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REVIEW ARTICLE

# Genesis of the nucleus from bacterial sporulation: A simple hypothesis of eukaryotic origin

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**Abstract**

The most complexed issue of eukaryogenesis is the origin of the nucleus. Many hypotheses have been forwarded to explain this. Most of them are complicated and intangible. Here, a new and relatively simple hypothesis to address this unresolved problem has been hypothesized. This hypothesis is denominated as "Theory of Nucleus Origin from Bacterial Sporulation" (TNOBS). The hypothesis points out that the nucleus may be derived from a bacterial endospore, particularly, when sporulation is arrested at stage 4 due to a gene mutation. At this stage, a double membrane structure containing a chromosome (foreospore) has developed, which is reminiscent of a nucleus. In addition to the forespore, the mother cell also contains an additional chromosome. This morphologically specific cell is referred as a proto-nucleate cell (PTC). The PTC requires additional energy to maintain their newly formed endomembrane compartment (protonucleus). This energy demand has the potential of driving the expression of genes for energy production from the cytosolic chromosome which finally evolves to mitochondria, whereas the forespore develops to the nucleus. This TNOBS considers the nucleus and mitochondrion having derived simultaneously in the same cell. Moreover, this scenario avoids the difficulty to explain how an α-proteobacterium (precursor of mitochondria) can be taken up by the host despite of lacking capacity for classic endocytosis. It is further suggested that PTC generation may not be an extremely rare event in nature due to the widely existing spore-forming bacteria and frequent mutations. TNOBS is comparably simple and may, in some of its principle traits, be even reproducible under laboratory conditions.

## INTRODUCTION

In the evolutionary tree of life, three domains are distinguished, i.e., bacteria, archaea and eukarya (= eukaryotes). Despite some difficulties concerning details in the distinction between genes of bacterial and archaeal origin, due to horizontal gene transfer (Schleifer 2009), this three-domains concept of life forms is accepted by the majority of scientists. Profound genetic differences concerning especially the machineries of gene expression clearly distinguish between bacteria and archaea, whereas eukaryotes differ by the presence of the nucleus (Forterre 2015; Zhou *et al.* 2018). Moreover, only eukaryotes possess multiple extensive endomembrane systems, such as endoplasmic reticulum (ER), Golgi apparatus, Golgi-derived vesicles, and mitochondria. However, several phylogenetically unrelated eukaryotic taxa are devoid of mitochondria. Instead, they often contain mitochondrion-related organelles (MROs), such as hydrogenosomes (Biagini *et al.* 1997; Jerlström-Hultqvist *et al.* 2013) or mitosomes (Heinz & Lithgow 2013), or even

lack MROs(Karnkowska *et al.* 2016). The amitochondrial state of eukaryotes is not primary, but represents secondary reductions of pre-existing mitochondria, typically due to parasitism. Similar considerations can be made in cases of absence of other organelles, such as the missing Golgi apparatus in Microsporidia, which is replaced by an avesicular functional analog (Beznoussenko *et al.* 2007).

With regard to the fact that eukaryotes contain homologs of genes and functional machineries from both archaea and bacteria, most researchers working in this field agree that eukaryogenesis took place by a merging process between an archaeon and one or even two bacteria (Koonin 2015; Martin *et al.* 2015; López-García & Moreira 2015; Eme *et al.* 2018). According to the endosymbiosis theory, non-bacterial protoeukaryotes were assumed to have accidently acquired mitochondria by endocytosis of a bacterium, around 1.8-2.0 billion years ago(Sagan 1967). This view had tacitly implied that the difference in size between the large protoeukaryotic cell and a bacterium has already existed at the time of merging. The protoeukaryote was, thus, assumed to have been a predator of much larger size than a bacterium, i.e., a heterotrophic organism feeding on bacteria, among which some of the preys escaped from digestion and turned to endosymbionts, thereby conveying the decisive advantage to the host cell of providing respiratory pathways for much more efficiently producing ATP from oxidizable substrates, as known from the extant mitochondria. The bacterial origin of mitochondria is nowadays largely undisputed, and α-proteobacteria have been shown to be the most likely mitochondrial ancestors, among which extant Rickettsiales have the highest degree of molecular similarity to mitochondria (Abhishek *et al.* 2011). By comparing the relatively large genome of the giant mitochondrion of the jacobid flagellate *Reclinomonas americana* with α-proteobacterial genomes, the highest degree of homology was found in *Rickettsia provazekii* (Abhishek *et al.* 2011). An alternative to the involvement of a rickettsiacean as the α-proteobacterial partner has been forwarded by assuming that instead a giant virus from the order of Megavirales may have acted as a mitochondrial precursor (Seligmann 2019).

While the identification of rickettsiaceans as closest extant bacterial relatives of mitochondria can be taken as granted, this conclusion leads to substantial practical consequences, because these organisms are actually intracellularly living parasites of animals. On the one hand, one might be inclined to see this biological trait of parasitism as a favorable precondition for an interaction with a non-bacterial host such as an archaeon, but, on the other hand, a respective rickettsiacean ancestor should have been free-living instead of parasitic and, with regard to the usually small size of extant archaea, doubts may arise as to whether such an ancient archaeon might have been large enough for serving as a host cell. This problem would also apply to the alternative idea that the α-proteobacterial ancestor of mitochondria was not taken up by endocytosis, but rather by bacterial invasion, a suggestion that likewise assumed a considerably larger size of the archaeal partner. Another possibility might be a different kind of fusion, as will be discussed below. Fusion along with membrane exchange instead of endocytosis or parasitic intrusion has been also suggested in syntrophy models, the last version of which has suggested a tripartite association of a hydrogen-producing archaeon, a facultatively aerobic, sulfide-oxidizing α-proteobacterium, and a sulfate-reducing δ-proteobacterium, a consortium that may have been based on coupled inter-species redox metabolism in microbial mats (López-García & Moreira 2020). A difficulty consists in the presence of bacterial and archaeal cell walls, which may be regarded as obstacles for hypotheses based on endocytosis or membrane fusion between or among these organisms. However, such interactions must have occurred, if an achaeal-bacterial association describes correctly the beginnig of eukaryogenesis. A more severe obstacle seems to exist in the assumption of classic endocytosis by membrane intrusion as the mechanism of bacterial uptake into an archaeal host, to which archaea seem to be incapable. As will be outlined below, this problem can be circumvented when assuming engulfment by means of protrusions emerging from the host cell.

While the mitochondrial origin had been traced back to α-proteobacteria like rickettsiaceans or closely related organisms, it took quite some time until an archaeal sister group of eukaryotes had been discovered. Based on the extensive presence of eukaryotic signature proteins (ESPs), the archaeal superphylum of Asgardarchaeota (Asgard archaea) was concluded to be more closely related to eukaryotes than other archaea (Zaremba-Niedzwiedzka *et al.* 2017; Spang *et al.* 2018). This conclusion was corroborated by the detection of ESCRT homologs (endosomal sorting complex required for transport) in Asgard archaea(Lu *et al.* 2020), findings that may be seen as a preadaptive advantage for later eukaryotic intracellular traffic, and by homologies concerning supersized expansion segments (ESs) of the large ribosomal subunit rRNA (Penev *et al.* 2020). Among them, the subgroup Heimdallarchaeota (Williams *et al.* 2020; Neveu *et al.* 2020) and, recently, the Lokiarchaeota-related *Candidatus Prometheoarchaeum* syntrophicum (Imachi *et al.* 2020) were discussed as being most closely related to eukaryotes. As far as archaea like these may be representatives of a sister group to eukaryotes, it seems worth-while to analyze whether they may possess properties that fit the existing theories of eukaryogenesis. Especially with regard to an endocytosis-based type of endosymbiosis theory, one may be inclined to dismiss this possibility, though with the reservation that the archaeon that gave rise to the eukaryotic ancestor might have been profoundly different from the extant species, at least, in terms of size and cell morphology. If this would not be the

case, the small size of extant archaea that can be studied speaks against an endocytotic uptake. Another argument against the classic view is provided by the genetically based conclusion that Asgard archaea are not phagocytotic(Burns *et al.* 2018). Moreover, archaea do not possess endomembranes as required for intracellular digestion of endosomal contents, an observation for which only a single, but highly unusual exception is known: in the crenarchaeote, *Ignicoccus hospitalis*. In this organism, numerous bended cytoplasmic protrusions are present within a voluminous intermembrane space between inner and outer membranes that surround the cytoplasm(Heimerl *et al.* 2017). This endomembrane system is fairly different from all others that are known to date.

A major problem related to the origin of eukaryotic endomembranes is that of the sequence in which the major organelles developed, in particular, nucleus, mitochondria, ER and Golgi apparatus. The original idea of endocytotic uptake of an α-proteobacterium as a mitochondrial ancestor had assumed that the protoeukaryote host cell already possessed a nucleus(Sagan 1967). The mitochondrial descendance from α-proteobacteria remains undisputed because of overwhelming evidence concerning the highly effective ATP production by oxidative metabolism, as present in this bacterial group, and several other bacterial traits such as circular chromosomes, low DNA methylation, N-formylmethionyl-tRNA as translational initiator, high cardiolipin levels in the inner mitochondrial membrane and numerous genes of bacterial origin in mitochondria. However, the presence of a nucleus prior to the uptake of the endosymbiont is by far uncertain. In fact, the origin of the nucleus has remained a mystery and lacks any commonly accepted concept. Understanding the emergence of the nucleus faces a number of problems. The nuclear envelope (NE) consists of two membrane layers interconnected at the nuclear pores and is also connected to the ER. Thus, the perinuclear space between them is merged with the ER lumen. In open mitosis as in animals, the reconstruction of the nuclear envelope after karyokinesis takes place by outgrowing of ER membranes, in conjunction with fusion of vesicles formed during disintegration of the previous envelope in early prometaphase (Kutay & Hetzer 2008). From this point of view, one might assume that the NE had originated from other ER-related endomembranes. However, this conclusion might be precocious, because of the existence of closed mitosis, in which no NE breakdown occurs (Sazer *et al.* 2014; Mori & Oliferenko 2020). This mode is present in some yeasts, algae and meta-algae (e.g., dinoflagellates). This mode may be secondary in reduced fungi like yeasts, in which a limited local NE disassembly occurs at the nuclear pores(Dey *et al.* 2020) and in which other yeast species exhibit a semi-open mitosis (Mori & Oliferenko 2020). However, closed mitosis as in other primitive organisms may reflect an ancient mode in primarily unicellular eukaryotes. Therefore, a deduction of the NE from other endomembranes remains entirely hypothetical. Additional problems concern the origin of the nuclear pores, of the nuclear lamina, and of the linear chromosomes.

At the current state of our knowledge, contrasting possibilities exist concerning the relationship between NE and other endomembranes as well as their origins. (1) If the nucleus had developed first, endomembranes might have derived from blebs outgrowing from the outer layer of the NE, a possibility that is actually not favored by evidence. (2) If mitochondrial ancestors had entered the host cell prior to the existence of a nucleus, both cytoplasmic endomembranes and NE might have derived from either α-proteobacterial membranes or invagination processes that took place during bacterial entry. More in particular, these membranes were assumed to have developed by (2a) inward budding of the inner membrane of the gram-negative α-proteobacterium (Jékely 2007)(Gould *et al.* 2016), or (2b) by formation of outer membrane vesicles (OMVs) (Vesteg *et al.* 2006). (3) In the case of the tripartite syntrophic model (López-García & Moreira 2020), NE and other endomembranes might have even derived from an invagination of a δ-proteobacterial membrane. (4) A further possibility, which is in a sense a variant of (2b), assumes that the cytoplasmic endomembranes as well as NE have been formed by outward budding of the (pre-)mitochondrial outer membrane, with reference to the fact that extant mitochondria still form such mitochondria-derived vesicles (MDVs). For instance, peroxisomal membranes generated in actually living cells have been reported to become composed of mitochondrial vesicles and pre-peroxisomal ER-derivatives (Sugiura *et al.* 2017).

Despite these multiple theoretical possibilities, the origin of the nucleus is still a most enigmatic issue. This does not only concern the nuclear membranes, pores and the lamina, but also the process by which the NE has circumfered the enclosed eukaryotic chromosomes, which are profoundly different from bacterial and mitochondrial chromosomes, especially with regard to linearity, chromatin proteins, organization of genes and control elements. Several popular hypotheses have been formulated to explain the origin of the nucleus. These hypotheses have their merits, but are not free from some shortcomings. Their commonality is their complexity and untestability, as will be discussed below. In this article, we propose a relatively simple and, to a certain degree, testable hypothesis on the origin of nucleus. We hypothesize that the assumed protoeukaryotic cells bearing a preliminary nucleus had originated in a process of bacterial sporulation, in which precursors of nucleus and mitochondria have been jointly introduced into the assumed protoeukaryotic cell. This would imply acquisition of precursors of the two most important organelles in a single, rapid transition event that leads to eukaryogenesis. We denominate

this hypothesis as the Theory of Nucleus Origin from Bacterial Sporulation (TNOBS).

## CURRENTLY POPULAR HYPOTHESES OF NUCLEUS ORIGIN

In this section, we will briefly review several popular hypotheses regarding the origin of the nucleus as well as their pros and cons.

*The endomembrane genesis hypothesis*

The earliest and, for some while, predominant hypothesis related to the origin of the nucleus is the endomembrane formation theory which suggests that the NE had originated from cell membranes or inner membranes of prokaryotic cells [8-10]. This was believed to be a gradually progressing process. For example, for survival advantages, the prokaryotes (bacteria or archaea) had developed endomembrane structures (Vesteg *et al.* 2006; Heimerl *et al.* 2017) resembling endosome vesicles or ER. Some of these endomembrane structures might have become located close to the chromosomes, fused more or less to completely circumfer the chromosomes, perhaps, leaving some gaps, and formed a preliminary nucleus. For details and sources of vesicle budding see the preceding section. It has been speculated that synthesis and maintenance of the sophisticated eukaryotic endomembrane system required mitochondrial participation to provide sufficient energy for ATP-consuming processes such as the necessary involvement of cytoskeletal motor proteins (Martin & Koonin 2006; Martin *et al.* 2017). From the energy requirement point of view, one would expect that mitochondria or, at least, mitochondrial precursors should have been present before emergence of the nucleus. However, the time frame from the preliminary endomembrane structure to the intact NE should have occurred in the period between the assumed first eukaryotic common ancestor (FECA) and the last eukaryotic common ancestor (LECA), along with co-evolution of the cytoskeleton, heterochromatin, and the nuclear pore complex (CAVALIER-SMITH 1987; Cavalier-Smith 1988), whereas mitochondria appeared after the LECA. In this case, mitochondria could not have contributed to NE formation, and the origin of the nucleus is not plausibly explained. According to this model, the FECA and LECA were still amitochondrial cells with preliminary endomembrane structures including NE. Mitochondria should have appeared after nuclear formation in the transition from protoeukaryotes to the eukaryote. Due to the extremely long period of time since eukaryogenesis, no clear description concerning the nature FECA and LECA is possible and evidence for their presence in the evolution is lacking.

*The endosymbiotic hypothesis*

In reverse correspondence to the origin of mitochondria, endosymbiosis has also been suggested to explain the origin of the eukaryotic nucleus, however by assuming the engulfment of an archaeon by a bacterium [17]. This reversion of host and endosymbiont, relative to the original endosymbiotic hypothesis for mitochondria, takes account of the poor or missing endocytotic capacity of archaea (Burns *et al.* 2018). In this case, it was hypothesized that a bacterium belonging to the PVC superphylum (Planctomycetes, Verrucomicrobia, Chlamydiae) engulfs a thaumarchaeon. According to this view, the thaumarchaeon provided both informational and operational proteins, whereas the PVC bacterium was the source of phospholipids, tubulin and membrane coat proteins required for the formation of the nucleus (Forterre 2011). However, according to more recent knowledge, neither PVC bacteria nor thaumarchaea are the likely partial ancestors of eukaryotes. Currently, López-García and Moreira (López-García & Moreira 2020) have reformulated this hypothesis and adapted it to more likely precursors, in tripartite syntrophic model. These authors believe that eukaryotes originated in early proterozoic microbial mats from the endosymbiosis of a hydrogen-producing Asgard archaeon within a sulfate-reducing, myxobacterial-like δ-proteobacterium, to which a methanotrophic α-proteobacterium contributed the future mitochondrion, In this model, the Asgard archaeon evolved into the nucleus, while the δ-proteobacterium turned into the future eukaryotic cytoplasm (López-García & Moreira 2020). Based on the lacking endocytotic capacity of archaea, Baum and Baum (Baum & Baum 2014; Baum 2015) proposed an "inside out" hypothesis to explain the alternative endosymbiosis and nuclear origination. Notably, this hypothesis infers that nucleus and mitochondria evolved simultaneously. According to this concept, membrane blebs containing cytoplasmic extrusions emerged from an archaeal cell and grew out to form double-membrane structures, in which the basal part of the bleb membrane closely approached the plasma membrane of the archaeal cell body. These growing membrane extrusions finally connected to each other. Thus, the fused peripheral membranes of the outgrown blebs became the eukaryotic plasma membrane and the central part of the original archaeon which had produced the extrusions remained to be located in the middle of the membrane enclosure and was converted to the nucleus, while the α-proteobacteria that had been captured by the membrane blebs evolved to mitochondria. However, the assumption of capturing an α-proteobacterium, which is gram-negative and therefore enclosed by an outer and an inner membrane, is affected by the same problem as the endocytosis-based endosymbiont theory of mitochondria, namely, that the bacterial cytoplasm, i.e., the future mitochondrial matrix, should have been initially surrounded by three membranes, one of which, e.g., the outer bacterial membrane, needs to eliminated to arrive at the typical mitochondrial appearance. This problem exists in many models. Nevertheless, this hypothesis has

the advantage of jointly providing some clues to explain the formation of a complex endomembrane system including ER, mitochondria and, particularly, the double-membrane structure of the NE. Although this hypothesis, as any other one, cannot provide certainty about processes that happened about two billion years ago, it gives answers to several critical issues not only concerning the origin of the nucleus, but also that of the eukaryotic cell as a whole. However, a problem remains in this version with regard to the relative sizes of archaeon and bacteria.

Current evidence seems to support various aspects of this "inside out" hypothesis of endosymbiosis. In this model, a crucial argument is that the small cell size of archaea implies a lack of sufficient machinery and energy to carry out phagocytosis. Theoretically, the small host archaeon engulfs the metabolically more efficient partner bacteria only by using extrusion structures as also described in the entangle–engulf–endogenize model (Imachi *et al.* 2020). In this version, too, the extruded segments of the small archaeon (specifically, an Asgard archaeon) capture the aerobic organotrophic partner, which is a future mitochondrion. In the inside-out model, the archaeon becomes surrounded by its own expanded extrusions and develops a nucleus-like structure with chromosomes, and maintains the original plasma membrane as inner layer of the NE. In the entangle–engulf–endogenize model (Imachi *et al.* 2020), this process is not definitely described, but might be likewise applicable. Most of the endosymbiotic hypotheses prefer the nucleus having originated from an archaeon rather than from bacteria. The strongest arguments for this conclusion are based on the striking similarities between eukaryotic and archaeal machineries of nucleic acid metabolism, which are distinct from those of bacteria. For example, this concerns the DNA replication systems (Samson & Bell 2016), the presence of histones and nucleosomes in archaeal chromosomes (Gehring *et al.* 2016), occurrence of introns in tRNA genes (Yoshihisa 2014), similarities between the archaeal RNA polymerase (RNAP) and the eukaryotic RNAPII, in terms of subunit number, composition and architecture, promoter elements and basal transcription factors required for initiation and elongation of transcription (Werner 2007).

*The viral origin hypothesis*

The hypothesis that the eukaryotic cell nucleus originated from a virus is not new and was already proposed decades ago (Livingstone Bell 2001). It seems that several characteristics of the eukaryotic nucleus may have derived from a viral ancestry, since several eukaryotic traits cannot be deduced from bacteria nor from archaea. These include mRNA capping, linear chromosomes, and separation of transcription from translation. The evidence related to the molecular phylogenetic analysis of DNA polymerases suggests that eukaryotic DNA polymerase-α is closely related to the DNA polymerases of poxviruses. However, the nucleus of the viral origin hypothesis has its weaknesses. For example, the genetic material can have been horizontally transferred between species and viral genes are also frequently identified in the prokaryotic DNA. Thus, DNA polymerase-α may have been transmitted to eukaryotes or their prokaryotic ancestors via a horizontal transfer route. With the discovery of giant viruses of the NucleoCytoplasmic Large DNA Viruses group (NCLDV; also known as Megavirales), the nucleus of viral origin hypothesis was revisited. According to this view, NCLDVs played an important role in the origin of modern eukaryotes and the nucleus might have originated from an ancient NCLDV-related virus (Forterre & Gaïa 2016). Notably, some NCLDVs exceed both archaea and bacteria in both particle and genome size, the latter sometimes amounting up to 2.5 megabases. The origin of NCLDVs has been differently discussed, as descendants of smaller viruses (with some genophyletic support) (Koonin & Yutin 2019), as remnants of a fourth, meanwhile extinct, domain of cellular life (Brandes & Linial 2019), and even as missing links between rickettsiae and mitochondria (Seligmann 2019). The existence of nucleus-like structures in prokaryotic viruses has been seen as a further support for the viral origin hypothesis of nucleus genesis. However, in the case of the bacteriophage 201φ2-1, which infects *Pseudomonas* spec., a nucleus-like "envelope", which may protect the viral genome from host cell attack, is not formed by membranes, but rather by viral proteins. Currently, Takemura (Takemura 2020) has updated the viral hypothesis by illustrating the following scenario. An ancestral giant virus constructed a viral factory (VF) which surrounded the viral genome using a cytoplasmic, inner membrane-derived membrane. This VF was present in an infected prokaryotic cell, the viral DNA was replicated very closely to the host genome, and the host cell developed an envelope as a defense system against viral DNA to protect its own genome, thereby forming a primordial nucleus. This interpretation was explicitly given for eukaryogenesis, but remained largely based on parallels in bacteria. The presence of eukaryotic/archaeal proteins in the hypothetical VF, such as DNA polymerase-δ, histones, and Ran GTPase, which are primarily absent in viruses, was considered as the result of horizontal gene transfer from host to virus. Notably, internal compartmentalization with an NE-like structure was described in a member of the PVC superphylum, the planctomycete, *Gemmata obscuriglobus* (Fuerst 2005; Sagulenko *et al.* 2014). This bacterium also exhibits eukaryote-like nuclear pores (Sagulenko *et al.* 2017), an incomplete separation of ribosomes, which are found in minor amounts in the genome-containing compartment, but mainly in an additional riboplasm that is enclosed by another membrane (Sagulenko *et al.* 2014). However, this remarkable parallel to eukaryotes cannot be taken without difficulty as a basis of

eukaryogenesis, since PVC bacteria are no longer regarded as partial ancestors of eukaryotes and since the contribution of archaea remains beyond this concept. Moreover, the role of a virus in the planctomycetal compartmentalization has not been demonstrated and, in particular, the participation of a Megavirus seems to be excluded, for reasons of size, including that of the genomes. Finally, host entry of extant NCLDVs strictly depends on endocytosis, which is unproblematic in large eukaryotic cells, such as the frequently studied amoebae, but seems impossible with extant, normally sized bacteria and archaea and, presumably, also with their extinct ancestors.

## A THEORY OF NUCLEAR ORIGIN FROM BACTERIAL SPORULATION (TNOBS)

*The general process of bacterial sporulation*

Sporulation (endospore formation) has been most thoroughly studied in gram-positive bacteria, especially in *Bacillus subtilis*. The bacterial endospore often occurs under conditions of nutrient deficiency, high cell density or other unfavorable environments. This is a self-protective mechanism of bacteria against the environmental insults and it is also a genetically programmed process (Errington 1993)(Ryan & Shapiro 2003). The matured bacterial spores can tolerate the harsh environments including desiccation, heat, cold, extreme pH changes and UV radiation due to their thick proteinaceous coats, peptidoglycan cortex, low water content, and high levels of dipicolinic acid (DPA) (Cho & Chung 2020). The bacterial spores can remain dormant for long periods of time and return to vegetative growth under favorable ambient conditions. The bacterial sporulation is monitored by a genetic program that controls this process step by step. In the typical sporulation of *Bacillus subtilis*, seven stages are distinguished (Hilbert & Piggot 2004) (Figure 1). Interruption of this sequence at different stages leads to specific consequences.

Stage 1: under an unfavorable condition, some structural alterations precede the duplication of the bacterial chromosomes. Phosphorylation of the transcriptional regulator, Stage 0 Sporulation Protein A (Spo0A), turns on the genes that govern forespore- and mother cell-specific transcription factors and genes that are required for switching on the assembly of the tubulin-like protein FtsZ, whose filaments form the cytokinetic Z-ring. The FtsZ assembly and its controlling factors are normally used in the equal division of bacteria, in which the Z-ring is, under control of MinCD proteins, positioned at midcell (Ghosal *et al.* 2014; Szwedziak

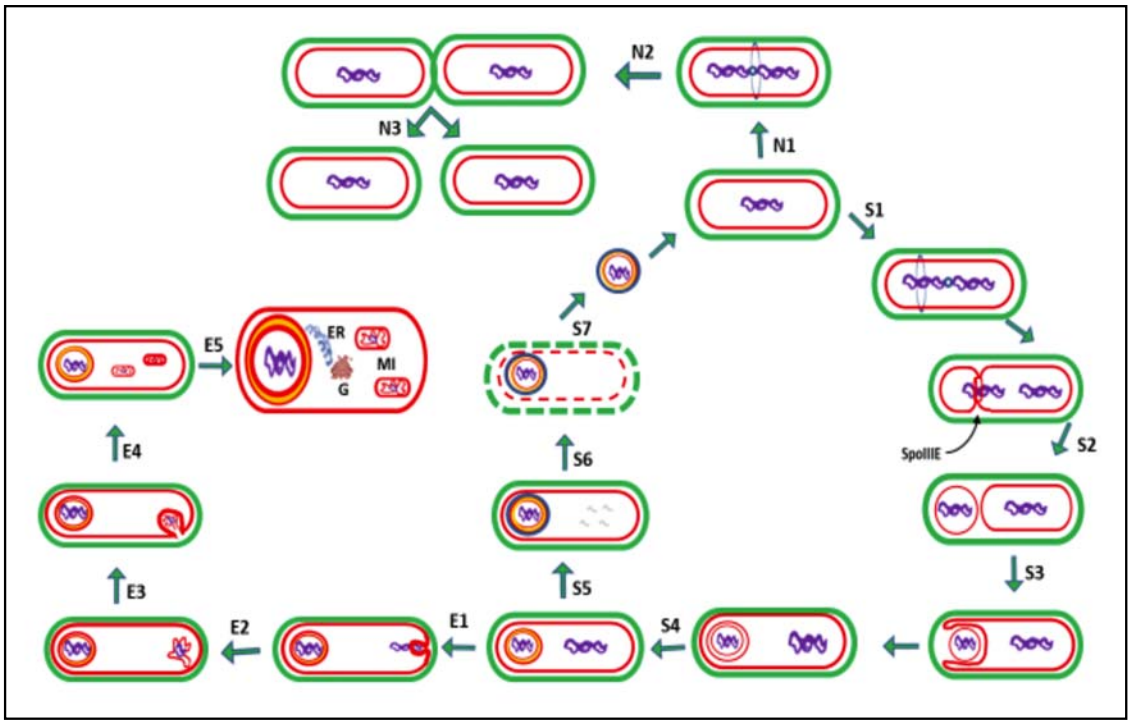


**Fig. 1. The stages of bacterial sporulation and hypothesized eukaryotic genesis.**
N1-N3: The bacterial normal vegetative growth, S1-S7: The sporulation stage 1-7, E1-5: A speculated eukaryotic genesis from the stage 4 forespore (PTC). E1: The self-endocytosis to engulf the part of chromosome which encoding the genes favoring the energy metabolism and ATP production. E2: The speculated protomitochondrion with single layer membrane. E3: The secondary self-endocytosis to engulf the protomitochondrion. E4:Mitochondrial formation. E5: Eukaryotic cell formation. Green cycle: cell wall, Red cycle: cell membrane, Blue ring: Z ring, Purple structure: chromosome, Yellow color between the two membrane cycle: peptidoglycan, Black cycle: spore coat, The disconnected cycle: lysed cell membrane and wall, ER: endoplasmic reticulum, G: Golgi apparatus, Mi: mitochondria, SpoIIIE: Stage III Sporulation Protein E

& Ghosal 2017). However, in sporulation stage 1, two Z-rings are positioned close to the two cell poles, presumably under control by SpoIIE, but usually only one of them leads to septum formation and, thus, later to asymmetrical cell division, a selection believed to be caused by unipolar accumulation of FtsA (Errington & Wu 2017). To ensure spatially correct DNA replication, the chromosome's replication origin, *oriC*, is trapped close to the selected Z-ring (Jameson & Wilkinson 2017). Stage 2: Asymmetrical (unequal) division of the cell membrane commences by tightening of the Z-ring. During this stage, the cellular pole portion (forespore) contains only the origin-proximal one-third of the chromosome, whereas the mother cell contains one complete chromosome and two-thirds of the chromosome destined to be placed in the forespore. At this moment, an ATP-dependent dsDNA translocase, Stage III Sporulation Protein E (SpoIIIE), located in the center of the septum pumps the remaining two-thirds of the forespore chromosome into the small compartment. Thereafter, septum formation proceeds and the separation of forespore and mother cell is finished by completion of their membranes. Stage 3: The mother cell membrane engulfs the forespore and, thus, the forespore receives a double membrane structure inside the mother cell. Stage 4: A peptidoglycan-containing cortex is formed between the membranes surrounding the forespore, while the basement layer of the coat (primordial germ cell wall) is initiated by the adapter protein, Sporulation Stage V Protein M (SpoVM), to which Sporulation Stage IV Protein A (SpoIVA) is attached (Decker & Ramamurthi 2017). Although the name SpoVM refers to stage 5, its action of membrane binding starts already at stage 4. During stage 4, the chromosome of the large cell is degraded, at least, in the normally proceeding sporulation program. Stage 5: Deposition of the protective layers of proteins starts with polymerization of SpoIVA and attachment of further coat proteins to complete the forespore coat. Stage 6-7 (Decker & Ramamurthi 2017): the spore experiences maturation. During these stages, the spore acquires its full resistance properties. The mother cell is lysed and the mature spore is released into the environment.

*Applicability of the concept to the putative karyogenesis*

Bacterial sporulation has been preferentially studied in gram-positive species, most often in *Bacillus subtilis*, from which the details described in the preceding sub-section have been obtained (Ghosal *et al.* 2014; Errington & Wu 2017; Szwedziak & Ghosal 2017; Jameson & Wilkinson 2017)(Decker & Ramamurthi 2017). As the putative partner in eukaryogenesis has most likely been an α-proteobacterium, presumably an extinct, free-living ancestor of Rickettsiaceae or a closely related species, and an Asgard archaeon, perhaps belonging to the Heimdallarchaeota or being related to Lokiarchaeota (Williams *et al.* 2020; Neveu *et al.* 2020)(Imachi *et al.* 2020), the applicability of our hypothesis to these groups of organisms has to be analyzed first. Although sporulation has been much less studied in gram-negative bacteria, the occurrence of this process has been unequivocally demonstrated in these diderm organisms. In *Acetonema longum*, endospore formation was shown to proceed in a widely similar way as in *Bacillus*, with the main difference of the existence of an outer membrane of the sporulating cell, but not more than the double membrane surrounding the spore, as formed by the engulfment of the early forespore by the mother cell's inner membrane (Tocheva *et al.* 2016). With regard to the diversity of gram-negative bacteria, the next question had been as to whether sporulation also occurs in Rickettsiaceae. The specific problem in this point results from the fact that all extant rickettsiae are endoparasites, and one may wonder why such an organism should experience adverse conditions that force it to sporulate. To date, we could not find any member of the genus *Rickettsia* that forms endospores. However, in another rickettsiacean species, *Coxiella burnetii*, formation of "terminal bodies" was described that strongly resemble endospores (Silverman 1991). As *Coxiella* widely lives in phagolysosomes, where it is exposed to rather low pH, this organism may be more easily confronted with adverse conditions. Whether or not this is a peculiarity of *Coxiella*, the finding indicates that Rickettsiaceae are in principle capable of endospore formation. Notwithstanding the fact that the biology of extinct, free-living rickettsiacean ancestors is and remains to be entirely unknown, a hypothetical concept of eukaryogenesis may be based on the assumption of sporulation in the α-proteobacteria. There is no evidence to show that the extant archaea have the capacity for sporulation, but one also cannot exclude this possibility for the ancient extinct archaea when they faced environmental stress such as nutrient deficiency.

*The hypothesized nucleus origin during sporulation*

Based on the above description and the illustration of Figure 1, the process of nuclear genesis (karyogenesis) in the subsequent steps is described as follows: In stage 2, an asymmetric division of the assumed α-proteobacterial cell results in two (a small and a large) daughter cells separated by membranes, but remaining encased by the same primary plasma membrane and the cell wall. However, at stage 3, the large cell that represents the major part of the mother cell engulfs the small cell (prospective forespore), in a process of self-endocytosis. This otherwise unusual step of self-endocytosis may have been an important achievement during evolution. Gould and Dring have hypothesized that bacterial endospore formation is one of the results from an early engulfment event that had also led to the development of complex eukaryotic cells (Gould & Dring 1979). After engulfment of the forespore, the relationship of these two daughter cells has changed to a cell inside of the other one, which

occupies in this stage the entire space of the previous mother cell. In literature, the large daughter cell that has engulfed the endospore is usually continued to be called "mother cell". Most importantly, the small cell inside the large one has acquired by the self-endocytosis a second membrane. Notably, these double membranes have been ultimately derived from morphological changes of the same plasma membrane of mother cell. As a result, this process leads to the consequence of two membranes, i.e., to the same result as in the earlier endosymbiotic hypotheses regarding origins of either nucleus or mitochondria, however, with some substantial differences. Any endocytosis-based assumption concerning interactions between different partners inevitably leads to two different membranes. Another fundamental difference concerns the genetic materials (chromosomes) of endocytosed and host cells, due to origination from two species. In stage 3 of sporulation, the endocytosed small cell has the exactly the same chromosome as the large cell, since they had been formed by replication in the initial stage of cell division. At stage 4, in addition to cortex formation between the membranes and the beginning of coat basement assembly by packed SpoIVA, another important change consists in the degradation of the chromosome of the large cell (Hosoya *et al.* 2007)(Shapiro & Losick 1997). If sporulation terminates in this stage, what will be the appearance of the large daughter cell containing the engulfed endospore? Morphologically, it resembles the assumed protoeukaryotic cell, i.e., a cell with a nucleus-like double-membrane boundary, but without mitochondria. For reasons of simplicity, we will refer to this cell as a proto-nucleate cell (PTC). In nature, genesis of a PTC should not be regarded as a low-probability event. Even mutation of a single gene, such as *X8* (Coote & Mandelstam 1973) or sporulation stage V protein G *(spoVG)* (Rosenbluh *et al.* 1981), can lead to an arrest of sporulation in stage 4, to generate a PTC under laboratory conditions, with a gross morphology that is reminiscent of the assumed protoeukaryote (or the LECA) (Figure 2).

Actually, sporulation-related gene mutations in bacteria frequently occur in nature and they influence the various stages of sporulation (Piggot & Coote 1976). We speculate that PTCs are generated frequently in different sporulating bacteria under natural conditions. The focus will particularly be given to the α-proteobacteria. A crucial aspect is, however, that of the viability of these cells. In beginning stage 4, the additional chromosome of the PTC is still present in the cytoplasm of the mother cell and could warrant its survival, but will be soon degraded. Therefore, inhibition of DNase expression is a prerequisite for survival of the PTC. If the chromosome in the endospore is already in a state preparing for dormancy, it will presumably be transcriptionally rather inactive, and the entire spore may remain for quite some time in the resting state without being damaged. More importantly, the fates of the cortex and of the primordial basement layer of the endospore coat may be decisive for survival. This is an aspect that is far from being banal. Usually, an inaccuracy of coat assembly, e.g., due to a *spoIVA* mutation, leads to the release of SpoIVA-CmpA heterodimers from the SpoVM adapter proteins, with the consequences of proteosomal lysis of SpoIVA-CmpA, inability to form a coat, but also of a lack of cortex formation. The endospore cells typically die and this may also be the fate of the mother cells that surround them(Decker & Ramamurthi 2017). However, all processes of autolysis can be expected to depend on the accuracy of cell death programs. Therefore, the existence of multiple mutations, in addition to those of Stage 4 arrestment, may allow both endospores and mother cells to survive. Unfortunately, information on this possibility is only marginally available and, moreover, confined to gram-positive bacteria, in particular, *Bacillus subtilis*. For further discussion of this important point see section 3.5.

Provided that a PTC can survive, it will, thereafter, demand more energy to maintain its endomembrane compartment, i.e., the assumed prospective nucleus. Since the spore chromosome is bounded by the newly

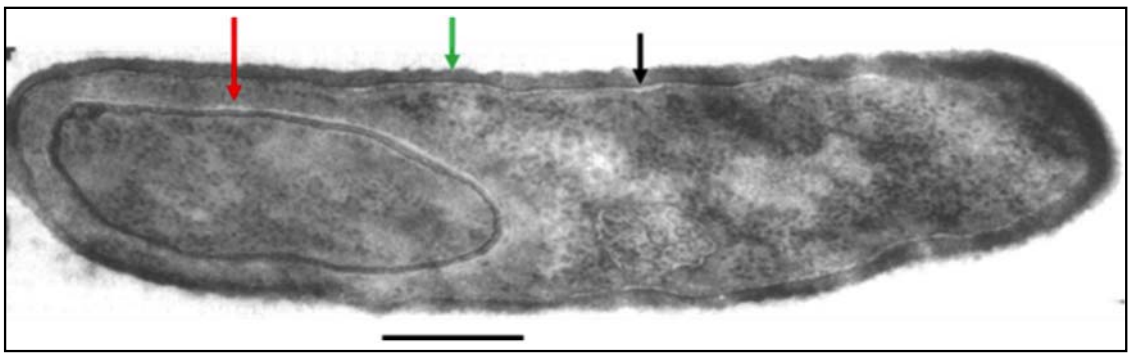

**Fig. 2. Stage 4 arrest of sporulation leads to a PTC-like morphology.**
The sporulation of Bacillus subtilis with X8 Mutation is terminated in the stage 4 with cortex formation and primordial germ cell wall. This is a typical PTC with nucleus-like structure. Cortex is visible as an electron-dense band between the forespore membranes. Red arrow: nucleus-like structure, green arrow: cell wall, black arrow: plasma membrane. The bar represents 0.2 µm, modified from Ref [68].

formed double membrane, gene activities including those required for replication, transcription and translation of the PTC will consume more energy than those of the non-compartmentalized chromosome present in its precursor. The potential energy deficiency with its consequences of slowing growth and replication of PTCs may limit their competition for propagation relative to their precursors and to other species. The necessity of sufficient ATP supply might have been irrelevant if we assume that PTC development has occurred in α-proteobacteria, especially in the ancestors of mitochondria. The discovery of sporulation-like process in *Coxiella* (Silverman 1991) may let this appear not entirely unlikely. As mentioned previously, endosymbiosis as a step in mitochondrial genesis was supported by the observation of the genetic similarity of mitochondria and α-proteobacteria (extant rickettsians). However, a major problem with this hypothesis is that of whether the assumed protoeukarytic cells or LECA had the capacity to conduct endocytosis as a means for capturing an entire bacterium. Such an endocytosis has never been identified either in bacteria or archaea. In a PTC which is supposedly derived from an α-proteobacterium, two identical chromosomes are present. One wrapped with double membranes is hypothesized to become the nucleus and may be metabolically conservative. The other chromosome in the cytoplasm of the PTC must be transcriptionally active to generate machineries for energy metabolism, thus, to maintain the PTC survival. If this holds, the machineries of energy metabolism encoded by the chromosome in the PCT may serve as a preliminary mitochondrion to provide energy. To improve the ATP productive capacity, the energy metabolic machineries and the essential part of the chromosome encoding them, become enclosed by double membranes to form mitochondria (Figure 1). The source of the mitochondrial membrane remains hypothetical and may be derived as similar as the forespore by self-edocytosis. This speculation keeps the core point that the mitochondria are derived from α-proteobacteria and avoids the dilemma related to the obstacle of the unidentified endocytosis in bacteria and archaea. Based on this hypothesis, the interval between karyogenesis and acquisition of mitochondria becomes negligible. This may be one of the reasons for why the assumed FECA and LECA have not been identified to date.

*Several issues related to this hypothesis*

1). *The nuclear membranes:* The process by which the double membranes of the nuclear envelope have been formed is the most critical issue for understanding karyogenesis. As mentioned above, most of the hypotheses on NE formation are largely speculation based on limited evidence and poorly tangible. In addition, no transitional structure of NE development has been identified in any putative precursor of eukaryotes to support most of the hypotheses. Nevertheless, compartmentalization by membranes does exist in non-sporulating bacteria, such as planctomycetes, in which riboplasm, paryphoplasm, and specialized compartments, e.g., the anammoxosome are separated (Neumann *et al.* 2011; van Niftrik & Jetten 2012; Grant *et al.* 2018). Contrary to the general perception of bacteria, the planctomycetal *Gemmata obscuriglobus* contains a nucleus-like structure bounded by a double-membrane that encloses the chromosome (Fuerst 2005; Sagulenko *et al.* 2014)(van Niftrik & Jetten 2012). However, there is no indication for a role of these organisms as ancestors of eukaryotes. At least, these findings show that bacteria are, in principle, capable of generating a persistent double-membrane structure that, when established, allows communication and exchange of macromolecular particles between the compartments. Of course, our own conclusions are also hypothetical, since a fusion process that has taken place almost 2 billion years ago cannot be judged on the basis of direct evidence, but rather on probabilities deriving from properties and cell biological potential of extant organisms. In our current hypothesis, the double layers of NE formation in the assumed proto-nucleus (the forespore engulfed by the mother cell) are not speculation but rather a reproducible, well-established fact (Figure 1, Stage 4).

2). *The nuclear pore complex (NPC):* NPCs are the protein structures used for information and selective material exchanges between nucleus and cytoplasm. In addition, the NPC plays an important role in gene expression and cellular homeostasis (Cho & Hetzer 2020). NPCs have not been identified in the double membrane of maturing forespores, what cannot be expected from a barrier that is destined to shield its contents from outside. However, a structure that allows controlled passage has been identified in the septum between forespore and mother cell at stage 2 of sporulation. At stage 2, the dsDNA translocase SpoIIIE is assembled to the newly formed septum membrane of the forespore as a major constituent of a large pore that allows transfer of one nascent chromosome from the cytosol of the mother cell into the forespore. Actually, the intercompartmental communications between the forespore and the mother cells are regularly taking place during all stages of sporulation. These communications are required for the profound morphological changes that also require global gene expression modifications in both compartments. All of these are mediated by activation of alternative RNA polymerase factors σ in both compartments (Shapiro & Losick 1997)(Xenopoulos & Piggot 2011). For example, $\sigma^F$ in the forespore is a master regulator of sporulation. When $\sigma^F$ is first activated, its downstream proteins cross the membrane to activate $\sigma^E$ in the mother cell and drive the mother cell to engulf the forespore.

These signaling pathways are two-directional. The $\sigma^E$ of the mother cell subsequently activates $\sigma^G$ in the forespore, whereupon $\sigma^G$ continuously relays $\sigma^K$ activation in the mother cell to instruct coat and cortex synthesis in stage 4 of sporulation. Intercompartmental communication continues until shortly before the release of the matured spore and the control part is obviously the forespore (a hypothesized protonucleus).

Despite some parallels between the septal pore and NPCs, these structures are dissimilar with regard to constituents, function and regulation of selectivity. Among NPC proteins, one has to distinguish between membrane nucleoporins, which are integrated in the pore membrane, scaffold nucleoporins and barrier nucleoporins (Onischenko & Weis 2011). Especially, the scaffold nucleoporins seem to share a common history with several eukaryotic coat proteins that are involved in membrane bending (Devos *et al.* 2004; Onischenko & Weis 2011). Importantly, these coat proteins must not be confused with those involved in spore coat formation. The homologies would imply that membrane-bending coatomers may have been at the basis of NPC development. A further implication may be deduced from the existence of forced membrane bending in both bacteria, e.g., in endospore engulfment of *Bacillus*, in endomembranes of planctomycetes (Neumann *et al.* 2011), and in archaea such as the presumably eukaryote-related Ca. *Prometheoarchaeum*, which develops long cell protrusions and membrane blebs (Imachi *et al.* 2020). The existence of these dynamic changes in cell morphology of prokaryotes is consistent with findings on the bacterial origin of sequences of NPC proteins(Koonin & Aravind 2009). Comparisons of NPC proteins from phylogenetically extremely distant eukaryotes have led to the conclusion that NPCs have been presumably present already in the LECA(DeGrasse *et al.* 2009). Interestingly, the similarities only concerned the scaffold and barrier nucleoporins, whereas transmembrane NPCs were absent in a rather primitive unicellular eukaryote, *Trypanosoma* (DeGrasse *et al.* 2009). If this is not a secondary loss, it might indicate that the primary step of NPC formation had been the repurposing of coat proteins for bending nuclear membranes to create pores. Of note, homologs of eukaryotic coat proteins that might have been repurposed have been detected in Asgard archaea, including Heimdallarchaeota(Zaremba-Niedzwiedzka *et al.* 2017).

3). *The chromosomes:* The proto-nucleus of a PTC contains the identical genome as the previous mother cell, now, the cellular cytoplasm. At this stage, the identity of constituents inside and outside the PTC's double membrane avoids any intracellular adverse reaction due to a primitive intracellular innate immune response (IIIR). IIIRs are obviously ancient, have been demonstrated in unicellular organisms (Ausubel 2005; Salminen *et al.* 2008), and seem to have evolved as a necessity of host defense against foreign nucleic acids. Such an IIIR will inevitably occur as soon as chromosomes from other species of prokaryotes (bacteria or archaea) come into play, by whatever mode of endosymbiosis, via endocytosis or other kind of fusion. The notion that archaea and eukarya are more closely related relative to the bacteria is mainly based on substantial traits concerning transcription and translation machineries. However, this does not justify a conclusion that the entire nucleus is derived from archaea, since chromosomes and machineries do not have to be confused with structural elements of organelles. In fact, bacteria have contributed in manifold ways to the eukaryotic genomes. For instance, the ribosome stimulating proteins called Ribosome Export Factors (REFs) may suggest an evolutionary history of inscribing the origin of eukaryotic nucleus. The non-membranous REFs (non-mREFs) which localize in nucleus originate exclusively from eubacterial (Gram positive bacterial) proteins, implying that the nucleus arose in a cell that contained chromosomes possessing a substantial fraction of eubacterial genes (Ohyanagi *et al.* 2008). The archaeal genes found in eukaryotes are not contradictory to a genesis of the nucleus via bacterial sporulation. Several possibilities exist for the entrance of archaeal genes to eukaryotic nucleus, such as horizontal gene transfer by plasmid translocation, virus infection, or extracellular vesicles from archaea (Gill *et al.* 2019). Based on phylogenetic analyses of aminoacyl-tRNA synthetases, Furukawa *et al.* have claimed that lateral gene transfers from several archaeal species of the DPANN superphylum have contributed to the formation of eukaryal cells. (Furukawa *et al.* 2017). In other words, some archaeal genes contained in eukaryotic chromosomes may descend from a distinct, ancient, and otherwise uncharacterized archaeal lineage that acquired some euryarchaeal and crenarchaeal genes via early horizontal gene transfer (Yutin *et al.* 2008).

4). *Nuclear lamins:* Lamins are constituents of the nuclear lamina on the karyoplasmic side of the inner nuclear membrane (Worman 2012). The contribution of these intermediate filaments to the integrity of the nucleus becomes obvious in laminopathies, such as Hutchinson-Gilford Progeria Syndrome, caused by a mutation in the *LMNA* gene which encodes lamin A and C proteins. The consequence of the lamina's structural instability is an early breakdown of nuclei followed by cell death (Piekarowicz *et al.* 2019). Therefore, the acquisition of such important NE stabilizers had been a highly relevant step in eukaryogenesis. To date, information on prokaryotic intermediate filament proteins, in particular, lamins, is scarce. In a bacterium, *Caulobacter crescentus*, an

intermediate-like protein, crescentin (CreS) has been discovered that is required for the curved membrane shape of this species (Celler *et al.* 2013). While this protein displays the property of curving membranes, which would be required for a nucleus, there is no direct evidence for a CreS-like protein as an ancestral lamin precursor nor for a bacterial origin of lamins. Lamins have been assumed to be present since the LECA (Gräf *et al.* 2015; Koreny & Field 2016), but this conclusion seems to have been precocious. Although a presumably phylogenetically ancient lamin, NE81, with some homology to mammalian lamins, has been described in the swarm-forming amoeba *Dictyostelium discoideum* (Batsios *et al.* 2019), lamins in the proper sense are not present in all eukaryotes, In trypanosomes (Rout & Field 2001) and in plants (Harder *et al.* 2000; Gindullis *et al.* 2002), laminal proteins are present that fulfill the same functions as lamins do, but they do not show homology to lamins. Therefore, it may not be worth-while to further search for lamin homologs in bacteria and archaea, but rather look for homologs of other long coiled-coil proteins in these domains. In fact, proteins of this category do exist in both archaea and bacteria, sometimes also with functions in DNA binding and DNA repair (Soh *et al.* 2015; Zabolotnaya *et al.* 2020). Whether some of them possess sufficient homology to lamina proteins of basal eukaryotes remains to be studied. In sporulation; however, the SpoIVA is the major structural protein in the basement layer of the forespore coat. It is anchored on the forespore surface by Stage IV Sporulation Protein M (SpoVM) and self-polymerizes in an ATP hydrolysis dependent manner to form a platform around the forespore. These static polymers are analogous to lamins to protect the integrity of the developing forespore (Decker & Ramamurthi 2017).

5). *Cell size:* The striking difference in size between large eukaryotic host cells and much smaller mitochondria as well as endosomes had led to the assumption that an endocytotic uptake of bacterial ancestors of mitochondria would have required a large protoeukaryote of similar dimension as a modern eucyte. This had further led to the conclusion that the protoeukaryote should have been an efficient predator that phagocytosed many bacterial cells, some of which escaped from intracellular digestion. However, such large cells are unknown among extant archaea, which are mostly in the range of bacteria. Relative to archaea, sporulating bacteria and PTCs deriving thereof can be fairly large and may attain 40 to 100 μm in length, such as in *Sporospirillum* species (Hutchison *et al.* 2014).

6). *Other endomembrane systems:* Based on our current hypothesis, other endomembrane systems including ER, Golgi apparatus and specialized Golgi-derived vesicles such as lysosomes and peroxisomes have developed after NE formation and appearance of proto-mitochondria.

*The survival odds for PTCs*

The PTCs are referred to as the cells whose sporulation has been terminated at sporulation stage 4. How long these cells can survive is an unanswered question. Since PTCs have lost the chance to escape from sporulation and also cannot further develop to mature spores, their fates are either to become lysed or to survive in the current status. Under the normal condition, the mother cells with matured endospores will be lysed at the final stage of sporulation (stage 6-7) (Figure 1). This is genetically programed by several temporally expressed genes (Smith & Foster 1995). This programmed lysis may not apply to the PTCs due to their arrest in stage 4. However, autolysis happens among various bacteria during sporulation and details may differ. In the majority of cases, sporulation occurs for the reason of limited nutrients available (Mueller *et al.* 1991). In some bacterial species, nutritional deficiency because of high cell density induces cell lysis (referred to as autolysis), to warrant a sufficient nutrient supply to the rest of the bacterial population. Sporulation induction assures formation of many viable matured spores (Liu *et al.* 2015). In addition, some bacteria practice cannibalism to lyse their siblings in favor of own survival (González-Pastor *et al.* 2003; Nandy *et al.* 2007). These two types of lysis are, in principle, applicable to PTCs. However, some PTCs can obviously bypass the lytic signals and survive. For example, the genetic variants of *B. subtilis* which have a terminated sporulation at stage 4 are still present in the nutrient-deficient medium and maintain an intact cell structure for at least 24 h after sporulation induction, whereas all wild-type mother cells with matured spores have autolysed to release the spores (Silvaggi *et al.* 2004). The question remains how long these PTCs will survive, when resuspended in the nutrient-enriched medium. Unfortunately, we could not identify any report on this issue. However, as discussed in subsection 3.3, the survival of PTCs has not yet been systematically studied in strains that carry mutations in autolysis control genes, in addition to sporulation arrest mutations. The reason for this gap is that the pertinent studies had been focused on sporulation efficiency rather than survival. Moreover, we believe that PTCs may have already survived under harsh conditions, such as nutrient deficiency, and there may be no reason for why they should not be able to survive and thrive under suitable conditions, as soon as the two threats of lysis are eliminated, in particular, by mutations in autolysis control genes. Theoretically, these PCTs still equip every machinery for survival as their precursors excepting having an additional "protonuclei". As mentioned above, for surviving and thriving the PTCs require to generate more energy maintaining the intact of their "protonuclei". This energy demanding finally

will drive the mitochondrial genesis from their cytosolic chromosome., a speculated eukaryogenesis.

## DISCUSSION

Eukaryotes have been hypothesized origin from the two prokaryotic domains, bacteria and archaea. This is currently common knowledge and this concept is accepted by the scientific community. However, the precise mode of interaction, fusion mechanisms and identification of interacting partners are still a matter of debate, partially due to the irreducible complexity of this process. Moreover, the reconstruction of interactions that have taken place almost 2 billion years ago in extinct organisms can never be achieved with absolute certainty. With regard to the archaeal and bacterial partners, extant species can only serve as models. This includes the problem that the nearest extant relatives of the assumed ancestors have meanwhile changed their properties, perhaps, profoundly. This is particularly the case in those α-proteobacteria that are believed to represent the group from which mitochondrial ancestors emerged. The Rickettsiaceae, among which these ancestors are assumed, are currently intracellular parasites of eukaryotes, whereas the ancient species that interacted, presumably with an Asgard archaeon, should have been a free-living organism, although it may have been constituent of a complex prokaryotic mat or biofilm.

The two most important organelles which represent unique hallmarks of eukaryotes are the nucleus and the mitochondria. The hypothesis of endosymbiosis for the mitochondrial origin is supported by genetic and biochemical evidence and is widely accepted by most scientists. However, the mode by which endosymbiosis was achieved, requires a critical reconsideration. The assumption that the uptake of the mitochondrial ancestor took place by classic endocytosis raises fundamental doubts, although this idea is outlined in textbooks and is still believed by numerous investigators. There are mainly two arguments that speak against this possibility [cf. sections 3.2 and 3.4.5)], namely, the small size and the absence of endocytosis in prokaryotes. However, an alternative to endocytosis has been described in an extant archaeon, Ca. *Prometheoarchaeum syntrophicum*, which is, according to current knowledge, the closest archaeal relative of eukaryotes (Imachi *et al.* 2020). Its mode of engulfing bacteria, based on extending cell protrusions and developing membrane blebs, eliminates the problems of both size and incapability of endosome formation by inward bending of the plasma membrane. Importantly, this process of protrusion formation requires the existence of proteins that act in a similar or even the same way as known from eukaryotes. Outward bending of the plasma membrane requires coat proteins (not to be confused with spore coat proteins) that act like those in filopodia formation, which contain I-bar domains (Yang *et al.* 2009). Moreover, the stability of extended protrusions is only conceivable with the involvement of cytoskeletal elements. The extension process indicates the existence of motor proteins. It would be of utmost importance to identify these three categories of proteins in *Prometheoarchaeum*. Their detection would even more strongly approximate this archaeon to eukaryotes and possibly shade some light on the unknown ancestor of the eukaryotes.

However, the origin of the nucleus has been much more difficult to explain and is still affected by considerable uncertainties. This is mainly due to the fact that no nucleus-like structures have been found in taxa containing putative eukaryotic ancestors. No such structures have been detected in archaea, whereas NE-like endomembranes have been described in planctomycetal bacteria, such as *Gemmata obscuriglobus* (Fuerst 2005)(Sagulenko *et al.* 2014)(van Niftrik & Jetten 2012). However, *Gemmata* is phylogenetically distant to those bacteria which have potentially contributed to eukaryogenesis, such as α- and δ-proteobacteria, and has consequently to be discarded as a potential source of the nucleus. Various hypotheses have been formulated to explain its origin, as discussed above (cf. section 2). The most difficult issue for these hypotheses is that the intermediate organisms between prokaryotes and eukaryotes suggested by any of the hypotheses cannot be identified in nature or reconstructed under laboratory conditions. Eucytes having a nucleus but are devoid of mitochondria do exist, but they can be shown to have lost their mitochondria secondarily (Karnkowska *et al.* 2016) and, as already stated, the bacterium *Gemmata* can be excluded for phylogenetic reasons. Eukaryotes harboring mitochondria but are devoid of a nucleus have never been discovered. The association of nucleus and mitochondria also suggests that they may have appeared either simultaneously or within a very short interval between their originations. This is also supported by the fact that the newly formed endomembrane compartments of eukaryotes, especially the nucleus, require sufficient ATP from mitochondria for maintenance and acquired additional functions. Otherwise, they would not have gained advantages in competing with prokaryotes, such as that or a considerably larger cell size, which enables phagocytosis and, thus, predation. It has been estimated that the energy demand of a eukaryotic cell is by orders of magnitude higher than that of a typical prokaryotic cell (Lane 2011). It would be inconceivable that nucleate cells could survive and thrive without multiple 'power stations' such as the mitochondria. Our hypothesis of nucleus origin has addressed some of these critical issues related to eukaryogenesis. First, we have identified cells with a nucleus-like structure, which we have referred to as PTC. PTCs are generated during the process of bacterial sporulation, especially, when sporulation is arrested in stage 4, because of mutations in sporulation-related genes. It has been reported that when

the environmental conditions alternate towards the beneficial direction, bacteria in an early stage of sporulation can still escape from this process and return to the non-sporulated form (Soufo 2016). However, once they have reached to stage 4 of sporulation, this process is irreversible (Freese *et al.* 1975). As a result, PTCs cannot revert back to their parent form and the majority of them are also unable to develop mature spores. Thus, they form a special type of cells that is reminiscent of the hypothetic protoeukaryotes with the "protonuclei", i.e., the double membrane structure with chromosome inside. We believe that the genesis of PTCs has occurred and still occurs countless times in nature, judging from the numbers of sporulating bacteria and the frequency of mutations in these fast-growing organisms. Notably, sporulation has not only been observed in gram-positive bacteria, which have served as model organisms for this process, but also in gram-negative species including α-proteobacteria. The energy demand for their survival will drive PTCs to generate more ATP supply as soon as possible. If PTC formation takes place in an α-proteobacterium related to the mitochondrial ancestor, the remaining chromosome in the cytosol of mother cell would be an excellent candidate for a future mitochondrial formation. It is logically that the chromosome containing inside of the "forespore" is metabolic and transcriptional conservation. This makes cytosolic chromosome must be transcriptional active to generate more and efficient machinery for ATP synthesis. During evolution, this cytosolic chromosome is evolved to mitochondrial specific ones by only maintaining the necessary gene for energy metabolism. In other hand, with extensive horizontal (lateral) gene transferring from the archaea or even from viruses, to the "forespore" chromosome, thus, then, by DNA recombination this "forespore" chromosome evolves to nuclear chromosome.

Our hypothesis of the nucleate genesis is based on the fact of the widely distributed spore-generating prokaryotes and the high mutation rates in bacteria that favor PTC genesis. The TNOBS does not repel the bacterial, archaeal and viral original theories of eukaryotes but it is a complementary work to them. In addition, TNOBS can plausibly explain the long lasting dilemmas as to the endosymbiosis that bacteria and archaea are relatively small size, lack the massive endocytosis capacity, as well as are absent for evidence of the LECA.

Nevertheless, the TNOBS hypothesis is a rather simple and the most tangible one, since some of its details may be reproducible under laboratory conditions.

## CONCLUSION

This new concept of eukaryogenesis delineates a mechanism by which a sporulating bacterium that is arrested in stage 4 of sporulation can provide both a nucleus-like double membrane and a precursor chromosome of a mitochondrion. Although most of the pertinent studies on sporulation have been conducted in gram-positive bacteria, this has also been found to exist in gram-negative bacteria including, with high likelihood, a member of the Rickettsiaceae, which are believed to be the closest extant phylogenetic relatives of mitochondria.

In summary, the immaturely arrested endospore that is already bounded by a double membrane is assumed to be transformed into a proto-nucleus, whereas the cytosolic chromosome in mother cell will become the chromosome of the mitochondrial precursor. These transformations require, of course, further changes in either organelle. For instance, the speculated mitochondrial chromosome requires extensive deletion to only preserve the genes with energy metabolic advantages while the speculated nucleus chromosome experiences the massive gene recombination by lateral gene transferring from archaea or even from viruses.

The main advancements of our TNOBS hypothesis consist in the combination of karyogenesis with concomitant of a mitochondrial genesis, along with the unnecessity of unidentified endocytotic uptake of an endosymbiont in prokaryotes and its reproducible feature under laboratory conditions.

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None.

## CONFLICT INTEREST

None.

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