Amoeba/Amoebal Symbiont Genetic Transfers: Lessons from Giant Virus Neighbours

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Key Words

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Abstract

Free-living amoebae serve as hosts for a variety of amoebae-resisting microorganisms, including giant viruses and certain bacteria. The latter include symbiotic bacteria as well as bacteria exhibiting a pathogenic phenotype towards amoebae. Amoebae-resisting bacteria have been shown to be widespread in water and to use the amoebae as a reservoir, a replication niche, a protective armour as well as a training ground to select virulence traits allowing survival in the face of microbicidal effects of macrophages, the first line of defense against invading pathogens. More importantly, amoebae play a significant role as a melting pot for genetic exchanges. These ecological and evolutionary roles of amoebae might also be at play for giant viruses and knowledge derived from the study of amoebae-resisting bacteria is useful for the study and understanding of interactions between amoebae and giant viruses. This is especially important since some genes have spread in all domains of life and the exponential availability of eukaryotic genomes and metagenomic sequences will allow researchers to explore these genetic exchanges in a more comprehensive way, thus completely changing our perception of the evolutionary history of or-

ganisms. Thus, a large part of this review is dedicated to report current known gene exchanges between the different amoebae-resisting organisms and between amoebae and the internalized bacteria.

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Introduction

The term ‘free-living amoebae’ encompasses a wide variety of monocellular eukaryotic species (fig. 1). They are widespread in nature and are normal inhabitants of freshwater and soil microbial ecosystems, where they feed by grazing on biofilms [1]. The complex phylogeny of free-living amoebae is still being investigated and the latest proposal for a phylogenetic classification is very recent. *Jakobidae* (comprising *Andalucia*, *Histiona*, *Reclinomonas*, *Jakoba* and *Seculamonas* genera) and *Heteroloboseae* (comprising *Naegleria*, *Sawyeria*, *Vahlkampfia* and *Willaertia* genera, among others) belong to the Excavata supergroup that also includes *Euglenozoa* (comprising *Bodo*, *Trypanoplasma*, *Leishmania*, *Phytomonas* and *Trypanosoma* genera, among others) [2]. The Amoebozoa supergroup roughly comprises all other free-living amoebae genera [3] (including *Acanthamoeba*, *Echinamoeba*, *Hartmannella*, and *Vannella*, among others). This super-

group also includes the soil amoeba *Dictyostelium discoideum* and parasitic species of critical medical importance such as *Entamoeba* spp. Although free-living amoebae from Excavata and Amoebozoa supergroups present many common ecological characters, they are phylogenetically very distant.

Given their behavior as professional phagocytes, free-living amoebae engulf huge amounts of foreign DNA, which is generally degraded along with internalized microorganisms in acidic phagolysosomes. However, some microorganisms have evolved to resist the amoebal microbicidal effectors. Amoebae can serve as hosts for a variety of amoebae-resisting microorganisms, including giant viruses and bacteria. The latter include a large array of species such as *Legionella* spp., *Mycobacteria* spp., new members of the *Chlamydiales* order and some alpha-proteobacteria that can be symbiotic species or species exhibiting a pathogenic phenotype towards amoebae [4–7]. Amoebae-resisting bacteria are widespread in water and can use amoebae as a reservoir and replicative niche, as well as a protective armour when amoebae-resisting bacteria are able to survive within amoebal cysts. As a consequence, amoebae are considered to play a significant role as a training ground for the selection of virulence traits needed to survive an attack of the microbicidal effectors of any phagocytic cells, including the macrophages that represent the first line of defense against invading pathogens. This concept not only applies to amoebae-resisting bacteria such as *Legionella*, *Mycobacteria* and *Parachlamydia*, but also to fungi, as demonstrated by the encapsulated *Cryptococcus neoformans* being able to resist amoebae and macrophages whereas non-virulent acapsular strains cannot [8, 9]. The growing body of evidence suggesting a pathogenic role of Mimiviridae [see review by Vincent et al., this issue, pp 58–63] tend to demonstrate that it might also apply to giant viruses.

More recently, amoebae have been suggested to represent a place where genetic exchanges take place and where facultative as well as strict intracellular microorganisms may replenish their gene content (see below). This is of primary importance since these exchanges may also have been at play in ancestral eukaryotic species, and a better understanding of the events that led to spreading of selected genes in all domains of life will change our perception of the evolutionary history of organisms. In this context, the intention of this review is to report currently known gene exchanges between the different amoebae-resisting microorganisms, while further highlighting the important ecological aspects that allow these exchanges.

Fig. 1. Trophozoite of *Acanthamoeba castellanii* strain ATCC 30010 observed using scanning electron microscopy. Scale bar = 2 μ m.

Gene Transfer: A Key Mechanism in the Evolutionary History of Organisms

Gene transfers between bacteria have been extensively reported and are thought to be of primordial importance in many aspects of microbial ecology, such as (1) establishment and maintenance of complex microbial communities [10]; (2) spread of antibiotic resistance mechanisms [11]; (3) evolution of pathogenic strains and species through transfer of virulence traits [12], and (4) acquisition of genes involved in symbiosis and improved host-microbe interactions [13]. Gene transfers from bacteria to eukaryotes, viruses to eukaryotes or eukaryotes to eukaryotes are less well described, likely due to the relatively lower abundance of eukaryotic microbial genomes in databases until recently. Gene transfer between related and unrelated eukaryotic organisms is still particularly under-reported [reviewed in 14]. Nevertheless, gene transfers are thought to occur frequently, notably in large phagotrophic eukaryotes which ingest various microorganisms, including other eukaryotic cells. In the following lines we give several examples of genetic exchanges from bacteria to eukaryotes as an introduction to the study of genetic exchanges occurring within free-living amoebae.

Many gene transfers from bacteria to eukaryotes occurred as a consequence of ancestral symbiotic events that led to the establishment of plant and animal cell lineages. The most plausible primary endosymbiotic event was invasion of a prokaryote related to archaeobacteria by

another prokaryote related to alpha-proteobacteria and presenting *Bdellovibrio*-like bacteriovory ability, leading to the formation of mitochondria and to the first primitive eukaryotic cells [15–17]. It has been proposed that a phage also took part in the establishment of mitochondria in addition to the alpha-proteobacteria, since several mitochondrial genes involved in replication and transcription are related to the T-odd lineage of bacteriophages [18]. Plastids were subsequently acquired, descending from a primary cyanobacterial symbiosis which occurred over 1.2 billion years ago [19]. Of note, cyanobacteria could themselves originate from a symbiosis between an ancient actinobacterium and an ancient clostridium [20]. Due to the high number of chlamydial genes with homologues in plant, it is hypothesized that a chlamydial endosymbiont or parasite facilitated the establishment of the cyanobacterial endosymbiont [21]. During the course of endosymbiosis, most genes were then lost from the symbiont's genomes and many were relocated to the host nucleus through endosymbiotic gene transfer, explaining why many genes with homologues in *Cyanobacteria* and *Chlamydiales* are present in *Plantae* [22]. Plastids have then moved between eukaryotic lineages by secondary and tertiary endosymbiotic events [19].

Gene transfer is a dynamic process and therefore continuously contributes to the evolutionary history of eukaryotes [23]. Amitochondriate (likely due to loss of mitochondria during evolution) micro-aerophilic protists which survive anaerobic conditions within the intestinal lumen have been demonstrated to have acquired at least part of their fermentation enzymes from anaerobic bacteria. The parasitic protozoa *Entamoeba histolytica* probably acquired 96 genes from bacteria (mainly from the *Cytophaga-Flavobacterium-Bacteroides* group), with a major impact in the area of carbohydrate and amino acid metabolism, where they have increased the range of substrates available for energy generation [24]. Genes transferred from bacteria have also been evidenced in *Giardia lamblia* [25] and *Cryptosporidium parvum* [26], and the same kind of study led to the identification of 153 probable genes transferred from bacteria (again mainly *Cytophaga-Flavobacterium-Bacteroides* group) to the flagellated parabasalid *Trichomonas vaginalis* [27]. A recent study has also investigated probable genes transferred from bacteria to *Plasmodium*, *Theileria*, *Toxoplasma*, *Cryptosporidium*, *Leishmania*, *Trypanosoma*, *Phytophthora*, diatoms, *Ostreococcus* and *Saccharomyces* [28]. In addition to plastid-related processes and genes implicated in the carbohydrate metabolism, a number of pathways that are rich in enzymes encoded by transferred

genes and potentially key to pathogenicity were found in eukaryotic parasites [28].

When published several years ago, the genome sequence of the soil amoeba *Dictyostelium discoideum* revealed at least 18 genes of bacterial origin. Some of them, such as the thymidylate synthase gene, have replaced the endogenous genes whereas others, such as the siderophore encoding gene, established new functions [29]. Since bacterial species from which these genes might have originated were not mentioned in the article by Eichinger et al. [29], we performed BlastP and phylogenetic analyses for these 18 genes. Potential donor genera varied according to the protein investigated, including *Bacteroidetes*, gamma-proteobacteria (including *Francisella* spp.), *Planctomycetales*, and cyanobacteria. In our analyses the thymidylate synthase gene presented homologues in alpha-proteobacteria but also in several eukaryotic species, in various phages and in two recently described nucleocytoplasmic large DNA viruses (NCLDV, family: Phycodnaviridae) infecting the smallest known marine photosynthetic eukaryote, *Ostreococcus tauri* [30, 31]. Phylogenetic analyses (fig. 2, table 1) suggest that the thymidylate synthase gene would have been transferred from a *Deinococcus* phage to the *O. tauri* virus (OsV) and then to various representatives of amoebae, including Amoebozoae (*Dictyostelium* spp. and *Physarum* spp.) and Jakobids (*Reclinomonas* spp.) that are very distant in the eukaryotic phylogeny. Demonstration that free-living amoebae that belong to *Jakobidae* can feed on *O. tauri* could explain the transfer from an OsV-infected *O. tauri* cell to its amoebal predator [32].

The examples given above show that genes may travel between organisms that belong to the different kingdoms of life, and that this is a permanent and dynamic process. In the second part of this review, we describe the range of possible interactions between some amoebae-resisting bacteria and free-living amoebae. We also discuss genetic exchanges between viruses, endosymbiotic bacteria and free-living amoebae, which are closely associated from an ecological and evolutionary point of view.

Ecology of Free-Living Amoebae and Their Interactions with Endosymbionts

Free-living amoebae live in close interaction with intricate microbial communities termed 'biofilms'. Biofilms are the predominant mode of growth for bacteria in aquatic ecosystems, with planktonic bacteria representing <0.1% of the total microbial community [33]. These

Fig. 2. Phylogenetic tree deciphering relationships of *Dictyostelium discoideum* thymidilate synthase (DictyBase ID: DDB0214905) with closest homologues retrieved by performing a BlastP analysis of the *D. discoideum* protein. Amino acid sequences were aligned using the Muscle software [112], the tree was constructed using the PhymI (PHYlogenetic inferences using Maximum Likelihood) software [113]. Eukaryotic sequences of amoebae cluster with sequences of *Ostreococcus* viruses (NucleoCytoplasmic Large DNA Viruses, family: Phycodnaviridae).

Table 1. Probable gene transfer events highlighted in this review

Gene coding for	Organism used to perform blast search	Gene length	Homologues retrieved in	Gene length	Identities	Positives	Gaps	E-value
7-dehydrocholesterol reductase	<i>Legionella drancourtii</i>	431	<i>Naegleria gruberi</i>	450	233/425 (54%)	309/425 (72%)	3/425 (0%)	1E-143
Thymidilate synthase	<i>Dictyostelium discoideum</i>	303	<i>Ostreococcus</i> virus	260	142/263 (53%)	177/263 (67%)	14/263 (5%)	1E-69
Methyltransferase	<i>Legionella dumoffii</i> plasmid	280	Chilo iridescent virus	265	64/228 (28%)	120/228 (52%)	3/228 (1%)	5E-24
			<i>Amsacta moorei</i> entomopoxvirus	270	60/223 (26%)	125/223 (56%)	6/223 (2%)	9E-24

biofilms are complex ecosystems that include many different microbial species that establish metabolic relationships. As a result, they are more and more being considered as ‘multicellular communities’ [10]. Conjugation and transformation allow gene exchanges within bacterial members of biofilms [34] and it has been demonstrated that extracellular DNA (termed ‘eDNA’) released by bacteria is involved in the formation of mature biofilm structures [35].

Although their roles in regulating composition/dynamics of biofilms are thought to be essential, the fate of

protozoa and phages in these communities is still under-reported. It is believed that protozoa play a major role in the regulation of biofilms: they graze on attached bacteria and can integrate into the biofilm community [36]. Lyso-genic phages can convert to a lytic cycle under stressful environmental conditions but also under the influence of acyl-homoserine lactones, the quorum sensing signalling molecules used by many Gram-negative bacteria to perceive their population density and regulate cell physiology accordingly [37]. Bacteriophages can also be embedded in biofilms, being protected from adverse environ-

mental conditions [38], interacting with local flora and being occasionally released from biofilms [39]. Thus, free-living amoebae live in permanent and close association with multiple bacterial species, extracellular DNA present in biofilms, phages associated to these biofilms and other microbial eukaryotes. In these ecosystems, many situations can favour gene exchanges.

During the feeding process, it is more than probable that various bacterial species will be ingested by the same protozoal cell and locate in the same cellular compartment, thereby facilitating lateral gene exchanges. Some studies have demonstrated that a single protozoal cell can contain two phylogenetically distinct bacterial symbionts [40, 41], thus suggesting that stable intracellular multi-partner associations could result in coevolution of intracellular microorganisms [41]. Gene transfer between bacteria located within digestive vacuoles of protozoa has been speculated for *Escherichia coli*: in a mixed population, containing donor and recipient *E. coli* cells, the presence of the eukaryotic predator *Tetrahymena pyriformis* increased conjugational gene transfer by three orders of magnitude [42].

Conjugation may also occur between strict intracellular bacteria, since a *tra* operon encoding a putative DNA conjugative transfer system is present in the genomes of different members of the *Chlamydiales* [43, 44] and in the genome of *Rickettsia bellii* [45]. Furthermore, it has been reported that intra-amoebal bacteria related to *Chlamydia* can be infected by phages [46, 47], and phages infecting *Legionella pneumophila* have also been recently described [48]. Such phages infecting intra-amoebal bacteria are likely to take part in genetic exchanges between bacteria, free-living amoebae and amoebal viruses if present. For example, it was demonstrated that *D. discoideum* cultured with *E. coli* cells infected with lambda phage can take up phage DNA, and that some fraction of phage DNA existed in intact form in amoebal cells [49]. It has also been demonstrated that bacterial phages can survive within the ciliate *Tetrahymena thermophila* [50], thus providing an extended opportunity for gene exchanges with the eukaryotic host and other intracellular microorganisms.

Interactions of Free-Living Amoebae with Legionella spp. (Gamma-Proteobacteria)

Since the first report describing interaction of *L. pneumophila* with free-living amoebae [51] more than 50 *Legionella* species have been described. All bacteria belonging to this genus are considered to be able to grow in amoebae, although clear demonstration of intra-amoebal

growth is still lacking for most of them. The biology of the pathogenic species *L. pneumophila* has been extensively studied and has revealed unique features, among which is a large number and wide variety of eukaryotic-like proteins that interact with the host cells, having roles in different regulatory pathways and in different stages of the intracellular cycle [52]. Degtyar et al. [53] demonstrated that distribution of effector-encoding genes is highly variable in *Legionella* species, with most genes of eukaryotic origin being present in different *Legionella* species whereas others are specific to *L. pneumophila*. Focusing on the *legS2* gene encoding a homologue of eukaryotic sphingosine 1-phosphate lyase, they demonstrated that the gene is probably of protozoan origin and that the LegS2 protein is translocated into host cells via the Icm/Dot secretion system but is not necessary for host infection. They then provided evidence that the gene can complement a deficient *Saccharomyces cerevisiae* mutant and that, unlike its eukaryotic homologues, LegS2 was mainly targeted to the host mitochondria. This interesting study thus demonstrated that host acquired genes can be modified by *Legionella* to encode for effector proteins that are relocated to cellular organelles different from their original locations in the eukaryotic cells. This is only one of the many genes likely acquired by *Legionella* during its intra-amoebal life.

When taking into account free-living amoebae ongoing genomes and expressed sequence tags databases, we found that at least 8 of the 30 *L. pneumophila* genes previously demonstrated to be of eukaryotic origin [see 54] present significant phylogenetic relatedness with free-living amoebae (data not shown). Two of these genes are the S-adenosylmethionine (SAM)-dependent methyltransferases *lpp2134* and *lpp2747* (*L. pneumophila* strain Paris). Interestingly, they present homologies with genes encoded by: (1) a NCLDV virus (Chilo iridescent virus); (2) a poxvirus (*Amsacta moorei* entomopoxvirus), and (3) the plasmid pLD-TEX-KL (originating from *Legionella dumoffii*). Further analyses confirmed phylogenetic affinity between methyltransferase homologues from these viruses and from the protein encoded in the *L. dumoffii* pLD-TEX-KL plasmid (fig. 3 and table 1). The gene may have been transferred from the bacteria to the Chilo iridescent virus ancestor when coinfecting a protist, and from the Chilo iridescent virus to the entomopoxvirus when coinfecting an insect. If true, this would support the hypothesis that both amoebae and insects represent places of common gene exchange, since both are largely exposed to microbial communities (see fig. 5).

Fig. 3. Phylogenetic tree depicting relationships of proteins presenting homology with the SAM-dependent methyltransferases *lpp2134* and *lpp2747* of the *Legionella pneumophila* strain Paris. Amino acid sequences were aligned using the Muscle software [112], the tree was constructed using the PhymI (PHYlogenetic inferences using Maximum Likelihood) software [113]. One of the

homologues was found in *Legionella dumoffii* plasmid pLD-TEX-KL and clusters with proteins from Chilo iridescent virus (NCLDV virus) and a poxvirus, *Amsacta moorei* entomopoxvirus. *L. pneumophila* SAM-dependent methyltransferase was used as an out-group.

Before the advent of molecular biology and genome sequencing, a series of pioneer studies deciphered metabolic interactions that can take place between a newly established symbiont and its host. Since their first paper in 1967 that demonstrated the presence of an intra-amoebal symbiont [55], Jeon et al. have published several reports to describe an endosymbiotic bacterium ('X-bacterium') which had infected the amoebae initially as an intracellular parasite. X-bacterium was further demonstrated to belong to the *Legionella* order, being proposed as *Candidatus Legionella jeonii* [56]. Briefly, authors first demonstrated that after several years of infection the presence of live endosymbionts was required for host cells survival. This resulted in many changes in host cell physiology. Among many interesting findings [for a review, see 57], they demonstrated that (1) infected amoebal cells became sensitive to high temperatures: exposure to 34° resulted in loss of two plasmids by *Ca L. jeonii*, disappearance of bacteria in amoebae and finally death of amoebae, and that (2) *Ca L. jeonii* can switch expression of host genes coding for S-adenosylmethionine synthetase (sams) from one sams-coding gene to another. The molecular mechanisms allowing bacteria to modulate amoebal gene ex-

pression remain to be determined but authors have formulated the hypothesis that *Ca L. jeonii* plasmids might be involved.

Temperature tolerance linked to the presence of plasmids in *Ca L. jeonii* is reminiscent of the recent work demonstrating that *L. dumoffii* (a non-obligate symbiont) replicates efficiently at low temperature (<30°) in *Acanthamoeba culbertsoni* only in the presence of the pLD-TEX-KL plasmid, and that the plasmid gene product TraA(Ti) is required for replication [58]. Such temperature tolerance mechanisms were also reported for other *Legionella* spp. [59]. Thus, rapid establishment and persistence of the symbiotic relationship between *Ca L. jeonii* and *Amoeba proteus* demonstrates that endosymbiosis can take place in a very short time, and those mobile genetic elements can be of importance in this process. Infection of the amoebae probably initially occurred through *Tetrahymena* cells used to feed the amoebae, and which are known to support the growth of various *Legionella* species [60].

Moliner et al. recently made available the genome of *Legionella drancourtii* [61]. This species was first thought to be strictly intracellular [62] but it has then been suc-

Fig. 4. Phylogenetic tree depicting relationships of *Legionella drancourtii* sterol delta-7 reductase with closest homologues retrieved by performing a BlastP analysis against NCBI protein database and *Naegleria gruberi* ongoing genome (<http://genome.jgi-psf.org>). Amino acid sequences were aligned using the Muscle software [112], the tree was constructed using the Phylml (PHYlogenetic inferences using Maximum Likelihood) software [113]. *L. drancourtii* sterol delta-7 reductase clusters with that of *Coxiella burnettii*, *Protochlamydia amoebophila*, *Parachlamydia acanthamoebae* and a putative protein of *N. gruberi*.

cessfully grown onto axenic BCYE medium [61]. They demonstrated that a malate synthase gene has probably been transferred from a Bacteroidetes species to *L. drancourtii*, then from *L. drancourtii* to amoebae [63]. Another example of genetic transfer revealed by the analysis of this genome is the evolutionary history of a sterol delta-7 reductase-encoding gene. Homologues of this gene were present in the genomes of (1) *Coxiella burnettii* (a gamma-proteobacteria), (2) *Protochlamydia amoebophila* (a Chlamydia-related bacteria), and (3) *Acanthamoeba polyphaga* Mimivirus, but were absent from the genomes of other *Legionella* spp. Based on phylogenetic analyses, Moliner et al. [61] proposed that this gene was acquired by the chlamydial ancestor from viridiplantae and then transferred to other intra-amoebal bacteria, or that it was directly transferred from the *Chlamydia* ancestor to the *Legionella* ancestor within amoebae, being then conserved only in several species after genome reduction. In an attempt to further characterize the evolutionary history of these genes, we performed additional BLAST analyses taking into account ongoing genomes and expressed sequence tags databases of free-living amoebae. A close homologue of the sterol delta-7 reductase-encoding gene was found in *Naegleria gruberi* and phylogenetic analyses including this new clade suggest a transfer be-

tween amoebae and amoebae-resisting bacteria (fig. 4 and table 1).

Interactions of Free-Living Amoebae with Candidatus Amoebophilus asiaticus (Bacteroidetes)

Ca Amoebophilus asiaticus is an as yet poorly investigated species and the availability of its genome will help understanding gene exchanges between free-living amoebae and their various microbial hosts. First described in 2001 [64], this bacterium was reported as an obligate, non-transferable endosymbiont of a T4 group *Acanthamoeba* spp. Since then, other *Acanthamoeba* have been described to host closely related endosymbionts [reviewed in 5].

Since *Ca Amoebophilus asiaticus* exhibits a host range similar to Mimivirus, we performed local BLAST analyses of one genome against another and we could identify a large number of significant BLAST hits. More importantly, the identified Mimivirus genes exhibited higher similarities with *Ca Amoebophilus asiaticus* than with any other bacteria, virus or eukaryote. Thus, among 172 best BLAST hits against bacteria, 64 were against *Ca Amoebophilus asiaticus*. Similarly, when considering hits against all organisms, 55 best BLAST hits were against *Ca Amoebophilus asiaticus*. Most of them (n = 44) were

genes containing a common, conserved ankyrin repeat domain. Ankyrin repeats are protein-protein interaction motifs; in intracellular microorganisms they are found in proteins implicated in host-pathogen interactions. An ankyrin-repeat-containing protein of *Ehrlichia chaffeensis* is translocated in host cells nuclei, with the effect of modulating host cell gene expression to promote survival of the pathogen [65]. A Dot/Icm translocated ankyrin protein has also been demonstrated to be required for intracellular multiplication of *L. pneumophila* [66]. The fact that most Mimivirus ankyrin repeats find their closest homologues in a bacterial symbiont infecting the same free-living amoebae genera further supports the hypothesis that genetic transfers have occurred between the virus and the bacteria.

Interactions of Free-Living Amoebae with Chlamydiales

The *Chlamydiales* order includes several human pathogens. *Chlamydia pneumoniae* is a causal agent of respiratory infection and *Chlamydia trachomatis* is responsible for urogenital tract infections and preventable blindness. *C. pneumoniae* and a species close to *C. trachomatis* have both been reported to be able to survive within amoebae [67, 68]. During the past decade, new *Chlamydiales* species representing at least five new families have been described: *Parachlamydiaceae*, *Simkaniaceae*, *Criblamydiaceae*, *Rhabdochlamydiaceae* and *Waddliaceae* [69, 70]. These 'Chlamydia-like' species are obligate intracellular bacteria that can naturally infect a large range of organisms including free-living amoebae, various invertebrates and vertebrates. They are thought to be widely spread in the environment because of their association with amoebae. Several species are potential emerging pathogens for humans and animals, being possibly responsible for pneumonia and miscarriage [70–72].

As mentioned above, an ancestral *Chlamydiales* related to *Protochlamydia amoebophila* (*Parachlamydiaceae*) has played an important role in the onset of plastids in the first representative cells of Plantae. Later studies demonstrated that *Chlamydiae* has contributed at least 55 genes with predominantly plastid functions to Plantae, leading to the hypothesis of a long-term symbiotic association that extends beyond plastid establishment [22]. One of these genes encodes for an ATP/ADP translocase, an enzyme that exchanges ATP for ADP. This protein is present in plant plastids and in the proteome of *Chlamydiae* and *Rickettsiae*. These strict intracellular bacteria use this enzyme to acquire energy from their host [73]. The most probable evolutionary scenario is that the ATP/ADP

translocase-encoding gene was duplicated in a *Chlamydiales* ancestor before the appearance of multicellular eukaryotes, being then horizontally transferred to *Rickettsiae* and plants [74]. This enzyme is also present in a delta-proteobacteria: *Lawsonia intracellularis*, an intracellular pathogen responsible for major diseases in swine; phylogenetic analyses suggest that it has been transferred from an ancestral *Rickettsia* [75].

Another interesting finding was the description of a genomic island encoding a potentially functional F-like conjugative DNA transfer system in the genome of *Protochlamydia amoebophila* (*Parachlamydiaceae*) [43]. This system was probably acquired from a phylogenetically-distant proteobacteria sharing the same amoebal vacuole [43], since it is also present in *R. bellii* [45].

Gene transfers have also been reported between actinobacteria and the common ancestor of the *Chlamydiales*, leading to the acquisition of a serine hydroxymethyltransferase (GlyA protein) and UDP-N-acetylglucosamine enolpyruvyl transferase (MurA, which is involved in the synthesis of cell wall peptidoglycan) by all *Chlamydiales* [76].

Interactions of Free-Living Amoebae with Rickettsiales (Alpha-Proteobacteria)

Among amoebae-resisting bacteria, most alpha-proteobacteria belong to the *Rickettsiales* order, including *Caedibacter* spp., *Paracaedibacter* spp., and *Ca. Odysella thessalonicensis* [77–79]. Interestingly, the *Amoeba proteus* strain from which Jeon et al. isolated *Ca. L. jeonii* (see above, [55]) also harbours *Rickettsia*-like endosymbionts (so called DNA-containing bodies) in their cytoplasm. These bacteria encode a cryptic plasmid originating from *Bacillus* species [80]. This plasmid (pAP3.9) has 3 open reading frames encoding proteins for mobilization, replication and a phosphoglycolate phosphatase supposed to modulate the rate of DNA repair of the bacterial host, thus allowing better survival in the hostile intracellular environment [80].

Due to high pathogenicity of most *Rickettsiales* species that are transmitted through arthropods, several genomes have been sequenced, highlighting gene exchanges between these bacteria, their hosts and other intracellular bacterial species. The genome of the early diverging species *R. bellii* revealed that it possesses a type IV secretion system encoded by a gene cluster with homologues found in *Protochlamydia amoebophila* [45] (see above). Homologues of this putative conjugal DNA transfer system are also present in *Rickettsia felis* and in *Rickettsia massiliae*, demonstrating that gene transfers

between different *Rickettsia* species are much more frequent than previously thought [81–83]. Phylogenetic analyses suggest a complex evolutionary scenario involving several transfers of genes that belong to this conjugative system between *Chlamydia*, *Rickettsia* and possibly other yet unidentified microorganisms [45, 84]. Survival of *R. bellii* for more than 3 weeks in *Acanthamoeba polyphaga* argue in favour of the hypothesis of a protozoon hosting both *Chlamydia* and *Rickettsia* ancestors, thus allowing gene exchanges to occur [45]. Further phylogenetic analyses also demonstrated that a *Rickettsiales* ancestor likely acquired other genes from *Legionella* (15 candidates for *R. bellii*) and *Chlamydia* (7 candidates for *R. bellii*) [45]. Among these potentially acquired genes, one is homologous to that encoding the *L. pneumophila* RalF protein [45] and was likely acquired from protozoa by *Legionella* before being transferred to *Rickettsia* [53, 85].

Thus, Rickettsial species such as *R. bellii* that are characterized by a sympatric lifestyle, i.e. that can coinfect several eukaryotic species (including amoebae), together with other intracellular bacteria exhibit a larger genome compared to Rickettsial species that present a narrow host range and consequently have no opportunity for gene exchanges with other bacterial species (allopatric lifestyle, e.g. for *R. prowazekii*) [86].

Insects: A Potential Niche for Transfer Events Affecting Intra-Amoebal Symbionts

It is noteworthy that genes potentially transferred between intra-amoebal organisms while within free-living amoebae might also have been transferred while in insects, since most bacterial species involved in these transfers are symbionts of both free-living protists and insects. These insect symbionts can co-exist in the cells specifically modified to house bacteria (bacteriocytes) and thus represent a unique intracellular ecosystem providing indeed ample opportunities for gene exchanges [87].

The endosymbiotic bacterial *Wolbachia* spp. have been extensively studied. These are symbiotic species living in ovaries of arthropods and having a role in induction of parthenogenesis. Major gene transfers have been demonstrated between *Wolbachia* spp. and insects and nematode hosts, ranging from nearly the entire bacterial genome (>1 megabase) to short (<500 bp) insertions [88]. Some of these genes are transcribed within eukaryotic cells lacking endosymbionts, thus demonstrating that lat-

eral gene transfer is a mechanism for acquisition of new genes and functions [88]. Furthermore, *Wolbachia* bacteriophages are widespread, present high recombination rates and can be laterally transferred between different *Wolbachia* species, thus playing a primordial role in exchange of genetic material between bacteria that coinfect the same intracellular environment [89]. It has been recently demonstrated that some *Wolbachia* prophage genes have experienced dynamic evolutionary trajectories, with homologues found across divergent bacterial phyla including various Proteobacteria (mainly species that resist amoebal digestion) but also *Ca Amoebophilus asiaticus* [90].

Among insects hosting symbionts, the whitefly *Bemisia tabaci* is a very promising model to study potential gene transfers between various intracellular microorganisms, insects and amoebae. In fact, it has been demonstrated to host many different symbiotic species that have also been described in amoebae [91]. Thus, among others, *B. tabaci* has been demonstrated to host:

- (1) the ovarian symbiont *Fritschea bemisiae* related to the new *Chlamydiales* family, *Simkaniaceae* [92], which comprises *Simkania negevensis*, a species that grow in amoebae;
- (2) a bacterial species, *Candidatus Cardinium hertigii*, with its closest homologue being the amoebal symbiont *Ca Amoebophilus asiaticus* [93]; its lifestyle compares to *Wolbachia* spp. endosymbionts and recent reports demonstrate that *Ca Cardinium hertigii* often coexist with *Wolbachia* spp. in the same insects [94];
- (3) *R. belli*, which also resist digestion by amoebae [45, 95];
- (4) a virus very close to the NCLDV invertebrate iridescent virus type 6 [96]. *Iridoviridae* are supposed to have evolved from *Phycodnaviridae* whereas *Ascoviridae* have likely evolved from *Iridoviridae* [97]. It is thought that other obligate endosymbiont viruses of parasitoid wasps, ichnoviruses (family: *Polydnaviridae*), emerged from *Ascoviridae* by lateral transfer of ascoviral genes into wasp genomes [98]. Transfers from the wasp genome to the virus symbiont are also likely to occur since it has recently been demonstrated by Desjardins et al. [99] that genes encoding sugar transporters in *Glyptapanteles indiensis* and *Glyptapanteles flavicoxis* bracoviral (family: *Polydnaviridae*) genomes likely originated from the wasp host.

It is also probable that some members of *Poxviridae* can infect *B. tabaci* since they have been recovered from other insects [100]. As is the case with other NCLDV, *Poxviridae* also acquired many genes from their hosts

Fig. 5. Demonstrated and hypothetical gene exchanges between intra-amoebal microorganisms and microorganisms associated with insects (whitefly *Bemisia tabaci* was taken as a model insect). (1) Intra-protzoon genes exchanges between ancestors of *Rickettsiales* and *Chlamydiales* [45]. (2) Probable intra-protzoon exchanges of genes coding for ankyrin-repeats proteins between *Ca* *Amoebophilus asiaticus* and *Mimivirus*. (3) Very close phylogenetic relationship between the amoebal symbiont *Ca* *Amoebophilus asiaticus* and the insect symbionts *Cardinium* spp. [93]. (4) 7-dehydrocholesterol reductase gene exchange between *Legionella drancourtii* and *Naegleria gruberii* (see fig. 3 and table 1). (5), (12) Probable gene exchange between a gene found in *Legionella dumoffii* plasmid pLD-TEX-KL, a NCLDV virus and a poxvirus (see fig. 2 and table 1). (6) Direct and indirect gene exchanges between NCLDV viruses and their eukaryotic hosts (see [114] and another example in fig. 1 and table 1: thymidylate synthase gene exchange between *Ostreococcus* virus and amoebae comprising *Reclinomona*

nas americana and *Dictyostelium discoideum*). (7) Gene exchanges between *Mimivirus* and its amoebal hosts (see [115] and table 1). (8) Gene exchanges between *Mamavirus* and its virophage, *Sputnik* virus [106]. (9) Iridoviridae evolved from an ancestor of NCLDVs infecting a protzoon. (10) Ascoviridae evolved from Iridoviridae [97]. (11) Ascoviridae integrated into their host's genome and evolved to Polydnnaviridae [98]. (12) Probable gene transfer between a poxvirus and an Iridovirus infecting the same host (see fig. 2 and table 1). (13) Poxviridae acquired genes from their hosts [102]. (14) Genes encoding sugar transporters in bracoviruses (family: Polydnnaviridae) genomes likely originate from the wasp host [99]. (15) Major gene transfers have been demonstrated between *Wolbachia* spp. and insects and nematodes hosts [88]. (16) *Wolbachia* bacteriophages are widespread, present high recombination rates and can be laterally transferred between different *Wolbachia* species [89].

[101, 102]. Some of these genes (homologues to interleukin-10, glutathione peroxidase and deoxyribodipyrimidine photolyase) are important for survival of the virus in or out of the host cell. Another eukaryote-acquired gene (thymidine kinase) increases susceptibility to some antiviral drugs [102].

In this context it is thus possible that genes that have been exchanged between microbial species in the insect can use one of these microorganisms (e.g. *R. bellii*) as a shuttle to amoebae, and that other genes can also travel from amoebae to insect using the same shuttle (fig. 5).

Conclusion

Close ecological interactions of free-living amoebae with a variety of microorganisms including pathogenic and symbiotic bacteria, other eukaryotic species and viruses make of them a place of choice for genetic exchanges between microorganisms. These gene exchanges already occurred very early in the evolution, probably just after the primary endosymbiotic events that led to the formation of the first protzoon cells and they likely played a significant role in the diversification of life. A

better knowledge of the interactions that took place between amoebae, other eukaryotic species, bacteria and viruses will help us to understand the evolutionary mechanisms that can lead to the selection of virulence traits and to the emergence of new pathogenic species. Mechanisms allowing bacteria to reprocess acquired eukaryotic genes and use them for their own life cycle also need to be investigated [53].

The publication of the Mimivirus genome in 2004 [103] triggered considerable interest to study the role of this new category of viruses as vehicles for genes traveling between bacteria, eukaryotes and viruses [104, 105]. The discovery of new viruses infecting amoebae, such as the recently published 'Marseillevirus' and the 'Sputnik' virophage infecting Mamavirus, brought new information [106, 107]. There is undoubtedly a huge diversity of new virus species remaining to be discovered; it is for example conceivable that viruses infecting the *Hartmannella vermiformis* amoeba can be found in the environment, and interestingly this amoebal species is thought to have higher rates of lateral gene transfers compared to *Acanthamoeba* spp. [108].

Important studies demonstrating horizontal gene transfer of an entire metabolic pathway between the eukaryotic alga *Emiliania huxleyi* and its NCLDV virus

EhV (family: Phycodnaviridae) were also recently published [109]. Authors have demonstrated transfer of seven genes involved in the sphingolipid biosynthesis pathway; it is not clear if this transfer occurred from virus to alga or from alga to virus. These genes are thought to play critical roles in the timing and physical processes of virion release from the host cells, and further research demonstrated a shift from *E. huxleyi* to EhV sphingolipid gene expression during the infection cycle, thus allowing viruses to regulate eukaryotic pathways necessary for their life cycle [110]. Of note, DNA fragments of another phycodnavirus, *Ectocarpus siliculosus* virus-I, were also described to be interspersed in the host's genome in regions containing DNA repeats, pseudogenes and genes involved in DNA replication, integration and transposition [111].

In conclusion, we are at a very early age of genomics of eukaryotes and available genomes are currently limited to only a few species, which limits opportunities to high-light gene transfer events. High-throughput sequencing techniques should be used in the future to provide insight into a more comprehensive set of unicellular eukaryote genomes, thus extending the opportunities for exciting research in the field.

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Note Added in Proof

The genome of *Ca Amoebophilus asiaticus* has been published during publication of this review and clearly demonstrates that many gene transfers occurred between this species and other obligate intracellular bacteria, mainly *Rickettsiales*. See: Schmitz-Esser S, Tischler P, Arnold R, Montanaro J, Wagner M, Rattei T, Horn M: The genome of the amoeba symbiont *Candidatus Amoebophilus asiaticus* reveals common mechanisms for host cell interaction among amoeba-associated bacteria. *J Bacteriol* 2010;192:1045–1057.