Biochimica et Biophysica Acta 1847 (2015) 495–504

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbabio

Review

Toward an understanding of the function of Chlamydiales in plastid endosymbiosis

Steven G. Ball *, Christophe Colleoni, Derifa Kadouche, Mathieu Ducatez, Maria-Cecilia Arias, Catherine Tirtiaux

Université des Sciences et Technologies de Lille, Unité de Glycobiologie Structurale et Fonctionnelle, Unité Mixte de Recherche 8576, Centre National de la Recherche Scientifique, Cité Scientifique, 59655 Villeneuve d'Ascq cedex, France

ARTICLE INFO

Article history:
Received 17 September 2014
Received in revised form 3 February 2015
Accepted 7 February 2015
Available online 14 February 2015

Keywords:
Plastid endosymbiosis
Chlamydiales
Cyanobacteria
Photosynthesis evolution

ABSTRACT

Plastid endosymbiosis defines a process through which a fully evolved cyanobacterial ancestor has transmitted to a eukaryotic phagotroph the hundreds of genes required to perform oxygenic photosynthesis, together with the membrane structures, and cellular compartment associated with this process. In this review, we will summarize the evidence pointing to an active role of Chlamydiales in metabolic integration of free living cyanobacteria, within the cytosol of the last common plant ancestor.

1. Introduction

Metabolic integration of established endosymbionts into novel organelles, such as the mitochondrion or plastids, defines events of the outmost rarity that have had far reaching consequences in the forging of the first eukaryotes and of all their photosynthetic derivatives. While the endosymbiont theory states that mitochondria and plastids derive from α-proteobacteria and cyanobacteria respectively, metabolic integration of these endosymbionts has evidently implied the massive participation of genes whose ancestry cannot be traced back to these two sole clades [1,2]. In particular, plastid endosymbiosis is known to correlate with a phylogenomic imprint from intracellular chlamydia pathogens specific and selective to all lineages derived from this unique event [3–10]. It has recently been shown that enzymes that are thought to have been responsible for photosynthetic carbon assimilation in the host cytosol, define metabolic effector proteins secreted by intracellular Chlamydiales pathogens ([10] highlighted by [11]; reviewed in [12]) the cytosol of their host. Hence the intracellular pathogens and incipient cyanobacterium were possibly tied together with their host in a tripartite symbiosis where the three partners coded essential components of a common photosynthetic carbon assimilation pathway [10]. This suggests that intracellular bacteria living as temperate pathogens or symbionts within eukaryotes may define major players down the path of metabolic integration of future organelles. Such intracellular bacteria are usually viewed as degenerate genomes that evolved from free living sister lineages by selective gene losses. (for review see [13]). However the intracellular lifestyle also implied the evolution of hundreds of protein effectors that ensures intracellular life either within phagocytosis derived vacuoles or more rarely in the cytosol. Because direct microinjection of free living bacteria in the eukaryotic cytosol, fails to yield any multiplication of the injected organisms unless they already define intracellular pathogens or symbionts [14], we believe that free-living cyanobacteria were driven into endosymbiosis thanks to helper intracellular symbionts. Recent work on the impact of chlamydia in plastid endosymbiosis has yielded an unexpectedly detailed molecular description of the early events that may have triggered plastid endosymbiosis, including the molecular nature of the symbiotic gene and the precise nature of the major carbon and ATP transporters involved. This speculative scenario is presently well sustained by a series of distinct phylogenetic and biochemical observations that, together, make a strong case for the implication of Chlamydiales in the initial steps of plastid endosymbiosis. In this review we will describe the evidence sustaining this hypothesis.

2. The chlamydial phylogenomic signal in the Archaeplastida genome

Chlamydiaceae, including genus Chlamydia and Chlamydophila are a family of obligate intracellular bacteria with a small size genome (<1 Mbp) that multiply in inclusion vesicles within the eukaryotic cytosol. Chlamydiae commonly infect animals, while related organisms from the order Chlamydiales, with a two to three-fold larger genomes, may infect a wider range of other eukaryotic phagotrophs. All Chlamydiales share a similar obligate intracellular life cycle (Fig. 1) consisting first of attachment of the infectious bacterium called the "elementary body" to an exposed membrane, followed by penetration through

* Corresponding author. Tel.: +33 320436543.
E-mail address: steven.ball@univ-lille1.fr (S.G. Ball).

http://dx.doi.org/10.1016/j.bbabio.2015.02.007
0005-2728/

endocytosis–phagocytosis, and then by modification of the endocytic vacuole to escape lysosomal digestion, and by active multiplication within this vacuole named the inclusion vesicle (for a review see [15]). This is followed by cell lysis or budding of the inclusion vesicles releasing novel infectious bacteria unable to replicate autonomously. In 1998, in the first genome description of a chlamydial intracellular pathogen infecting human cells (*Chlamydia trachomatis*), the authors surprisingly reported that a majority of the cases of LGT (lateral gene transfer), uniting Chlamydiae with eukaryotes, did not translate in the capture of genes from their animal target cells [3]. In fact, for some unknown reason, a majority of the 37 cases documented at that time united the pathogens with the green plants! This came as a total surprise since no extant plants are known to be susceptible to chlamydial infection, an observation which correlates with the requirement for exposed membranes in order to initiate infection. In their genome description, the authors proposed that the LGTs discovered in the *C. trachomatis* genome were ancient and dated back to the time when Chlamydial ancestors infected the amoebal ancestors of both the plant and animal lineage [3]. Because of their phagotrophic habit, such organisms were not covered by a continuous cell wall. This interpretation proved in part to be correct as the LGTs can indeed be traced back to over a billion years of evolution of these very ancient pathogens. However the assumed directionality of gene transfer, which was thought to consist of the capture of amoebal genes by the evolving pathogens, proved to be partly incorrect, as a majority (but by no means all) of these LGTs are now suspected to reflect the transfer of Chlamydial intracellular pathogen genes to the amoeba-like phagotroph that defines the common ancestor of plants, rather than the opposite. This common ancestor is the founder of the Archaeplastida, a group of eukaryotes containing the ancestor of all plastids (for review see [12]). Archaeplastida diversified into three major lineages: the glaucophytes, consisting of a small number of unicellular freshwater algae containing a peptidoglycan containing plastid called the muroplast; the Rhodophyceae, known as the red algae, a very diverse group of freshwater and marine unicellular and multicellular organisms, containing a rhodoplast, and the Chloroplastida, an equally diverse and complex set of marine and freshwater organisms harboring the chloroplast. Within the Chloroplastida, a particular lineage later established itself on land and gave birth to all "true plants". In 2007–2008, three distinct groups published that a specific chlamydial imprint could be evidenced in all Archaeplastida lineages [6–8]. The presence of at least a third of these Archaeplastida-specific LGTs in several of three (red algae, green algae and glaucophytes) genomes hinted that these LGTs happened in the common ancestor of the Archaeplastida. Because the common ancestor can be defined as the cell that resulted from plastid endosymbiosis, Peter Gogarten first proposed that the pathogens took an active role in metabolic integration of the protoplastid [6]. This hypothesis was also sustained by the two other studies [7,8] and by a more recent study that integrated the genomes of the major families of the order Chlamydiales [9]. It must be stressed that the phylogenomic signal, which is recovered by imposing a minimal bootstrap value of 70 in maximum likelihood phylogenies uniting Archaeplastida and chlamydial lineages at the exclusion of all other lineages, may not be powerful enough to distinguish issues of transfer directionality or to ascertain that the LGTs do relate to plastid endosymbiosis, especially when the LGTs are clade specific (that is when only one of the three Archaeplastida lineages is concerned). LGTs from bacteria are common in all eukaryotic lineages and we can predict a background of LGTs within the chlamydial phylogenomic signal which is most probably not related to plastid endosymbiosis. We presently estimate between 1/10 to at most 1/3 the number of LGTs within the chlamydial phylogenomic signal not related to plastid endosymbiosis [10]. This would leave us with a lower pessimistic and restrictive figure of 30 genes concerned with plastid endosymbiosis, and a maximum more optimistic figure of 50 genes. This figure is certainly dwarfed by the cyanobacterial phylogenomic signal which comes out one to two orders of magnitude stronger but it is nevertheless robust and only matched in plastid proteins by proteobacteria, as a whole, which define a far more prevalent and more diverse group of bacteria [1]. Phylogenomic approaches did not leave us any clues as to how and why this signal was generated at plastid endosymbiosis.

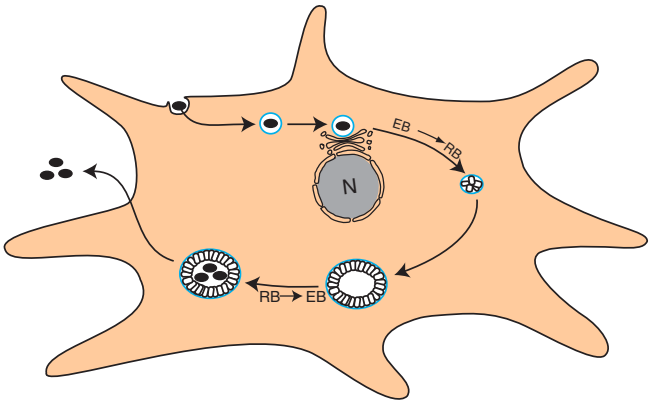

Fig. 1. Life cycle of chlamydiales pathogens. A small infectious chlamydia cell, called an elementary body (EB in solid black), interacts with the plasma membrane of an amoeba-like eukaryotic host. The endocytosis–phagocytosis vacuole is reprogrammed by the pathogen to become an inclusion vacuole thereby avoiding acidification and destruction. The EBs differentiate into actively multiplying reticulate bodies (RBs), which are thought to be attached to the inclusion vesicle through their TTS (type three secretion system, not displayed in this drawing). Some EBs detach from the inclusion vesicle, and redifferentiate into EBs. Progeny infectious EBs are released into the extracellular medium through lysis or fusion of the inclusion vesicle with the plasma membrane. EBs never divide in the extracellular medium.

3. Working out the plastid endosymbiosis symbiotic flux

Plastid endosymbiosis can be distinguished from mitochondrial endosymbiosis by a good knowledge of the setting and preexisting conditions. The nature of the host is universally accepted as being a standard heterotrophic and phagotrophic flagellate, while that of the future plastid was most certainly an ancestor of extant diazotrophic cyanobacteria. Because phagotrophy was observed in very early diverging prasinophyte green algae, we and many others reason that phagotrophy in the case of plastids defines a very obvious candidate mechanism for penetration of the plastid's ancestor into its eukaryotic host [16–18].

Such a good knowledge of the starting conditions certainly does not apply to mitochondrial endosymbiosis where the status of the host, and the entry mechanism remain obscure, further precluding inference of the very nature of the metabolic symbiosis that prompted this event [2]. Having accepted the phagotrophic and classical eukaryotic status of the host of plastid endosymbiosis, we are faced with a more restricted number of possibilities. Phagotrophy in all cases had to abort, and a symbiotic flux had to be installed between the two partners that give a selective advantage to this partnership. Because cyanobacteria are not reported to have the ability to live within eukaryotes, it is reasonable to assume that this unlikely partnership was selected because only cyanobacteria could provide the required metabolic traits. This of course leaves us with oxygenic photosynthesis, and to a lesser extent diazotrophy, as possible candidates for the installment of the symbiotic flux.

We have reviewed elsewhere the metabolic reasons explaining why maintenance of diazotrophy in a symbiont exporting photosynthetic carbon was metabolically impossible [19]. Briefly, ancestors of extant single-cell diazotrophic cyanobacteria, which we have hypothesized to define the plastid source [21], display a very tight circadian-clock regulation of cellular metabolism. Indeed Nitrogenase being equisitively

sensitive to oxygen, these organisms, that could not resort to physical separation of photosynthesis and nitrogen fixation, have evolved mechanisms confining diazotrophy to the dark phase. By uncoupling diazotrophy from oxygenic photosynthesis in time, these organisms had also to evolve processes that maximize energy storage for its delayed use at night by the costly process of Nitrogen fixation. These unicellular diazotrophic cyanobacteria indeed store larger amounts of carbohydrates in the light, than their nondiazotrophic sisters. In addition, in the dark, these very large amounts of carbohydrates are degraded through respiration thereby further consuming O_2, and decreasing its local concentration to the point where anoxia is reached and Nitrogenase can operate. In such a context, if we now imagine a cyanobiont exporting photosynthate rather than storing it, the latter would not have been able to reach anoxia through respiration of carbohydrate stores and would not have been able to supply the large amounts of reducing power and ATP required by Nitrogenase. The universal presence of phototrophy in Archaeplastida and the absence of diazotrophic eukaryotes argue for an early symbiosis relying on the export of photosynthetic carbon from the cyanobiont to its host. Most importantly the symbiotic flux defines the onset of plastid endosymbiosis: it had to be optimal and necessarily relied on the recruitment of preexisting components since there was no time to wait for the evolution of novel functions. How could this challenging agenda of plastid endosymbiosis be met?

Because we had a selective interest in starch metabolism (for a general review on starch metabolism see [20], we published in 2008 a study aimed at reconstructing the storage polysaccharide metabolism of the common ancestor of the Archaeplastida ([21] reviewed in [22]). This was done by working out a vertical inheritance model for the genes of storage polysaccharide metabolism, which in their majority, displayed a monophyletic origin, in agreement with the monophyletic nature of plastids and host. We ended up with a cytosolic pathway consisting of a complete set of eukaryotic glycogen metabolism enzymes with only three prokaryotic genes (Fig. 2): a cyanobacterial ADP-glucose pyrophosphorylase, located within the cyanobiont, responsible for the synthesis of the bacterial specific glycosyl nucleotide ADP-glucose and two non-cyanobacterial yet prokaryotic enzymes which were the ancestors of extant isoamylases (found in all Archaeplastida) and of the SSIII–IV starch synthases (found in green algae and plants and glaucophytes) ([21] reviewed in [22]). For storage polysaccharide metabolism to be active from ADP-glucose, the ancestor had to export this nucleotide-sugar to the cytosol. Evidence for an ancient host-

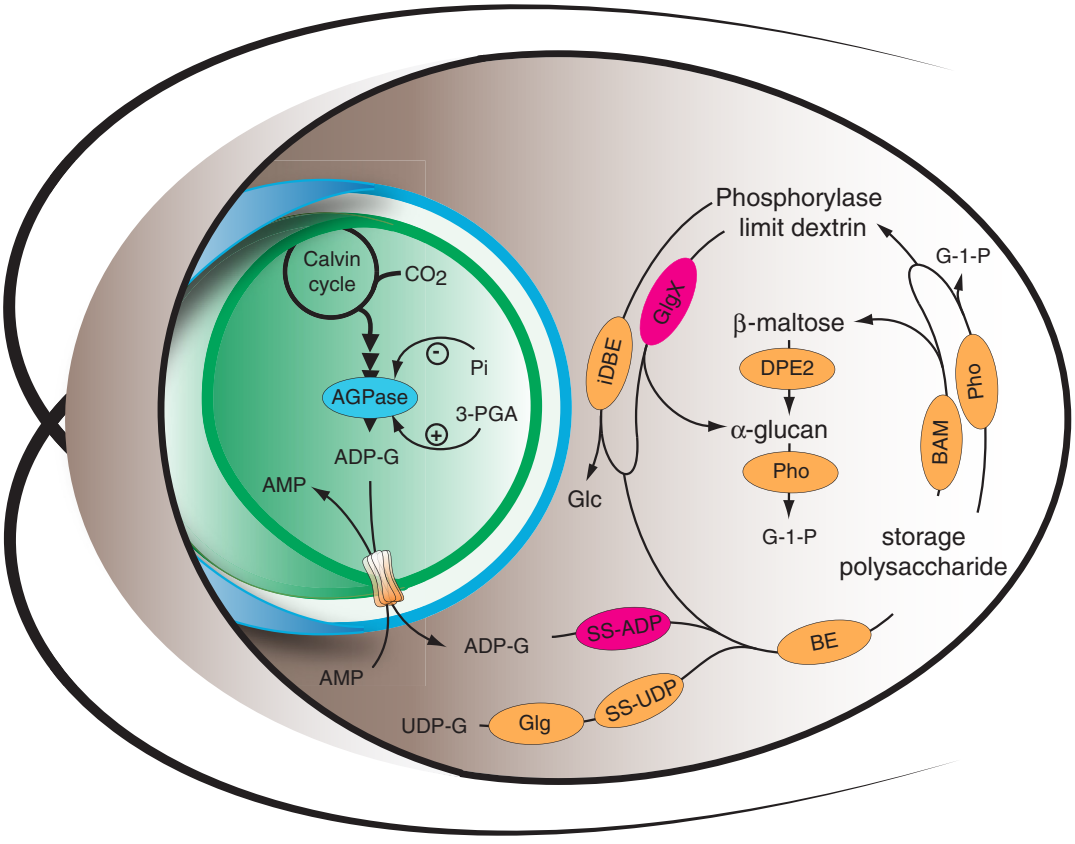

Fig. 2. Reconstruction of storage polysaccharide metabolism in the last Archaeplastida common ancestor. Red algae, glaucophytes and green algae display monophyletic phylogenies for most of storage polysaccharide metabolism. We have thus deduced the minimum number of genes and enzymes present in the Archaeplastida ancestors that suffice to explain their present distribution in extant organisms. A cytosolic localization similar to that evidenced today in red algae and glaucophytes is deduced for several reasons outlined in Ball et al. (2011) [22]. AGPase (ADP-glucose pyrophosphorylase) an enzyme of cyanobacterial origin (in blue) is proposed to synthesize the bacterial specific glycosyl-nucleotide ADP-Glc which is neither produced nor used by eukaryotes. The sugar nucleotide is thought to have been transported by an NST that originated from the host golgi (see text) where its normal function would have been to transport analogous purine sugar nucleotides (GDP-mannose for instance) [23]. Because the host enzymes (in orange) do not recognize it the ADP-Glc in the cytosol would have been incorporated into the host glycogen pool by a bacterial glucan synthase whose descendants is nowadays found in the glaucophyte cytosol and in green plants and algae. This glucan synthase can be considered together with the transporter as the symbiotic gene. A complete suite of eukaryotic glycogen metabolism is found in the cytosol of the host including an UDP-Glc specific glucan synthase (eukaryotes polymerize glycogen from UDP-Glc only) which together with the branching enzyme (BE) synthesizes glycogen. Glycogen is degraded through the actions of both β-amylase and glycogen phosphorylases that produce respectively β-maltose and glucose-1-P. However these enzymes cannot degrade the α-1,6 branch and therefore leave out glycogen whose outer chains have been digested (called Phosphorylase or β-limit dextrin). The limit dextrin must be debranched by "indirect debranching enzyme" (iDBE) which in eukaryotes produces glucose. Note the presence in this reconstruction of the bacterial GlgX protein which has a function similar to that of iDBE, but displays a different product, generating debranched oligosaccharides such as maltotetraose (displayed as α-glucan). α-glucans and maltose are metabolized by a combination of host amylomaltase and glycogen phosphorylase. Both the symbiogenic gene (SS-ADP) and GlgX are of chlamydial phylogenetic affiliation (displayed in purple).

derived nucleotide sugar transporter came subsequently, by demonstrating that the common ancestors of the extant plant plastidial carbon translocators that export photosynthetic carbon from the plastid to the cytosol are sisters to extant Golgi derived purine *N*ucleotide *S*ugar *T*ranslocators (referred to as Golgi purine NSTs), and that the eukaryotic ancestors still display an innate ability to translocate ADP-glucose efficiently [23]. Hence, such transporters may have preexisted in the host and may have been recruited to the protoplastid inner membrane at an early stage when the machinery of protein targeting to plastids had not yet appeared. Indeed Loddenkoetter et al. (1993) [24] have suggested that one of the extant plastidial carbon transporters deprived of its transit peptide displays the innate ability to reach the inner membrane of organelles such as the yeast mitochondria. In these experiments however the authors could not definitively distinguish between a mitochondrial or/and a possible rough ER localization of this protein [24].

Hence, all components of this ancient reconstructed carbon flux to storage polysaccharide preexisted in both partners of endosymbiosis, with the noticeable exception of the two non-cyanobacterial prokaryotic components. Upon examination of the flux generated by the proposed reconstruction, the biochemical logics of the connection become enlightening. The carbon that escapes the cyanobiont and is exported to the cytosol is only that part of cyanobacterial metabolism which was anyhow devoted to storage, as this defines the only outlet for ADP-glucose synthesis which is devoted to storage. Few penalities are thus expected in the light from such an escape. Upon arrival in the cytosol the only fate for this metabolite which is unrecognized by eukaryotic metabolism is to be incorporated into glycogen with little or no impact on the osmotic pressure of the host cytosol. Once within the host glycogen pool this carbon will be mobilized exclusively through host biochemical networks according to host needs. The system was thus immediately optimal It must be stressed however that, in darkness, the cyanobiont would have suffered from ATP depletion, because of the absence of carbon stores. It is indeed well known that cyanobacteria defective for glycogen accumulation require continuous light for growth [25,26]. Carbon export would have rendered the cyanobiont similar to such mutants. We will underline below how Chlamydiales have provided a function that obviates this problem in the form of the gene coding for the ATP translocator. We have outlined above, that Golgi purine NSTs (GDP-mannose translocators for instance) may have been recruited to import the analog substrate ADP-glucose at plastid endosymbiosis. However polymerization of photosynthetic carbon from ADP-glucose into the host glycogen pools still required the presence in the host cytosol of a non cyanobacterial yet prokaryotic glucan synthase (the ancestor of the SSIII–IV group), whose presence in the cytosol could not be explained. This glucan synthase gene defines the key to metabolic symbiosis and its phylogenetic origin must be addressed and understood.

4. Closing the loop: understanding both the chlamydial signal and the symbiotic flux

In 2012, it became clear to us that the prokaryotic gene that we proposed to have triggered the symbiotic flux of plastid endosymbiosis was a gene of chlamydial phylogeny [27]. It occurred to us that, if enzymes of glycogen metabolism defined virulence effectors secreted by the intracellular pathogens in the cytosol of their host, then the symbiotic flux will have initially depended on the coding of three genomes, thereby explaining both the origin of the required glucan synthase and the presence of a chlamydial phylogenetic signal in the Archaeplastida genomes. We tested this prediction, and found that most of the glycogen enzymes were indeed important virulence effectors secreted by the type three secretion systems of chlamydiae and not housekeeping genes as previously thought. [10] While the initial demonstration relied on the use of a semi-in vitro system involving a heterologous *Shigella* system [28], a full in vivo demonstration has been since provided for animal cells infected by Chlamydiae [29]. From the Chlamydiae's point of view, secretion of their enzymes in the host cytosol ensures massive glycogen synthesis at the beginning of their infection cycle at a time where cytosolic ATP and hexose phosphate are abundant (Fig. 3). Unregulated breakdown of the glycogen stores at later stages through chlamydial catabolic enzymes would have generated glucose-1-P and maltotetraose [10]. The latter defines a substrate that only bacteria can effectively metabolize. Quite interestingly, maltotetraose is generated through the bacterial GlgX gene effector product. GlgX encodes a direct glycogen debranching enzyme which does not exist in heterotrophic eukaryotes which use a different indirect mechanism of glycogen debranching (reviewed in [30]). It should be noted that isoamylase in all three archaeplastida lineages is phylogenetically derived from the chlamydial glgX enzyme ([10] reviewed in [30]). This observation renders the possibility of a coincidental nature of the presence of these two effector proteins in the storage polysaccharide pathway of Archaeplastida extremely remote. We believe this provides strong evidence for our hypothesis.

Because the chlamydial compartment did not, by contrast to the cyanobiont, carry out an essential biochemical function, lateral gene transfer to the host nucleus of the Chlamydial symbiotic gene would have rendered the pathogen that had previously contained the required gene dispensable to the partnership. Hence the chlamydial partner would have been maintained as long as it evolved a minimum of one useful effector to the tripartite symbiosis, thereby accelerating metabolic integration of the protoplastid, through the use of all possible genes from the former pathogen's genes repertoire. This very conveniently explains both the chlamydial imprint of the Archaeplastida genome, and the uniqueness of plastid endosymbiosis.

In 2012, the sequence of the Cyanophora genome confirmed the presence of the SSIII/IV chlamydial glucan synthase in the cytosol of the glaucophyte lineage [27]. However in 2012–2013, the report of the glaucophyte plastid proteome established that these peptidoglycans containing plastids contained a one order of magnitude lesser number of transporters in their plastid inner membrane by comparison to the green plants and algae, with one third of these defining transporters of chlamydial phylogenetic origin [31,32]. Not only does this testify to the importance of these pathogens in plastid endosymbiosis, but it also showed the unexpected presence of UhpC a chlamydial carbon transporter exporting hexose phosphates from the plastid to the host cytosol [31]. In a recent review [32], we examine the consequences of this finding on our understanding of plastid endosymbiosis (Fig. 4). To summarize, we discuss in this review the relative merits of two alternative models that involve chlamydia intracellular pathogens in plastid endosymbiosis [32]. The first model states that the cyanobiont penetrated the cytosol independently from the pathogen. It also states that the aforementioned Golgi derived purine NST was the initial carbon translocator, present on the cyanobiont inner membrane, while UhpC was recruited later and specifically in the glaucophyte lineage. This is the model that we initially proposed. In a second model, we propose that both the pathogen and the cyanobiont co-existed in the same inclusion vesicle [32], and that the UhpC chlamydial translocator defined the original carbon translocator of the cyanobiont. In this model, the pathogen extracts photosynthetic carbon from the cyanobiont within the chlamydial inclusion vesicle and the host gets the overflow of intravesicular glycogen synthesis, through the aforementioned golgi derived NST for host cytosolic glycogen synthesis (Fig. 4A). This second model requires the secondary escape of the cyanobiont from the inclusion vesicle.

Of the two alternatives we prefer the scenario depicted in Fig. 4B because it offers many additional advantages, and kills many birds with a single stone, which undoubtedly makes it a more parsimonious scenario. It indeed offers a straightforward explanation for phagocytosis abortion, since co-infection with a chlamydial elementary body would have enabled the cyanobiont to benefit from all the chlamydial effectors responsible for remodeling the phagocytosis vesicle into an active and

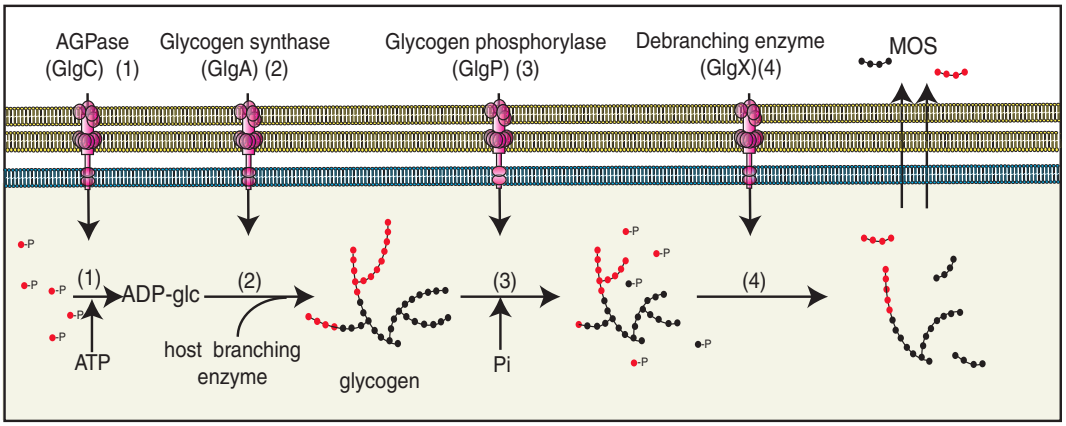

Fig. 3. Manipulation of the glycogen pools by Parachlamydiales. Parachlamydiales display the whole suite of bacterial glycogen metabolism genes, including ADP-glucose pyrophosphorylase (GlgC) responsible for ADP-Glc synthesis, an ADP-Glc specific glucan synthase (glycogen synthase — GlgA) a branching enzyme (GlgB) and the catabolic enzymes bacterial glycogen phosphorylase (GlgP), bacterial glycogen debranching enzyme (GlgX) and amylomaltase (MalQ). All enzymes of glycogen metabolism with the exception of GlgB and MalQ have been shown in Parachlamydiales [10] to define effector proteins secreted into the cytosol (in gray) by the TTS (in purple), through the two bacterial membranes (in yellow) and through the host derived inclusion vesicle (in blue). In the host cytosol, all the enzymes of eukaryotic glycogen metabolism, as displayed and discussed in Fig. 2, are present and active. (1) At the beginning of the infection cycle the chlamydial effector ADP-glucose pyrophosphorylase uses the host glucose-1-P and ATP pools to generate ADP-Glc. (2) The chlamydial effector glycogen synthase will incorporate this glucose (in red) into the host glycogen pools (in black). Branching will occur in the cytosol, thanks to the host glycogen branching enzyme. (3) When the concentrations of orthophosphate rise and those of cytosolic glucose-1-P and ATP decrease, the chlamydial effector glycogen phosphorylase will bypass the tight regulation of the host phosphorylase and yield massive breakdown of the host glycogen pools thereby generating glucose-1-P and phosphorylase limit dextrin. (4) The phosphorylase limit dextrin will be further degraded by the chlamydial GlgX debranching enzyme that will generate maltotetraose which cannot be degraded by host enzymes. This Figure was used by permission from reference 10.

stable inclusion vesicle. Second, the inclusion vesicle offers a less dense physical environment than the host cytosol more akin to the extracellular environment, yet more stable and provided with abundant minerals and metabolites, facilitating division and growth of the cyanobiont. Third, early endosymbiosis relied on important novel transporters such as the chlamydial UhpC and the chlamydial ATP import protein. The latter would have been required early on in darkness, because of the loss of storage polysaccharides that followed photosynthate export (see above). Such proteins define unlikely cargo for the chlamydia TTS (type three secretion system), yet the corresponding chlamydial genes could have been readily transferred in such a confined environment, thanks to the type IV conjugation machinery that Chlamydiales have been recently reported to contain [9]. In a similar fashion, other chlamydial genes may have transited early in the cyanobiont genome before they were transferred to the host nucleus. Fourth, it buys time for the cyanobiont before it escapes from the inclusion vesicle to the cytosol and evolves a plastid protein targeting machinery, in effect priming the symbiont for intracellular life. Fifth, it was recently found that at least in *C. trachomatis*, the intracellular pathogens direct glycogen accumulation in both its own inclusion vesicle and in the host cytosol. Sixth, the continuity existing between the inclusion membrane and the Golgi offers an easy explanation for the very early recruitment of the host Golgi purine NSTs to tap ADP-glucose from within the chlamydial inclusion.

The scenario depicted in Fig. 4B nevertheless makes two predictions which are presently not verified. First Chlamydiales have yet to be observed together with other bacteria in the same inclusion vesicle. Second, conjugative transfer of chlamydial DNA into the cyanobiont genome suggests that the protoplastid genome was a mosaic of cyanobacterial and chlamydial genes. However the extant plastomes show very little evidence of LGTs from sources other than cyanobacteria and only two such comparatively more recent cases have been reported [33,34].

One striking observation, that could be relevant to this issue, is the simultaneous presence of the same group I intron in the 23S rDNA of green alga plastid DNA, of Simkaniaceae (one of the 6 currently proposed families within Chlamydiales) of amoebal mitochondrial DNA and of extant cyanobacteria including those suspected to be closer to the protoplastid ancestor [35,36]. Another striking observation consists in a similar organization of the translational machinery in Chlamydiales and cyanobacteria as outlined by Brinkman et al. (2002), that could have facilitated expression of chlamydial genes in the protoplastid [4]. Equally striking is the presence of several cases of LGTs from Chlamydiales to Archaeplastida that could affect the control of protein translation [9]. These could suggest that Chlamydiales may have controlled protein translation in the protoplastid. However the compiled extant evidence makes for a rather weak case supporting extensive ancient gene transfers to the cyanobiont genome.

5. Ten percent of the LGT cases can be fully explained biochemically

Strong support for a role of Chlamydiales in plant genome evolution came first from those studying the intracellular pathogens of animals and amoebae. In the case of both the *C. trachomatis* and the *Protochlamydia amoebophila* genome descriptions, there is little doubt that plants stand out as the most prevalent eukaryotic group that displays a close relationship to these Chlamydiales. This striking and completely unexpected result, at first, must have come as a disappointment to those studying these pathogens. These researchers were probably hoping to reveal the capture and use in the chlamydia life cycle of genes from the Chlamydiales natural hosts: the animals and amoebas (or other protists). Some cases of ancient LGTs from eukaryotes to Chamydiales have indeed been documented [37]. Yet the Archaeplastida stood out in their analysis as the major eukaryotic signal in the chlamydial genomes. This explains why researchers studying these pathogens uniformly agree that Chlamydiales did have a major role to play in plastid endosymbiosis.

This observation is further strengthened by the phylogenomic study carried out by Ball et al. [10]. In this analysis performed in 2012, LGTs uniting chlamydiae with diverse eukaryotic groups were scored using a 75% bootstrap cutoff. This type of approach gives useful information only if the groups investigated are documented through enough genomic data for the comparisons to be relevant. This was certainly the case at the time of the analysis for the green algae and land plants (506,307 proteins at the time of the analysis), the fungi (451,434 proteins) and animals (976,563 proteins). However this was certainly not the case for other important eukaryotic groups such as the Amoebozoa or Excavata. At this level of bootstrap cutoff, 64 protein families were identified at that time that united Chlamydiales with Archaeplastida while respectively 14 protein families united fungi to Chlamydiales

and 36 protein families united them to animals. Although the precise numbers may continue to evolve with the ever expanding databases the outcome of the comparison is unlikely to change. Hence Archaeplastida are the major recipients of LGTs uniting Chlamydiales and eukaryotes in such studies. To us, of particular significance is the fact that two equally sampled and studied extant groups: the fungi and Chloroplastida (green algae and land plants), that are both nowadays immune to chlamydial infection, display vastly different numbers of LGTs uniting them to Chlamydiales, the signal being nearly 5-fold more prevalent in Archaeplastida. Yet, both fungi and Archaeplastida

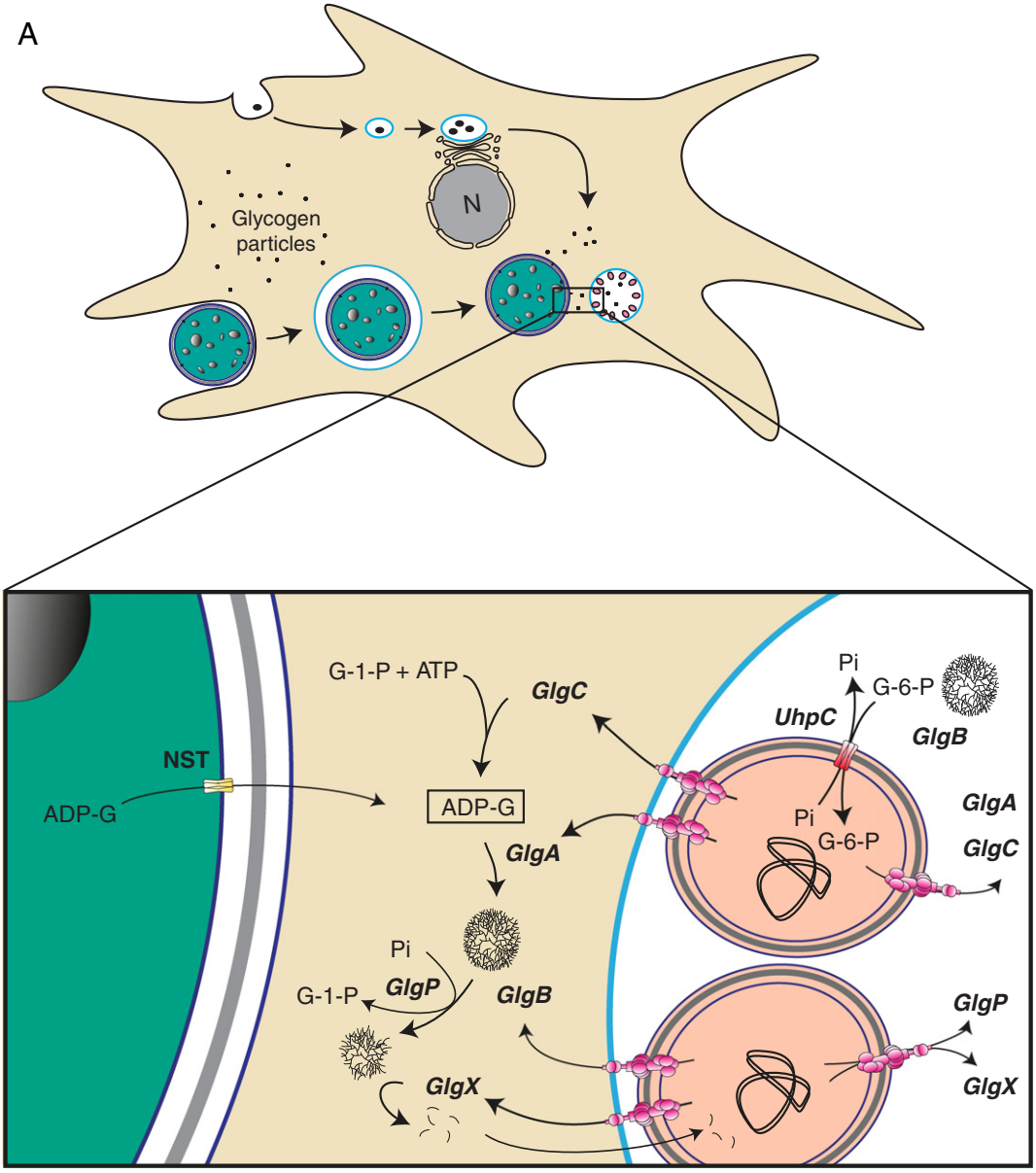

Fig. 4. Alternative models explaining chlamydial assisted plastid endosymbiosis. A. This model hypothesizes that both the chlamydia (in black and pink) and the future cyanobiont (in blue) have entered independently the eukaryotic host through distinct phagocytosis events. For symbiosis to occur the cyanobiont must have accidently escaped the phagocytosis vesicle. A section of the host cytosol (in beige) enclosing a part of the cyanobiont and of an inclusion vesicle is enlarged. Intact glycogen particles and glycogen limit dextrins are depicted as black granules. Targeting of the host NST (in yellow) to the cyanobiont inner membrane allows escape of ADP-Glc to the cytosol. In the cytosol all the enzymes of host glycogen metabolism depicted (in orange) in Fig. 2 are active and present (not displayed in Fig. 4A or B). In addition to these the chlamydiae secreted through their TTS (in purple-pink) GlgC (ADP-glucose pyrophosphorylase), GlgA (ADp-Glc specific glycogen synthase), GlgP (glycogen phosphorylase) and GlgX. The system functions exactly as in Fig. 3. However the massive escape of ADP-Glc from the cyanobiont rescues the otherwise cytotoxic effect of chlamydia-induced glycogen synthesis by inhibiting chlamydia-induced ADP-Glc synthesis. The chlamydial GlgA is nevertheless required to polymerize photosynthate into the host glycogen pools instantly changing a pathogenic into a symbiogenic interaction. The chlamydia which encodes the symbiogenic gene (GlgA) must still be fed through the import of GlgX-generated maltotetraose. However both the chlamydial GlgC and GlgP are no longer desirable in the tripartite symbiosis and will soon be lost after installment of the tripartite symbiosis. B. This model hypothesizes that the cyanobiont and the chlamydia have both entered simultaneously. A single large-size cyanobiont is displayed surrounded by multiple chlamydial reticulate bodies within a single inclusion vesicle. In the cytosol the situation is exactly as described in Fig. 4A with the same set of chlamydial effectors and host enzymes. However the ADP-Glc now flows from the inclusion vesicle (and not directly from the cyanobiont) through the same host NST transporter. Nevertheless the situation is different within the vesicle. The chlamydia is able to exchange genes with the cyanobiont thanks to its conjugation machinery (type IV secretion system displayed in light green and blue). The chlamydial UhpC now present on the cyanobiont inner membrane thanks to direct transfer of its gene from the chlamydia to the cyanobiont genome catalyzes glucose-6-P to orthophosphate exchange. An efflux of glucose-6-P will be generated within the inclusion vesicle and allow in this compartment the massive synthesis of glycogen thanks to the same suite of chlamydial effectors as that present in the host cytosol. When the supply of glucose-6-P exceeds the sink abilities of the inclusion vesicle the inclusion ADP-Glc concentration will increase to the point where it is transported out by the low affinity host NST thereby feeding host glycogen synthesis as detailed in Figs. 3 and 4A. Hence in this hypothesis, the host initially gets only the overflow of carbon. Fig. 4B requires that the cyanobiont escapes the inclusion vesicle at a later stage of plastid endosymbiosis. The relative merits of both hypothesized models are discussed in the text. This Figure was used by permission from reference 32.

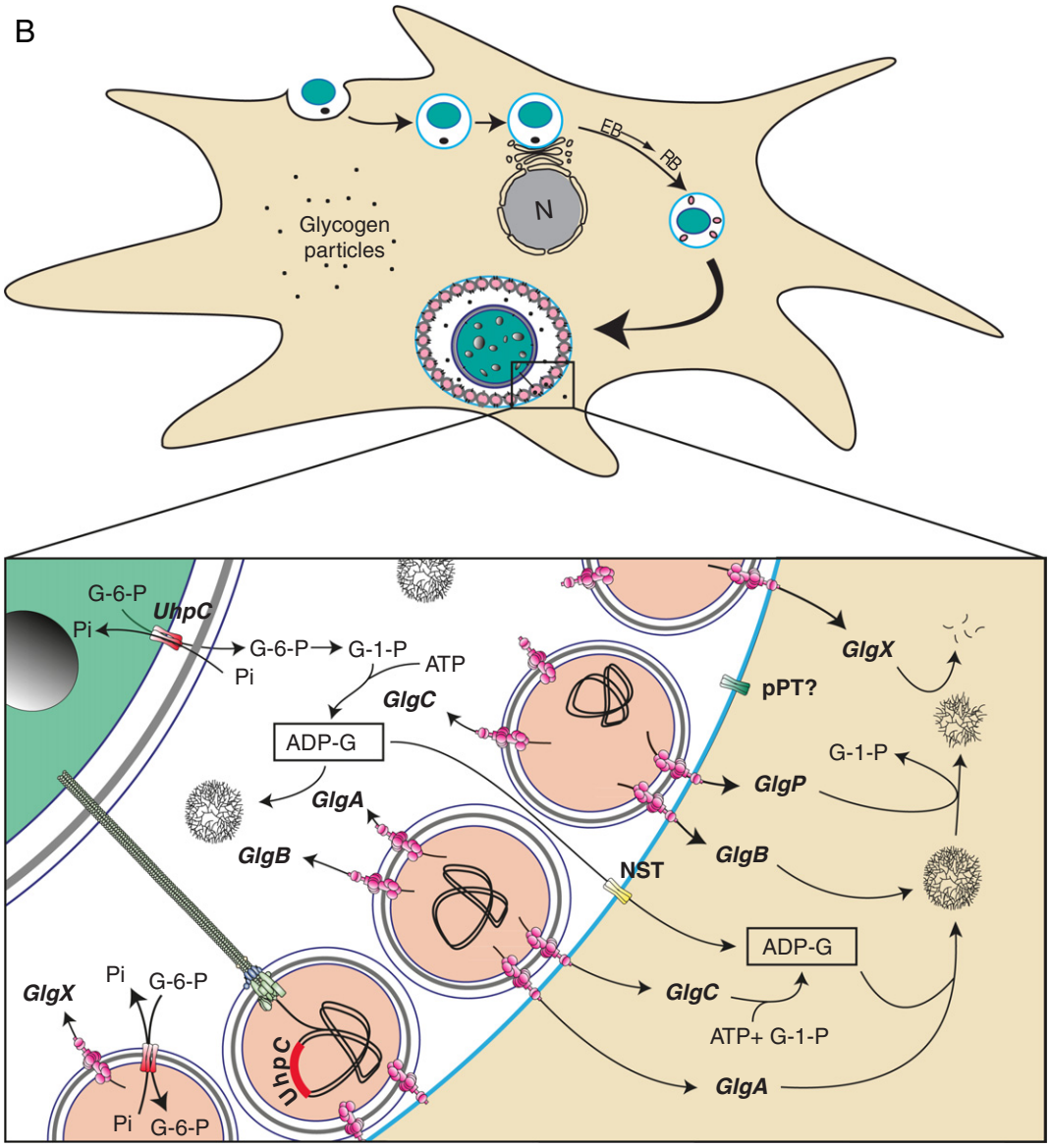

Fig. 4 (*continued*).

have closed the door to chlamydial infection by building a continuous solid cell wall and not having outer membranes exposed. In addition to this and significantly, the intensively studied and thoroughly sampled animals, despite having been the subject of continuous infection by Chlamydiales during their whole evolutionary history, display significantly less LGTs uniting them to Chlamydiales when compared to the Archaeplastida. Hence there is no question that Archaeplastida are selectively enriched in LGTs from Chlamydiales and this enrichment relatively to other well studied and sampled eukaryotic groups needs to be explained. These comparisons also tell us that the phylogenomic signal in Archaeplastida is certainly a composite one. A minor yet significant part of the signal may be common with other eukaryotes and completely unrelated to plastid endosymbiosis. This background of LGTs found in all eukaryotic clades results from either very ancient LGTs from Chlamydiales to eukaryote ancestors or possibly also the reverse. This may also explain the fungal signal although this needs to be further studied. In metazoa, an animal specific component enriching the latter in LGTs relatively to the fungi can be explained by their constant exposure to Chlamydiales infections. A 2.5 fold higher level of LGTs scored in animals relatively to fungi reflects the fact that parasitism and the physical presence of the pathogen as "roommate" does impact the LGT frequency at variance with what is stated in Deschamps (2014) [40]. Looking at the Chlamydiales phylogenetic signal, one can safely distinguish 24 LGTs that have happened in the common ancestor of Archaeplastida as they impact several of the 3 Archaeplastida lineages [40]. This is certainly an underestimate as Glaucophyta and to a lesser extent Rhodophyceae have been insufficiently sampled. There is in addition to this figure of 24, an additional ancient component of the phylogenetic signal that is specific to only one of the three Archaeplastida clades and that may result from gene losses in the two other clades. If we nevertheless restrict the analysis to the aforementioned 24 cases, a majority of functions are either plastid related (found in plastids today) or functionally tied to plastid endosymbiosis. Hence we are probably looking here at component of the signal specific to Archaeplastida which is of a very different nature when compared to those evidenced in other eukaryotic lineages.

Although the community of scientist studying Chlamydiales are convinced that these bacteria are major players in plastid endosymbiosis, the same cannot be said of those studying the evolution of eukaryotes where the debate is currently unsettled. This is because looking at the phylogenetic signal from the Archaeplastida genome only, rather than from the pathogen's perspective, gives a more complex, and perhaps

less convincing picture of the role of Chlamydiales in plant evolution. Indeed, Chlamydiales do give a significant phylogenetic signal in the Archaeplastida genome but it is not the prevalent one. The phylogenetic signal due to cyanobacteria is over one order of magnitude stronger and that of proteobacteria as a whole (among others) is comparable to Chlamydiales in the plastid proteome and probably stronger in the total cell proteome [1,38]. It is very difficult to assess the significance of comparing such diverse groups of bacteria through mere numbers. This, and the complexity of some of these phylogenies, have led some to question the relevance of the hypothesized role of Chlamydiales in Archaeplastida evolution [38–41]. According to some [38,41], LGTs of Chlamydiales to Archaeplastida are all basically of the same nature as those that concern other groups such as proteobacteria [38,41]. It is well known that during organelle evolution, genes coding for proteins of similar function as those encoded by the organelle DNA often get transferred to the nucleus and replace the organellar copy. Hence a chlamydial signal could have been generated during the process of metabolic integration of the protoplastid in a fashion very similar to the signal seen with proteobacteria, or other prokaryotic groups. In these reports [38,41], mainly concerned with other topics, no effort was made to explain the prevalence of Archaeplastida among all eukaryotic clades as recipient of LGTs from Chlamydiales. Nor was any effort made to either understand or propose alternative explanations for the numerous biochemical observations in favor of an active role of Chlamydiales in plastid endosymbiosis.

From our point of view, each tree must be analyzed individually, in the light of the function of the protein it encodes and of the evolutionary history of the gene. Two other studies more thoroughly address these questions [39,40]. In the first study the authors question the interpretation of the signal on the basis of tree topologies and the second study questions both the tree topology interpretation and the biochemical evidence. Interestingly both of these interesting but critical studies did recover the ancient Archaeplastida-specific chlamydial signal which was ignored in the two other studies [38,41]., However, we do not believe, as suggested Moreira and Deschamps (2014) [39], that the absence of observed present interaction of Chlamydiales with Archaeplastida do not support a role of the latter in endosymbiosis. Nor do we believe that an atypical topology of Chlamydiales with other eukaryotes or with bacterial lineages unrelated to the PVC (Planctomycetes Verrucomicrobia and Chlamydia superclade) disqualifies a clear LGT of the Chlamydiales to the Archaeplastida ancestor. As emphasized above, the vast majority of Archaeplastida have lost the ability to perform phagocytosis and are presently surrounded by a continuous cell wall, conditions which are known to preclude the entry of Chlamydiales in their eukaryotic hosts On the contrary, it is precisely because of this absence that the presence of archaeplastidal genes in the Chlamydiales genomes as the major eukaryotic contribution was hypothesized initially to be very ancient [3]. This contribution was subsequently tracked back to the time of plastid endosymbiosis.

As mentioned above there are cases of LGTs from eukaryotes to bacteria [37], particularly in the case of those bacteria that live and multiply within eukaryotes, so topologies uniting eukaryotes to Chlamydiales with an LGT from the latter to Archaeplastida should not been discounted. Nor do they weaken our argument.

Similarly, the presence of unexpected clades such as cyanobacteria instead of PVC members in tree topologies that support a particular LGT to Archaeplastida, does not weaken the argument. Indeed extensive LGTs between bacteria are sufficient to explain the absence of close relatives of the PVC superclade in the vast majority of chlamydial trees. In addition, cyanobacteria as other bacteria are indeed suspected to also have extensively shared genes with Chlamydiales [35,4].

Finally LGTs from Chlamydiales to that are not shared by the other Archaeplastida clades (Glaucophyta or Rhodophyceae) do not make a case against an ancient role of Chlamydiales in protoplastid integration as hypothesized by Moreira and Deschamps. Indeed for instance 4-hydroxy-3-methylbut-2-en-1-yl diphosphate synthase, involved in carotenoid biosynthesis is known to be of chlamydial origin in Chloroplastida and of cyanobacterial origin in Rhodophyceae. It is very easy to envision how a chance replacement of the cyanobacterial gene by a chlamydial version, could have occurred selectively in the green lineage. This chance replacement was nevertheless facilitated, as mentioned above, by the required presence of the chlamydial symbiont during early divergence of the evolving Archaeplastida.

Had the phylogenomic analysis of Archaeplastida genomes been the sole argument for a specific role of Chlamydiales in plastid endosymbiosis, we would agree with Moreira and Deschamps that the question would indeed remain open to alternative interpretations. However, the more recent developments in the understanding of the evolutionary history of biochemical pathways are now beginning to give a very detailed picture of the early events that may have prompted plastid endosymbiosis. This includes the function of the major enzymes and transporters involved and the identity of the biochemical fluxes at work. 4 chlamydial LGTs (roughly 10% of the total number of Chlamydial LGTs: glycogen/starch synthase; glgX debranching enzyme, NTT (the ATP import protein) and UhpC) can be explained by the same narrative related to carbon export from the cyanobiont to the host cytosol which is at the core of the metabolic symbiosis. The key element is the presence as a cytosolic effector protein secreted by Chlamydiales of the glycogen/starch synthase responsible for incorporating photosynthate in the host carbon stores. As long as the gene coding for the symbiotic enzyme was encoded by the pathogen, the chlamydia was a full partner of a tripartite symbiosis. Deschamps [40] questions the biochemical interpretation of the ménage à trois hypothesis. In this study an attempt is made to question the fact that the Chlamydiales and cyanobiont were ever room-mates in the same host. To downplay the presence of the aforementioned 24 genes common to several of the archaeplastida lineages Deschamps proposes that these LGTs could have preceded plastid endosymbiosis, a proposal difficult to accept in face of the functions and(or) present plastidial localization of the proteins encoded by these genes. What would be the purpose of such LGTs in the heterotrophic ancestor? We thus have very little doubts that the pathogens and the cyanobiont were indeed roommates. We would like to further emphasize the very high level of coincidences that would have to be inferred to reject the biochemical evidence sustaining the tripartite symbiosis hypothesis. First the finding that the glucan synthase that was hypothesized to have fed photosynthate into the cytosolic storage carbohydrate stores is a protein of chlamydial provenance has to be assumed to be coincidental. The finding that the protein could have been located in the host cytosol at the onset of endosymbiosis only because it was, indeed, found to be a cytosolic effector must be considered coincidental. The finding of 2 out of 6 transporters in the inner membranes of the glaucophyte plastids as chlamydial proteins, with the sole protein in charge of exporting photosynthate being a chlamydial protein, must be considered coincidental. Finally, deduction of a requirement for the maintenance of GlgX to feed carbon into the pathogen and the finding of GlgX as an LGT common to all Archaeplastida and a chlamydial effector must also be considered coincidental. Knowing that the frequency of LGTs from Chlamydiales to Archaeplastida may not exceed 30 to 50 genes such a cascade of coincidences can be considered as truly astonishing.

As mentioned above, the tripartite symbiosis would have disappeared as soon as the chlamydial gene was transferred successfully to the host nucleus and correctly expressed. As long, as the symbiotic gene remained within the pathogen there was a strong selection pressure for maintenance of the chlamydial genome. This maintenance allowed time for the chance replacement of cyanobacterial genes by their chlamydial counterpart during metabolic integration of the protoplastid. This kind of LGT is very similar to the chance replacement of cyanobacterial enzymes by proteins from diverse sources, within the evolving plastid. It thus does not necessarily tie with any functional importance in the maintenance of the tripartite symbiosis, but it was significantly facilitated by it.

As pointed out above, constant evolution of novel beneficial effectors secreted in the host cytosol would have been the only way for the pathogen to ensure its maintenance. Among all chlamydial LGTs witnessed today in Archaeplastida those that define suspects for playing such a role will be those for which an ancestral cytosolic localization can be suspected. The chlamydial GlgX debranching enzyme that was donated to Archaeplastida and in effect switched glycogen to starch metabolism defines a prime suspect in this respect, but after all this may define yet another coincidence.

Conflict of interest

We have no conflict of interest to report concerning the following items:

(1) All third-party financial support for the work in the submitted manuscript. (2) All financial relationships with any entities that could be viewed as relevant to the general area of the submitted manuscript. (3) All sources of revenue with relevance to the submitted work who made payments to you, or to your institution on your behalf, in the 36 months prior to submission. (4) Any other interactions with the sponsor of outside of the submitted work should also be reported. (5) Any relevant patents or copyrights (planned, pending, or issued). (6) Any other relationships or affiliations that may be perceived by readers to have influenced, or give the appearance of potentially influencing, what you wrote in the submitted work.

We do have a copyright issue to resolve with respect to the use of Fig. 4.

Acknowledgements

This work was supported by the CNRS, the Université des Sciences et Technologies de Lille, the Région Nord Pas de Calais, and the ANR grants Ménage à trois (ANR-12-BSV2-0009) and Expendo (ANR-156E0029N).

References

[1] K. Suzuki, S.Y. Miyagishima, Eukaryotic and eubacterial contributions to the establishment of plastid proteome estimated by large-scale phylogenetic analyses, Mol. Biol. Evol. 27 (2010) 581–590.
[2] M.W. Gray, Mitochondrial evolution, Cold Spring Harb. Perspect. Biol. 4 (9) (2012) a011403. http://dx.doi.org/10.1101/cshperspect.a011403.
[3] R.S. Stephens, S. Kalman, C. Lammel, J. Fan, R. Marathe, L. Aravind, W. Mitchell, L. Olinger, R.L. Tatusov, Q. Zhao, E.V. Koonin, R.W. Davis, Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*, Science 282 (1998) 754–759.
[4] F.S. Brinkman, J.L. Blanchard, A. Cherkasov, Y. Av-Gay, R.C. Brunham, R.C. Fernandez, B.B. Finlay, S.P. Otto, B.F. Ouellette, P.J. Keeling, A.M. Rose, R.E. Hancock, S.J. Jones, H. Greberg, Evidence that plant-like genes in Chlamydia species reflect an ancestral relationship between Chlamydiaceae, cyanobacteria, and the chloroplast, Genome Res. 12 (2002) 1159–1167.
[5] M. Horn, A. Collingro, S. Schmitz-Esser, C.L. Beier, U. Purkhold, B. Fartmann, P. Brandt, G.J. Nyakatura, M. Droege, D. Frishman, T. Rattei, H.W. Mewes, M. Wagner, Illuminating the evolutionary history of Chlamydiae, Science 304 (5671) (2004) 728–730.
[6] J. Huang, P. Gogarten, Did an ancient chlamydial endosymbiosis facilitate the establishment of primary plastids? Genome Biol. (2007)http://dx.doi.org/10.1186/gb-2007-8-6-r99.
[7] B. Becker, K. Hoef-Emden, M. Melkonian, Chlamydial genes shed light on the evolution of photoautotrophic eukaryotes, BMC Evol. Biol. 8 (2008) 203.
[8] A. Moustafa, A. Reyes-Prieto, D. Bhattacharya, Chlamydiae has contributed at least 55 genes to Plantae with predominantly plastid functions, PLoS One 3 (5) (2008) e2205. http://dx.doi.org/10.1371/journal.pone.0002205.
[9] A. Collingro, P. Tischler, T. Weinmaier, T. Penz, E. Heinz, R.C. Brunham, T.D. Read, P.M. Bavoil, K. Sachse, S. Kahane, M.G. Friedman, T. Rattei, G.S. Myers, M. Horn, Unity in variety: the pan-genome of the Chlamydiae, Mol. Biol. Evol. 28 (2011) 3253–3270.
[10] S.G. Ball, A. Subtil, D. Bhattacharya, A. Moustafa, A.P.M. Weber, L. Gehre, C. Colleoni, M.C. Arias, U. Cenci, D. Dauvillée, Metabolic effectors secreted by bacterial pathogens: essential facilitators of plastid endosymbiosis? Plant Cell 25 (2013) 7–21.
[11] D. Baum, The origin of primary plastids: a Pas de Deux or a Ménage à Trois? Plant Cell 25 (2013) 4–6.
[12] G.I. McFadden, Origin and evolution of plastids and photosynthesis in eukaryotes, Cold Spring Harb. Perspect. Biol. 6 (4) (2014) a016105. http://dx.doi.org/10.1101/cshperspect.a016105.
[13] G. Greub, D. Raoult, Microorganisms resistant to free-living amoebae, Clin. Microbiol. Rev. 17 (2004) 413–433.
[14] M. Goetz, A. Bubert, G. Wang, I. Chico-Calero, J.A. Vazquez-Boland, M. Beck, J. Slaghuis, A.A. Szalay, W. Goebel, Microinjection and growth of bacteria in the cytosol of mammalian host cells, Proc. Natl. Acad. Sci. U. S. A. 98 (2001) 12221–12226.
[15] Y.M. Abdelrahman, R.J. Belland, The chlamydial developmental cycle, FEMS Microbiol. Rev. 29 (2005) 949–959.
[16] Z.M. McKie-Krisberg, R.W. Sanders, Phagotrophy by the picoeukaryotic green alga Micromonas: implications for Arctic Oceans, ISME J. (2014) 1–9. http://dx.doi.org/10.1038/ismej.2014.16.
[17] J.A. Raven, J. Beardall, K.J. Flynn, S.C. Maberly, Phagotrophy in the origins of photosynthesis in eukaryotes and as a complementary mode of nutrition in phototrophs: relation to Darwin's insectivorous plants, J. Exp. Bot. 60 (2009) 3975–3987.
[18] S. Maruyama, E. Kim, A modern descendant of early green algal phagotrophs, Curr. Biol. 23 (2013) 1081–1084.
[19] S.G. Ball, Evolution of Storage Polysaccharide Metabolism in Archaeplastida Opens an Unexpected Window on the Molecular Mechanisms That Drove Plastid Endosymbiosis, in: Wolfgang Loffelhardt (Ed.), Endosymbiosis, Springer, Wien, Heidelberg, New York, Dordrecht, London, 2014, pp. 111–134.
[20] S.G. Ball, M.K. Morell, From bacterial glycogen to starch: understanding the biogenesis of the plant starch granule, Ann. Rev. Plant. Biol. 54 (2003) 207–233.
[21] P. Deschamps, C. Colleoni, Y. Nakamura, E. Suzuki, J.L. Putaux, A. Buléon, S. Haebel, G. Ritte, M. Steup, L.I. Falcon, D. Moreira, W. Loeffelhardt, J. Nirmal Raj, C. Plancke, C. D'Hulst, D. Dauvillée, S.G. Ball, Metabolic symbiosis and the birth of the plant kingdom, Mol. Biol. Evol. 25 (2008) 536–548.
[22] S.G. Ball, C. Colleoni, U. Cenci, J. Nirmal Raj, C. Tirtiaux, The evolution of glycogen and starch metabolism in eukaryotes gives molecular clues to understand the establishment of plastid endosymbiosis, J. Exp. Bot. 62 (2011) 1775–1801.
[23] C. Colleoni, M. Linka, P. Deschamps, M.G. Handford, P. Dupree, A.P.M. Weber, S.G. Ball, Phylogenetic and biochemical evidence supports the recruitment of an ADP-glucose translocator for the export of photosynthate during plastid endosymbiosis, Mol. Biol. Evol. 27 (2010) 2691–2701.
[24] B. Loddenkötter, B. Kammerer, K. Fischer, U.I. Flugge, Expression of the functional mature chloroplast triose phosphate translocator in yeast internal membranes and purification of the histidine-tagged protein by a single metal-affinity chromatography step, Proc. Natl. Acad. Sci. 90 (1993) 2155–2159.
[25] M. Gründel, R. Scheunemann, W. Lockau, Y. Zilliges, Impaired glycogen synthesis causes metabolic overflow reactions and affects stress responses in the cyanobacterium Synechocystis sp. PCC 6803, Microbiology 158 (2012) 3032–3043.
[26] Y. Xu, L.T. Guerra, Z. Li, M. Ludwig, G.C. Dismukes, D.A. Bryant, Altered carbohydrate metabolism in glycogen synthase mutants of *Synechococcus* sp. strain PCC 7002: cell factories for soluble sugars, Metab. Eng. 16 (2013) 56–67.
[27] D.C. Price, C.X. Chan, H.S. Yoon, E.C. Yang, H. Qiu, A.P.M. Weber, R. Schwacke, J. Gross, N.A. Blouin, C. Lane, A. Reyes-Prieto, D.G. Durnford, J.A.D. Neilson, B.F. Lang, G. Burger, J.M. Steiner, W. Löffelhardt, J.E. Meuser, M.C. Posewitz, S.G. Ball, M.C. Arias, B. Henrissat, P.M. Coutinho, S.A. Rensing, A. Symeonidi, H. Doddapaneni, B.R. Green, V.D. Rajah, J. Boore, D. Bhattacharya, Cyanophora paradoxa genome elucidates origin of photosynthesis in algae and plants, Science 335 (2012) 843–847.
[28] A. Subtil, C. Delevoye, M.E. Balañá, L. Tastevin, S. Perrinet, A. Dautry-Varsat, A directed screen for chlamydial proteins secreted by a type III mechanism identifies a translocated protein and numerous other new candidates, Mol. Microbiol. 56 (2005) 1636–1647.
[29] C. Lu, L. Lei, B. Peng, L. Tang, H. Ding, S. Gong, Z. Li, Y. Wu, G. Zhong, Chlamydia trachomatis GlgA is secreted into host cell cytoplasm, PLoS One 8 (7) (2013) e68764. http://dx.doi.org/10.1371/journal.pone.0068764.
[30] U. Cenci, F. Nitschke, M. Steup, B.A. Minassian, C. Colleoni, S.G. Ball, Transition from glycogen to starch metabolism in Archaeplastida, Trends Plant Sci. 19 (2014) 18–28.
[31] F. Facchinelli, M. Pribil, U. Oster, N.J. Ebert, D. Bhattacharya, D. Leister, A.P. Weber, Proteomic analysis of the *Cyanophora paradoxa* muroplast provides clues on early events in plastid endosymbiosis, Planta 237 (2013) 637–651.
[32] F. Facchinelli, C. Colleoni, S.G. Ball, A.P.M. Weber, Chlamydia, cyanobiont, or host: who was on top in the ménage à trois? Trends Plant Sci. 18 (2013) 673–679.
[33] D.W. Rice, J.D. Palmer, An exceptional horizontal gene transfer in plastids: gene replacement by a distant bacterial paralog and evidence that haptophyte and cryptophyte plastids are sisters, BMC Biol. 4 (2006) 31.
[34] J. Janouškovec, S.L. Liu, P.T. Martone, W. Carré, C. Leblanc, J. Collén, P.J. Keeling, Evolution of red algal plastid genomes: ancient architectures, introns, horizontal gene transfer, and taxonomic utility of plastid markers, PLoS One 8 (3) (2013)http://dx.doi.org/10.1371/journal.pone.0059001.
[35] K.D.E. Everett, S. Kahane, R.M. Bush, M.G. Friedman, An unspliced group I intron in 23S rRNA links Chlamydiales, chloroplasts, and mitochondria, J. Bacteriol. 181 (1999) 4734–4740.
[36] P. Haugen, D. Bhattacharya, J.D. Palmer, S. Turner, L.A. Lewis, K.M. Pryer, Cyanobacterial ribosomal RNA genes with multiple, endonuclease-encoding group I introns, BMC Evol. Biol. 7 (2007) 159. http://dx.doi.org/10.1186/1471-2148-7-159.
[37] V. Thomas, G. Greub, Amoeba/amoebal symbiont genetic transfers: lessons from giant virus neighbours, Intervirology 53 (2010) 254–267.
[38] T. Dagan, M. Roettger, K. Stucken, G. Landan, R. Koch, P. Major, S.B. Gould, V.V. Goremykin, R. Rippka, N. Tandeau de Marsac, M. Gugger, P.J. Lockhart, J.F. Allen, I. Brune, I. Maus, A. PÜhler, W.F. Martin, Genomes of stigonematalean cyanobacteria

(subsection V) and the evolution of oxygenic photosynthesis from prokaryotes to plastids, Genome Biol. Evol. 5 (1) (2013) 31–44.
[39] D. Moreira, P. Deschamps, What Was the real contribution of endosymbionts to the eukaryotic nucleus? insights from photosynthetic eukaryotes, Cold Spring Harb. Perspect. Biol. 6 (7) (2014) a016014. http://dx.doi.org/10.1101/cshperspect.a016014.
[40] P. Deschamps, Primary endosymbiosis: have cyanobacteria and Chlamydiae ever been roommates? Acta Soc. Bot. Pol. 83 (4) (2014) 291–302.
[41] C. Ku, M. Roettger, V. Zimorski, S. Nelson-Sathi, F.L. Sousa, W.F. Martin, Plastid origin: who, when and why? Acta Soc. Bot. Pol. 83 (4) (2014) 281–289.