FEMS Microbiology Letters, 365, 2018, fny021

doi: 10.1093/femsle/fny021
Advance Access Publication Date: 29 January 2018
Research Letter

RESEARCH LETTER – Environmental Microbiology

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

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One sentence summary: A new mutualism based on carbon-nitrogen exchange between the alga *Chlamydomonas* and *Methylobacterium* spp. improves the growth of both microbes on amino acids and peptides.
Editors: David Richardson

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, this new model of mutualism may facilitate insights into the ecology and evolution of plant–bacterial interactions and design principles of synthetic ecology.

Keywords: Algal–bacterial mutualism; *Chlamydomonas*; *Methylobacterium;* metabolic complementation; nitrogen assimilation

INTRODUCTION

Nitrogen (N) is one of the major limiting nutrients for primary producers like plants and phytoplankton in terrestrial and aquatic ecosystems. Although elemental N is abundant, it is often not bioavailable for most organisms. Plants and phytoplankton depend on the abilities of other microorganisms to transform inaccessible N sources into useable forms like ammonium and nitrate (Hirsch and Mauchline 2015; Pajares and Bohannan 2016). Often, these ecological dependencies evolve and yield stable interactions between organisms with improved efficiency in nutrient exchange. A well-known example is the formation of nodules by N-fixing

Received: 5 November 2017; **Accepted:** 24 January 2018

bacteria in plant roots, which provides an excellent environment in which N_2 from the air can be efficiently transformed into assimilable N for plants. This symbiosis has allowed plants to thrive in N-limiting environments. In contexts where inorganic N is limiting but organic N is present, microorganisms can play a fundamental role in mineralizing N into bioavailable forms for photosynthetic organisms. Such microbes include plant growth-promoting bacteria (PGPB), which have been well studied because of their positive influence on plant fitness (for a review, see de Souza, Ambrosini and Passaglia 2015). Such mutualistic bacterial interactions are not limited to land plants but also extend to the green algae (Xie *et al.* 2013; Hom *et al.* 2015; Tanabe *et al.* 2015). The dominant bacterial species within the plant rhizosphere are mostly shared with those found in the planktonic analogue or 'phycosphere', suggesting that algal–bacterial associations might have coevolved (Goecke *et al.* 2013; Kim *et al.* 2014; Ramanan *et al.* 2015, 2016; Seymour *et al.* 2017). Furthermore, the ability of bacteria to benefit algae has been a recently exploited strategy to advance biotechnological applications (Wu *et al.* 2012; Li *et al.* 2013; Cho *et al.* 2015; Mahdavi *et al.* 2015; Wirth *et al.* 2015). Thus, a better understanding of such interactions will not only cast light on the evolutionary and ecological aspects of plant–microbe associations but will have practical ramifications for the biotechnological industry. The use of model organisms towards this end could be particularly effective given their experimental tractability.

Chlamydomonas reinhardtii (*Chlamydomonas*) has been a powerful model system for studying N metabolism in algae and plants (Fernández and Galván 2007; Sanz-Luque *et al.* 2015). Although *Chlamydomonas* preferentially uses inorganic N (i.e. ammonium, nitrate and nitrite), it can use some organic N sources such as amino acids and purines, albeit with significantly lower efficiency (Kirk and Kirk 1978). For *Chlamydomonas*, assimilation of N from amino acids occurs via a periplasmic L-amino acid oxidase (CrLAO1) that deaminates amino acids to generate an α-keto-acid and ammonium, which is then efficiently assimilated. Some amino acids such as L-Pro and L-Hyp are not deaminated by CrLAO1 (Vallon *et al.* 1993), and very little is known about the ability of *Chlamydomonas* to grow on peptides as a sole N source. To date, only high-throughput screening results based on cellular respiration reactions of *Chlamydomonas* grown on a few di- and tripeptides have been published (Chaiboonchoe *et al.* 2014). However, it is unclear from this study how or whether *Chlamydomonas* is able to grow on peptides as sole N sources. In this work, we demonstrate the inability of *Chlamydomonas* to grow on certain amino acids or peptides as a sole N source and show how this inability can be complemented by PGPB *Methylobacterium* species in co-culture.

Methylobacteria are pink-pigmented, facultative methylotrophs (PPFMs) that are found on the surface of most plant species where they grow using the methanol released by plant stomas as a by-product of cell wall degradation during plant cell growth (Abanda-Nkpwatt *et al.* 2006). PPFMs have been extensively reported to benefit plant hosts by fixing atmospheric nitrogen or by inhibiting plant pathogens (Sy *et al.* 2001; Madhaiyan *et al.* 2006). Evidence suggests that *Methylobacterium* species may have co-evolved as photosymbionts of bryophytes, which are ancestral to land plants (Kutschera 2007). Here, we describe a new interaction between *Chlamydomonas* and *Methylobacterium* that could provide key insights into the evolution of plant–PPFM interactions and serve as a new model system for studies in synthetic ecology that may be of biotechnological relevance.

MATERIALS AND METHODS

Strains, culture media and pre-culture conditions

Wild-type CC-1690 (Sager 21 gr) *Chlamydomonas* cells were pre-cultured in Tris-acetate-phosphate (TAP) medium (Gorman and Levine 1965), containing 8 mM of ammonium chloride, for 2–3 days (exponential growth phase) at 25°C under continuous light and agitation (120 rpm).

Methylobacterium strains (summarized in Table 1) were pre-cultured in a modified CYB minimal salts medium containing (per liter): 0.125 g $MgSO_4 \bullet 7H_2O$, 0.074 g $CaCl_2 \bullet H_2O$, 0.4 g K_2HPO_4, 0.18 g NaH_2PO_4, 29.2 mg EDTA, 6.6 mg KOH, 1.2 mg $MnCl_2 \bullet 4H_2O$, 0.3 mg $ZnCl_2$, 0.06 mg H_3BO_3, 0.2 mg $CoCl_2 \bullet 6H_2O$, 0.4 mg $CuCl_2 \bullet 2H_2O$, 0.05 mg $Na_2MoO_4 \bullet 2H_2O$, 0.018 mg KBr, 0.003 mg KI, 0.0018 mg Na_3VO_4, 0.0018 mg Na_2SeO_3 (Hom and Murray 2014) that we refer to as MeM (*Methylobacterium* Medium), for which potassium nitrate (1 g L^{-1}), peptone (2.5 g L^{-1}) and methanol (0.5%) are added. Pre-cultures were grown for 2–3 days at 25°C and agitation (120 rpm) to ensure that cells were in exponential growth.

Potential culture contamination was routinely checked by streaking each liquid pre-culture on modified TAP–gellan gum (1.7%) plates supplemented with tryptone (2.5 g L^{-1}) for *Chlamydomonas* cultures or on MeM–gellan gum (1.7%) plates for *Methylobacterium* cultures, incubated for 2 weeks at 25°C, and examined under a microscope.

Growth on amino acids and peptides

Mono- and co-culture experiments were performed in CYB medium with the indicated L-amino acid or peptide (8 mM) as sole N and/or C source. All L-amino acids and peptides were dissolved in CYB and filter-sterilized (0.2 μm). Prior to filtering, pH was adjusted as needed to 7.2 $\pm$ 0.1 using KOH or HCl.

Chlamydomonas and *Methylobacterium* pre-cultures in exponential growth were each harvested by centrifugation for 2–3 min at 2090 *g*, washed three times using CYB and used independently (monocultures) or in combination (co-cultures) to achieve a final absorbance at 750 nm (A_{750}) of 0.025 for algal cultures and A_{600} of 0.002 for bacterial cultures in a final volume of 250 μL in sterile V-bottom 96-well culture plates (BRANDplates®). These cultures were incubated at 25°C under continuous light and after 8–12 days cell cultures in the plates were pelleted by centrifugation (5 min, 2090 *g*) for imaging from below.

All growth experiments were performed at least twice including two biological replicates per experiment.

Identification of methylobacterial contamination

Methylobacterium contamination 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 primers U1F (5′-CTY AAA KRA ATT GRC GGR RRS SC-3′) and U1R (5′-CGG GCG GTG TGT RCA ARR SSC-3′) (NCBI accession number: MG287145).

Co-culture growth quantification by quantitative PCR

To simultaneously quantify *Chlamydomonas reinhardtii* and *Methylobacterium aquaticum* growth, species-specific primers were designed using Primer3 (http://primer3.ut.ee/) and BLAST to amplify single-copy genes. To quantify algal growth, 213 bp of

Table 1. *Methylobacterium* spp. used in this work.

Species name	Strain	ID	Isolated from	Source	Reference
M. oryzae	CBMB20	18207	Stem tissues of *Oryza sativa*	DSMZ	Madhaiyan *et al.* 2007
Methylobacterium sp.	88A	–	Lake Washington	Kindly provided by Prof. Ludmila Chistoserdova	–
M. organophilum	XX	ATCC 27886	Lake sediment	BCCM/LMG	Patt, Cole and Hanson 1976
Methylobacterium sp.	M017	–	Solid culture of *Chlamydomonas reinhardtii*	This work	This work
M. marchantiae	JT1	21328	Thalus of *Marchantia polymorpha*	DSMZ	Schauer *et al.* 2011
M. hispanicum	GP34	16372	Drinking water	CECT	Gallego, García and Ventosa 2005
M. nodulans	–	21967	Nodules of *Crotalaria podocarpa*	BCCM/LMG	Jourand *et al.* 2004
M. aquaticum	GR16T	16371	Drinking water	DSMZ	Gallego, García and Ventosa 2005
M. aerolatum	5413S-11	19013	Air sample	DSMZ	Weon *et al.* 2008
M. extorquens	AM1	–	Airborne contaminant	Kindly provided by Prof. Cecilia Martinez-Gomez	Nunn and Lidstrom 1986

DSMZ, German Collection of Microorganisms and Cell Cultures–Leibniz Institute; BCC/LMG, Belgian Coordinated Collections of Microorganisms; CECT, Spanish Type Culture Collection–University of Valencia.

the *Chlamydomonas* centrin gene (Cre11.g468450.t1.2) were amplified using primers Cen1CreU (5′-TTA CAA GAT GGG ACA GCC CG-3′) and Cen1CreL (5′-CAG CCC GCA GAG GAA CTA AC-3′). To quantify bacterial growth, 239 bp of *rpoB* gene (Maq22A˙c27070) were amplified using primers rpoBMaqU (5′-TAG ATG TAG CCG ACC GTG AC-3′) and rpoBMaqL (5′- ATG AAG GCG ATC TAC AGC GA-3′). To generate the calibration curves, each PCR product of the two above-mentioned gene fragments were cloned into pSpark® I vector (Canvax Biotech S.L.) and used to transform *Escherichia coli* DH5α. Individual clones were selected and confirmed by DNA sequencing. The corresponding pDNA was quantified 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). For each quantitative PCR (qPCR) run, 1 μL of each standard aliquot (centrin 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. qPCR was performed using SsoFast EvaGreen Supermix (Bio-Rad, Madrid, Spain), and run and detected in a MyiQ2 (Bio-Rad) detection system. The conditions used for qPCR were: initial denaturation at 98°C for 2 min and 40 cycles of 5 s of denaturation at 98°C, and 10 s of annealing and extension at 61°C. One milliliter of sample was used to extract gDNA using a standard phenol–chloroform extraction protocol (Sambrook, Frish and Maniatis 1989).

To validate cell number quantification by qPCR, gDNA of serially diluted *C. reinhardtii* and *M. aquaticum* cultures was isolated and used for single-copy gene quantification by qPCR. Each of the serially diluted cell cultures was also plated to quantify colony-forming units (CFUs) to confirm the results obtained by qPCR. CFU and qPCR quantifications of three biological replicates of three different dilutions of cell cultures resulted in an r^2 value of 0.992 for *C. reinhardtii* and 0.993 for *M. aquaticum* cultures.

Metabolite quantification

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). One milliliter of bacterial cultures was harvested by centrifugation and 100-μL samples of cell-free supernatants were transferred to flat-bottom 96-well 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 were included in every measurement and samples were diluted as needed.

Glycerol accumulation in the medium was analyzed by HPLC (Agilent series 1200, Agilent Technologies, Spain) using a previously established protocol (Jurado-Oller *et al.* 2015) involving 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.). A 100-μl volume of culture supernatant recovered after centrifugation was filtered (0.2 μm) and analyzed by high performance liquid chromatography (HPLC) using a Refractive Index Detector (Agilent G 1362A, Agilent Technologies).

Phylogenetic analysis

A *Methylobacterium* spp. tree was constructed with MEGA7 software (Kumar, Stecher and Tamura 2016) by using the Maximum Likelihood method with default settings. Sequences 500 bp long corresponding to the highly conserved UARR region of 16S rDNA were used to construct the tree.

RESULTS AND DISCUSSION

Methylobacterium complements the inability of *Chlamydomonas* to grow using peptides

We analysed *Chlamydomonas* growth on a number of dipeptides under axenic conditions that led to a typical N-starved phenotype and inability to grow. In the course of experimentation, we observed an unexpected 'pink' contamination of an L-Ala-Ala culture that enabled *Chlamydomonas* to grow efficiently. We proceeded to isolate this contaminant through repeated colony re-streaking and sequenced PCR-amplified product of the conserved UARR region of 16S rDNA from a single

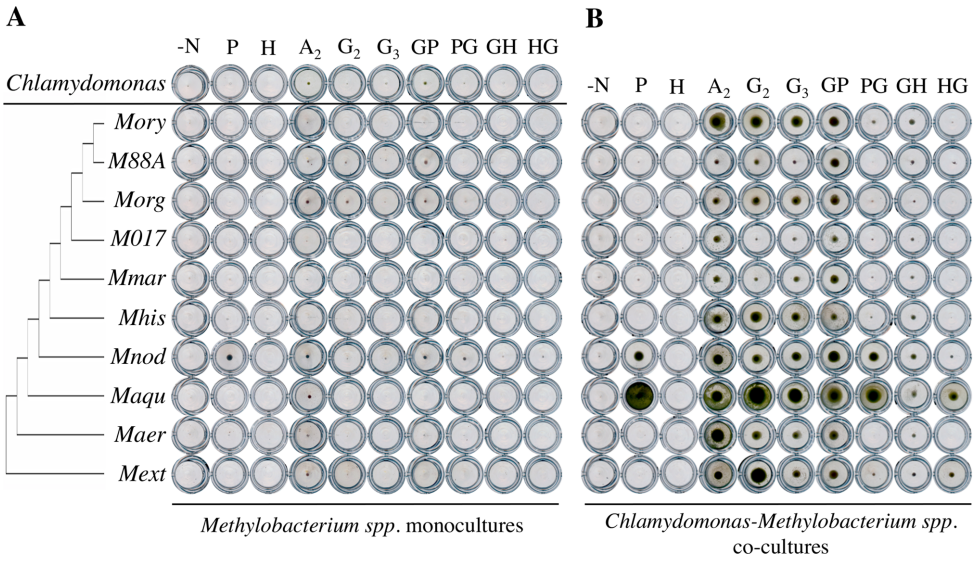

Figure 1. *Chlamydomonas* growth complementation by different *Methylobacterium* species. Maximum likelihood tree was performed using 16S rDNA sequences. *Chlamydomonas* and *Methylobacterium* species were grown on monocultures (**A**) and co-cultures (**B**) on 8 mM of the indicated L-amino acids or peptides as the sole added N and C source. –N, without any N source; P: L-proline; H, 4-hydroxy-L-proline; A_2: L-Ala-Ala; G_2, Gly-Gly; G_3, Gly-Gly-Gly; GP, Gly-Pro; PG, L-Pro-Gly; GH, Gly-Hyp; HP, L-Hyp-Gly. *Mory*, *M. oryzae*; *M88A*, *Methylobacterium* sp. 88A; *Morg*, *M. organophilum*; *M017*, *Methylobacterium* sp. isolated in this work (M017); *Mmar*, *M. marchantiae*; *Mhis*, *M. hispanicum*; *Mnod*, *M. nodulans*; *Maqu*, *M. aquaticum*; *Maer*, *M. aerolatum*; *Mext*, *M. extorquens*. Liquid cultures on V-bottom 96-well plates were centrifuged to pellet the cells prior to imaging, after 8 days for amino acids and 12 days for peptides. Green spots correspond to pelleted algal growth.

colony. This sequence showed a 99% identity with *Methylobacterium* spp. (*M. marchantiae* and *M. bullatum*).

Methylobacterium species are considered plant growth-promoting bacteria (PGPB), so it was not entirely surprising that these bacteria might also interact with *Chlamydomonas*, a green alga with lineage ancestral to the land plants (Lewis and McCourt 2004). We explored the potential of *Chlamydomonas* to interact with *Methylobacterium* spp. in relation to the use of amino acids and dipeptides. Specifically, we tested 10 different species of *Methylobacterium* covering the main branches of the methylobacterial phylogeny (Fig. 1; Dourado *et al.* 2015) in their ability to complement *Chlamydomonas*'s lack of growth on dipeptides as a sole N source. We used the dipeptides L-Ala-Ala, Gly-Gly, L-Gly-Pro, L-Pro-Gly, L-Gly-Hyp, L-Hyp-Gly, and the tripeptide L-Gly-Gly-Gly (Fig. 1), along with the amino acids L-Pro and L-Hyp, which have been shown not to be deaminated by CrLAO1 (Vallon *et al.* 1993). These peptides were chosen in particular because they do not appear to support metabolic activity in *Chlamydomonas* (Chaiboonchoe *et al.* 2014). *Chlamydomonas* growth was not observed with any of these N sources when grown alone (Fig. 1A). In contrast, co-culturing with different *Methylobacterium* species supported *Chlamydomonas* growth with three major patterns observed (Fig. 1B). First, complementation specifically by *M. nodulans* and *M. aquaticum* allowed algal growth on N sources such as L-Pro and L-Pro-Gly. Second, all *Methylobacterium* species tested led to algal growth in N sources like the dipeptides L-Ala-Ala and Gly-Pro. Third, lack of growth on L-Hyp could not be complemented by any of the *Methylobacterium* species assayed (Fig. 1B).

A *Chlamydomonas*–*Methylobacterium* obligate mutualism underlying growth on L-Pro

Both *M. aquaticum* and *M. nodulans* are phylogenetically closely related and were able to complement *Chlamydomonas* growth on L-Pro (Fig. 1; Dourado *et al.* 2015). *Methylobacterium nodulans* is a N_2-fixing organism (Jourand *et al.* 2004) and was able to grow alone with L-Pro and a sole N source. In contrast, *M. aquaticum* was unable to grow on L-Pro alone (Fig. 1A). We found that co-culturing with *Chlamydomonas* permitted both *M. aquaticum* and *Chlamydomonas* to grow on L-Pro as a sole N source in a mutually dependent fashion. Using qPCR-based cell quantification, we did not detect cell growth in either algal or bacterial monocultures, while growth was observed for both cell types in co-culture (Fig. 2A and B). When observed under the microscope, aggregates of *M. aquaticum* were observed as previously reported (Gallego, García and Ventosa 2005); in co-cultures, *Chlamydomonas* cells appear to be embedded and attached to these bacterial aggregates (Fig. 2C and D). This physical association likely facilitates the exchange of metabolites between both organisms.

Chlamydomonas can fix CO_2 under autotrophic conditions so N is expected to generally be the limiting nutrient for algal growth. We hypothesized that *M. aquaticum* might metabolize L-Pro and produce ammonium to sustain *Chlamydomonas* growth in co-culture. We incubated *M. aquaticum* cells on L-Pro as sole N or C source for 48 h and quantified ammonium in the supernatant; we indeed found that ammonium was produced by *M. aquaticum* and accumulated in the culture medium (Fig. 3A). We then asked whether *Chlamydomonas* might provide some reciprocal benefits, specifically, a source of carbon that might be exuded during photosynthetic growth. Glycerol plays a key role in coral nutrition and growth during symbiosis with dinoflagellates, and is the major end product of fixed CO_2 that these algae provide to the coral host (Muscatine 1967; Suescún-Bolívar, Iglesias-Prieto and Thomé 2012). Under stressful conditions (e.g. osmotic stress), *Chlamydomonas* is known to produce glycerol and release it into the media (Husic and Tolbert 1986; León and Galván 1994). Thus, we hypothesized that *Chlamydomonas* might release glycerol under our culture conditions, and analyzed the supernatants from *Chlamydomonas* cells incubated in L-Pro. As expected, glycerol was detected after 24 h in the supernatants

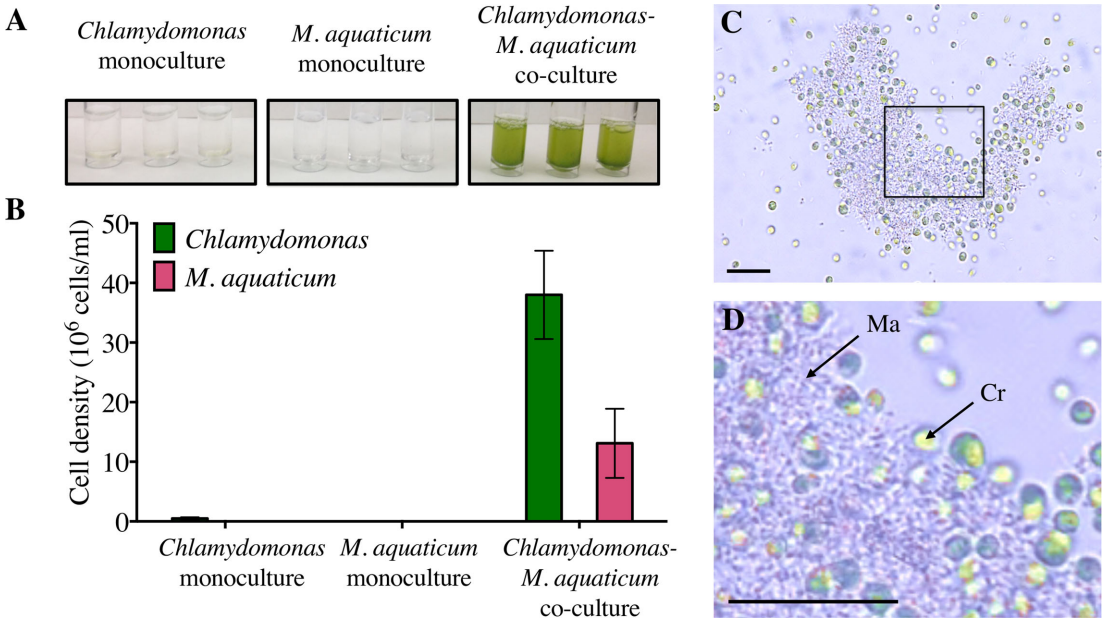

Figure 2. *Chlamydomonas* and *Methylobacterium aquaticum* growth on L-proline. (**A**) Growth of *Chlamydomonas* and *M. aquaticum* monocultures and co-cultures on L-Pro after 11 days. The initial optical density was A_{600} 0.002 for the bacteria and A_{750} 0.025 for the alga. (**B**) Cell quantification by qPCR from cultures in (A). (**C**) Light microscope image of *Chlamydomonas–M. aquaticum* co-culture in (A). ×40 magnification (C), and zoomed image (**D**) corresponding to the black framed area. Colourless aggregates correspond to bacterial cells (Ma) and rounded green cells stuck to the aggregates correspond to algal cells (Cr). Scale bars correspond to 50 μm.

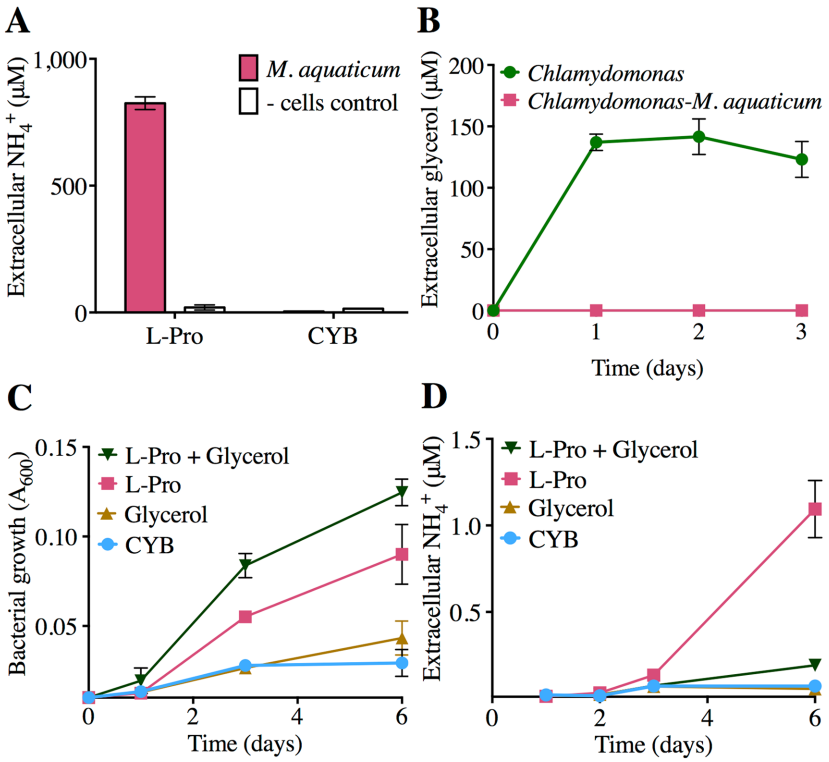

Figure 3. Ammonium and glycerol exchange between *Chlamydomonas* and *M. aquaticum*. (**A**) Ammonium was determined in the supernatant of axenically grown *M. aquaticum* after 48 h on 8 mM L-Pro, and without any added N. Control without cells to account for any ammonium generated by spontaneous L-Pro degradation (– cells control). (**B**) Glycerol quantification from supernatants of *Chlamydomonas* and *Chlamydomonas–M. aquaticum* cultures on L-Pro. (**C**) Effect of glycerol (8 mM) supplementation to *M. aquaticum* growth on L-Pro (8 mM). Initial cell density was A_{600} 0.01. (**D**) Ammonium quantification from the cultures in (C).

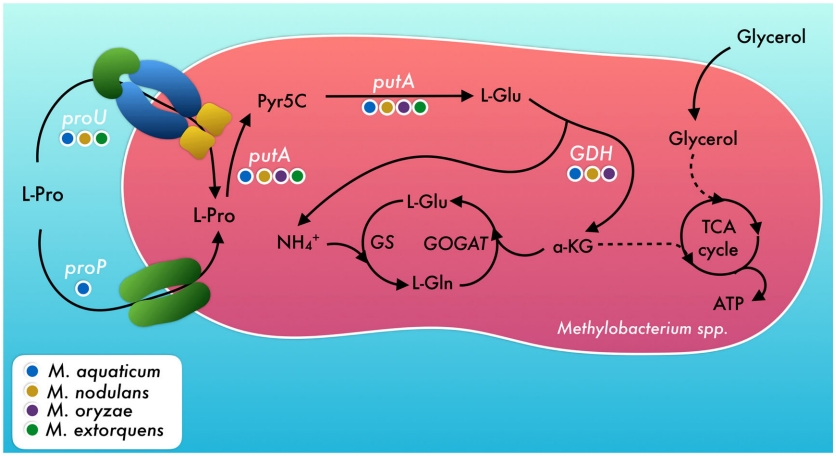

Figure 4. Putative L-proline utilization pathways by *Methylobacterium* species. Putative genes coding for each enzyme are listed in white. *proU*, glycine betaine/proline ABC transporter; *proP*, proline permease (MFS transporter); *putA*, bifunctional proline dehydrogenase; Pyr5C, Δ^1-pyrroline-5-carboxylate; *GDH*, glutamate dehydrogenase; *GS*, glutamine synthetase; *GOGAT*, glutamine oxoglutarate aminotransferase; TCA, tricarboxylic acids. Coloured dots correspond to orthologous genes found on each indicated *Methylobacterium* spp. genome by a reciprocal BLAST hit approach.

of *Chlamydomonas* cultures supplemented with L-Pro (Fig. 3B). In contrast, no glycerol accumulated in the media in co-cultures, consistent with the fact that methylobacteria can metabolize glycerol (Gallego, García and Ventosa 2005).

Our data show that L-Pro was not used efficiently and that it may not be a stoichiometrically balanced source of carbon and nitrogen for *M. aquaticum*. For example, *M. aquaticum* growth on L-Pro led to a curious release of ammonium into the media. We asked whether extra carbon input, in the form of glycerol, could balance the carbon:nitrogen nutrient ratio leading to efficient *M. aquaticum* growth on L-Pro. Indeed, glycerol promoted bacterial growth on L-Pro (Fig. 3C) and also limited ammonium excretion (Fig. 3D).

Methylobacterium genome analysis for L-Pro metabolism

To understand the complementation patterns of different *Methylobacterium* co-cultured with *Chlamydomonas*, we analyzed the (four) available *Methylobacterium* genome sequences: *M. aquaticum* and *M. nodulans*, which complemented *Chlamydomonas* growth on L-Pro and L-Pro-Gly, and *M. oryzae* and *M. extorquens*, which did not (Fig. 1). We focused on the set of putative genes involved in the L-Pro assimilation and identified through reciprocal BLAST analysis with well-annotated genes from *E. coli* and *Serratia marcescens* (Fig. 4 and Table S1 in the online supplementary material). In bacteria, L-Pro can be imported into the cell through different transport systems proP, proU and putP (Culham *et al.* 1993). Putative genes coding for SSS transporters (sodium solute symporters) were present in all four genomes, but no *putP* orthologues were found. The putative *proU* ABC transporter was found in all species but *M. oryzae*. A putative *proP* major facilitator superfamily (MFS) transporter was only found in *M. aquaticum* (Fig. 4).

Once inside the cell, L-Pro is oxidized to glutamate in a two-step process carried out by one single enzyme encoded by the gene *putA* (Tanner 2008) (Fig. 4). Glutamate can be then oxidized by glutamate dehydrogenase generating ammonium and α-ketoglutarate that feeds into the central glutamine synthetase-glutamine oxoglutarate aminotransferase (GS-GOGAT) cycle. The α-ketoglutarate also feeds into the tricarboxylic acids cycle (TCA) for energy production. A putative orthologue for *putA* was found in all four *Methylobacterium* species. Therefore, the presence of the L-Pro transporter *proP* could explain *M. aquaticum*'s specific ability to complement *Chlamydomonas*'s growth on L-Pro relative to the other *Methylobacterium* spp. examined. The *M. aquaticum* genome also contains two genes coding for putative prolyl peptidases that support catabolism of dipeptides (e.g. prolyl-glycine). The evidence for prolyl peptidase genes existing in the other *Methylobacterium* genomes, however, was not conclusive.

From our gene inventory analysis, the metabolism of L-Pro by *M. aquaticum* is expected to generate α-ketoglutarate and ammonium. Since ammonium is released into the medium, we speculate that α-ketoglutarate shunted to the TCA cycle may result in a carbon:nitrogen imbalance vis-à-vis the GS-GOGAT cycle and ammonium assimilation. Supplementation with glycerol carbon backbones, which also feeds into the TCA cycle, may restore balance and permit L-proline-derived α-ketoglutarate to be directed towards nitrogen assimilation instead.

A carbon–nitrogen metabolic circuit between *Chlamydomonas* and *Methylobacterium*

A carbon-for-nitrogen metabolic exchange circuit has been reported for *Chlamydomonas* and fungi (Hom and Murray 2014). Under those culturing conditions, the fungus metabolizes glucose to produce CO_2, which serves as a carbon source for the alga, while the alga reduces NO_2 to NH_3 as a source of N for the fungus. In the system described here, the flow of carbon and nitrogen to and from *Chlamydomonas* is reversed: *Methylobacterium* provides N (NH_4^+) to *Chlamydomonas* (from L-Pro or dipeptides), while *Chlamydomonas* provides carbon (glycerol) to the bacteria (Fig. 5). This flow of carbon and nitrogen relative to the alga is also commonly observed in lichens (Seneviratne and Indrasena 2006), the dinoflagellate–coral symbioses (Muscatine 1967; Suescún-Bolívar, Iglesias-Prieto and Thomé 2012) and bacteria–plant symbioses (Vance *et al.* 1998).

Although glycerol was the only form of carbon that we detected by HPLC under our conditions (i.e. continuous light and aerobiosis), *Chlamydomonas* can also release other small carbon compounds (Moroney, Wilson and Tolbert 1986; Mus *et al.*

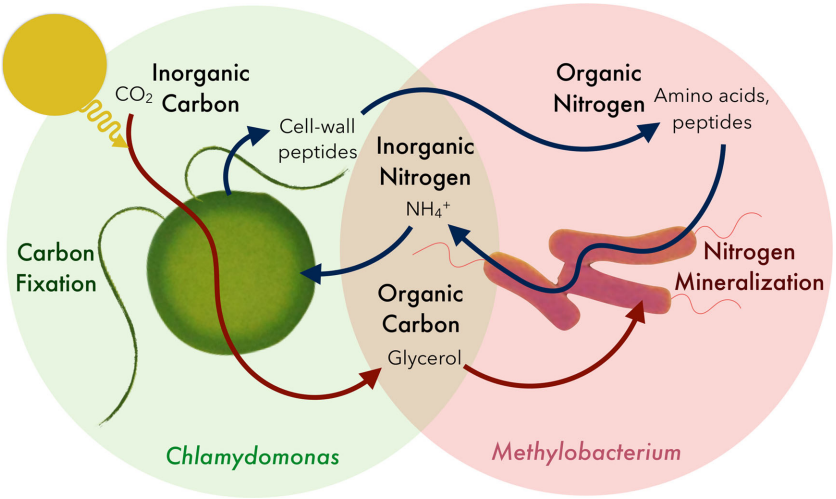

Figure 5. Hypothesized carbon–nitrogen metabolic exchange circuit between *Chlamydomonas* and *Methylobacterium* spp. *Chlamydomonas* may provide *Methylobacterium* spp. organic carbon sources that are by-products of photosynthesis (e.g. glycerol). The cell wall composition of the alga, rich in glycoproteins containing glycine, proline and hydroxyproline, may serve to attract associations with *Methylobacterium* spp. In turn, *Methylobacterium* spp. can transform the Gly-, Pro-, Hyp-containing peptides (which *Chlamydomonas* cannot use) into a bioavailable N (e.g. ammonium). This carbon–nitrogen metabolic exchange could establish a mutualistic interaction between *Chlamydomonas* and *Methylobacterium* spp.

2007) that could in theory feed methylobacteria (Green 1992). For example, this alga can excrete formate, acetate and ethanol in the dark (Mus *et al.* 2007). During normal light/dark cycles, different carbon sources could be released by the alga, leading to different temporally phased nutrient exchanges with bacteria in natural contexts.

The aggregation of *Chlamydomonas* and methylobacteria (Fig. 2D) raises the question of whether this might be chemotactically driven: are there molecular signals or motifs that would attract *Methylobacterium* to *Chlamydomonas*? Plant–methylobacterial interactions have been understood based on properties of the plant cell wall, which is composed of a complex cellulose–xyloglucan network. During plant cell growth, methyl-esterified pectins in the cell wall are de-methyl-esterified by pectin methylesterases, which releases methanol and is a primary carbon source for *Methylobacterium* spp. In contrast, the *Chlamydomonas* cell wall does not contain cellulose but is composed of crystalline glycoproteins, predominantly hydroxyproline-rich glycoproteins (Imam *et al.* 1985; Goodenough *et al.* 1986; Adair *et al.* 1987). Similarly to plant cell walls, however, turnover of this cell wall framework takes place during *Chlamydomonas* growth and proline- and hydroxyproline-rich polypeptide fragments are released into the culture medium (Voigt 1985, 1986). We hypothesize that methylobacteria may take advantage of these peptide by-products of *Chlamydomonas* cell growth, and that this serves as the incipient basis for mutualistic carbon and nitrogen exchange.

Our newly described mutualism between *Chlamydomonas* and *Methylobacterium* may be a useful model for understanding algal–bacterial interactions for further synthetic ecology studies of biotechnological relevance (Hom *et al.* 2015). Moreover, given the simplicity and experimental tractability, this new algal–methylobacterial system may be a useful model for understanding the evolution of plant–PGPB associations and elucidating plant–PGPB signalling.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSLE* online.

ACKNOWLEDGEMENT

We thank Michael Clear for designing the centrin primers used in this study, and Hamilton Moore and Aitor Gómez for assistance with experiments.

FUNDING

This work was funded by MINECO (Grant BFU2015- 70649-P), the European FEDER program, Junta de Andalucía (BIO-502), the Plan Propio de la Universidad de Córdoba, the U.E.INTERREG VA POCTEP-055_ALGARED_PLUS5_E, and by a travel grant (*Beca de Movilidad Internacional de la Universidad de Córdoba*) to VC to pursue aspects of this work in the lab of EFYH.

Conflict of interest. None declared.

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