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ORIGINAL ARTICLE

Metabolic analysis of *Chlorobium chlorochromatii* CaD3 reveals clues of the symbiosis in '*Chlorochromatium aggregatum*'.

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A symbiotic association occurs in '*Chlorochromatium aggregatum*', a phototrophic consortium integrated by two species of phylogenetically distant bacteria composed by the green-sulfur *Chlorobium chlorochromatii* CaD3 epibiont that surrounds a central β-proteobacterium. The non-motile *chlorobia* can perform nitrogen and carbon fixation, using sulfide as electron donors for anoxygenic photosynthesis. The consortium can move due to the flagella present in the central β-protobacterium. Although *Chl. chlorochromatii* CaD3 is never found as free-living bacteria in nature, previous transcriptomic and proteomic studies have revealed that there are differential transcription patterns between the symbiotic and free-living status of *Chl. chlorocromatii* CaD3 when grown in laboratory conditions. The differences occur mainly in genes encoding the enzymatic reactions involved in nitrogen and amino acid metabolism. We performed a metabolic reconstruction of *Chl. chlorochromatii* CaD3 and an *in silico* analysis of its amino acid metabolism using an elementary flux modes approach (EFM). Our study suggests that in symbiosis, *Chl. chlorochromatii* CaD3 is under limited nitrogen conditions where the GS/GOGAT (glutamine synthetase/glutamate synthetase) pathway is actively assimilating ammonia obtained via N₂ fixation. In contrast, when free-living, *Chl. chlorochromatii* CaD3 is in a condition of nitrogen excess and ammonia is assimilated by the alanine dehydrogenase (AlaDH) pathway. We postulate that '*Chlorochromatium aggregatum*' originated from a parasitic interaction where the N₂ fixation capacity of the *chlorobia* would be enhanced by injection of 2-oxoglutarate from the β-proteobacterium via the periplasm. This consortium would have the advantage of motility, which is fundamental to a phototrophic bacterium, and the syntrophy of nitrogen and carbon sources.

Introduction

Symbiosis is widespread in the microbial world (Moya *et al.*, 2008). Symbiotic interactions can fluctuate between parasitism and mutualism through time (Toft and Andersson, 2010). These interactions are a source of evolutionary innovation through genetic rearrangements that give rise to metabolic capabilities and emergence of syntrophy to exploit resources, stabilizing in a mutualistic relationship (Margulis and Fester, 1991; Moya *et al.*, 2008; Moya and Peretó, 2011).

The phototrophic consortium '*Chlorochromatium aggregatum*' is a model of symbiotic interactions involving prokaryotes. It was isolated from Lake Dagow in Germany (Fröstl and Overmann, 1998) and is formed by green-sulfur bacteria *Chlorobium chlorochromatii* (epibionts) that surround a central, colorless and motile β-proteobacterium, phylogenetically related to Comamonadaceae (Kanzler *et al.*, 2005). *Chl. chlorochromatii* CaD3 belong to the *Chlorobia* phylum. They are Gram-negative, non-motile, anaerobic photosynthetic and diazotrophic bacteria. These bacteria perform anoxic photosynthesis in the chlorosoma, which contains a photosystem I (PSI) reaction center. Their major photosynthetic pigment is bacteriochlorophyll c, and sulfide is used as electron donor. CO₂ is fixed via the reverse tricarboxylic acid cycle (rTCA) (Vogl *et al.*, 2006). In natural conditions they are exclusively found in symbiosis although they can be

cultured without their symbiont, which is not the case for the obligate-symbiotic β-proteobacterium (Müller and Overmann, 2011). Previous studies have identified genes that code for virulence factors in *Chl. chlorochromatii* CaD3 that seem to be relevant in the symbiosis, one of them seems to have been acquired by horizontal gene transfer (Vogl *et al.*, 2008).

A recent study compared the proteome and gene expression of symbiotic and free-living states in *Chl. chlorochromatii* CaD3 (Wenter *et al.*, 2010). It showed that ∼350 genes of *Chl. chlorochromatii* CaD3 have differential expression patterns between both states (Wenter *et al.*, 2010). A significant fraction of these correspond to genes involved in nitrogen and amino acid metabolism. Among these there were genes coding for a nitrogen regulator protein PII, a gene coding a glutamine synthetase (GS) enzyme and the *nifH* gene coding for nitrogenase reductase. These genes seem to be having a regulatory role in nitrogen assimilation between symbiotic and free-living states (Brown and Herbert, 1977; Magasanik, 1982; Forchhamer, 2004; Texeira *et al.*, 2010). In this study, we performed the metabolic network reconstruction of *Chl. chlorochromatii* CaD3 and an elementary flux mode (EFM) analysis (Schuster *et al.*, 1999; Jevremovic *et al.*, 2011) of the reconstructed amino-acid metabolism sub-network, to better understand the metabolic shift between symbiotic and free-living states.

Materials and methods

Genomes used
The following genomes were downloaded from the Genbank database: *Chlorobium chlorochromatii* CaD3 (NC_007514.1), *Chlorobium tepidum* TLS (NC_002932.3), *Chlorobium phaeobacteroides* BS1 (NC_010831.1), *Chlorobium phaeobacteroides* DSM 266 (NC_008639.1), *Chlorobium luteolum* DSM 273 (NC_007512.1), *Chlorobium phaeovibrioides* DSM 265 (NC_009337.1), *Chlorobium limicola* DSM 245 (NC_010803.1), *Pelodictyon phaeoclathratiforme* BU-1 (NC_011060.1), *Prosthecochloris aestuarii* DSM 271 (NC_011059.1), *Chloroherpeton thalassium* ATCC 35110 (NC_011026.1), *Chlorobaculum parvum* NCIB 8327 (NC_011027.1) and *Escherichia coli* K-12 (MG1655) (NC_000913.2).

Reconstruction of the metabolic capabilities of Chl. chlorochromatii CaD3
We first reconstructed the metabolism of *Chl. chlorochromatii* CaD3 with Pathway-Tools software (Karp *et al.*, 2010). The Pathway-Tools software inferred all metabolic pathways directly from the Genbank file. Thereby, we obtained a Pathway/ Genome Database (PGDB) to be manually curated. As *C. tepidum* is the most studied species of *chlorobia*, we also performed the automatic reconstruction of its metabolism with Pathway-Tools

software from its annotated genome downloaded from Genbank database (Eisen *et al.*, 2002). The automatic reconstruction was curated with overall experimental information, and metabolic function was assigned only if it could be confirmed in the literature. Then, the metabolic reconstruction of *C. tepidum* was used to cure the metabolic reconstruction of *Chl. chlorochromatii* CaD3 (see below).

To improve and curate the initial metabolic reconstruction we performed a search for orthologous proteins between *Chl. chlorochromatii* CaD3, *C. tepidum* and the other species of *Chlorobia* listed under 'Genomes used'. We also searched for orthologous proteins between the *Chlorobia* and *E. coli* and used BranchClust software to find orthologs between those genomes (Poptsova and Gogarten, 2007). We used the *E. coli* genome assuming that it is the best curated bacterial genome and is available through EcoCyc database (Keseler *et al.*, 2011). The genomes of the others species of *Chlorobia* were added to the analysis to improve the performance of BranchClust. For the 12 genomes included in this study, we asked BranchClust to consider as orthologs all monophyletic groups of sequences having at least eight different species represented. For those peptide sequences of *Chl. chlorochromatii* CaD3 that had no orthologs, we looked for conserved protein domains against the Pfam database (Finn *et al.*, 2010) with HMMER3 software (Johnson *et al.*, 2010) by using an *E*-value cutoff of 0.01. Based on the orthologous search with BranchClust and domain identification with Pfam, we assigned 382 enzymatic reactions to the metabolism of *Chl. chlorochromatii* CaD3 (Supplementary Material 1). These reactions were obtained from KEGG (Kanehisa *et al.*, 2012) and BRENDA databases (in their WWW available versions in 2011–2012) (Scheer *et al.*, 2011). Finally the reactions were integrated within YANA software (Schwarz *et al.*, 2005).

EFM analysis of symbiotic and free-living states
We performed an EFM analysis of the amino-acid metabolism in the symbiotic and free-living states of *Chl. chlorochromatii* CaD3. The EFM algorithm decomposes the metabolic network in EFMs, where each EFM is a minimal set of enzymes operating in steady state with all reactions proceeding in the direction dictated by its thermodynamic restrictions (for theoretical details, see Schuster *et al.*, 1999 and Wagner, 2004) being equivalent to a metabolic pathway that supports the network structure in a specific growth condition. The inferred metabolic network of the amino-acid metabolism consists of 99 enzymatic reactions from the whole reconstruction. These reactions were identified following the metabolic pathway classification of KEGG database. Then, based on the expression profiles published by Wenter *et al.* (2010), we removed with YANA software 5 and 10 reactions not transcribed in symbiotic and free-living states respectively, thus

generating two sub-networks: one with 94 reactions corresponding to the symbiotic state and the other with 89 reactions corresponding to the free-living state (see Supplementary Material 1, 2 and 3).

To identify the EFM of the symbiotic and free-living states, the two sub-networks in METATOOL file format (Schuster *et al.*, 1999; Supplementary Material 1) were analyzed with ElMo-comp-1.0.4 software (Jevremovic *et al.*, 2011). For each sub-network there were two output files: one containing the EFMs found, and the other containing the reactions of each EFM. These output files were analyzed with scripts in python v2.7 to see whether the EFMs in both states (free-living and symbiosis) were the same. We further analyzed the EFMs with ACoM-c software (Pérès *et al.*, 2011). This software groups the EFMs in motifs, which are clusters of EFMs that share at least three reactions. Motifs were visualized with Celldesigner (Funahashi *et al.*, 2007) (Supplementary Material 2). Finally, to prove that the obtained EFM reconstructions were independent of chance, we performed 100 random simulations of the symbiotic and free-living networks as follows: from the whole network of amino-acid metabolism containing 99 reactions, we randomly removed 5 and 10 reactions to simulate symbiotic and free-living states with 94 and 89 reactions each. We repeated this procedure 100 times for each simulated state. The 200 resulting networks were analyzed with ElMo-comp-1.0.4 and the EFMs obtained were compared against each other by a python script. All scripts are available upon request.

Results and discussion

EFM analysis of amino-acid metabolism in free-living and symbiotic states

The automatic metabolic reconstruction with Pathway-Tools software assigned approximately 1000 enzymatic reactions to *Chl. chlorochromatii* CaD3. From this initial reconstruction, only 382 reactions remained after manual curation (Supplementary Material 1). As mentioned above, several enzymes related to amino acid and nitrogen metabolism change their expression profile between free-living and symbiotic states (Wenter *et al.*, 2010). To better understand the metabolic changes related to both states, we selected those reactions belonging to amino acid metabolism for further EFM analysis. In brief, EFM analysis identifies all unique and non-decomposable steady state flux distributions in a metabolic pathway, which represents the metabolic potential of the network in a specific condition (Klamt and Stelling, 2002). The amino-acid metabolic networks of the free-living and symbiotic states contained 89 and 94 reactions, respectively. These networks overlap by 84 reactions.

Although in EFM analysis, the amount of EFMs normally increases according to the number of reactions (Klamt and Stelling, 2002), in this study the EFM analysis of amino-acid metabolism showed that the free-living state network containing 89 reactions has more EFMs (7069 EFMs), than the network in symbiotic state with 94 reactions (5075 EFMs). This is easily explainable because five of the reactions of the symbiotic state are consecutive in two different pathways (three in the biosynthesis of isoleucine and two in the biosynthesis of leucine). In contrast, in the simulated networks, the number of EFMs increased with the number of reactions. The random networks simulating the number of enzymes in the free-living state had a mean of 7500 EFMs, which approximated the 7069 EFMs obtained for the free-living state (Figure 1). In contrast, the random sampling simulating the symbiotic state had a mean of 12 446 EFMs, approximately two standard deviations ($\sigma = 4029.2$) higher than the EFMs obtained in the symbiotic network (5075 EFMs). These results suggest that there is a low probability of obtaining this number of EFMs in a random configuration of 94 reactions and that the symbiotic state is clearly constraining the structure of the metabolic network of amino acids in *Chl. Chlorochromatii* CaD3.
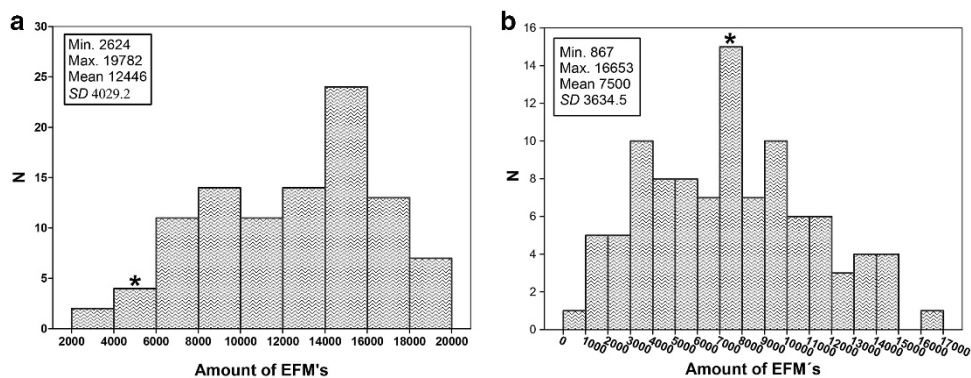

Figure 1 Histograms showing the distribution of EFMs in the randomly resampled networks of the symbiotic (**a**) and free-living (**b**) states of *Chl. chlorochromatii* CaD3. Asterisks indicate the bin on which the amount of EFMs obtained in symbiosis (**a**) and free-living (**b**) states would be located.

Experimental data showed that GS was present only in symbiosis (Wenter *et al.*, 2010). Accordingly, the motifs obtained with ACoM-c software showed that the EFMs were clearly different in hotspots of NH_3 assimilation between symbiotic and free-living states (Figure 2). In free-living state 5805 EFMs (82%) contained a reaction performed by alanine dehydrogenase (AlaDH) EC:1.4.1.1:

$$H_2O + L\text{-Alanine} + NAD^+ \rightleftarrows H^+ + NADH + NH_3 + Pyruvate$$

Whereas in symbiosis, 3438 EFMs (68%) contained a reaction performed by GS EC:6.3.1.2:

$$ATP + L\text{-Glutamate} + NH_3 \rightarrow ADP + L\text{-Glutamine} + Phosphate$$

The reaction performed by AlaDH in the symbiotic state was only present in 3258 EFMs (64%). Random simulations of the free-living and symbiotic states gave very different percentages of the number of EFMs containing the reaction performed by AlaDH (Table 1).

Is nitrogen assimilated via AlaDH in free-living state?
Previous work has shown that N_2 fixed to ammonia by the green-sulfur bacteria is assimilated via GS/GOGAT and the GDH (glutamine dehydrogenase) pathways (Brown and Herbert, 1977; Wahlund and Madigan, 1993). Both mechanisms are regulated by nitrogen availability. The GS/GOGAT pathway is a high-affinity mechanism that is active when there is low availability of nitrogen, and in the case of diazotrophic bacteria, when they are fixing nitrogen (Rudnick *et al.*, 1997; Schulz *et al.*, 2001). On the contrary, the assimilation of ammonia by GDH is a mechanism of low affinity that is active when there is high availability of nitrogen (Brown and Herbert, 1977; Rudnick *et al.*, 1997).

Unlike the other green-sulfur bacteria species, *Chl. chlorochromatii* CaD3 lacks GDH, and expresses GS only when growing in symbiotic state. How does *Chl. chlorochromatii* CaD3 assimilate nitrogen when growing in a free-living condition? One possibility is by the use of alanine dehydrogenase AlaDH (gene Cag_1878). The alanine dehydrogenase pathway is another N assimilation mechanism. This enzyme has aminase–deaminase activity and is active in conditions of high nitrogen availability. Although this role for AlaDH has not been identified in green-sulfur bacteria, it has been observed in diazotrophic-photosynthetic bacteria that lack GDH such as *Rhodopseudomonas capsulata* (Johansson and Gest, 1976), *R. acidophila* (Herbert *et al.*, 1978), *Anabaena cylindrica* (Rowell and Stewart, 1975), and in non-photosynthetic diazothophic bacteria such as *Bradyrhizobium sp.* (Allaway *et al.*, 2000). The increase of EFMs containing AlaDH in the metabolic network corresponding to the free-living state supports this possibility.
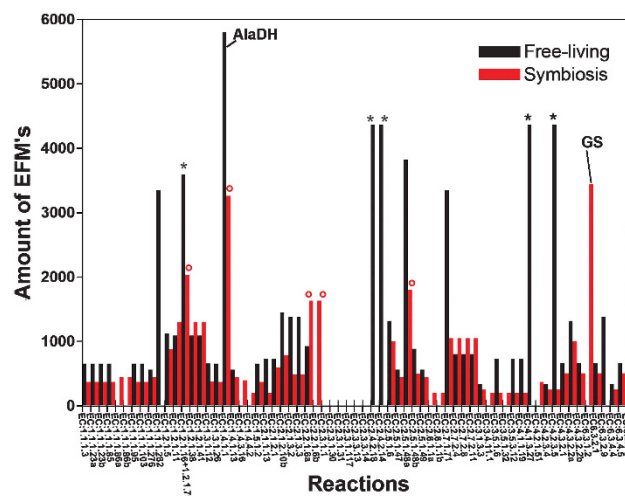

Figure 2 Distribution of EFMs in the metabolism of amino acids of *Chl. chlorocromatii* CaD3 in free-living and symbiotic states. Reactions that contain 1-0 EFMs were not included in this plot. The reactions represented by bars with circles are involved in EFMs of the GS and by bars with stars are involved in EFMs of the AlaDH pathways.

Table 1 Number of times the reactions performed by GS and AlaDH were found in free-living and symbiotic states

Enzyme	Symbiosis	Symbiosis (random)[a]	Free-living	Free-living (random)[a]
GS	3438 (68%)	42%	—	50%
AlaDH	3258 (64%)	90%	5805 (82%)	64%

[a]The percentage of the mean number of times the reaction was found in 100 random simulations of free-living and symbiotic states.

The EFMs analysis suggests that in symbiosis, ammonia is assimilated via the GS pathway (Figure 2). It is known that GS is a hotspot in nitrogen metabolism, distributing nitrogen in glutamine form to the whole metabolic network (Tyler, 1978; Reitzer, 2003). Likewise, there is a significant ratio of EFMs that goes through the AlaDH pathway. However, in free-living condition, GS is not present and AlaDH is present in a larger number of EFMs (Figure 2). These shifts in the mechanisms of nitrogen assimilation suggest that the availability of nitrogen in *Chl. chlorocromatii* CaD3 changes between symbiosis and free-living states. Wenter *et al.* (2010) and Overmann (2010) postulate that this bacterium in symbiosis is in a condition of low nitrogen availability, where the assimilation of ammonia becomes fundamental. The shift in the metabolic networks observed in our analysis supports this hypothesis. In symbiosis, the *chlorobia* seem to be in a condition of low nitrogen availability and the high-affinity mechanism is active; while in free-living state, where ammonia is available in high concentrations, the *chlorobia* use the low-affinity mechanism (Figure 3).
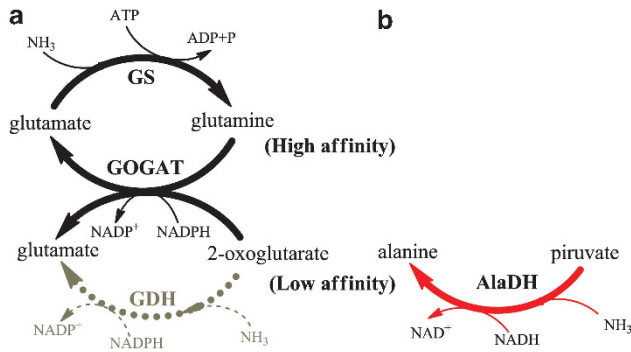
Figure 3 Mechanism of nitrogen assimilation of *Chl. chlorochromatii* CaD3. The GS/GOGAT and GDH pathways (modified from the study by Yan (2007)) characteristic of the green-sulfur bacteria are shown in (**a**); GS/GOGAT (black solid arrow) is assimilating nitrogen in symbiosis, however a gene coding for GDH (gray dashed arrow) is not present in *Chl. Chlorochromatii* CaD3, instead in (**b**) the AlaDH (red solid arrow) is assimilating nitrogen in free-living state.

Does 2-oxoglutarate mediate N₂ fixation in 'C. aggregatum'?

Several observations indicate that highly specific regulation mechanisms exist between *Chl. chlorochromatii* CaD3 and the central β-proteobacterium in 'C. aggregatum' (Müller and Overmann, 2011). For instance, in '*Pelochromatium roseum*', a closely related consortium, incorporation of 2-oxoglutarate by the central β-proteobacterium seems to be controlled by the physiological state of the *Chlorobia* (Glaeser and Overmann, 2003b). This is because incorporation of 2-oxoglutarate is strictly dependent on the simultaneous presence of light and sulfide, which is used by the epibiont as an electron donor in photosynthesis. Besides, addition of 2-oxoglutarate was necessary for the isolation of 'C. aggregatum', and this compound is pivotal to culture maintenance where the entire consortium 'C. aggregatum' seems to be unable to grow photo-lithoautotrophically or chemotrophically (Fröstl and Overmann, 1998). However, the nature of the molecules responsible for communication between both bacterial species is still unknown.

The PII protein, a regulatory protein of nitrogen metabolism, is exclusively expressed in symbiosis in *Chl. chlorochromatii* CaD3 (Wenter *et al.*, 2010). All PII-signaling proteins known so far, can bind up to three molecules of ATP/ADP and 2-oxoglutarate, thereby sensing the intracellular energy and carbon status (Ninfa and Jiang, 2005; Forchhammer 2008, 2010). PII proteins are also central in regulating processes related to nitrogen assimilation (Forchhammer 2010). In *E. coli*, PII signal protein GlnB is uridylynated (GlnB-UMP) by GlnD. This modification allows the sensing of the cellular nitrogen status directly by GlnD, assessed as the glutamine concentration (Forchhammer, 2007). Also in *E. coli*, PII protein GlnB controls the expression of nitrogen-regulated genes through the histidine kinase nitrogen regulation protein B (NtrB) and the

activity of GS through GS adenylyltransferase GlnE (Forchhammer 2008). The result is that high concentration of 2-oxoglutarate stimulates ammonia assimilation through GS (Ninfa and Jiang, 2005).

In cyanobacteria and methanogenic *Archaea* that have an incomplete TCA, because of the lack of 2-oxoglutarate dehydrogenase, the concentration of 2-oxoglutarate is the signal for PII proteins to sense the carbon/nitrogen status of the cell (Forchhammer 2008; Zhang and Zhao, 2008). This is because the cellular concentration of 2-oxoglutarate depends only on the rate of its formation and its consumption for ammonia assimilation, in most cases through the GS-GOGAT cycle. By contrast, in species with a complete TCA cycle, 2-oxoglutarate is an intermediate and its cellular concentration depends only partially on nitrogen assimilation. Therefore, measuring of 2-oxoglutarate concentration by PII indicates the level of available carbon skeletons for ammonia assimilation, and glutamine is used as a primary nitrogen status signal (Forchhammer 2007, 2008). *Chl. chlorochromatii* CaD3 has a complete reverse rTCA and 2-oxoglutarate is also an intermediate, and its concentration depends partially on the GS-GOGAT cycle.

As mentioned above, PII regulates GS in bacteria by a post-transcriptional modification via adenylation and deadenylation according to nitrogen availability (Magasanik, 1982; Rudnick *et al.*, 1997; Zhang *et al.*, 2005). In cyanobacteria, PII senses the concentration of 2-oxoglutarate, determining its modified state (phosphorylated) in response to nitrogen availability (Forchhammer *et al.*, 1999; Zhang and Zhao, 2008). In a similar way, in *Rhodospirillum rubrum*, another phototrophic bacterium, the GS pathway reaches its higher ratio of deadenylation when 2-oxoglutarate is present, as the PII protein and 2-oxoglutarate form a complex to allow the deadenylation of GS. Further, 2-oxoglutarate is detected by the PII protein and stimulates ammonia assimilation and N₂ fixation (Forchhammer 2004; Jonsson *et al.*, 2007). In *Azotobacter vinelandii*, 2-oxoglutarate stimulates the transcription of *nifA*, which is the master regulator of the *nif* operon, containing all genes involved in N₂ fixation (Dixon and Kahn, 2004).

As reviewed above, the sensing by PII of a high concentration of 2-oxoglutarate stimulates N₂ fixation and assimilation through the GS-GOGAT cycle. Could it be possible for the β-proteobacterium to use 2-oxoglutarate to stimulate nitrogen assimilation by the epibiont? This hypothesis is attractive because it links previous knowledge on the mechanisms of regulation of N₂ fixation and assimilation, with the observation that proteins involved in nitrogen metabolism, like PII and GS, are only expressed in symbiotic state. It also links the chemotactic behavior of 'C. aggregatum' towards 2-oxoglutarate with N₂ fixation and assimilation. However, this hypothesis has to overcome some observations:

First, the membranes of phototrophic bacteria are impermeable to 2-oxoglutarate, this allows a fine

regulation of this important regulator and effector in the assimilation of ammonia and N₂ fixation (Vásquez-Bermúdez *et al.*, 2000; Texeira *et al.*, 2010). In addition, none of the known species of green sulfur bacteria utilize 2-oxoglutarate for growth (Glaeser and Overmann, 2003b). *Chl. chlorochromatii* CaD3 is not the exception; it cannot assimilate 2-oxoglutarate when free-living (Vogl *et al.*, 2006). This limitation could be overcome if there is a transporter protein for 2-oxoglutarate that only expresses in the symbiotic state. *Chl. Chlorochromatii* CaD3 codes indeed for a homolog of the 2-oxoglutarate transporter in *E. coli*. The protein coded by the gene Cag_1339 is expressed only in the symbiotic state (Wenter *et al.*, 2010) and is homologous to five transporter proteins in *E. coli*, among these the 2-oxoglutarate transporter (gene *kgtP*). However, its best reciprocal hit in the *E. coli* genome is another predicted transporter coded by the gene *yhjE*. Therefore, it is not clear if Cag_1339 could function as a 2-oxoglutarate transporter in *Chl. chlorochromatii* CaD3.

Secondly, 2-oxoglutarate has to travel from the β-proteobacterium to the epibiont. Ultrastructural characterizations of '*C. aggregatum*' suggest that there is a common periplasmic space between both bacteria (Wanner *et al.*, 2008). This would facilitate the diffusion of 2-oxoglutarate from the central bacterium to the *chlorobia*. However, recent experiments suggest that the proposed periplasmic space is not a place where molecules can freely diffuse (Müller and Overmann, 2011). Anyway, it has been shown that *Chl. chlorochromatii* CaD3 when free-living, excretes a variety of amino acids and sugars (Pfannes, 2007), and according to the proposed role of the epibiont in '*C. aggregatum*', these molecules have to reach the β-proteobacterium, likely through the periplasmic space.

Although *Chl. chlorochromatii* CaD3 does not assimilate 2-oxoglutarate when free-living, '*C. aggregatum*' shows chemotactic behavior towards 2-oxoglutarate (Fröstl and Overmann, 1998). Several lines of evidence suggest that the β-proteobacterium and not the *chlorobia*, assimilates this metabolite from the medium (Fröstl and Overmann, 1998; Overmann *et al.*, 1998; Glaeser and Overmann, 2003b; Vogl *et al.*, 2006). However, it is not known whether the central bacterium transfers organic carbon to the epibiont (Müller and Overmann, 2011). Previous studies analyzed the $\Delta^{13}C$ ratio (that is, the difference between the $\delta^{13}C$ values of biomarkers and the $\delta^{13}C$ of ambient) to elucidate the carbon source used by the epibionts in '*P. roseum*' (Glaeser and Overmann, 2003a). It was found that the $\Delta^{13}C$ ratio of the *chlorobia* in '*P. roseum*' is consistent with photoautotrophic growth (Glaeser and Overmann, 2003a). As *Chl. chlorochromatii* CaD3 has a complete rTCA, 2-oxoglutarate can be used to build piruvate in addition to glutamine (Madigan *et al.*, 2009). Therefore, if 2-oxoglutarate enters the *chlorobia* from the β-proteobacterium it would end up forming biomass, contradicting the $\Delta^{13}C$ ratio observations in '*P. roseum*', but in accordance with the fact that the entire consortium requires of 2-oxoglutarate supplementation to grow (Fröstl and Overmann, 1998).

If the theoretical difficulties described above can be overcome, we propose that the β-proteobacterium diffuses 2-oxoglutarate to the *Chlorobia* via their shared periplasm (Wanner *et al.*, 2008). Figure 4 summarizes what this study proposes, where in free-living state *Chl. chlorochromatii* CaD3 can assimilate ammonia via the AlaDH, where ammonia is a low-affinity substrate, and perhaps also by the GOGAT pathway because this enzyme can use ammonia instead of glutamine as a low-affinity
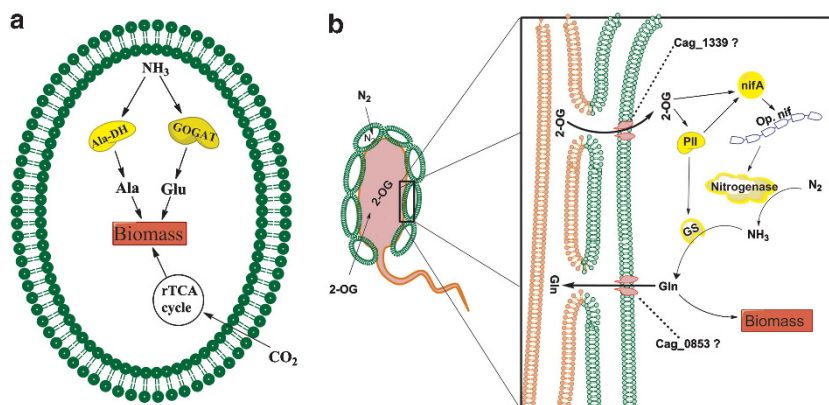

Figure 4 Diagram showing the proposed mode of nitrogen assimilation in symbiosis and free-living states. (**a**) *Chl. chlorochromatii* CaD3 in free-living state is assimilating ammonia by alanine dehydrogenase (AlaDH) and likely by glutamate synthetase (GOGAT) pathways. (**b**) *Chl. chlorochromatii* CaD3 in symbiotic state where 2-oxoglutarate (2-OG) is assimilated by the β-proteobacterium and diffused to *Chl. chlorochromatii* CaD3 through the shared periplasm that is, periplasmic tubules (Wanner *et al.*, 2008). 2-oxoglutarate interacts with the PII and NifA proteins, stimulating the expression of the *nif* operon (Op. nif) for N₂ fixation, and transformation of NH₃ to glutamine (Gln) by GS, a portion of which is transported to the β-proteobacterium. Cag_1339 is shown as the probable gene coding for a 2-oxoglutarate permease and Cag_0853 as probable gene coding for a glutamine transporter, both only expressed in symbiosis (Wenter *et al.*, 2010).

substrate (Mäntsälä and Zalkin, 1976; Adachi and Suzuki, 1977; Matsuoka and Kimura, 1986). In symbiosis, the increase in the intracellular concentration of 2-oxoglutarate produces a regulatory shift in nitrogen assimilation, stimulating the PII and NifA proteins and increasing GS activity. *Chl. chlorochromatii* CaD3 in symbiosis has high rates of N_2 fixation and GS activity (Wenter *et al.*, 2010). We postulate that this regulatory shift is induced by the 'injection' of 2-oxoglutarate by the β-proteobacterium, and part of the glutamine synthesized by *Chl. chlorochromatii* CaD3 is diffused to the β-proteobacterium through the periplasmic tubules. The above is supported by experimental evidence that shows the expression of an ABC transporter of amino acids by *Chl. chlorochromatii* CaD3 only in symbiosis coded by gene Cag_0853, and directly influenced by the β-proteobacterium (Wenter *et al.*, 2010).

Last but not the least, *Chl. chlorochromatii* CaD3 like cyanobacteria lack GlnD homologs (Arcondéguy *et al.*, 2001). However, differing from cyanobacteria that have only GlnB, *Chl. chlorochromatti* CaD3 has three PII proteins. One of them (Cag_1998) is coded next to an ammonium transporter, similar to GlnK in *E. coli*. The other two PII proteins are coded next to each other in an operon containing the *nifH* gene that codes for nitrogenase reductase. *Chl. chlorochromatii* CaD3 also lacks the gene *pphA* (sll1771 in *Synechocystis* sp. PCC 6803) responsible for the phosphorylation of PII in cyanobacteria (Forchhamer, 2004). Therefore, it is likely that regulation of nitrogen assimilation in the epibiont differs in the details from what has been described so far.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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