Part I
General Aspects

Intracellular Niches of Microbes. Edited by Ulrich E. Schaible and Albert Haas

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Introduction: The Evolution of Intracellular Life Forms and their Niches

Ulrich E. Schaible and Albert Haas

> "As species are produced and exterminated by slowly acting and still existing causes, and not by miraculous acts of creation and by catastrophes; and as the most important of all causes of organic change is one which is almost independent of altered and perhaps suddenly altered physical conditions, namely, the mutual relation of organism to organism, – the improvement of one being entailing the improvement or extermination of others". Charles Darwin

1.1
A Short History of Theories and Discoveries

The complex mutual relationship between intracellular microbes and their host cells is a challenging field of research and requires the perspective of evolution biology. The individual host–microbe interactions covered in this book all raise the following questions: how do microbes enter, survive and proliferate in, and how do they exit host cells? And how can intracellular niches be characterized and what are the benefits of intracellular life for the microbes and its consequences for the host cell? The question, however, is how and under what selective pressure did these interactions evolve? The year 2009 marks the 200th birthday of Charles Darwin (1809–1882; 12th February 1809), and, more importantly, the 150th anniversary of the publication of his most important book *The Origin of Species by Means of Natural Selection* (24th November 1859) [1]. In this eminent and highly disputed and provocatively revolutionary work, Darwin outlined the concept of evolution by natural selection in the struggle of life. The concept of interspecies competition as the driving force for the evolution of all bacterial, animal and plant species laid the basis for modern day biology.

Louis Pasteur (1822–1895) and others proved that microbial life did not arise spontaneously and miraculously, but rather due to the omnipresence of microorganisms, an important fact for food preservation and the consequential establishment of sterilization techniques. The seminal work of the nineteenth-century microbiologists

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set the path to study the novel complexity of interspecies interactions in natural science and medical research. Although infectious diseases were an important determinant for human history, causing migration, settlement and conflict behavior, it was not until the nineteenth century that infectious agents were identified as causative agents for certain diseases rather than the diseases being of mysterious origins. The time between the end of the nineteenth and the beginning of the twentieth century was the high season of bacteriology, during which a huge number of microbial species were identified using newly developed culture techniques. Many of these microbes were associated with humans, animals or plants, and they were either pathogens, beneficial symbionts or commensals. A number of those microbes had chosen other unicellular or multicellular organisms as their ecological niches. Finally, infectious diseases were recognized as the driving force for the evolution of the innate and, in vertebrates, the acquired immune systems (Chapter 12).

Robert Koch (1843–1910) and his colleagues identified the first intracellular pathogenic bacterium, the tubercle bacillus (*Mycobacterium tuberculosis*). In the late nineteenth century tuberculosis was the prime cause of death in the metropolitan areas of Europe and North America, stirring up intensive medical and scientific interest. At around the same time, an important virulence trait of the tubercle bacillus, that is, living in macrophages, was described by Elie Metchnikoff (1845–1916), the founder of phagocyte biology. This is still a prime topic in tuberculosis research today (see Chapter 19). Metchnikoff was the first to observe the phagocytosis of bacteria by phagocytes in 1883 during his time at the Viennese Institute of Zoology and he also pointed out the importance of these cells in host response and inflammation [2, 3]. The term macrophage was attributed to him and made him the founder of innate immunity. In 1908, he received the Nobel Prize for his achievements. Metchnikoff was also the first to observe tubercle bacilli thriving intracellularly in macrophages (Figure 1.1) [4]. However, it was not until the last quarter of the twentieth century that scientists started to study the virulence factors of pathogens, and that intracellular pathogens (and symbionts) were highlighted for their unique capabilities to survive within and manipulate their host cells.

The identification of intracellular survival mechanisms was made possible by novel techniques in cell biology and the arrival of modern molecular genetics. J. A. Armstrong and Philip D'Arcy Hart [5, 6] were the first to show inhibition of phagolysosome fusion by the tubercle bacillus. Similar peculiarities of *Toxoplasma gondii*- and *Chlamydia psittaci*-containing vacuoles were published in 1979 and 1981, respectively [7, 8]. In the last decade of the twentieth century, many virulence traits of intracellular microbes were elucidated. Genome analyses and molecular techniques, paired with novel model systems such as yeast two-hybrid screening technology, uncovered pathogenicity islands and plasmids, virulence factors, as well as host cell target structures. It was discovered that throughout evolution there must have been a tremendous horizontal gene transfer between different microbes as well as between bacteria and eukaryotes (Chapter 2). Many of those pathogens and their virulence traits will be covered in this book. Some important intracellular microbes, such as *M. leprae*, *Chlamydia* and *Rickettsia*, are not yet accessible to

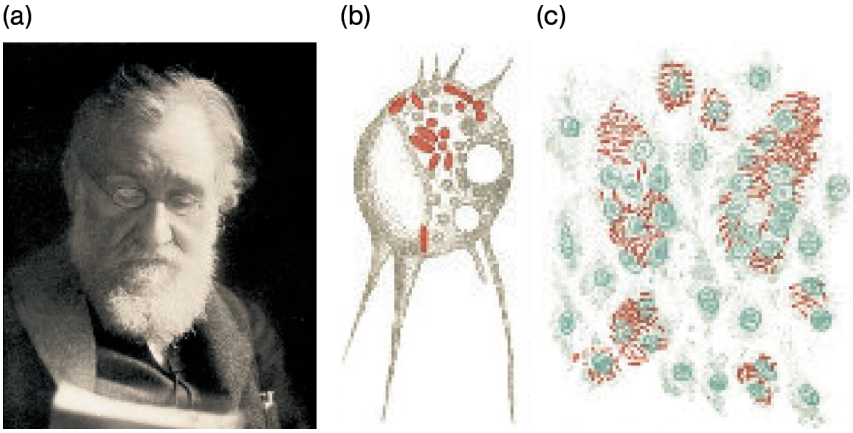

Figure 1.1 (a) Elie Metchnikoff (1845–1916) in his later life. He was the founder of phagocyte biology and coined the term "macrophage." Metchnikoff was also the first scientist to observe tubercle bacilli within macrophages and suggested they be able to survive within these cells, which otherwise are able to kill microbes. (b) Metchnikoff's depiction of a pigeon macrophage containing mycobacteria. (c) Macrophage culture infected with mycobacteria as observed by Elie Metchnikoff. These pictures were kindly provided by Stefan H. E. Kaufmann, Max-Planck-Institute of Infection Biology, Berlin, Germany.

manipulation by molecular genetics and future attempts will focus on generating targeted mutants in such organisms. A peek into the book of evolution of intracellular microbe genomes suggests that many of these virulence traits were established early in evolution, though probably not exactly for the purposes they are used for today.

1.2
A Look Through the Microscope of Evolution

> *Now, here, you see, it takes all the running you can do, just to keep in the same place*. The Red Queen in Lewis Carroll's *Alice in Wonderland*. (*The Origin of Species by the Means of Natural Selection*, 1859)

According to Darwin, life may have started in some "warm little pond." Rather than in warm ponds, it is believed today that primitive *Bacteria* and *Archaea* arose in the vicinity of hot vents in the ancient oceans some 4 billion years ago; and microbes still rule the earth today. It was only after the first half of life's history that microbes started to share the world with eukaryotes, and at this point they successfully explored these larger organisms as ecological niches. The *Cyanobacteria* laid the groundwork for the development of higher (aerobic) life forms by inventing photosynthesis and the production of oxygen, and α-proteobacteria contributed to the formation of eukaryotes by providing the ancestors of mitochondria. According to the (now widely accepted) endosymbiont hypothesis, mitochondria, plastids and hydrogenosomes

originated from free-living bacteria. Without these essential endosymbiotic organelles, unicellular eukaryotes and subsequently larger multicellular organisms would probably never have seen the light of day.

Mitochondria and plastids probably represent the most intimate relationship between pro- and eukaryotic cells, and are the prime example of intracellular life. During the initial invasion step the host cell was likely just a membrane sac with a membrane compartment to concentrate the genetic information – an ancient nucleus precursor. Most likely it was still a fellow prokaryote. In 1967, Lynn Margulis (b.1938) reintroduced the endosymbiont theory to the field of evolution biology [9]. According to this hypothesis, mitochondria and plastids originate from ancient bacterial and cyanobacterial symbionts, respectively (Figure 1.2). This hypothesis was supported by the presence of two membranes surrounding these organelles,

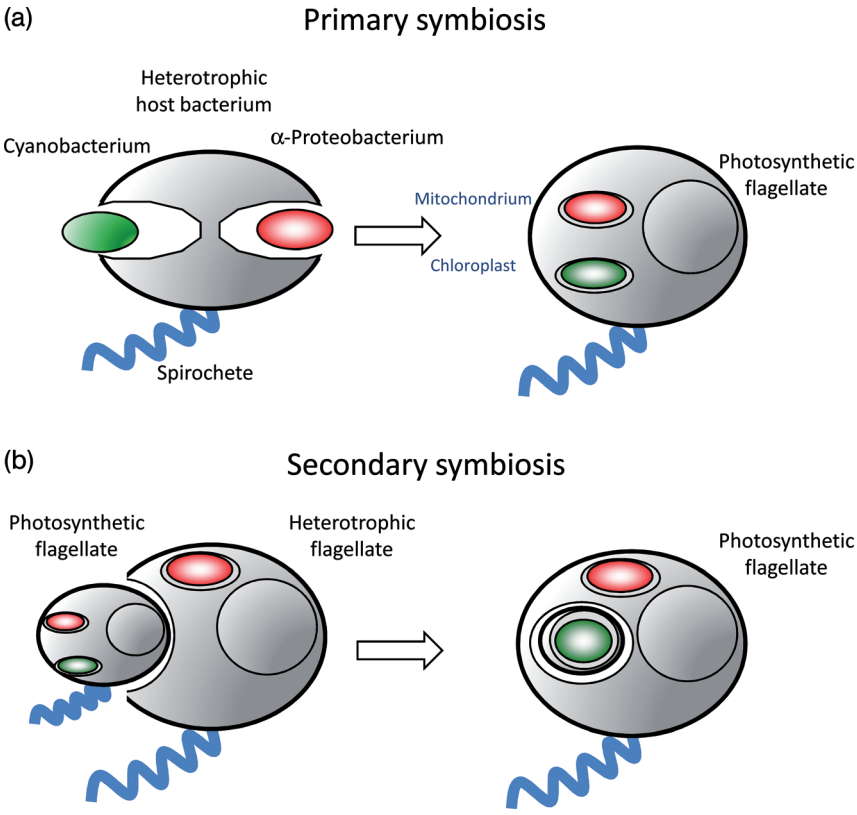

Figure 1.2 (a) Schematic drawing on the symbiont hypothesis how primary symbiosis between two (or three, or more) prokaryote microbes led to eukaryote cells with mitochondria, chloroplasts and flagellae. (b) Secondary symbiosis arose between heterotrophic and photosynthetic eukaryotes namely flagellate species. In both scenarios, the ultimate relationship lead to full dependency of both partners on each other and their loss of autonomy.

many structural similarities and the presence of bacterial DNA in these organelles. The theory dates back to 1883, when the German botanist Andreas Franz Wilhelm Schimper (1856–1901) postulated that chloroplasts are derived from photosynthetic bacteria, and was renewed by Konstatin Sergejewitsch Merschkowski (1855–1921) in 1905. Our current understanding is that more than 1.5 billion years ago an α-proteobacterium-like microbe invaded an ancient host cell, which was most likely another bacterium with compartmentalized chromosomes similar as found in *Gemmata obscuriglobus* and other δ-proteobacteria [10].

According to scenarios based on the argument that metabolic needs may have promoted formation of symbiosis between two prokaryote species, host cell and invader may have been a methanogenic *Archaea* and a methanotroph, respectively (reviewed in Dyall *et al.* [11]). In this scenario, the essential event of eukaryote evolution is set in the anoxic era, whereas others placed it in the aerobic age where an anaerobic archaeal host was protected from toxic oxygen by an aerobic symbiont.

Another version suggests that mitochondria are derived from photosynthetic bacteria due to the following arguments: (i) they release photosynthates such as glycollate for metabolic use by the heterotrophic partner (through peroxisomes as realized in higher plants); (ii) their morphological features resemble cristae of mitochondria; and (iii) 31 of the most conserved mitochondrial genes are closely related to genes in the phototrophic bacterium *Rhodospirillum rubrum* [12].

Whichever microbe the mitochondrial ancestor was, it is most likely that the process of mitochondrial endosymbiogenesis succeeded just once since all known current eukaryotes contain a number of original genes from the α-proteobacterial ancestor.

During a process starting some 3.5 billion years ago, atmospheric oxygen accumulated through the metabolic activity of photosynthetic bacteria. Shortly after eukaryotes with mitochondria started roaming the earth, another invasion event by cyanobacteria led to the emergence of the ancestors of green algae and higher plants (*Chlorophyta*) and subsequently of red (*Rhodophyta*) and brown algae (*Glaucophyta*) (see below) [13]. Amitochondrial amoebal, trichomonad, ciliat and anaerobic fungal species still exist today. In those organisms, ATP-producing organelles, the hydrogenosomes, play a role similar to mitochondria. Although hydrogenosomes do not contain a genome, proteomic analyses suggest their relationship with mitochondria, but this is controversially discussed [11]. In some other amitochondrial organisms, such as *Giardia*, *Entamoeba* and *Microsporidia*, in which mitochondria-like remnants have been found, it is not clear whether their loss is a secondary event. The emergence of eukaryotes from a get-together of different bacterial species probably represents the first type of intracellular life on earth.

It should, however, be mentioned that the bacterium *Bdellovibrio bacteriovorus* is a specialized parasite of other bacteria and invades their periplasmic space. A symbiotic α-proteobacterium, *Midichloria mitochondrii*, has recently been described residing in the mitochondria of tick ovary cells [14]. These facts may lead to the bold hypothesis that intrabacterial parasites/symbionts may have preceded the rise of eukaryotes, and that they were the first intracellular life forms.

The current view that interbacterial symbiosis formed the basis for the evolution of *bona fide* organelles is further corroborated by the identification of more recent "domestication" events of (cyano-)bacterial symbionts by eukaryotes. The filose amoeba *Paulinella chromatophora* harbors photosynthetic *Synechococcus*-type cyanobacteria as symbionts, which have totally lost their autonomy thereby forming a primary symbiont [13] (Figure 1.2).

Subsequently to endosymbionts becoming mitochondria and chloroplasts, evolution led to further examples of endosymbioses. After green, red and brown algae emerged, secondary endosymbiosis (Figure 1.2) was born when aplastid flagellates incorporated red algae cells, thus joining the photosynthetic community. The genera *Cryptophyta*, *Dinophyta*, *Heterokontaphyta* and *Haptophyta* were the results of these joint ventures. Also, the parasite phylum *Apicomplexa* originated from such an endosymbiosis, which explains why parasites such as *Plasmodium* and *Toxoplasma* are affected by herbicides that target plastid enzymes [15]. Moreover, unicellular eukaryotes, probably heterotrophic flagellates, incorporated green algal cells and gave rise to *Euglenophyta* and *Chlorarachniophyta*. A very recently evolved secondary endosymbiosis is the union between the colorless flagellate "Hatena" and a green algae of the genus *Nephroselmis* [16]. Upon engulfment of free-living flagellated *Nephroselmis* cells by Hatena, the symbiont loses flagellae, cytoskeleton and endomembranes but retains nucleus, plastides, mitochondria and eyespot. The complex feeding apparatus of the colorless host flagellates disappears after uptake of the symbiont. This event seems to coincide with the host cell's switch from heterotrophic predator to autotrophic algae. After cell division, the daughter cell lacking the symbiont becomes heterotrophic again and develops a feeding apparatus to catch a new symbiont. It has been suggested that "Hatena" could be a model for the early development of secondary symbiosis. This example shows that not only the symbiont but also the host cell may go through cellular changes upon formation of endosymbiosis. The latter notion is also corroborated by findings from the genome of the pathogenic filarial nematode *Brugia malayi*, which revealed adaptations, which had most probably evolved in response to the presence of *Wolbachia* symbionts [17].

On several occasions later in evolution, α-proteobacteria such as *Wolbachia*, *Rickettsia* and *Ehrlichia* species, as well as members of the *Chlamydiales* became settlers of eukaryotic cells as highly specialized obligate intracellular mutualists or pathogens. In free-living amoeba, more than 20 bacterial symbionts have been identified so far, belonging to the α-proteobacteria, β-proteobacteria, *Bacteroidetes* and *Chlamydiales* [18–20]. Interestingly, symbiotic *Chlamydia* species in amoebae have a biphasic lifecycle between metabolically active reticulate and inactive elementary bodies similar to that of pathogenic species in mammals, suggesting common ancestry between the groups. Among amoebae symbionts, differentiation between symbiosis and parasitism is difficult. In the case of *Parachlamydia*-related symbionts, their association with amoebal partners can also be detrimental to the host cell as they lyse their hosts at temperatures above ambient. In contrast, *Neohartmanella hartmanellae* is a *bona fide* mutualist since this bacterium promotes growth of its amoebal host [21, 22]. This suggests that there can be fine

lines between mutualistic and parasitic companionships between bacteria and eukaryotes, depending on factors such as environmental conditions.

Some hypotheses of intermicrobial symbiosis go beyond metabolic mutualisms. The evolution of motility and cytoskeleton elements has been suggested to originate from a hypothetical spirochetal symbiont forming a consortium with an archea [10]. It should be noted that "living fossils" for such a scenario exist in the form of *Chlorochromatium aggregatum* and *Mixotricha paradoxa*. *C. aggregatum* evolved from a consortium comprising green sulfur bacterial epibionts surrounding a central motile β-proteobacterium. *M. paradoxa* is a flagellate in the gut of the termite *Mastotermes darwiniensis* and is coated with *Bacteroides* species and spirochetes for motility [23, 24].

Box 1.1
Definitions of interspecies relationships

Symbiosis: Partnership between two different species. Often used synonymously with mutualism, meaning that advantages usually outweigh the disadvantages for both partners.
Mutualism: Partnership between two different species with benefits for both partners.
Parasitism: Relationship between two different species, in which the smaller one, the parasite, lives in or on a larger host organism, gaining benefits and causing harm to the host.
Commensalism: Relationship between two different species benefiting one partner, the commensal, but without (known) benefits or disadvantages for the other one.
Pathogen: Microbe that induces one or more infectious diseases.

1.3 Continuous Exchange of Information

Information – Inspiration, Shuggy Otis, musician, 1971

Stable symbiosis with the newly formed eukaryotes caused a drastic reduction in the sizes of the mitochondrial and plastid genomes [25]. Genes missing from mitochondria and plastids were either totally lost or transferred to the host cell genome. The genome of free-living cyanobacteria is typically between 2000 and 4000 kb in size, whereas plastids have 200 kb coding for fewer than 200 genes. Horizontal gene transfer from the mitochondrial to the host cell genome is reflected by the presence of around 400 proteins in this organelle's proteome that are encoded by nuclear genes [26]. Consequently, the proper localization of mitochondrial or plastid proteins synthesized by the host cell requires mechanisms of transport

into these organelles. Between 15 and 25 protein import translocases have been identified in mitochondria and plastids, and some of them have striking homology to bacterial protein exporters [11]. A significant reduction in genome size is also observed in obligate intracellular bacteria such as *Rickettsiae*, *Chlamydiae* and *M. leprae* (see Chapters 15, 19 and 26). Lateral gene transfer has occurred frequently between bacteria and eukaryotes. Genes of the intracellular symbiont *Wolbachia pipientis* have been found in the fruit fly (*Drosophila melanogaster*) genome but also in other host genomes, including those of wasps and nematodes [27]. *W. pipientis* is a maternally inherited endosymbiont in at least 20% of arthropod species. Relatives of this α-proteobacterium live in the germline of filarial nematodes (see Chapter 30). With respect to host–microbe interaction, one can envisage that bacterial genes inserted within host genomes may promote mutualism, but may also be employed by the host for its own purposes. Therefore, lateral gene transfer during microbe–host interactions may be a motor of evolution in both partner organisms.

1.4 Evolution of Intracellular Parasitism

> "Many microorganisms of diverse phylogenetic beginnings have adapted to intracellular life, each in its own unique way, and sometimes remarkable resemblances in behaviour among intracellular parasites are best ascribed to convergence in similar intracellular habits rather than to divergence from a common origin. Finally it should be remembered that adaptation to intracellular life, although by no means rare, is not easy. After all, most parasites still live extracellularly". James W. Moulder (1985) Comparative biology of intracellular parasitism, *Microbiological Reviews* 49, 298.

About 2 billion years ago, probably in a shallow laguna, bacteria encountered for the first time malicious shapeless little eukaryotes eating them. Some heterotrophic eukaryotic cells started a new business in life and became predators, whereas bacteria faced a new challenge, not to fall prey. The prototype of a phagocyte, an amoeba that feeds on bacteria, was probably the first to impose on bacteria the selection pressure to maintain or evolve new genes that facilitate survival within the predators (Figure 1.3). Today, still, amoebal creatures roam the world in search of bacterial prey. One of them, *Hartmanella*, is a notorious settler in cooling water systems and showerheads, an opportunistic human parasite itself, and one of the natural hosts for the human pathogen *Legionella pneumophila* (Chapter 18). The slime mold, *Dictyostelium discoideum*, has become a prime model organism for the study of some intracellular pathogens such as *Mycobacterium marinum*, *M. avium* and *L. pneumophila* (Chapter 4). It can be hypothesized that these free-living bacteria, which can survive and possibly even multiply in amoebae, carry preadaptations to divert phagosome trafficking, which may help to counter microbi-

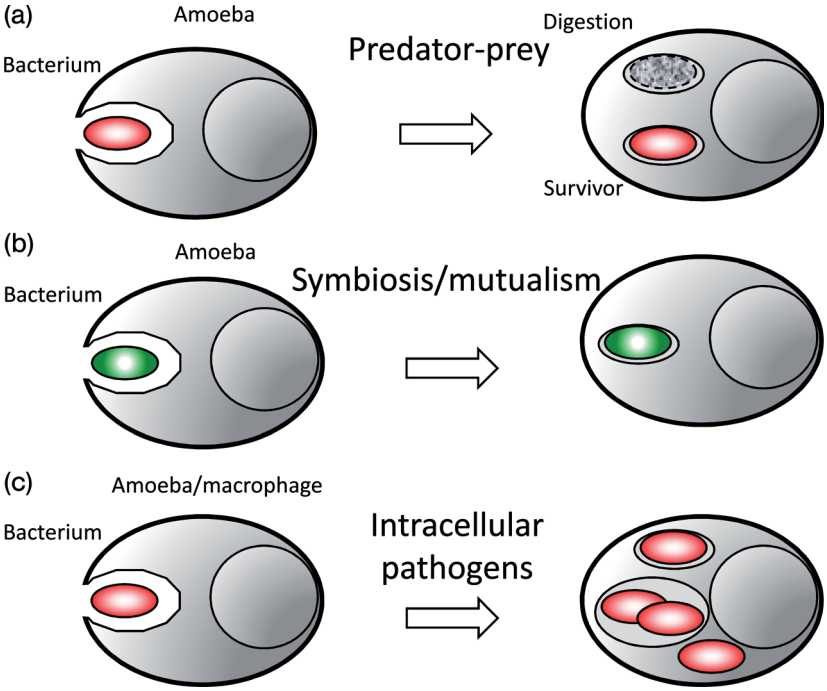

Figure 1.3 Drawings depicting the hypothetical transitions from bacteria falling prey to amoebal predators (a) to symbionts or parasites in amoeba (b). (c) The latter then give rise to intracellular pathogens of multicellular organisms including humans. This was due to preadaptations selected and sharpened during interactions with amoebal hosts.

cidal macrophages. These bacteria have the potential to become either pathogens of cold-blooded animals (e.g., *M. marinum*, a natural pathogen of fish and amphibians) or, when able to replicate at 37 °C, pathogens of mammals (e.g., *L. pneumophila*).

L. pneumophila is a prime example of how a prey of freshwater protozoa became a parasite within a unicellular host and thereby gained the prerequisites – or pre-adaptations to stay with the terminology of evolution biology – to survive inside mammalian phagocytes and become a pathogen (see Chapter 18). *Legionella* apparently benefited from the fact that mechanisms and factors of phagocytosis and intracellular trafficking are highly conserved throughout the animal kingdom. In principal, this allows intracellular bacteria to parasitize any phagocytic cell in the world. *L. pneumophila* lives in fresh water, especially artificial warm water distribution systems. When inhaled, it can cause a severe pneumonia called legionnaires' disease in immunocompromised and elderly patients. This pathogen seems to have a complex lifestyle which is regulated by nutrient availability and characterized by distinct gene expression patterns and switching from flagellated free forms to

intracellular nonflagellated and spore-like stages [28]. Inside amoebae and macrophages it survives and proliferates in compartments made of endoplasmic reticulum (ER) membranes (see Chapter 18) [29]. It has been suggested that during coevolution between *Legionella* species and protozoa the bacteria acquired a significant number of eukaryotic-like genes that are not found in most other bacterial genomes and that are possibly involved in protein–protein interactions with host cell structures [30–32].

Interestingly, the *L. pneumophila* genome contains an unusual number of ankyrin-like domains as well as F-box and serine/threonine protein kinase genes. Eukaryotic ankyrin repeat proteins are typically involved in protein–protein and protein–cytoskeleton interactions. F-box proteins play a role during ubiquitination, and protein Ser/Thr kinases are eukaryotic signaling molecules. It is fascinating to note that other intracellular pathogens share these genomic features of *L. pneumophila*. *Wolbachia*, *Coxiella* and *Rickettsia* species also carry ankyrin-like domain genes, and *M. tuberculosis* and the extracellular *Yersinia pseudotuberculosis* have Ser/Thr protein kinase genes involved in their virulence [33]. Homologs of these genes are found in protozoa, suggesting that these virulence traits may have been gained through lateral transfer of protozoan genes, thereby preadapting *Legionella* to become a mammalian pathogen [34]. It will be interesting to find out whether other mammalian pathogens that dwell within protozoa, such as *M. marinum*, *M. avium*, *Listeria monocytogenes*, *Francisella* species or *B. pseudomallei*, have developed similar virulence features during coevolution with ancient predatory protozoa. Unlike specific pathogens, these ubiquitous and opportunistic organisms require a broad genetic flexibility to thrive in different environments.

Unlike other bacterial genomes, over 7% of the *L. monocytogenes* genome encodes regulatory proteins, whereas the similar-sized genome of the extracellular pathogen *S. aureus* has half as much regulatory DNA. This suggests that *L. monocytogenes* needs flexibility in its gene regulation in response to sudden environmental changes [35]. Development of the *L. monocytogenes* PrfA-regulated virulence gene cluster, which is essential for intracellular survival and spread from cell to cell, was an important prerequisite to becoming an intracellular pathogen in mammals (see Chapter 24). Interestingly, this gene cluster is also present in the nonpathogenic *L. seeligeri*, a close relative of *L. monocytogenes* but does not (yet) give *L. seeligeri* the license to become a mammalian pathogen. However, it has been suggested that it may allow freshwater listeria to survive in invertebrate hosts and, therefore, the PrfA system may represent an ancient preadaptation to becoming a pathogen [36].

It is also noteworthy that intracellular pathogens are rarely found in higher plants. This may be either because of the robust plant cell wall, which restricts entry or because of the inability of plant cells to phagocytose. The enigmatic protists of the order *Plasmodiophorida*, such as *Plasmodiophora brassicae* and *Spongospora* species, are intracellular plant pathogens of economical importance [37]. Although these microbes have recently became a focus of more detailed genetic studies, an understanding of the invasion pathways, intracellular survival and niches of these pathogens is still lacking.

1.5
Intracellular Symbionts: Tamed or Acclimatized Parasites?

Give it to me. Timbaland, *Timbaland Presents Shock Value* album, 2007.

Symbiosis is an important factor in the earth's ecosystem. Bacteria and cyanobacteria form symbiont–host relationships with protozoa, plants and invertebrates. Marine organisms, such as mussels, clams and tubeworms, within the dense animal communities at deep-sea hydrothermal vents often depend entirely on chemoautotrophic methano- or thiotrophic bacterial symbionts for carbon fixation [38]. The vesicomyid clams carry their symbionts in gill epithelial cells and transmit their endosymbionts to the next generation through their eggs. Vestimentiferan tubeworms, however, have to ingest their symbionts during the juvenile stage. The worm's sulfur-oxidizing bacteria are contained within bacteriocytes concentrated within a specialized organ, the trophosome. Another vent settler, the mussel *Bathymodiolus* seems to access its thiotrophic symbionts, which also live in gill epithelial cells, from the environment [39]. The entire hydrothermal vent community probably depends on these symbionts, which strongly influence the local marine ecology. However, very little is known about their intracellular lifestyle.

Unicellular algae such as zooxanthellae as well as green algae of the genus *Chlorella* also became intracellular symbionts in plants, protozoa and invertebrate animals. These symbioses are of prime ecologically importance. Cyanobacterial or algal symbionts provide photosynthates to the heterotrophic host and remove its carbon dioxide. *Chlorella* forms a symbiotic relationship with the freshwater polyp *Hydra viridis*, and is transmitted to its offspring through the eggs. *Chlorella* avoids digestion in the polyp's cells by inhibiting phagolysosome formation [40–42], as do several intracellular pathogens. Zooxanthellae of the genus *Gymnopedium* are intracellular symbionts of coral reef polyps, which have formed the biggest natural sculptures in the world. *Gymnopedia* are close relatives of dinoflagellates. They provide up to 90% of the polyp's energy and are therefore essential for the growth of the coral reefs in the otherwise oligotrophic oceans of the tropics.

The close relatives of some intracellular symbionts are important bacterial pathogens, which employ the intracellular niche as a survival mechanism, though with the outcome of disease. *Legionella*-, *Francisella*-, *Chlamydia*- and *Rickettsia*-like species have been found in free-living amoeba. Under experimental conditions, *L. pneumophila* can infect up to 14 amoeba species, including *Hartmanella* and *Acanthamoeba* spp., and two species of ciliated protozoa, whereas its close relative, the rarely pathogenic *L. micdadei*, only infects *Hartmanella* amoebae [33, 43]. Compared with *L. pneumophila*, *L. micdadei* inhabits a different intra-amoeba compartment and does not exit through pore formation and cytolysis [44]. *L. micdadei* may therefore represent an earlier stage during the evolution of *L. pneumophila* to become a successful survivor and ultimately a parasite of amoeba.

Establishment of a symbiosis is likely "a large step for the microbial species," yet "a small one for an individual microorganism." Transition from a pathogenic to a

symbiotic microbe and vice versa could happen frequently, but selective pressure can stabilize the respective relationship. It has been demonstrated in the laboratory that transition from an extracellular microbe to an intracellular mutualist can be observed within several hundred generations time, which corresponds to a few years [45]. The partner organisms in this experiment were *Legionella*-like bacteria and *Amoeba proteus*. The resulting symbiosis was stable in that removal of the bacteria killed the host cells [45]. This feature of a mutualistic relationship often goes hand in hand with the fact that symbiotic microbes cannot be cultivated on their own in the laboratory, at least not with the currently known culture methods.

Relatives of pathogenic *Rickettsia*, *Coxiella* and *Francisella* spp. – causative agents of louse- or tick-borne zoonoses – have been found in arthropods, including ticks [18, 36]. Mutagenesis of *Wolbachia* can reprogam this fruit fly symbiont to become a parasite, suggesting that it can be a small step between both types of relationships [46]. *Wolbachia* spp. in nematodes are symbionts, whereas those dwelling in arthropods can cause disease, including sexual morphological alterations, cytoplasmic infertility and reduced reproductivity [47, 48]. An unexpected benefit for the insect host, however, is that *Wolbachia* promotes innate immunity in *Drosophila*, protecting against infection with the *Drosophila C virus* [49].

Rhizobium meliloti, a symbiont of legumes, depends on homologous genes, *bacA* and the *bvrR-bvrS* two-component regulatory system, for intracellular survival in root cells, both of which are also essential for another α-proteobacterium, the zoonotic *Brucella abortus* living inside macrophages [50]. This suggests that general adaptations are required for intracellular survival in organisms as distant as plants and mammals, and are independent of whether a pathogen or a mutualist is involved. Mutualism between symbiont and host is a fine balance and often the equilibrium is not far from parasitism. The distinction between symbiosis and parasitism is hard to make since both partners gain benefits as well as exploit each other.

1.6 An Ecological View of Intracellular Life

> *Pathogens lack malice; they are just trying to survive.*
> Arno Karen, in *Biography of a Germ*, 2000.

We have argued that bacteria developed ways to escape or survive the attack of predatory amoeba in the early ponds of evolution. However, successful intracellular survivors did not only gain the benefit of not being digested but also were rewarded with protection from environmental conditions and the possibility of accessing novel food sources. Exploiting the newly inhabited intracellular niche for survival and growth was a profitable result of novel adaptations to avoid or resist phagocyte digestion (Figure 1.3). The intracellular niche can provide microbes with nutrients, including essential micronutrients they otherwise have to compete for with fellow microbes in the extracellular environment and/or have to capture or synthesize by themselves:

Rickettsiae graze on the host cell's energy sources including ATP [51]. Mycobacteria inhibit phagosome maturation in macrophages and thus inhabit early phagosomes where they can scavenge iron from transferrin because this compartment intersects with the iron import pathway of the host cells (i.e., the transferrin–transferrin receptor uptake system into early endosomes) [52]. *Coxiella burnetti* and *Leishmania* amastigotes exploit the harsh lysosomal environment for growth and probably feast on hydrolytic degradation products such as amino acids [53–55]. *L. mexicana* amastigotes probably exploit autophagolysosomes to access purines [56]. *Leishmania* species are purine auxotrophs and require host cell-derived purine sources such as autophagosomes. Certain *C. trachomatis* strains are unable to synthesize the amino acid tryptophan.

Nutrient limitation – the bacteria's Achilles heel – is targeted by the host response in an interferon γ (IFNγ)-induced manner. Activated macrophages express the gene for indoleamine 2,3-dioxygenase (IDO), which depletes tryptophan by degradation to kynurein [57]. Sequestration of *C. trachomatis* from tryptophan drives the bacteria to differentiate into the nongrowing residual body form and causes latent infection. The genome of *C. psittaci* contains a more complete tryptophan synthesis machinery and resistance to IDO of this *Chlamydia* species is due to efficient recycling of the amino acid [57].

Genome reduction is a common consequence of colonization for pathogens or symbionts which became highly adapted to the intracellular lifestyle and entirely dependent on their host cells. This is seen in diverse obligate intracellular microbes such as the insect symbionts of the *Buchnera* genus, or *M. leprae*, *Rickettsia* or *Chlamydia* species, as well as in extracellular *Mycoplasma* species. The host provides a pretty constant environment as well as nutrients and metabolic resources. As a consequence of this close relationship, obligate intracellular microbes lost their ability to survive and proliferate outside of the host cell and become metabolically dependent. This often leads to loss of genes required for the synthesis of organic molecules such as amino acids and, ultimately, to the inability to generate ATP.

In an ecological view of interspecies relationships, interactions between two partners also determine interactions beyond this partnership. Thus, simple coevolution is unlikely, because the broad ecological context with its entire range of interacting factors, including food competition and predator–prey interactions, also influence host–parasite/symbiont interactions [58]. Intracellular microbes probably also influence each other. In the broader ecological context it can be hypothesized that intracellular symbionts such as *Rhizobium* or *Wolbachia* species enhance the fitness of legumes or parasitic nematodes, respectively. Studies on the evolution of virulence have found that the more virulent parasites are, the higher their transmission rates and the less they are controlled by immunity. It has been shown that immune pressure selects for more virulent parasites [59, 60]. These studies, however, never took into account that hosts with higher parasite loads may be an easier prey for predators and are therefore removed more quickly from the population [58]. This would increase the resistance to the parasite within the host population and would eventually lead to an equilibrium in an individual host–pathogen relationship (i.e., between defense and virulence). It may become disturbed, however, when the

predator is removed, for example, or a new pathogen is introduced that affects herd immunity. A good example is the increased tuberculosis rate as boosted by the HIV pandemic.

1.7 The Immunologist's View

> *Thus spears and swords gave rise to shields and body armour, and radar defenses to the Stealth bomber.* Nesse and Williams, in *Why We Get Sick*, 1994.

Just as in warfare, acquisition of a new weapon by one party will cause development of a defense system by the opposition, allowing them to counter, a microbial challenge promotes the development of sophisticated defense mechanisms in the host organisms. Microbicidal effectors of amoebae comprise acidic pH, porins, bactericidal peptides and lysozymes. Multicellular invertebrates also primarily depend on defensin-like microbicidal peptides but additionally employ motile amoeboid phagocytes as eliminators of pathogenic invaders such as the haematocytes within the arthropod's hemolymph. Cytokines such as interleukin 1 and tumor necrosis factor (TNF) mediate phagocyte activation. Invertebrate cells sense microbial stimuli. *Drosophila* and other arthropods use Toll-like receptors (TLR) to distinguish between pathogenic types such as fungi, viruses or bacteria [61]. The immune deficiency (IMD) signaling pathway, homologous to the TNF receptor signaling pathway, is also essential for the fruit fly to survive infection [62].

In higher vertebrates, particularly in mammals, phagocytes have become diverse in function. Macrophages still fulfill their ancient functions of eliminating microbial invaders from tissue and clearing away dead cells. Interferons, in particular IFNγ, facilitate macrophage activation in order to better regulate expression of highly effective antimicrobial mechanisms, which can be destructive to normal tissue. Neutrophils – microphages as Elie Metchnikoff called them – became specialized in rapid recruitment to sites of microbial invasion and killing. These cells, however, also came with the danger of collateral tissue damage and therefore required control mechanisms, such as their short lifetime. Finally, dendritic cells took over the job of processing foreign antigens in order to present them in the context of self-molecules to the acquired immune system. In vertebrates, and particularly in mammals, the acquired immune system, comprising B cells, T cells, immune memory functions and a complex regulatory cytokine network, confronted pathogenic microbes with a totally novel challenge. Nevertheless, many pathogens thrive within the mammalian host. The diversity of tissues and entry ports opened up new niches, immune privileged sites and host cells within the multicellular organism such as Schwann cells for *M. leprae* or erythrocytes for *Bartonella*, *Plasmodium* and *Babesia* species. Active invasion at the epithelial interface and entry mediated by arthropod vectors provided new ways to access different host cells. Inflammation-mediated tissue damage opened up novel paths to exit the host in order to facilitate transmission to a

new one. Lung lesions in people with active tuberculosis allow tubercle bacilli to be spread through coughing [63]. The development of a highly complex and versatile adaptive immune system by large multicellular organisms such as mammals is most likely the result of coevolution arising from continuous interactions with pathogenic microbes.

1.8 The Public Health View

> *Also, because of their rapid evolution and constantly changing circumstances of human life, they [pathogenic microorganisms] continue to present threats of future pestilence.*
> Cedric A. Mims in *The Pathogenesis of Infectious Disease*, 1988.

Protozoan or invertebrate hosts are likely to provide breeding grounds for newly arising human pathogens as they must have in the past. *L. pneumophila*, trained by predatory amoebae, became a relatively recent addition to the list of human pathogens due to the development by humans of extensive water distribution and air condition systems within large building complexes, including hospitals and pools. *Legionella*'s protozoan hosts thrive under these ecological conditions, making the bacterium a pathogen and public health issue. In the light of this, human infection with *L. pneumophila* can be thought of as an "accident," but it also shows how far preadaptations can get you as a pathogen, once you arrive in a permissive environment.

Preadaptations for survival within protozoan and invertebrate hosts can yield more infectious agents for humans in the future due to environmental changes and novel ecological niches generated by humankind. For pathogens such as *B. pseudomallei*, *Afipia felis* and the extracellular *M. ulcerans*, which recently gained more public health coverage, it is also hypothesized that reservoir hosts – freshwater amoeba or insects – exist, which may have been the training ground for those human pathogens [64–66]. Finally, mutualists, which are already equipped with genes for intracellular survival, could also become pathogens when the mutualist's behavior changes towards uncontrolled growth and pathology, or when it is transmitted to a new host. More importantly, mutualists living in ectoparasites of mammals such as blood-sucking insects, ticks and leeches can become future infectious burdens to humans and livestock.

It can also become a public health issue when free-living protozoa support pathogenic bacteria in surviving and persisting in the environment. Already established intracellular pathogens of mammals can "re-employ" their virulence factors to survive within free-living protozoa. The anaerobic pathogen and causative agent of vaginitis, *Mobiluncus curtisii*, does not normally replicate under aerobic conditions but does when amoeba are added to the cultures [67]. The facultative intracellular opportunist, *M. avium*, can even survive encystation of its host amoeba [68]. More important for hygiene measures are reports that residency within amoebae protects

microbes from common water disinfectant procedures such as chlorination, as shown with *M. avium* ssp. *paratuberculosis* [2]. An experiment using an *Arabidopsis* model has due to their virulence-associated type III secretion system shown the unexpected result that *S. typhimurium* is able to invade and survive within plant cells. This makes lettuce cells a potential reservoir for typhoid fever when exposed to *Salmonella*-contaminated water [69].

1.9 The Book

> *If none of the microorganisms associated with man did any damage, and none was notably beneficial, they would be interesting but relatively unimportant objects.* Cedric A. Mims in *The Pathogenesis of Infectious Disease*, 1988.

Since Elie Metchnikoff's first observations of phagocytosed bacteria, intracellular pathogens have attracted the attention of microbiologists and attempts have been made to describe their biology in a systematic and comparative way [70]. In this book, specialists studying the different species, pro- and eukaryotes, parasites and symbionts give detailed insight into the intracellular lifestyle of these microorganisms, their ways of entering, surviving and proliferating within host cells, the diseases they cause and the benefits they have for partners in symbiosis. The main theme is discussion of the evolutionary and ecological aspects of this fascinating field of interspecies interactions.

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