5413S-11	19013	DSMZ	7
M. extorquens	AM1	-	*(2)	8

*(1), Kindly provided by Prof. Ludmila Chistoserdova. *(2), Kindly provided by Prof. Cecilia Martinez-Gomez.1, (Madhaiyan et al., 2007); 2, (Patt et al., 1976); 3, (Calatrava et al., 2018); 4, (Schauer et al., 2011); 5, (Gallego et al., 2005); 6, (Jourand et al., 2004); 7, (Weon et al., 2008), 8, (Nunn and Lidstrom, 1986). DSMZ, German Collection of Microorganisms and Cell Cultures-Leibniz Institute. BBC/LMG, Belgian Co-ordinated Collections of Microorganisms. CECT, *Colección Española de Cultivos Tipo*.

M.2 Microbiology Techniques

M.2.1 Culture media and conditions

M.2.1.1 Stock solutions for culture media

Different culture media were prepared according to Harris (2009) using the following stock solutions containing, per liter:

- **Tris Solution:** 242 g Tris.
- **A Solution**: 5 g $CaCl_2{\cdot}2H_2O$, 10 g $MgSO_4{\cdot}7H_2O$, 40 g NH_4Cl.
- **A-N Solution**: Similar to A solution but without NH_4Cl.
- **B Solution**: 115 g K_2HPO_4, 46 g KH_2PO_4.
- **Trace Elements Solution**: 50 g EDTA (free acid), 11.4 g H_3BO_3, 22 g $ZnSO_4{\cdot}7H_2O$, 5.1 g $MnCl_2{\cdot}4H_2O$, 5 g $FeSO_4{\cdot}7H_2O$; 1.6 g $CoCl_2{\cdot}6H_2O$, 1.6 g $CuSO_4{\cdot}5H_2O$, 0.214 g $MoO_4Na_2{\cdot}2H_2O$. First, EDTA was dissolved in 250 mL distilled water (dH_2O) with 21 g of KOH. Separately, salts were added in the mentioned order and dissolved in 200 mL dH_2O. Once dissolved, both solutions were mixed and heated up to 80-90°C and the pH adjusted with KOH to 6.8. Then dH_2O was added up to 500 mL, transfer to an amber glass bottle and let stand for at least two days under darkness before use.

M.2.1.2. *Chlamydomonas* culture media

M.2.1.2.1 TAP (Tris-Acetate-Phosphate) medium

Standard **TAP medium** containing ammonium **(TA)** was prepared using (per liter): Tris Solution (10 mL), A solution (10 mL), B solution (1 mL), Trace Elements Solution (1 mL) and acetic acid (glacial) (0.95 mL). This medium was sterilized

by autoclave and used for *Chlamydomonas* pre-cultures under mixotrophic conditions.

Modified **TAP medium without nitrogen (T-N)** was prepared as TAP medium, omitting A Solution and using A-N Solution instead. When indicated, this medium was supplemented with the specified filter-sterilized nitrogen source (amino acids or peptides).

M.2.1.2.2. Minimal medium

Standard **Minimal Medium** containing ammonium **(MA)** was prepared using (per liter): A Solution (10 mL), B solution (10 mL) and Trace Elements Solution (1 mL). The media was sterilized by autoclave. When indicated, this medium was bubbled with CO_2 (5%) and it was used for autotrophic conditions.

Modified **Minimal Medium without nitrogen (M-N)** was prepared as MA medium, omitting A Solution and using A-N Solution instead. When indicated, this medium was supplemented with the specified filter-sterilized nitrogen source (amino acids or peptides).

M.2.1.3 Bacteria culture media

M.2.1.3.1 *Methylobacterium* Medium (MeM).

For *Methylobacterium* spp. growth, a basal M-N media was prepared supplemented with 1 $g \cdot L^{-1}$ KNO_3, sterilized by autoclave, cooled down to room temperature and supplemented with 0.5% filter-sterilized methanol (HPLC grade). This media was used for the selective growth of *Methylobacterium* spp. Otherwise, a modified MeM medium supplemented with tryptone or peptone (MeM^P) was used for rapid *Methylobacterium* growth of pre-cultures.

M.2.1.3.2 LB medium.

LB (Lysogeny Broth) medium was used for *Escherichia coli* cells growth (Sambrook et al., 1989). This medium contained (per liter): 10 g bactotryptone, 5 g yeast extract and 5 g NaCl. For solid plates, the medium was supplemented with 1.6% agar (w/v).

M.2.1.3.3 PSI medium

PSI medium for competent cells preparation (M.4.10.2.1.) contained (per liter): yeast extract (5 g), bactotryptone (20 g), $MgSO_4$ (5 g) and pH was adjusted to 7.6 with KOH.

M.2.1.3.4 CYM:MH medium.

For wild bacteria samples, a modified CYB (*Chlamydomonas*-Yeast Basal) medium was used (Hom and Murray, 2015). This medium, lacking carbon or nitrogen sources consists on the following components (per liter): 0.125 g $MgSO_4 \cdot 7H_2O$, 0.074 g $CaCl_2 \cdot H_2O$, 0.4 g K_2HPO_4, 0.18 g NaH_2PO_4, 29.2 mg EDTA, 6.6 mg KOH, 1.2 mg $MnCl_2 \cdot 4H_2O$, 0.3 mg $ZnCl_2$, 0.06 mg H_3BO_3, 0.2 mg

$CoCl_2 \cdot 6H_2O$, 0.4 mg $CuCl_2 \cdot 2H_2O$, 0.05 mg $Na_2MoO_4 \cdot 2H_2O$, 0.018 mg KBr, 0.003 mg KI, 0.0018 mg Na_3VO_4, 0.0018 mg Na_2SeO_3 without vitamins supplementation. The pH of this medium is 6.8. For preparation, a concentrated 25x stock solution containing CYB Salts was diluted in dH_2O and sterilized by autoclave. After autoclave, a concentrated stock of filter-sterilized phosphate solution (1000x) was added. To this basal medium the following filter-sterilized compounds were added to favor the growth of Methylobacteria (final concentration): methanol (0.5%), formaldehyde (50 uM), and NH_4Cl (10 mM), KNO_3 (10 mM) or urea (10 mM).

M.2.1.3.5 BB Medium.

BB (Bold's Basal) Medium is a freshwater algae medium that has been used to culture a variety of green algae (Nichols and Bold, 1965).

M.2.1.3.6 KS Medium

KS medium (KSM) from the Hom Lab is formulated for improved cultivation of green algae and cyanobacteria (unpublished).

M.2.1.4 Co-cultures media

Prior to co-culturing experiments, *Chlamydomonas* strains were pre-cultivated for 2 days on TA under continuous light and agitation at 23°C. *Methylobacterium* strains were pre-cultivated on MeM^P for 2 days under continuous agitation at 28°C. Experimental co-cultures and control mono-cultures were performed in CYB or M-N medium, as indicated, supplemented with the specified source of nitrogen. The nitrogen source (amino acids or peptides) was dissolved in the specific media to obtain a final concentration of 10 mM, the pH adjusted to 7-7.2 if necessary (with KOH or HCl), and filter-sterilized (0.2 µm pore-size).

M.2.2. Incubation for gene expression induction

Chlamydomonas WT and *nit2* strains (Table M.1) were incubated under ammonium or nitrate, under autotrophic and mixotrophic conditions to further study transcriptional changes. Cells were cultivated for 2-3 days on TAP or MA (with 0.5% CO_2). Then, cells were washed twice with the corresponding media (T-N or M-N, supplemented with 4 mM NH_4Cl or 4 mM KNO_3) and then incubated for 60 min under continuous light and agitation (TAP) or CO_2-bubbling (Minimal) (0.5% CO_2). All experiments were carried out three times. After incubation, 50 mL of each culture were harvested by centrifugation and treated for RNA extraction (M.4.1).

M.2.3. Growth Tests

M.2.3.1. Liquid Media

Cell growth on the different proteinogenic amino acids and peptides was performed in CYB or M-N (without acetate and nitrogen) supplemented with the indicated L-amino acid or peptide at 8 or 4 mM, respectively, (final concentration) as the sole nitrogen/carbon source, with the exception of L-

tyrosine, which was used at 2 mM due to poor solubility. All L-amino acids and peptides were purchased from SIGMA-ALDRICH (Madrid, Spain), dissolved in CYB/M-N, filter sterilized (0.2 µm) and pH-adjusted if necessary using KOH or HCl to 7.2±0.1.

Chlamydomonas growth on amino acids and peptides: Mid-log phase cell pre-cultures were harvested by centrifugation for 2 min at 3,000 x *g*, washed three times using CYB/M-N and adjusted to a final A_{750} of 0.025 in 250 µL final volume in sterile V-bottom 96-wells culture plates or in 1 mL final volume in flat-bottom 48-well plates (BRANDplates®, Madrid, Spain) and incubated at 23 °C under continuous light in the presence of the indicated amino acids, di- or tripeptides. After 7 days, cells cultures in the V-bottom 96-wells plates were pelleted by centrifugation (5 min, 2,000 x *g*) for imaging from below. Otherwise, the flat-bottom 48-wells plates were resuspended prior to imaging. All growth experiments were performed at least twice, with two replicates per experiment.

Chlamydomonas co-cultures with wild bacteria on L-serine were performed as *Chlamydomonas* mono-cultures in V-bottom 96-wells plates adding bacterial biomass to the co-cultures by picking and resuspending in the cultures with a sterile stick.

For *Chlamydomonas-Methylobacterium* spp. co-culture experiments, mid-log phase pre-cultures of both cell types were prepared as described above. Pelleted pre-cultures were washed two times with CYB/M-N and cell densities adjusted separately (*Chlamydomonas* and *Methylobacterium* mono-cultures) or in combination (*Chlamydomonas*-Methylobacterium co-cultures) to a final A_{750} of 0.025 for algae and A_{600} of 0.002 for bacteria in a 250 µL final volume in V-bottom 96-wells culture plates. Co-cultures were cultivated and imaged as described above for mono-cultures.

M.2.3.2. Solid Media

Mid-log phase pre-cultures of both cell types were prepared as described above. Pelleted pre-cultures were washed twice with CYB/M-N and cell densities adjusted separately (*Chlamydomonas* and *Methylobacterium* mono-cultures) or in combination (*Chlamydomonas*-Methylobacterium co-cultures) to an A_{750} of 0.125 for algae and A_{600} of 0.012 for bacteria. 5 µL of each culture were plated on 1.6% agar (w/v) plates. Plates were incubated at 23ºC under continuous horizontally-installed light or darkness.

M.2.4. *Chlamydomonas* genetic cross

A *Chlamydomonas* WT strain for nitrate assimilation (CC-1690, Sager 21gr) was crossed with a double mutant *nia1nit2* (CC-5325) by the random spore plating method (Levine and Ebersold, 1960). Zygotes were matured on ammonium-containing minimal medium (MA) plates with 4% agar under continuous light. After six days, plates were treated with chloroform vapour to eliminate vegetative cells. Agar blocks containing

around 80 zygotes were cropped, transferred to TA plates with 1.6% agar and irrigated with 0.1 mL of liquid medium to allow germination. After 16 hours of incubation under continuous dim light, 0.7 mL of liquid medium was used to spread the germinated zygotes all around the plates. Plates were then incubated under continuous light for a week. A hundred randomly selected colonies were plated on different TAP agar plates containing: ammonium (8 mM), nitrate (4 mM) or nitrite (2 mM) as sole nitrogen source for genotype analysis. Resulting segregants were named as '2J', followed by the number of plate (1-2) and individual segregants (1-50).

M.2.5. Bacteria isolation and identification

M.2.5.1. *Chlamydomonas* contamination isolation and identification

The contaminating *Methylobacterium* sp. (named here as strain M017) was isolated by plate streaking a *Chlamydomonas* culture growing on L-Ala-Ala to generate single colonies and identified by colony PCR amplification and subsequent amplicon sequencing of the highly conserved UARR region of 16S rDNA as described by Rivas et al. (2004) using *U1F* and *U1R* primers (Table M.3). The alignment of the resulting sequence using the basic local alignment search tool (BLAST) showed 99% of identity to *Methylobacterium marchantiae* and *M. bullatum.* This sequence was deposited in the NCBI database (accession no. *MG287145*) and this strain was named as *Methylobacterium* sp. M017.

M.2.5.2. Field sampling, isolation and identification of bacteria

Samples were obtained from leaves, water ponds, wet green rocks at the Field Station of the University of Mississippi (Oxford, MS, USA), in collaboration with the Hom Lab. Samples were plated in different media (CYM:MH, BBM and KSM) and resulting bacterial colonies were isolated by plate streaking on the same media. Single colonies were used to identify the species by colony PCR amplification of a region of the 16S rDNA using ReadyMade™ 16S rDNA primers from IDT (Integrated DNA Technologies, catalog nos. 51-01-19-06 and 51-01-19-07) (Forward, 5'-AGA GTT TGAT CCT GGC TCA G-3'; Reverse, 5'-ACG GCT ACC TTG TTA CGA CTT-3'). The obtained amplicons were sequenced, and the resulting sequences were aligned using BLAST to find the species identity.

M.2.6. Microorganisms cell quantification.

M.2.6.1. Micro-cell counter

50-100 µL-samples of *Chlamydomonas* cultures (and co-cultures) were used to quantify the algal cell density using a microcell counter (Sysmex F-500).

M.2.6.2. Absorbance determination

Cell density of algal and bacterial mono-cultures was quantified spectrophotometrically measuring the absorbance at 750 nm for *Chlamydomonas* and 600 nm for *Methylobacterium*.

M.2.6.3. Colony Forming Units (CFU) counting

Chlamydomonas and *Methylobacterium* CFUs (Colony-Forming Units) were quantified to estimate cell density of both cell types. From each culture, serial 10-fold dilutions were made and 200 µL of each dilution were plated. Plates were incubated for at least 7 days until colonies reached a considerable size enough to be easily counted by naked eye. Then, those plates containing 50-100 colonies were selected, counted and the corresponding dilution factor was used for further calculations. *Chlamydomonas*-containing cultures were plated on MA plates (without organic carbon to avoid bacterial growth) and incubated under continuous light at 23°C. Otherwise, *Methylobacterium*-containing cultures were plated on MeM^{P} and incubated under darkness at 28°C to avoid algal growth. Thus, for simultaneous estimation of algae and bacteria in the co-cultures, two samples of each dilution were separately plated on both MA and MeM^{P} plates to quantify both cell types independently.

M.2.6.4. PCR-based Multi-Species Cell Counting

PCR-based Multiple Species Cell Counting (PCR-MSCC) (Huang et al., 2015) was used for simultaneous quantification of algal and bacterial cell types during co-culture. Genus-specific primers were designed to amplify single-copy genes. To quantify algal growth, 213 bp of the *Chlamydomonas* centrin gene (*cen*) (Phytozome id. *Cre11.g468450.t1.2*) was amplified using primers *Cen1CreU* and *Cen1CreL*. To quantify bacterial growth, 239 bp of *rpoB* gene (KEGG id. Maq22A_c27070) was amplified using primers *rpoBMaqU* and rpoBMaqL. To generate the calibration curves, each PCR product of the two above-mentioned gene fragments was cloned into a vector and used to transform *E. coli* DH5αF′ (M.4.10). Individual clones were selected and confirmed by DNA sequencing (M.4.6). The corresponding plasmid DNA was quantified (M.4.4) and 3-µL aliquots (containing 10^{10} copies μL^{-1}) were stored at −20°C on TE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA). To calculate the number of copies of pDNA in each sample, the DNA Copy Number and Dilution Calculator (ThermoFisher Scientific) online tool was used. For each quantitative PCR (qPCR) run, 1 µL of each standard aliquot (*cen* and *rpob*) was serially 10-fold diluted, from 10^{9} to 10^{1} copies, and loaded in the same qPCR plate to quantify gene copies (Figure M. X).

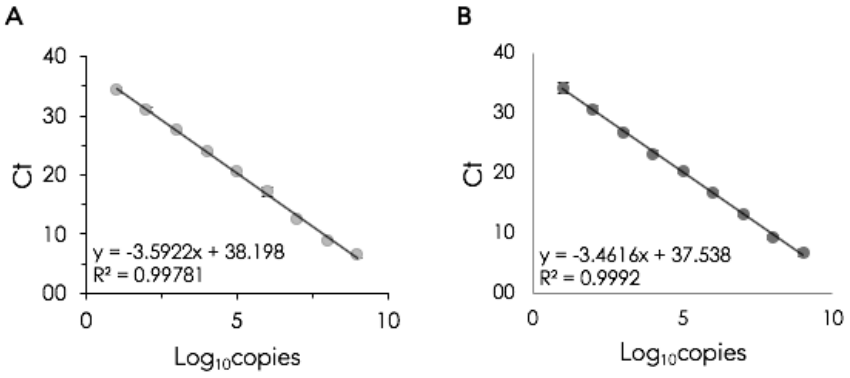


Figure M.1. Standard curves for *Chlamydomonas cen* (A) and *M. aquaticum rpoB* (B). Ct is the threshold cycle obtained by qPCR for each pDNA ten-fold dilution (copies). Error bars represent standard deviation of three replicates.

To validate cell number quantification by qPCR, genomic DNA (gDNA) extracted from serially diluted *C. reinhardtii* and *M. aquaticum* mono-cultures was isolated and used for single-copy gene quantification by qPCR. Each of the serially diluted cell cultures was also plated to quantify CFUs (M.2.5.3.) to confirm the results obtained by qPCR (Figure M.2, A-B). Moreover, possible interference by the presence of non-target DNA was tested by adding dilutions of bacterial gDNA to the algal gDNA and vice versa (Figure M.2, C-D).

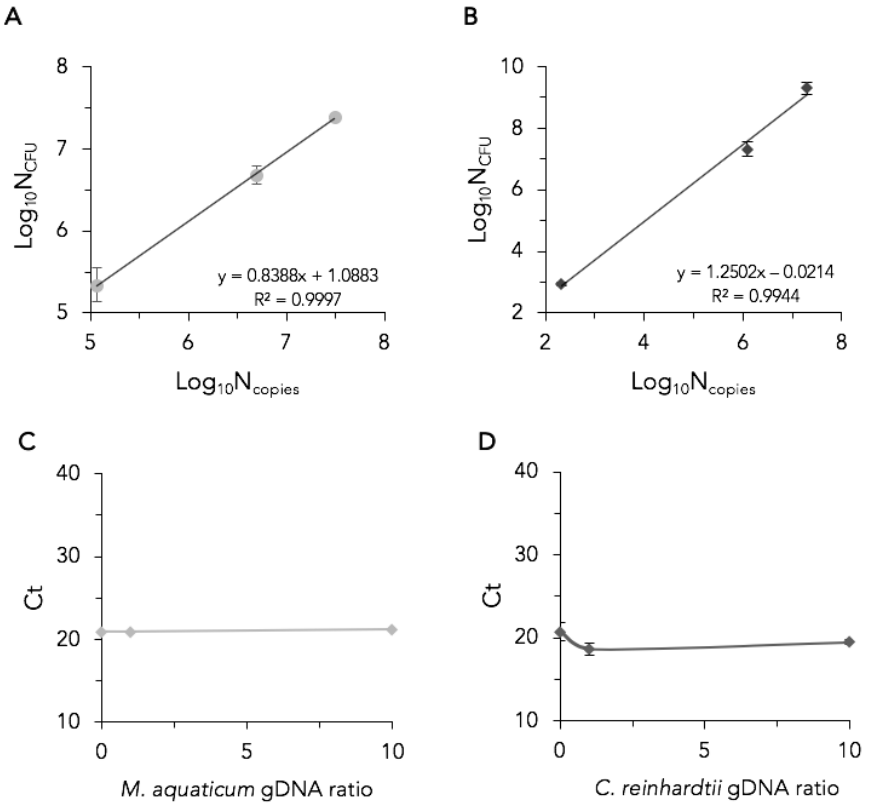


Figure M.2. qPCR regression curves and influence of non-target DNA. Regression curves generated from the number of plasmids (N_{copies}) containing *cen* (A) and *rpoB* (B) fragments and the number of counted CFUs (N_{CFU}); and the influence of non-target DNA upon the performance of qPCR. 5 ng of *Chlamydomonas* gDNA was mixed with 5 (ratio 1) and 50 (ratio 10) ng of *M. aquaticum* gDNA (C); 5 ng of *M. aquaticum* gDNA was mixed with 5 (ratio 1) and 50 (ratio 10) ng of *Chlamydomonas* gDNA (D).

M.3. Biochemical Assays.

To determine metabolites concentrations in the culture supernatants, 1 mL of cultures was harvested by centrifugation for 5 min at 15,000 x *g* and cell-free supernatants were stored at -20°C before use.

M.3.1 Ammonium determination

Ammonium concentrations in the culture supernatants were determined by the use of Nessler's reagent (Koch and McMeekin, 1924). A Nessler's reagent mixture was prepared by mixing equal volumes of Nessler reagents A and B (MERCK 109011 and 109012, respectively). 100-µL samples of cell-free supernatants were transferred to flat-bottom 96-wells microtiter plates. A volume of 100 µL of freshly prepared Nessler's reagent mixture was added, incubated for 2 min and A_{410} was read using a microplate reader (iMark, Bio-Rad). Ammonium calibration curves containing at least ten points of known concentrations of NH_4Cl, ranging from 50 to 1,000 µM, were included in every measurement and samples were diluted as needed.

M.3.2 Nitrite determination

Nitrite concentrations in the culture supernatants were determined by a colorimetric assay based on Griess reaction (Giustarini et al., 2008; Griess, 1879) . A working reagent mixture was prepared mixing equal volumes of 10 $g \cdot L^{-1}$ sulfanilamide (SIGMA, S-9251), dissolved in 2.4 N HCl, and 200 $mg \cdot L^{-1}$ N-(1-naphthyl)ethylenediamine dihydrochloride (MP Biomedicals, LLC, 152549) dissolved in dH_2O. One milliliter of algal cultures was harvested by centrifugation and 50-µL samples of cell-free supernatants were transferred to flat-bottom 96-wells microtiter plates. A volume of 100 µL of freshly prepared working reagent mixture was added, incubated for 2 min and A540 was read using a microplate reader (iMark, Bio-Rad). Nitrite calibration curves containing at least ten points of known concentrations of KNO_2, ranging from 5 to 100 µM, were included in every measurement and samples were diluted as needed.

M.3.3 Nitrate determination.

Nitrate was determined by its reduction to nitrite with acidic vanadium(III)-solution (Schnetger and Lehners, 2014) and subsequent determination of total concentration of nitrite by the Griess assay mentioned above. 80 µL of free-cell samples were mixed with 80 µL of vanadium(III) solution and 80 µL of Griess working reagent mixture in flat-bottom 96-wells microtiter plates, incubated for 30 min at 37°C and A_{540} was read using a microplate reader (iMark, Bio-Rad). To infer nitrate concentration, nitrite determination without the vanadium solution was parallelly determined and the resulting absorbance was subtracted to that obtained in the presence of the reducing agent, which represents the combination of the originally present nitrite and the one obtained by nitrate conversion to nitrite. The saturated acidic vanadium(III)-solution was prepared by dissolving 40 mg of VCl_3 in 5 mL HCl 1 N. The solution was filtered (0.2 µm pore-size) to remove salt excess and stored at 4°C under darkness for a maximum of two weeks.

M.3.4 Indoles determination.

Indoles concentration in the supernatant were determined using the Salkowski reagent (Glickmann and Dessaux, 1995; Salkowski, 1885), which consisted on 12 $mg \cdot mL^{-1}$ $FeCl_3$ in 7.9 M H_2SO_4. 100-µL samples of cell-free supernatants were mixed with 100 µL of freshly prepared Salkowski reagent, incubated for 30 min under darkness at room temperature and A_{540} was read using a microplate reader (iMark, Bio-Rad).

M.3.5 L-arginine determination.

L-arginine in the media was analyzed by HPLC (Agilent series 1200, Agilent Technologies, Spain) using a modified established protocol by an automated, online precolumn derivatization using o-phtalaldehyde (OPA) (Henderson et al., 2000). 100 µL of cell-free samples were filtered (0.2 µm pore size) and derivatized using freshly-prepared OPA reagent. The OPA reagent was prepared by dissolving 10 mg of OPA (SIGMA P0657) in 200 µL of methanol (HPLC-grade) and mixing with 800 µL of borate buffer (25 $g \cdot L^{-1}$ sodium tetraborate decahydrate, pH 10.2) and 10 µL of 3-mercaptopropionic acid (SIGMA 63768). The reagent was filtered (0.2 µm pore size), the head space purged with N_2 and stored at 4°C protected from light for less than one week before use. The amino acid was separated using a C18 reverse phase column (Agilent Zorbax Eclipse XDB-C18 (4.6 x 150 mm, 5 um pore-size) at 50°C and eluted using a gradient of two freshly-prepared and filtered solvents: solvent A, consisting of 40 mM NaH_2PO_4 pH 7.8 (5.5 g NaH_2PO_4, monohydrate in one liter of MQ water and pH adjusted with 10 N NaOH); and solvent B, an organic phase containing HPLC-grade acetonitrile:methanol:water (45:45:10, v/v/v). Solvents were delivered at a flow rate of 2 $mL \cdot min^{-1}$. Methanol and acetonitrile were HPLC-grade reagents and water was purified using a Millipore Milli-Q system (MQ). A linear gradient from 7.2 to 38% B was pumped for 5 min, followed by an isocratic hold at 100% for 3 additional minutes. The conditions were reestablished to initial conditions and held for 3 min. The signal was detected with an Agilent G 1362A fluorescence detector (Agilent Technologies) (Excitation wavelength 229 nm, Emission wavelength 454 nm). The retention time for L-arginine was 3.98±0.5 min. To determine the concentration of this compound, a calibration curve with samples containing known concentrations of L-arginine (SIGMA, A5131), ranging from 0.3 to 3 mM, were analyzed under the same analytical conditions. This curve was lineal within this range and the r^2 was 0.999 using six different concentrations with three technical replicates.

M.3.6 Organic acids determination.

Glycerol, pyruvic acid and acetic acid accumulation in the media were analyzed by HPLC (Agilent series 1200, Agilent Technologies, Spain) using a previously established protocol (Jurado-Oller et al., 2015) that involves isocratic elution of sulphuric acid (5 mM) at 55°C in an ion-exchange column (Agilent Hi-Plex H, 300 × 7.7 mm, 6 µm i.d.). Signals were detected using a Refractive Index Detector (Agilent G 1362A, Agilent Technologies). The retention time for glycerol, pyruvic acid and acetic acid were 13.8, 15.9 and 10.5 min, respectively. To determine the concentration of these compounds, calibration curves with samples containing known concentrations of each compound, ranging from 0.125 to 4 mM for glycerol, from 0.05 to 10 mM for pyruvic acid, and from 0.1 to 10 mM for acetic acid, were analyzed under the same analytical conditions. These curves were lineal within this range and the r^2 was 0.999 for all of them using at least six different concentrations with three technical replicates.

M.4 Molecular Biology

M.4.1 RNA extraction

For total RNA extraction from *Chlamydomonas* cells, 50 mL of cultures were harvested by centrifugation for 2 min at 3,000 x *g* and the pelleted cells were resuspended in 800 µL of lysis buffer (50 mM Tris·HCl pH 8.0; 0.3 M NaCl; 5 mM EDTA, pH 8.0) and sodium dodecyl sulfate (SDS) to a final concentration of 2%. Then, samples were frozen and stored at -80°C until processed for phenol-chloroform extraction (Sambrook et al., 1989). For extraction, samples were thawed at 4°C and extracted using an equal volume of a phenol solution containing phenol (pH 4.3): chloroform:isoamyl alcohol (25:24:1) saturated with 50 mM Tris·HCl, and vortexing vigorously for 1 min. Then, the samples were centrifuged for 5-10 min at 15,000 x *g* to separate both phases. The aqueous phase was transferred to a new tube and the extraction step with phenol solution was repeated for 2-3 times until no interphase was observed. Then, a last extraction step with chloroform was performed and the obtained aqueous phase after separation by centrifugation was mixed with LiCl (4 mM). The samples were incubated at 4°C for at least 4 hours and were centrifuged for 30 min at 15,000 x *g*. The resulting RNA-containing pellets were washed with 70% ethanol and resuspended in 35 µL of sterile MQ-water. Nucleic acids concentration was quantified spectrophotometrically (M.4.4). These samples were subsequently used for cDNA synthesis or stored at -80°C. All extraction steps were performed at 4°C.

For transcriptomics analysis, RNA samples were treated with DNAse to remove contaminant gDNA using DNase I recombinant, RNase-free (ROCHE, catalog no. 04 716 728 001) and further purified using the RNeasy® MinElute® Cleanup Kit (Qiagen, catalog no. 74204). The RIN (RNA Integrity Number) obtained was higher than 8 and was analyzed by a BioAnalyzer 2100 (Agilent Technologies) carried out by the centralized service SCAI at the University of Córdoba (Spain). These RNA samples were shipped to the company *Sistemas Genómicos* (Valencia, Spain) for transcriptomic analysis.

M.4.2 Genomic DNA extraction

For *Chlamydomonas* and *Methylobacterium* (mono- and co-cultures) total gDNA extraction, a standard phenol–chloroform extraction protocol (Sambrook et al., 1989) similar to the above-mentioned for total RNA extraction was carried out with some modifications: only 1 mL of cultures was harvested for gDNA extraction and, after the chloroform extraction step, precipitation was achieved with 0.9 volumes of isopropanol for 1 hour at room temperature. The obtained gDNA was treated with RNase H.

M.4.3 Plasmid DNA isolation

Plasmid DNA (pDNA) of over-night grown *E. coli* clones containing *pSpark-cen* or *pSpark-rpoB* was isolated using E.Z.N.A. plasmid DNA Mini Kit D6942 (OMEGA bio-tek). The isolated pDNA was quantified (M.4.), digested (M.7.) and confirmed by gel electrophoresis (M.4.5).

M.4.4 Nucleic acids quantification.

Spectrophotometrically, nucleic acids were quantified measuring absorbance at 260 nm taking into account that the average extinction coefficients are 0.02, 0.027 and 0.025 $(\mu g \cdot cm^{-1}) \cdot mL^{-1}$ for double-stranded, single-stranded DNA and single stranded RNA, respectively.

Alternatively, a Quantus™ Fluorometer (Promega) was used for specific determination of DNA following the supplier instructions.

Non-purified nucleic acids were semi-quantitatively estimated by gel electrophoresis by comparing fluorescence intensity emitted by DNA standards with known concentrations of commercial lambda DNA (ThermoFischer Scientific).

M.4.5 Gel electrophoresis

Nucleic acids were separated using agarose gels containing 1% agarose (w/v) in TAE buffer (4.84 $g \cdot L^{-1}$ Tris; 1.1 $mL \cdot L^{-1}$ acetic acid; 50 mM EDTA pH 8.0), using 0.5 $\mu g \cdot mL^{-1}$ ethidium bromide to visualize them using an UV-transilluminator. Prior to load the samples in the gel, 1µL of loading buffer (50% glycerol; 0.25% bromophenol blue; 0.25% xylene cyanol) was added to the samples in a final volume of 10 µL. As molecular weight marker, 1 kb Ladder Plus marker (Invitrogen) was used.

Once the electrophoresis was run, gels were visualized using UV light and data analyzed with the image system Gel Doc 2000 (Bio-Rad).

The observed bands corresponding to DNA fragments of interest were obtained from the gel by cropping the gel with a scalpel, freezing the obtained gel block at -80°C and harvesting by centrifugation for 15 min at 3,000 x *g*. Then, the agarose-free supernatant was used for subsequent purposes.

M.4.6 DNA sequencing.

DNA samples were sequenced using Sanger technology by the centralized service SCAI at the University of Córdoba (Spain) or by GenSript (USA).

M.4.7 DNA digestion

Extracted pDNA (M.4.3) was digested with the restriction enzyme BamHI (Takara) following the supplier instructions to confirm the transformation of *E. coli* with the *cen-/rpoB* fragments-containing vectors (M.4.10.2.). 2-5 U of enzyme per µg of DNA were used for 30 min at 37°C. The size of the digested pDNA was analyzed by gel electrophoresis and those clones which showed the expected molecular weight bands were sequenced (M.4.6) to further confirm the presence of the *cen* and *rpoB* fragments.

M.4.8 Complementary DNA synthesis

For gene expression quantification by qPCR, complementary DNA (cDNA) was synthesized from purified RNA samples. 1 µg of DNA-free RNA was used for reverse transcription using the iScript™ (high fidelity) cDNA Synthesis Kit (Bio-Rad), following the supplier indications. This kit is RNase H+ and the enzyme is provided preblended

with RNase inhibitor. The provided reaction mix contains a mixture of oligo(dT) and random hexamer primers and allows the production of less than 1 kb long targets. The reaction was performed in a total volume of 20 µL using a thermocycler (5 min at 25°C, 20 min at 56°C and 1 min at 95°C). After synthesis, the samples were diluted 2.5 times (50 µL final volume). The samples were stored at -20°C until use for RT-qPCR (M.4.9.4.).

M.4.9 PCR

M.4.9.1 Primers design

Specific primers to amplify a fragment of the single-copy gene *rpoB* of *Methylobacterium aquaticum* (KEGG id. *Maq22A_c27070*) were designed using Primer3 (http://primer3.ut.ee/) and BLAST online tools, and amplification parameters were analyzed by EditSeq program (M.4.11.4).

M.4.9.2 Standard PCR

For the characterization of the purchased *lao1* mutant, single colonies were picked as recommended by the supplier (*Chlamydomonas* Library Project, CLiP), cultured on TA to obtain biomass and extract gDNA (M.4.2.). PCR was carried out using iProof™ High-Fidelity DNA Polymerase (Bio-Rad) following the supplier instructions. To confirm the location of the inserted CIB cassette, *CIB-5'*, *CIB-3'*, *LAO1F* and *LAO1R* primers were used as recommended by the supplier (*Chlamydomonas* Library Project, CLiP) and further detailed in Results and Discussion section (Chapter 2). The run protocol started with 30s at 98°C, followed by 35 cycles of 10 s at 98°C, 30 s at 60°C and 60 s at 72°C, and a final step of 10 min at 72°C. Resulting bands with expected sizes (0.7 kb for LAO1F-CIB-5', 0.8 ok for CIB-3'-LAO1R, and 1.5 kb for LAO1F-LAO1R, approximately) were cropped and sequenced to confirm the location of the mutation (M.4.6.).

The single-copy genes *centrin (cen)* (Phytozome id. *Cre11.g468450.t1.2*) and *rpoB* (KEGG id. *Maq22A_c27070*) fragments were amplified from *Chlamydomonas* CC-1690 (21gr) and *M. aquaticum* GR16T gDNAs, respectively, by PCR using iProof™ High-Fidelity DNA Polymerase (Bio-Rad) following the supplier instructions. Primers used to amplify *cen* fragment in *Chlamydomonas* gDNA were *Cen1CreU* and *Cen1CreL*, and *rpoBMaqU* and rpoBMaqL (Table M.3.) to amplify *rpoB* fragment in *Methylobacterium* gDNA. The amplification protocol was 98°C for 30 sec, followed by 35 cycles of 98°C for 10 sec, 61°C for 30 sec and 72°C for 9 sec; and a final step of 72°C for 10 min. PCR products were analyzed by gel electrophoresis (M.4.5.) and cloned into a vector (M.4.10.).

M.4.9.3 Colony PCR

To identify the species of the bacteria isolated in this work (M.2.4.1), colony PCR method was used to amplify a conserved region of the 16S rDNA (M.2.4) using PCRBIO HiFi Polymerase (PCR BIOSYSTEMS Ltd.) following the supplier instructions. To obtain bacterial DNA, single colonies were picked and resuspended in 20 µL of sterile water, heated for 15 min at 98°C and harvested by centrifugation for 5 min (15,000 x *g*). Then, the cell-free supernatants were

transferred to clean tubes and used as DNA templates for amplification with the 16S ReadyMade(R) primers from IDT (Table M.3). The amplification run protocol started with 2 min at 95°C followed by 30 cycles of 15 s at 95°C, 15 s at 66.4°C and 60 s at 72°C, and a final step of 5 min at 72°C. Amplified DNA was analyzed by gel electrophoresis (M.4.5) and those products with the expected size were purified with the PCR cleanup kit DNA Clean & Concentrator (DCC) (Zymo Research) and quantified by Quantus™ Fluorometer (Promega). The samples were then sequenced by Sanger technology (M.4.6) by GenScript (USA).

M.4.9.4 Quantitative PCR for relative gene expression quantification

Real time-quantitative PCR (RT-qPCR) to estimate the quantity of mRNA corresponding to a specific gene by measuring the fluorescence signal emitted by the dsDNA-binding EvaGreen dye using the SsoFast™ EvaGreen Supermix (Bio-Rad). 1 µL of cDNA samples and 10 µM of the specific primer pairs was used per 10 µL of reaction following supplier instructions. The reactions were carried out in 96-wells plates in an MyiQ2 (Bio-Rad) detection system. Control reactions without added DNA to discard DNA contamination were included in every run for each primer pair. At least two replicates per sample were run and reactions containing specific primers to amplify the *Chlamydomonas* ubiquitin-ligase gene were included in the same plate. Ubiquitin ligase was used as a housekeeping gene since its expression remains unaltered by the source of nitrogen and/or carbon for at least 24 hours (González-Ballester et al., 2004), and the expression of the gene of interest was calculated in relation to the one detected in this control gene. The run protocol for cDNA was 95°C for 30 sec followed by 30 cycles of 95°C for 5 sec and 60°C for 10 sec, and a melt curve from 65 to 95°C for 10 sec per step. Threshold cycle (Ct) values obtained were normalized to the housekeeping gene control and expressed as fold-changes ($2^{-\Delta Ct}$, where $\Delta Ct = Ct_{sample} - Ct_{control}$).

M.4.9.5 Quantitative PCR for cell quantification (PCR-MSCC)

The quantification of *Chlamydomonas* and *Methylobacterium* cell density during co-culture was achieved using amplification by qPCR of single-copy genus-specific genes (M.2.5.4). qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad) following supplier instructions, and run and detected in a MyiQ2 (Bio-Rad) detection system. The run protocol started at 98°C for 2 min followed by 40 cycles of 98°C for 5 sec and 60°C for 10 sec, and a melt curve from 65 to 95°C for 10 sec per step. A calibration curve for each gene (*cen* and *rpoB*) was included and used to calculate the number of copies detected for each obtained Ct.

M.4.9.6. Primers used in this work

Table M.3. Primers used in this work.

Name	Sequence (5'-3')	Gene	Application	Ref.
LAO1QC1	GAG ACT GTG ATG CCC AAA AAG TG	*LAO1*	Real Time qPCR	1
LAO1QR1	*GCT TGC CCA GGC CGC GAA TGG AA*			
Amt1upper	*GCACGGGAGGGCAAGAGGTTC*	*AMT1.1*		2
Amt1lower	*ATGTGCCGCAGTCAAGAAGGATTT*			
Amt2upper	*GGCTCGCCACCTGCAAGAGACAAC*	*AMT1.2*		
Amt2lower	*GGCTCGCCACCTGCAAGAGACAAC*			
Amt4upper	*GGGAAATGCCGGGCTTTTAGACCA*	*AMT1.4*		
Amt4lower	*TTTTTTTTTTTTTTTTTTTTCACTAGACAG*			
Amt5upper	*TAAGGACGGCTACACCAACGGTTTC*	*AMT1.5*		
Amt5lower	*CCGGCGTAGTCGATTGCTCCAATG*			
UbiUp	*GTACAGCGGCGGCTAGAGGCAC*	*Ubiquitin -ligase*		
UbiLo	*AGCGTCAGCGGCGGTTGCAGGTATCT*			
CIB-5'	*GCA CCA ATC ATG TCA AGC CT*	*CIB*	*Lao1* mutant confirmation	3
CIB-3'	*GAC GTT ACA GCA CAC CCT TG*			
LAO1F	*TCG AAG CAA GTA CAC GCA AC*	*LAO1*		
LAO1R	*AAC TGT GGG AGT GTG GGA AG*			
U1F	*CTY AAA KRA ATT GRC GGR RRS SC*	*16S/18S*	Species identification	4
U1R	*CGG GCG GTG TGT RCA ARR SSC*			
16S rRNA For	*AGA GTT TGA TCC TGG CTC AG*	*16S*		5
16S rRNA Rev	*ACG GCT ACC TTG TTA CGA CTT*			
Cen1CreU	*TTA CAA GAT GGG ACA GCC CG*	*Centrin*	PCR-MSCC	6
Cen1CreL	*CAG CCC GCA GAG GAA CTA AC*			
rpoBMaqU	*TAG ATG TAG CCG ACC GTG AC*	*rpoB*		7
rpoBMaqL	*ATG AAG GCG ATC TAC AGC GA*			

1, (Wei et al., 2014); 2, (González-Ballester et al., 2004); 3, (Li et al., 2016). 4,(Rivas et al., 2004); 5, ReadyMade® from IDT. 6, designed and provided by the Hom Lab (University of Mississippi, MS, USA). 7, This work.

M.4.10. Cloning

Centrin and *rpoB* PCR-amplified fragments were cloned into the cloning vector pSpark® I DNA cloning system (Canvax), following the supplier instructions.

M.4.10.1 *E. coli* transformation:

M.4.10.1.1 Competent cells preparation

To generate competent cells for high efficiency transformation, 5 mL of PSI medium were inoculated with an over-night grown colony of *Escherichia coli* strain DH5αF' (Sambrook et al., 1989) and incubated over-night at 37°C. After incubation, the cells were diluted 20 times with fresh PSI medium and cultured until reaching A_{550} = 0.45-0.5. Then, the culture was cooled on ice for 5 min and then harvested by centrifugation at 4°C for 5 min (3,000 x *g*). Then, the cells were gently resuspended in 40 mL of pre-cooled TFB-1 solution and maintained on ice for 5 min. Later, the cells were again harvested by centrifugation and the pellet was resuspended in 4 mL of TFB-2 solution and

maintained on ice for 15 min. Finally, cells were frozen down in liquid nitrogen in 150 µL-aliquots and stored at -80°C.

TFB-1 solution: 30 mM potassium acetate, 100 mM KCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$ and 15% glycerol (v/v). The pH was adjusted to 5.8 with 0.2 M acetic acid, filter-sterilized (0.2 µm) and store at 4°C.

TFB-2 solution: 10 mM MOPS (3-(*N*-morpholino)-propanesulfonic acid), 75 mM CaCl2, 10 mM KCl, 15% glycerol (v/v). The pH was adjusted to 6.5 with KOH, filter-sterilized (0.2 µm) and stored at 4°C.

M.4.10.2.2 Bacterial transformation

An aliquot of competent E. coli cells (M4.10.2.1) was thawed on ice. Immediately after thawing, 1 µL of 1.8% β-mercaptoethanol per 10 µL of cells and the centrin-/rpoB-pSpark ligation were added and maintained on ice for 30 min. Also, an empty vector and a DNA-free controls were included in the transformation. Then, a heat shock was carried out by incubating in a 42°C-bath for 45 sec and immediately transferred to ice (Sambrook et al., 1989). Later, 800 µL of LB medium were added to the mixture and the cells were incubated for 50 min at 37°C and gentle agitation for cell recovery. Finally, transformed cells were plated in LB media plates containing 100 µg·mL-1 ampicillin, 134 µM IPTG (isopropyl-β-D-1-tiogalactopyranoside) and 0.005% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and incubated over-night at 37°C. After incubation, no colonies were found in the control without DNA and few blue colonies were found in the empty vector control. From the sample plates, six white isolated colonies of each plate were picked and inoculated in LB liquid media containing ampicillin (100 µg·mL-1). After over-night incubation at 37°C, pDNA was isolated (M.4.3).

M.4.11 Bioinformatic Tools

M.4.11.1 Databases

- Algae and plants genome databases:
  - Phytozome v. 12, The Plant Genomics Resource (JGI) *https://phytozome.jgi.doe.gov/pz/portal.html*
  - JGI Genome Portal https://genome.jgi.doe.gov/portal/
- *Methylobacterium* spp. genome database: KEGG GENOME Database *https://www.genome.jp/kegg/genome.html*
- General database for DNA and protein sequences search: Nacional Center for Biotechnology Information (NCBI) https://www.ncbi.nlm.nih.gov/

M.4.11.2 Alignment tools

- Basic Local Alignment Search Tool (BLAST): https://blast.ncbi.nlm.nih.gov/Blast.cgi
- Multiple Sequence Alignment: Clustal Omega https://www.ebi.ac.uk/Tools/msa/clustalo/
- Multiple sequence alignment editing program Jalview http://www.jalview.org/

M.4.11.3 Cellular location prediction tool

- Multi-subcellular location prediction tool dedicated to Algae: PredAlgo Program (Tardif et al., 2012). https://giavap-genomes.ibpc.fr/cgi-bin/predalgodb.perl?page=main

M.4.11.4 Primers design tools

- Primer3 online tool: http://bioinfo.ut.ee/primer3-0.4.0/
- Primer-BLAST online tool: https://www.ncbi.nlm.nih.gov/tools/primer-blast/

M.4.11.5 Phylogenetic analysis

Evolutionary trees were constructed using the Maximum Likelihood method and default settings in MEGA7 (Kumar et al., 2016). Alignments were performed using the Clustal method, and the evolutionary distances were computed using the Poisson correction model (Zuckerkandl and Pauling, 1965). The bootstrap consensus tree was inferred from 500 replicates (Felsenstein, 1985). The analysis of L-amino acid oxidases involved 60 amino acid sequences. The *Methylobacterium* spp. tree was performed using 500 bp long nucleotide sequences corresponding to the highly conserved UARR region of 16S rDNA.

M.4.11.6 Transcriptomic analysis

The bioinformatic transcriptomic analysis was performed by the company Sistemas Genómicos (Valencia, Spain) as follows: the initial whole transcriptome paired-end reads obtained from sequencing were mapped against the version 5.0 of *C. reinhardtii* using the bowtie2 algorithm (Langmead and Salzberg, 2012). Low-quality reads were eliminated using Picard Tools software version 1.83, remaining only high-quality reads. The expression levels were calculated using the htseq software, version 0.5.4p3 (Anders and Huber, 2010). The DESeq2 method (Love et al., 2014) was applied for differential expression analysis among conditions. Differentially expressed genes were established in those genes with a fold-change lower or higher than −2 or 2, respectively, with a /p/ value adjust to 0.05

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