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The Parasitic Bacterium *Wolbachia* and the Origin of the Eukaryotic Cell

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Abstract—The acquisition of endosymbiotic alphaproteobacteria that gave rise to mitochondria was one of the key events in the origin of eukaryotic cell. To reconstruct this process, it is important to analyze relationships that developed later between eukaryotes and other alphaproteobacteria. *Wolbachia pipientis*, a bacterium that inhabits cells of numerous terrestrial invertebrates and exerts diverse effects on its hosts, is used as a model. Although *Wolbachia* is similar to mitochondria in many important features (basic metabolism, small molecule membrane transport, envelope structure, etc.), their relationships with the nucleocytoplasm are different. Mitochondria import most of their required proteins from the nucleocytoplasm and are controlled by the nucleocytoplasmic regulatory systems. On the contrary, *Wolbachia* exports its proteins into the host's cytoplasm, thus causing dramatic aberrations in the ontogeny and reproduction of the host. This difference may be due to the fact that most of the protomitochondrial genes had been transferred into the central (nuclear) genome at the early stages of the development of the endosymbiotic system, while *Wolbachia* genes were not transferred into the nucleus. This fits well with the previously suggested hypothesis that there was a period of rapid lateral gene transfer in the evolution of proto-eukaryotes; the acquisition of mitochondria took place during this period. Later, eukaryotes, and especially metazoans, developed powerful mechanisms for prevention of lateral gene transfer. Therefore, the genes of the newly acquired endosymbionts cannot be transferred into the central genome, and the endosymbionts retain the capacity for selfish evolution.

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INTRODUCTION

The appearance of eukaryotes was one of the most important events in the history of life on Earth, which significantly changed the structure of the biosphere and of biogeochemical cycles, and opened new opportunities for progressive evolution of organisms (Schopf, 1983; Martin and Russell, 2003). It is believed that, for the majority of its existence (until 1 Ga), the biosphere was mainly prokaryotic (Zavarzin, 2001). However, more recent data suggest that eukaryotes apparently appeared and began playing a significant role in communities much earlier, which suggests the existence of an intermediate (prokaryotic–eukaryotic) biosphere in the interval from 2 to 1 Ga (Rozanov, 2003). The very early appearance of eukaryotes is supported, for instance, by the remains of sterols founds in the 2.7 Ga-old beds (Brocks *et al.*, 1999), and so-called eukaryotic-like microfossils described from 2.9–3.0 Ga-old beds (Timofeev, 1982).

The acquisition of bacterial endosymbionts that gave rise to mitochondria was one of the major events in the early evolution of the eukaryotic cell. Some authors suggest that the acquirement of mitochondria, rather than the nucleus, was the key event in the evolution of eukaryotes (Vellai *et al.*, 1998; Vellai and Vida, 1999). The coexistence of two different genomes within the same cell required the development of an efficient system for their coregulation and coordination. Hence, the acquirement of intracellular symbionts could have become an important stimulus in the development of the nucleus and genetic regulatory system (Markov, 2005). The analysis of mitochondrial genomes shows a monophyletic origin of the mitochondria of all modern eukaryotes (Dyall and Johnson, 2000; Litoshenko, 2002; Boussau *et al.*, 2004). Protozoans lacking mitochondria apparently evolved from ancestors that had mitochondria because their nuclear DNA contains genes of mitochondrial origin. Interestingly, eukaryotes were shown to have nuclear genes, although coding for cytoplasm proteins, but having a nucleotide sequence similar to the genes of Proteobacteria (ancestors of mitochondria). This indicates that the mitochondrial symbiont could have played a more important role in the development of genetic and biochemical structure of the eukaryotic cell than was previously thought. Many genes of mitochondrial origin

became adapted to perform nuclear–cytoplasmic functions (Roger, 1999; Gabaldon and Huynen, 2003).

Mitochondria evolved from Alphaproteobacteria, which include some purple bacteria performing anaerobic photosynthesis. It is still debated which group of Alphaproteobacteria was ancestral to mitochondria. Some authors place the probable ancestor close to the Rickettsiales (Andersson *et al.*, 1998; Emelyanov, 2003). It has been shown recently that the mitochondrial genome of yeast is most closely related to that of the purple nonsulfurous alphaproteobacterium *Rhodospirillum rubrum* (Esser *et al.*, 2004). The electron transport chain that was originally formed in these bacteria as part of the photosynthetic apparatus was later adapted for oxygen respiration. This was performed by the symbiont scavenging toxic oxygen, a function that was the main incentive to group the anaerobic ancestor of the nucleocytoplasm with the protomitochondrion (Gupta, 1998; Kurland and Andersson, 2000; Dolan *et al.*, 2002).

Soon after the ancestors of mitochondria became endosymbionts, many of their genes were transferred into the nucleus, where they were controlled by nuclear-cytoplasmic regulatory systems. Genes were transferred in large complete blocks (Martin, 2003). In parallel, many alphaproteobacterial proteins were replaced or supplemented by new eukaryotic proteins, the genes of which were formed in the nuclear genome and were never included in the genomes of mitochondria, or their ancestors (Karlberg, 2004).

The reconstruction of the initial phase of symbiotic relationships of the mitochondria and the nucleocytoplasm is of key importance in understanding the entire process of the appearance of the eukaryotic cell. One possible approach to such a reconstruction is the study of relationships that later developed between eukaryotes and prokaryotic endosymbionts, especially alphaproteobacteria.

This paper discusses *Wolbachia pipientis*, one of the most interesting endosymbiotic alphaproteobacterium that has broad effects on its various hosts, as a model of endosymbiosis. We compare the systems of interactions of the nucleocytoplasm with mitochondria and *Wolbachia*. The discussion of possible reasons for similarities and differences revealed allows the recognition of several key factors, which determined the relationships of the nucleocytoplasm with mitochondria in the initial stage of the formation of eukaryotes. In addition, *Wolbachia* alone is an organism of great theoretical interest as an example of so far poorly known factors capable of having a significant effect on the evolution of metazoan eukaryotes.

Wolbachia inhabits cells of a vast diversity of land invertebrates. It infects many insects (Diptera, Lepidoptera, Coleoptera, Siphonaptera, Psocoptera, Mallophaga, Orthoptera, Isoptera, Hemiptera, Odonata, Hymenoptera, Collembola, etc.). According to different estimates, as many as 20 to 75% of insect species may be infected by *Wolbachia*. *Wolbachia* is also found in many spiders, ticks, crustaceans (land, littoral, and freshwater isopods, and littoral amphipods), and filarial nematodes. The number of known *Wolbachia* hosts grows continuously. It is becoming clear that *Wolbachia* infections in land invertebrates occur widely (Stouthamer *et al.*, 1999).

Wolbachia has different relationships with different hosts. It may be a parasite, a commensal, or a symbiont. *Wolbachia* has adapted to finely control the reproduction and development of its hosts. Therefore, it is referred to as the microbial manipulator (Stouthamer *et al.*, 1999). The wide distribution of *Wolbachia* and its large theoretical and practical importance explain the growing interest being taken in this bacterium. The time of origin of *Wolbachia* can be estimated only by the molecular phylogenetic methods because no fossil record of it has been found. Apparently, two main divisions of *Wolbachia* strains (A + B and C + D, living in arthropods and nematodes, respectively) diverged about 100 Ma (in the mid-Cretaceous), while the divisions A and B are found to have diverged about 58–67 Ma (approximately at the Cretaceous–Tertiary boundary) (Werren *et al.*, 1995b; Bandi *et al.*, 1998). Apparently, *Wolbachia* did not descend from free living ancestors, but rather from certain other intracellular alphaproteobacteria over 100 Ma (most likely in the Early Cretaceous) (Stouthamer *et al.*, 1999). Apparently, the initial phases of the evolution of *Wolbachia*, the stages of the evolution of *Wolbachia* were connected to the expansion of angiosperms and insects at the beginning and in the mid-Cretaceous.

EFFECTS OF *WOLBACHIA* ON HOSTS

Diverse manipulations performed by *Wolbachia* on its hosts pursue the single goal of spreading the bacterium in the infected population. *Wolbachia*, like mitochondria, can live only in the cells of other (eukaryotic) organisms. It is not possible to culture this bacterium in artificial media, and only recently was it successfully cultivated in vitro in insect and mammalian cell lines (Noda *et al.*, 2002). *Wolbachia* (like mitochondria) is mainly vertically-transmitted (maternally inherited), by penetrating into the oocyte cytoplasm and, thus, infecting the offspring. Lateral transmission is also possible, although it is relatively rare (Werren *et al.*, 1995a; Heath *et al.*, 1999; Van Meer *et al.*, 1999), because of the inability of *Wolbachia* to live outside their hosts. This determines the main targets facing this parasite. To spread as much as possible *Wolbachia* has to (1) be of very little harm to the infected female, or even increase its viability; (2) increase the fecundity of infected females; (3) decrease fecundity in unaffected females (see below about infected males that are used as a tool); (4) shift the sex ratio in the host population to increase the proportion of females. *Wolbachia* cannot be transmitted with spermatozoids (Bressac and Rousset, 1993). Bacteria that enter a male do not have

a chance to transmit their offspring to the next host generation. Therefore, males are unnecessary ballast for *Wolbachia*.

Effects of *Wolbachia* on its host is firstly determined by the strain of the bacterium, and only secondly by the biology of the host. The main types of effects of *Wolbachia* are summarized below.

Cytoplasmic incompatibility (CI) is the most widespread and apparently evolutionary the earliest effect of *Wolbachia*. CI is induced when the infected male fertilizes a uninfected female and the parental chromosomes in a fertilized egg behave abnormally, and are eventually destroyed (Richardson *et al.*, 1987; Callaini *et al.*, 1997). As a result, the egg remains haploid, and the embryo soon dies. The molecular mechanism of CI is still not completely resolved. According to the best supported hypothesis, *Wolbachia*, living in the male gonads, somehow modifies ("labels") the chromosomes of spermatozoids. This label is the reason why chromosomes are destroyed after fertilization. However, if the female is infected by the same strain of *Wolbachia*, paternal chromosomes are not destroyed, and a normal (although infected) individual is developed from the egg. Apparently, bacteria present in the cell recognize the label and rescue chromosomes from destruction. This recognition and rescue in most cases is **strain-specific**, i.e., *Wolbachia* only save chromosomes labelled by the same strain (Werren, 1997).

Parthenogenesis. In some Hymenoptera, Thysanoptera, Collembola, and ticks, *Wolbachia* causes parthenogenesis. Normally in Hymenoptera, unfertilized eggs produce males, while fertilized eggs produce females. *Wolbachia* interferes with the process of ontogeny and destroys the normal development of the insects. When an unfertilized egg (from which a male should normally develop) begins the first mitosis, *Wolbachia* stops this process in the anaphase when the chromosomes are already doubled, but the daughter nuclei are still undivided. As a result, the egg becomes diploid, and a female develops from it (Stouthamer and Kazmer, 1994).

Feminization. *Wolbachia* transforms genetic males into females in Isopoda. The mechanism of this phenomenon is studied in the woodlouse *Armadillidium vulgare*. It was found that *Wolbachia* affects the production of the androgenic hormone. In the absence of this hormone, the embryo develops in the female, while in its presence, it becomes a male. In male woodlice, *Wolbachia* suppresses the development of the androgenic gland producing this hormone (LeGrand *et al.*, 1987). If an adult male woodlouse with an androgenic gland already developed is deliberately infected by *Wolbachia*, partial feminization will take place, although the function of the androgenic gland will continue. In the case of the partial feminization, intersexes (individuals with a combination of male and female characters) appear. In isopods, females are heterogametic (i.e., have two different sex chromosomes (WZ), while males have ZZ). If a "neofemale" woodlouse (an infected genetic male with the chromosomes ZZ) is cured, it may partially or completely become male (if the "female" was young), or remain as a female, if it was old, but capable of producing males only (because it lacks a "female" chromosome W).

Male killing (androcide). *Wolbachia* causes death of male embryos in the two-spotted ladybug *Adalia bipunctata* (Hurst *et al.*, 1999, Zakharov *et al.*, 2000), the butterflies *Acraea* (Jiggins *et al.*, 1998) and *Ostrinia*, the flies *Drosophila bifasciata* (Hurst *et al.*, 2000) and *D. innubila* (Dyer and Jaenike, 2004), and, possibly, in some ants (Van Borm *et al.*, 2001). Death of males is caused by other bacteria (*Rickettsia, Ehrlichia*, or *Spiroplasm*) and parasitic protozoans. It is possible that, occasionally, partial death of males may be beneficial to the insects (Zakharov, 1999). In this case, this effect can be genetically selected by the host, i.e., the host transfers the regulation of the sex ratio in its population to the symbiotic bacteria. Perhaps, the death of males in insects is a response to the factors common for *Wolbachia* and other male-killing agents. It is important that, among insects in which *Wolbachia* causes androcide, there are species both with heterogamete females (females WZ, males ZZ in butterflies) and males (females XX, males XY in *Drosophila* and ladybugs), and also haplodiploids (females XX, males XO in ants). Hence, *Wolbachia* does not recognize males by their chromosome sets, but by some other means.

Increase in fecundity and viability. Some insects that have been cured from a *Wolbachia* infection live significantly less and their total adaptability (including fecundity, competitive ability of their larvae, etc.) is lower. In the wasp *Trichogramma bourarachae*, the only known effect produced by *Wolbachia* is a twofold increase in the fecundity of females (Girin and Boulétreau, 1995). In other flies and wasps, this effect is combined with SI. Sometimes, *Wolbachia* increases the fecundity of males (Hariri *et al.*, 1998). In the wasp *Asobara tabida*, all individuals of which are infected by all three strains of *Wolbachia*, one of these strains is absolutely necessary for normal oogenesis, whereas the other two cause SI.

In filarial nematodes, *Wolbachia* is apparently a useful symbiont. Nematodes cured of *Wolbachia* with tetracycline showed a considerable increase in mortality and lower fertility. However, these effects may be caused not only by the fact that *Wolbachia* is essential for normal development of nematodes, but also by toxins freed after the death of the endosymbiotic bacteria. Attempts to develop an effective method of filariasis treatment based on the use of antibiotics to kill the symbionts of the pathogenic nematodes have not yet been successful because not every single nematode is killed (Chirgwin *et al.*, 2003), although some success in this field has been achieved (Mal'tseva, 2004).

Apparently, all the diverse effects of *Wolbachia* are based on the same molecular-genetic basis and can rel-

atively easily transfer from one to another. There is a possibility of a phage transduction of genes responsible for various effects from one strains of *Wolbachia* to another. In contrast to other endosymbiotic bacteria, *Wolbachia* shows a high level of infestation by phages, which are relatively often transmitted laterally between the strains (Bordenstein and Wernegreen, 2004; Gavotte *et al.*, 2004).

Mobile genetic elements and evolution of *Wolbachia*. The genome of *Wolbachia* is small and very simplified, which is characteristic of both endosymbiotic bacterial parasites and mitochondria (Schneider and Ebert, 2004). However, *Wolbachia* was found to contain unusually many repeated sequences and mobile genetic elements (MGE). This unique feature of *Wolbachia* makes it different not only from mitochondria, but from all other endosymbiotic bacteria. The genome of *Wolbachia* carries the traces of numerous and frequent reorganization related to the translocation of MGE. Apparently, mobile elements played a significant role in the evolution of *Wolbachia* (Wu *et al.*, 2004). Possibly, numerous MGE allow *Wolbachia* to survive evolutionary crises occurring in its environment, because of the rapid evolutionary saltation of their hosts (e.g., *Drosophila*), in which stress can cause activization of their own MGE and explosive mutagenesis (Ratner and Vasilyeva, 1993; Vasilyeva *et al.*, 1999). MGE may be "receptors of external stressing signals initiating explosions of transpositional variability in critical periods of the population evolution" (Ratner and Vasilyeva, 1993, p. 57). Probably, the abundance of active MGE is one of the features facilitating the extreme evolutionary plasticity of *Wolbachia*.

WOLBACHIA AND MITOCHONDRIA

Size of genome and proteome. Compared to free-living bacteria, the size of the genome of *Wolbachia* is small (1268 thousand base pairs; for comparison, the genome of *E. coli* contains 5528 thousand base pairs). In alphaproteobacteria, the size of the genome ranges from one million base pairs (in rickettsias) to over nine million base pairs in *Bradyrhizobium japonicum* (Boussau *et al.*, 2004).

The mitochondrial genomes of different eukaryotes considerably differ in size, but in general they are significantly smaller than in *Wolbachia*. Relatively large mitochondrial genomes are characteristic of higher plants (in *Nicotiana tabacum*, 431 thousand base pairs; in *Beta vulgaris*, 369 thousand base pairs; in *Arabidopsis thaliana*, 367 thousand base pairs; in *Marchantia polymorpha*, 187 thousand base pairs); considerably smaller genomes are identified in protozoans, algae, and fungi (in *Saccharomyces cerevisiae*, 86 thousand base pairs; in *Chara vulgaris*, 68 thousand base pairs; in *Saprolegnia ferax*, 47 thousand base pairs; in *Paramecium aurelia*, 40 thousand base pairs; in *Laminaria digitata*, 38 thousand base pairs); and the smaller genomes are shown to be present in Metazoa (in *Loxodonta africana, Mytilus edulis, Arbacia lixula, Bos taurus*, and *Homo sapiens*, 16–17 thousand base pairs, while in *Brugia malayi*, 14 thousand base pairs). Apparently, the increase in the size of the mitochondrial genome in higher plants, as well as its extreme decrease in animals, is secondary. A mass transduction of the mitochondrial genes into the nucleus and simultaneous simplification of the mitochondrial genome apparently happened in the earliest eukaryotes, while the size of their mitochondria was apparently initially stabilized at approximately the level that is presently observed in lower (protozoan) eukaryotes.

Recent estimates suggest that the size of the proteome (number of proteins) in the hypothetical common ancestor of alphaproteobacteria was from 3000 to 5000. The genome of *Wolbachia* encodes slightly more than 1000 proteins. Apparently, a considerable number of genes and proteins (2300–3800) were lost by the common ancestor of *Wolbachia* and rickettsias as a result of endosymbiotic parasitism; after the lineages of *Wolbachia* and rickettsias diverged, the former lost an additional 200–700 proteins (Boussau *et al.*, 2004).

In eukaryotes, the number of mitochondrial proteins is usually smaller, but not much smaller. In the reconstructed proteome of a "protomitochondrion," the minimum number of proteins is 630 (Gabaldon and Huynen, 2003). The most important difference is that the overwhelming majority of the latter are encoded by nuclear genes. For instance, in the fungus *Saccharomyces cerevisae*, humans, *Arabidopsis thaliana*, and the flagellate *Reclinomonas americana*, only 8, 13, 25, and 64 mitochondrial proteins, respectively, are encoded by mtDNA. All other mitochondrial proteins (more than 600) are encoded by nuclear genes (Karlberg, 2004; Reichert and Neupert, 2004). All proteins of *Wolbachia* are naturally encoded by its own genome, while no evidence has been found suggesting transportation of the host's proteins to the bacterium.

Membrane. The external membrane responsible for material and informational exchange between organisms plays an important role in the relationship with the eukaryotic cell.

Wolbachia, living in the host's cytoplasm, are surrounded by a double membrane (like mitochondria). The internal membrane belongs to *Wolbachia*, whereas the external one is the product of the host's cell. The double membrane of the mitochondria supposedly has such a double origin.

Free-living gram-negative bacteria (including alphaproteobacteria) usually have a complex membrane (cell wall), composed of three layers: internal cytoplasmic membrane, periplasmic space containing peptidoglycan, and external membrane differing in its composition from the cytoplasmic membrane and containing lipopolysaccharides. Ancestors of mitochondria became obligatory symbionts and lost the external layers of their membrane, retaining the internal membrane only, while the

Wolbachia and mitochondrion: major similarities and differences

Similarity	Difference
• Aerobic energy metabolism (Krebs cycle, oxidative phosphorylation)	• Localization of the majority of genes: in the nucleus (mitochondrion) or in the own chromosome (*Wolbachia*)
• Transport of small molecules	• Protein transport direction: export (*Wolbachia*), or import (mitochondrion)
• Membrane (internal membrane is inherent, external membrane is from the host)	• Direction of regulatory effects (coincides with the direction of the protein transport)
• Basic information systems (ring chromosome, replication, transcription, translation, ribosomes, etc.)	• Direction of the gene transfer: export (mitochondrion) or import (*Wolbachia*)

nucleocytoplasm formed the external membrane of the newly acquired organelles.

Wolbachia and its ancestors have been obligatory endosymbionts or parasites for hundreds of millions of years, and, during this time. they lost many genes, necessary for the synthesis of a normal cell wall. For instance, the system of lipopolysaccharide synthesis is virtually lost. Supposedly, it stopped functioning in the common ancestor of *Wolbachia* and its closest relatives, i.e., the intracellular alphaproteobacteria *Ehrlichia* and *Anaplasma* (Wu *et al.*, 2004). Instead of its own external membrane, to form which lipopolysaccharides are necessary, these bacteria use the external membrane supplied by their host.

However, in contrast to mitochondria, *Wolbachia* retains a middle layer of the cell wall containing peptidoglycan. Its genome contains genes participating in the metabolism of the components of this layer (D-alanine-D-alanine ligase, (D-alanyl-D-alanine-carboxypeptidase, UDP-N-acetylmuramate-alanine ligase, N-acetylmuramoyl-alanine amidase, phospho-N-acetylmuramoyl-pentapetide transferase, etc.). Thus, there is no apparent similarity between the middle layer of the *Wolbachia* membrane and the intermembrane space of mitochondria.

Metabolism and transport. *Wolbachia*, an aerobic bacteria that is similar in its structure of the basic systems of energy metabolism to mitochondria, could have become a useful symbiont. However, this is not the case (table). Although metabolism of *Wolbachia* is generally organized in such a way as to do the least harm to its host, it provides no apparent metabolic benefit.

The main function of mitochondria is to synthesize ATP through the oxidation of pyruvate, the final product of the glycolysis in the cytoplasm. The ATP produced by the mitochondria is transported to the cytoplasm.

Rickettsias, relatives of mitochondria and *Wolbachia*, pump ATP out of their host's cytoplasm in exchange for ADP using TLC proteins (Andersson *et al.*, 1998). Thus, rickettsias behave like "energy vampires," in direct contrast to mitochondria. No genes responsible for such membrane transport of ATP are found in the genome of *Wolbachia*. *Wolbachia* is apparently less harmful to its host, pumping only some amino acids and carbohydrates from its cytoplasm. However, it certainly does not supply energy in the form of ATP to its host.

Although the metabolism of carbohydrates in *Wolbachia* is generally restricted, it still has the necessary minimum set of proteins responsible for the complete cycle of catabolism of carbohydrates to receive energy. Hexoses (glucose, fructose) are transported from the host's cytoplasm. The set includes: (1) a membrane protein responsible for the phosphorylation-based transport of hexoses into the cell; (2) glycolysis beginning from fructose-1.6-diphosphate; (3) nonoxidative pentose phosphate pathway; (4) complete cycle of tricarboxylic acids; and (5) respiratory electron transport chain of oxidative phosphorylation.

The fact that it has its own genes responsible for glycolysis distinguishes *Wolbachia* from mitochondria and rickettsias (Andersson *et al.*, 1998; Wu *et al.*, 2004) and shows that it is even less complimentary to the nucleocytoplasm from the point of view of metabolism. At the same time, it is potentially less harmful because it seizes the host's metabolites at the earlier stages of their biochemical processing and provides not only the most energy profitable (aerobic) stages of carbohydrate catabolism, but also the less profitable aerobic stages.

The large number of amino acid transporters suggest that *Wolbachia* receives a considerable portion of its energy from them. In addition, this allows *Wolbachia* to survive without many enzymes of amino acid metabolism. The same features (presence of amino acid transporters and extremely incomplete set of enzymes of amino acid metabolism) are also characteristic of the reconstructed "protomitochondria", the hypothetical ancestor of mitochondria (Gabaldon and Huynen, 2003), and for rickettsias.

Apart from glycolysis, *Wolbachia* has several more metabolic pathways absent in rickettsias (including the synthesis of nucleotide-monophosphates, riboflavin, degradation of threonine, metabolism of pyrimidines, etc.) Perhaps, the somewhat increased biochemical independence of *Wolbachia* compared to rickettsias is one of the reasons for the greater evolutionary plasticity in producing various means to manipulate the host. In general, the metabolic systems of *Wolbachia* are reduced considerably less than those of rickettsias and

retain more similarities with the hypothetical common ancestor of alphaproteobacteria (Boussau *et al.*, 2004).

Apparently, the ancestor of mitochondria, like *Wolbachia*, had several metabolic pathways which were not directly connected to the synthesis of ATP (e.g., metabolism of fructose and mannose, synthesis of lipids, nucleotides, and vitamins). Many of the genes of mitochondrial origin were transmitted to the nuclear genome; hence, new enzymes and metabolic pathways became functional in other parts of the eukaryotic cell (Gabaldon and Huynen, 2003). *Wolbachia*, in contrast, retained all such metabolic pathways for itself. There is no evidence of a large-scale gene transfer to the host's genome.

A fundamental difference between *Wolbachia* and mitochondria is in the organization of the protein transport (table). *Wolbachia* uses the type IV secretory system to export its proteins into the host's cytoplasm (Masui *et al.*, 2000; Wu *et al.*, 2004), whereas the mitochondrion, in contrast, imports proteins, encoded by the nuclear genes and synthesized in the cytoplasm. The external and internal membranes of the mitochondrion have specific protein complexes (TOM and TIM, respectively) responsible for this import (Rehling *et al.*, 2001). There is no similarity in the protein transport system in *Wolbachia* and the mitochondrion. It is very interesting that those mitochondrial proteins that are encoded in the nucleus but have bacterial origin are synthesized mainly in the ribosomes associated with the external membrane of mitochondria, whereas those mitochondrial proteins that have solely eukaryotic origin are more often synthesized on the cytoplasmic ribosomes some distance from the mitochondria (Marc *et al.*, 2002).

Signal regulatory systems. The eukaryotic cell has a number of effective means to regulate mitochondria (Cummins, 2001). For instance, signals are transferred to the mitochondrion using ions Ca^{2+}. The signal role of calcium ions in the nucleocytoplasm is usually performed by calmodulins (special proteins). In the mitochondria, calcium ions can immediately affect enzymes without any mediation by other proteins. The regulation of mitochondria is also achieved using cyclic AMP, which acts through protein kinase A, or by binding the receptor protein on the external side of the internal membrane of a mitochondrion. Some data suggest that the mitochondrial function is regulated by cGMP and protein kinase (Kulinskii, 1997). The mitochondria contain a considerable amount of proteins responsible for reception and transfer of signals, including GTP- and Ca^{2+}-binding proteins and a number of various protein kinases (Reichert and Neupert, 2004).

None of these methods can be used by the host's cell to regulate *Wolbachia* because the latter lacks necessary receptors, protein kinases, or ion channels (*Wolbachia* lacks membrane channels for calcium ion transport, protein kinase A, receptors of cAMP, etc.).

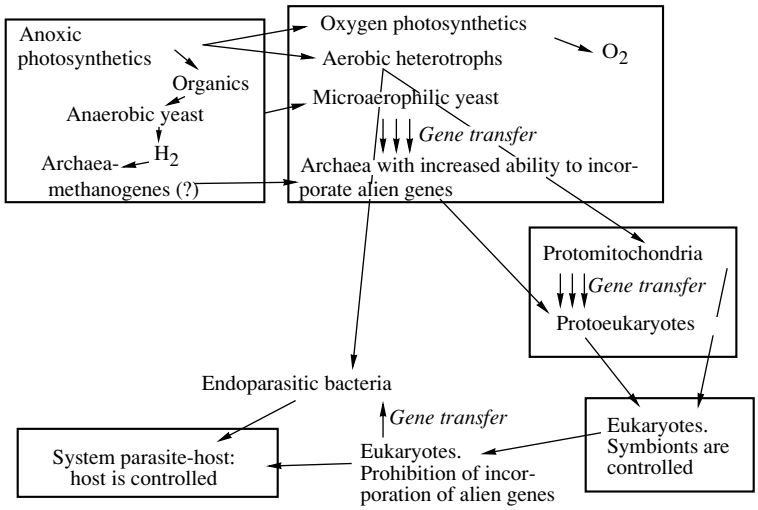
The main means that provides the nucleocytoplasm with complete control over mitochondria is certainly based on the fact that the largest part of the mitochondrial genome is encoded by nuclear genes. Most of those have alphaproteobacterial origin and were initially located in the chromosome of the ancestor of mitochondria, but were later transferred into the nuclear genome. This also explains the small size of the mitochondrial genomes compared to that of *Wolbachia*. The expression of these genes followed by the transport of corresponding proteins to the mitochondria is certainly controlled by the nucleocytoplasmic signal, regulatory, and transport systems. As mentioned above, no evident traces of *Wolbachia* gene transfer to the nuclear genome have been found. Thus, this powerful means cannot be used by cell to affect the parasitic bacterium.

If the means working for regulating mitochondria are useless in the case of *Wolbachia*, could it be assumed that the host can regulate in some other ways, affecting the signal regulatory systems of the parasite? This pathway is virtually impossible in the case of *Wolbachia's* hosts because, in this bacterium, signal regulatory systems are virtually absent.

Only three genes responsible for the reception and transfer of signals, and only six supposed regulators of transcription have been revealed in *Wolbachia*. A low number of regulators is also recorded in other endosymbionts and is usually explained by the constancy of their environment, which makes adaptive modifications unnecessary for survival (Andersson *et al.*, 1998; Seshadri *et al.*, 2003; Wu *et al.*, 2004). This may be not the only reason. The reduction of the signal regulatory systems makes intracellular bacteria less dependent on various signals coming from outside, i.e., from the host, and considerably reduces the probability of the host elaborating ways of controlling the symbiont. *Wolbachia* practically controls the reproduction, growth, and even evolution of its hosts, and does it in a way beneficial to itself. To achieve this it uses various and complex signal regulatory systems of the eukaryotic cell, which provide it with numerous potential points of action by its regulating agents. *Wolbachia* itself virtually lacks such points.

WOLBACHIA AS AN "ANTIMODEL" OBJECT FOR THE RECONSTRUCTION OF THE ORIGIN OF EUKARYOTES

Thus, the relationships of the cell with mitochondria, or with *Wolbachia* are in many ways completely opposite (table). So, why do such closely related microorganisms as *Wolbachia* and the ancestor of mitochondria have such fundamentally different relationships with the eukaryotic nucleocytoplasm? Or, more precisely, why were mitochondria completely subjected to the nucleocytoplasm, while *Wolbachia* in many ways controls its functions?



Scheme illustrating the origin of eukaryotes and later acquirement of the endoparasitic bacteria.

We suggest that the explanations should be based on the fact that most protomitochondrial genes were transferred to the nuclear genome at the early stages of eukaryotization (Martin, 2003), whereas the genes of *Wolbachia* were apparently virtually not transferred to the host's genome (Wu *et al.*, 2004). In addition, many mitochondrial proteins encoded by nuclear genes do not have traces of alphaproteobacterial origin. Apparently, they appeared in eukaryotes after the acquisition of the mitochondrial endosymbiont (Karlberg, 2004). The inclusion of mitochondrial genes in the nuclear genome, their integration in the nuclear-cytoplasmic gene network and signal regulatory systems, and the introduction of "alien" eukaryotic proteins (genes of which originated from the nucleus) into mitochondria excluded any possibility of selfish evolution for mitochondria (Martin and Herrmann-Reinhold, 1998; Rand *et al.*, 2001).

Wolbachia, in contrast, retained a relatively high level of independence not only regarding the expression of its genes, but also at the metabolic level. This allows lateral transfer of this bacterium from time to time between different hosts, and a rapid selfish evolution by working out various means to regulate its hosts, which are sometimes far from being harmless.

This fundamental difference in the localization of most genes required for survival of *Wolbachia* and mitochondria is closely connected with another fundamental difference, i.e., in the direction of protein transport. While *Wolbachia* exports its proteins to the host's cytoplasm, which allows it to regulate its cytoskeleton and other molecular systems, mitochondria import necessary proteins from the cytoplasm. Apparently, the systems responsible for this import should have appeared very early, because in their absence the transfer of mitochondrial genes into the nucleus would have led to the death of endosymbionts.

According to our hypothesis of the origin of eukaryotes (Markov and Kulikov, 2005), the evolution of the ancestor of the nucleocytoplasm included a period of active incorporation of alien genetic material (figure). We suggest that the ancestor of the nucleocytoplasm was an archaeobacterium (possibly methanogenic), which in the environment of crisis caused by the increased concentration of oxygen, acquired an ability to quickly and efficiently incorporate DNA of unrelated microorganisms into its genome. This archaeobacterium possessed genes and genetic complexes from various bacteria, primarily anaerobic, and from microaerophilic yeast. The resulting microorganism had combined archaeobacterial systems of replication, transcription, translation, and basic regulations, and with bacterial metabolism and peripheral signal regulatory systems. This microorganism later acquired endosymbionts, i.e., aerobic alphaproteobacteria, which became mitochondria.

According to this theory, the inclusion of the protomitochondrial genes in the central (nuclear) genome, could have happened even earlier than the endosymbiotic system was formed, i.e., when the ancestor of the nucleocytoplasm and promitochondria still existed separately, but in close contact within the same microbial community. In this period, the metabolism and signal regulatory systems of the two microbes were mutually calibrated, which provided grounds for their subsequent combination in an integral organism. Possibly, at that time, the system of transport of nucleocytoplasmic proteins into future mitochondria began to be formed. After the integration, the mitochondrial genes continued to pass to the central genome.

Thus, the relationships of the ancestor of the nucleocytoplasm with the future mitochondria and those of the hosts with *Wolbachia* were from the beginning based on completely different principles. This contrast is especially clearly visible in how the protein transport is directed. In this respect, the ancestor of the nucleocy-

toplasm behaved with protomitochondria approximately in the same way as *Wolbachia* behaved with the cells of its host. In this respect it is necessary to remember that the direction of the membrane transport of ATP observed in mitochondria and rickettsias (closest relatives of *Wolbachia*) is also contrasting. Rickettsias pump the energy in form of ATP out from the host's cytoplasm, while mitochondria are themselves victims of the energy vampirism of the nucleocytoplasm. Perhaps there was a relationship between the ancestor of the nucleocytoplasm and promitochondria, as was suggested by Karlberg (2004), although it is more likely that it was not the protomitochondrion that was the parasite, but the large organism, ancestor of the nucleocytoplasm.

The existence of a hypothetical organism with an abnormally high ability to incorporate alien genes could be neither long nor stable. Due to the endosymbiosis with an aerobic bacterium, the primary reason of such a strategy was removed, and the "oxygen" crisis generally resolved. Subsequently, eukaryotes apparently developed powerful defenses against lateral transfer replaced by more complex and well-controlled means of recombination connected to the sex process (meiosis, fusion of gametes, interspecific reproductive isolation).

The prevention of lateral transfer, especially strongly developed in Metazoa (including all hosts of *Wolbachia*), resulted in the transfer of the genes of the newly acquired endosymbionts into the central genome, under the control of the regulatory systems of the nucleocytoplasm), becoming very difficult. As a result, new endosymbionts (and endoparasites) retain not only relative independence, but also the capacity for selfish evolution.

Furthermore, because prokaryotes do not have such rigid restrictions of lateral transfer, these new inhabitants of the eukaryotic cell can include various regulatory genes of their hosts in their genomes and later use them for their own purposes. Apparently, this was how endosymbiotic bacteria acquired ankyrine proteins, eukaryotic regulators of the cytoskeleton, mitosis, and other important functions, including apoptosis (programmed cell suicide) (Shohat *et al.*, 2002; Dong, 2004; Wu *et al.*, 2004). This resulted in a situation opposite to that occurring at the initial stage of the evolution of endosymbiosis with protomitochondria.

Thus, in our opinion, one of the defining factors in the evolutionarily successful symbiosis of protoeukaryotes with protomitochondria was, as we previously stated, increased ability of the ancestor of the nucleocytoplasm to incorporate alien genetic material, which developed alongside the concentration of free oxygen in the environment, caused by the shift of some photoautotrophic bacteria to oxygenic photosynthesis. Paleontological data allow the appearance of eukaryotes to be dated from approximately 3.0 to 2.5 Ga (Timofeev, 1982; Brocks *et al.*, 1999; Rozanov, 2003).

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