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FEMS Microbiology Reviews 22 (1998) 255–275

FEMS MICROBIOLOGY REVIEWS

Genome evolution within the alpha *Proteobacteria*: why do some bacteria not possess plasmids and others exhibit more than one different chromosome?

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Received 13 May 1998; received in revised form 22 July 1998; accepted 21 August 1998

Abstract

Animal intracellular *Proteobacteria* of the alpha subclass without plasmids and containing one or more chromosomes are phylogenetically entwined with opportunistic, plant-associated, chemoautotrophic and photosynthetic alpha *Proteobacteria* possessing one or more chromosomes and plasmids. Local variations in open environments, such as soil, water, manure, gut systems and the external surfaces of plants and animals, may have selected alpha *Proteobacteria* with extensive metabolic alternatives, broad genetic diversity, and more flexible and larger genomes with ability for horizontal gene flux. On the contrary, the constant and isolated animal cellular milieu selected heterotrophic alpha *Proteobacteria* with smaller genomes without plasmids and reduced genetic diversity as compared to their plant-associated and phototrophic relatives. The characteristics and genome sizes in the extant species suggest that a second chromosome could have evolved from megaplasmids which acquired housekeeping genes. Consequently, the genomes of the animal cell-associated *Proteobacteria* evolved through reductions of the larger genomes of chemoautotrophic ancestors and became rich in adenosine and thymidine, as compared to the genomes of their ancestors. Genome organisation and phylogenetic ancestor–descendent relationships between extant bacteria of closely related genera and within the same monophyletic genus and species suggest that some strains have undergone transition from two chromosomes to a single replicon. It is proposed that as long as the essential information is correctly expressed, the presence of one or more chromosomes within the same genus or species is the result of contingency. Genetic drift in clonal bacteria, such as animal cell-associated alpha *Proteobacteria*, would depend almost exclusively on mutation and internal genetic rearrangement processes. Alternatively, genomic variations in reticulate bacteria, such as many intestinal and plant cell-associated *Proteobacteria*, will depend not only on these processes, but also on their genetic interactions with other bacterial strains. Common pathogenic domains necessary for the invasion and survival in association with cells have been preserved in the chromosomes of the animal and plant-associated alpha *Proteobacteria*. These pathogenic domains have been maintained by vertical inherence, extensively ameliorated to match the chromosome G+C content and evolved within chromosomes of alpha *Proteobacteria*.

Keywords: Bacterial genome; *Proteobacteria*; *Afipia*; *Agrobacterium*; *Anaplasma*; *Bartonella*; *Bradyrhizobium*; *Brucella*; *Mycoplana*; *Ochrobactrum*; *Phyllobacterium*; *Rhizobium*; *Rhodobacter*; *Rickettsia*; *Sinorhizobium*

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0168-6445/98/$19.00 © 1998 Published by Elsevier Science B.V.
PII: S0168-6445(98)00016-3

Contents

1. Introduction

Selfish accessory elements of bacterial genomes are plasmids, lysogenic phages, transposons, insertion sequences and retrons [1,2]. These elements, besides being transmitted vertically during bacterial replication, are also capable of horizontal transfer by means of conjugation, transportation transformation, transduction, capsduction or retrotransposition. Under natural conditions, the basic trend is that genes carried by accessory elements constitute a particular sample of the bacterial genome that is required occasionally rather than continually. By contrast, genes needed for normal functions located in chromosomes are the 'housekeeping' stock that is indispensable [3]. However, there are a number of exceptions to these rules.

Transposition and other mobilisation actions can shuttle specific genes and sequences between chromosomes and plasmids as well as group different genes into a single replicon [1]. During this process, some additional genes can be transferred by a particular phenomenon known as 'DNA hitchhiking'. The generalisation of this event has led to the suggestion that all bacteria might share a pool of genetic information that is accessible to virtually every other bacterial cell. Therefore, the bacterial community could be envisioned as a single, heterogeneous multicellular organism, with elements continually moving from one group of cells to another. In this regard, the bacterial universe would be a superorganism with a network structure rather than single domain constituted by entities with a branching family organisation [4]. On the other hand, evolutionary biologists have maintained that genetic identity is a reality in the bacterial world as it is in the eukaryotic domain, and that gene flux among species has not been of major general significance as far as chromosomal genes are concerned [5–7]. Although horizontal gene flux is possible under laboratory and natural conditions [1,4,8,9], the patterns of relatedness determined for many different proteins and nucleic acid sequences have generated a robust and consistent genealogy. Phylogenetic trees have been derived independently from sequences extracted from different parts of the genome, such as those from ribosomal RNAs, chaperonins, cytochrome *c*, nitrogenase, elongation factor, ATPase synthase and glutamine synthase, among others [5–7,10–14]. Another argument against the superorganism hypothesis is the existence of bacteria without plasmids or, to be more precise, where plasmids and lysogenic phages have not been found (Fig. 1). Under certain local circumstances, horizontal gene flux between bacteria, although possible, seems an improbable natural event. The still unknown processes that have generated bacteria without plasmids are important not only for those interested in the evolution and population genetics of prokaryotes, but also for those devoted to the investigation of hostile bacteria and endosymbionts.

2. Local adaptations and plasmids

Among *Bacteria* a particular cluster of Gram-negatives known as *Proteobacteria* constitutes a phylogenetic cohort divided into different groups called the alpha, beta, gamma, delta and epsilon subclasses [6,7]. The neisseriae, enterobacteriae, and pseudomo-

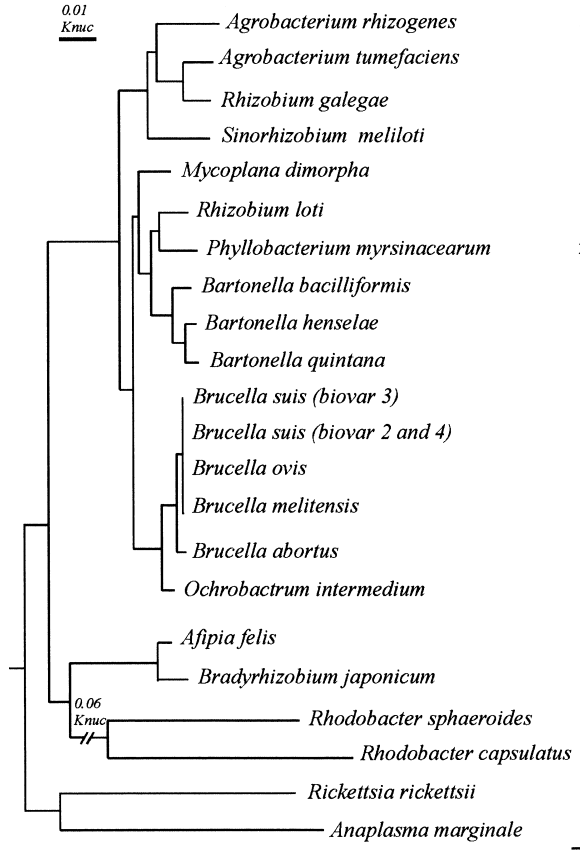
nas, as well as many other bacteria of medical and biological importance are located in the beta/gamma subclasses. The delta/epsilon branch harbours the sulphur and sulphate-reducing bacteria, the myxobacteria and the bdellovibrios. Within the alpha subclass are photoautotrophic and chemoautotrophic bacteria as well as species associated with plants and animals. Most of the members of the beta/gamma have been described as bacteria harbouring multiple copies of small to medium sized plasmids (20–250 kb) and one circular chromosome [3,15]. However, this is not straight forward since exceptions within the genus *Burkholdeira* (beta subclass) harbouring multiple replicons have been described [16]. Bacteria with more than one chromosome have been also described in spirochaete [17], microorganisms that do not belong to the class *Proteobacteria*. In contrast, several members of the alpha subclass harbour more than one essential replicon. Some of these additional megareplicons have a collection of 'housekeeping' genes and are non-curable and non-self-transmissible. Therefore, they have been designed as chromosomes (Fig. 1). Likewise, members of the alpha subclass have few medium sized or large plasmids (100–1400 kb) or no plasmids at all (Fig. 1). However, similar to their plasmid-bearing relatives, the genomes of some of these plasmid-free bacteria have been found to contain insertion sequences as accessory elements [18,19].

The Local Adaptation Hypothesis provides a framework for explaining the selective forces that generated phylogenetically related bacteria, some with plasmids and others without [20]. This hypothesis states that ''many of the characters that tend to occur on plasmids are adaptations to local variations in environmental conditions that occur only sporadically in time or space''. In some instances, such as local adaptations to antibiotics, which are generally restricted to the immediate vicinity of antibiotic-producing organisms like fungi and actinomycetes in soil, ''this kind of sporadic selection makes the maintenance of local adaptations more likely when genes are on plasmids than when they are on chromosomes''. This circumstance also applies to the production of virulent factors, medical usage of antibiotics, heavy metal resistance, inactivation of poisons or degradation of unusual substrates, among others.

The intracellular life of facultative intracellular bacteria of the gamma subclass (e.g. *Salmonella typhi, Shigella dysenteriae* and *Legionella pneumophila*) seems to be more of a transitional event than an obligatory state [21–24]. These bacteria, besides dealing with intracellular conditions, also have to deal extensively with a variable contaminated extracellular environment, such as the gut, manure, soil or water. Therefore, the presence of plasmids necessary to deal with local conditions seems to be an evolutionary advantage in this bacterial group. In these bacteria, plasmids coding for metal resistance, antibiotic resistance, defence mechanisms against other microbes or iron capture correlates well with the fact that the rapid adaptation of these microorganisms to different environments depends largely upon horizontal gene flux [20].

In the Bacterial domain, under certain conditions, intracellular life seems to take place through fast adaptation [21,25]. In other instances, fast adaptation to intracellular life may be followed by gradual adaptation. Many similarities in genes and gene clusters encoding virulence factors have been found in closely and distantly related bacteria. These genes are often located on plasmids or bacteriophages in one organism but in the chromosome in others [25]. Although a subset of plasmid coded virulence genes have been identified among several facultative intracellular animal pathogenic gamma and delta *Proteobacteria*, the distribution of chromosomal versus plasmid virulence genes is largely unbalanced in favour of the former [23,26–28]. In symbionts having part of their life cycle outside the host, such as the squid symbiont *Vibrio fischeri*, the symbiotic determinants of the light organ are encoded on the chromosome, and the plasmids found in this bacterial species carry genes that are important for survival outside of the symbiotic association [29]. Finally, among facultative intracellular Gram-positives, such as *Listeria monocytogenes* and *Mycobacterium tuberculosis*, the virulence genes implicated are located on the chromosome and not on a plasmid [23].

Characterisation of the protein export systems of Gram-negative bacteria has shown that animal and plant pathogens use remarkably similar machinery to deliver proteins into host cells [27,30]. The genes encoding type III secretion systems are conspicuous DNA segments within the bacterial chromosome which confer a variety of virulence traits, such as



	Plasmid size in kb	Major chromosome in kb	Minor chromosome in kb	G+C %	Preferred habitat and host	References
Agrobacterium rhizogenes	200-600	4000	2700[a]	62	Soil and pericellular pathogen of dicotyledones[b]	[40, 49, 57, 78]
Agrobacterium tumefaciens	200-500	3000	2100[c]	62	Soil and pericellular pathogen of dicotyledones[b]	[49, 50, 57, 147]
Rhizobium galegae	400-600	5850	1200[a]	63	Soil and intracellular symbiont of legumes	[49, 57, 151]
Sinorhizobium meliloti	500-140	3400	1700[d]	63	Soil and intracellular symbiont of legumes	[41, 49, 55, 57]
Mycoplana dimorpha	100-300	3200	none	64	Soil free living	[40, 57]
Rhizobium loti	400-600	5500	1200[a]	62	Soil and intracellular symbiont of legumes	[57, 109]
Phyllobacterium myrsinacearum	250-700[e]	5330	none	60	Soil and peri- and intracellular pathogen of sugar beets	[40, 50, 103]
Bartonella bacilliformis	none	1540	none	40	Peri- and intracellular pathogen of humans and sand flies	[94-96]
Bartonella henselae	none	1400	none	41	Intracellular pathogen of cats, humans and lice	[94, 95, 152, 153]
Bartonella quintana	none	1700	none	39	Intracellular pathogen of humans and lice	[40, 94, 95]
Brucella suis (biovar 3)	none	3100	none	57	Intracellular pathogen of suines	[97, 103, 111, 123]
Brucella suis (biovar 2 and 4)	none	1850	1350	57	Intracellular pathogen of suines and reindeer	[97, 103, 111, 123]
Brucella ovis	none	2100	1150	57	Intracellular pathogen of ovines	[97, 103, 111, 123]
Brucella melitensis	none	2100	1150	57	Intracellular pathogen of caprines	[97, 103, 123, 155]
Brucella abortus	none	2100	1150	57	Intracellular pathogen of bovines	[97, 103, 111, 123]
Ochrobactrum intermedium	50-200	2700	1900	59	Soil and opportunistic of humans and nematodes	[40, 103, 104]
Afipia felis	40-100	Unknown	Unknown	63	Opportunistic of cats and humans	[77, 82]
Bradyrhizobium japonicum	200-800[e]	8700	none	65	Soil and intracellular symbiont of legumes	[47-49, 57, 65]
Rhodobacter sphaeroides	40-200	3046	914	65	Water free living photoheterotroph	[42, 43, 45, 46]
Rhodobacter capsulatus	100-200	3800	none	66	Water free living photoheterotroph	[40, 42, 112, 154]
Rickettsia rickettsii	none	1300	none	33	Intracellular pathogen of rodents and ticks	[75, 98, 99,156, 157]
Anaplasma marginale	none	1200	none	51	Intracellular pathogen of rodents and ticks	[73, 158]

the ability to acquire iron, to adhere, to invade and to replicate within eukaryotic cells. Many of these type III secretion genes appear in phylogenetically unrelated bacteria, are located in a variety of genomic domains, display protein identities seldom higher than 45%, and often show G+C contents significantly different from the remaining chromosomal DNA [31,32]. These data suggests that these virulent sequences are transmitted horizontally as 'pathogenicity islands' from plasmids or bacteriophages to chromosomes. The ability to obtain complex virulence traits in one event, rather than by undergoing natural selection for many generations, provides a mechanism for sudden radical changes in bacterial host interactions, leading to a fast adaptation mechanisms for parasitism [24]. It seems that many of the genes necessary for bacterial intracellular survival become permanent chromosomal genes. Over time, these foreign sequences have the tendency to ameliorate and to reflect the overall DNA composition of the primordial chromosome [33]. In conclusion, it seems that all the above-mentioned bacteria rely mainly on chromosomal housekeeping genes for invasion, survival and replication within animal cells and that plasmid coded virulent genes are complementary and dispensable depending upon the local conditions surrounding these parasites.

Obligate intracellular *Coxiella burnettii*, phylogenetically related to *Legionella* spp. (gamma subclass), possesses a small genome and what is believed to be a cryptic plasmid encoding housekeeping genes which may be essential for virulence [34]. However, there are plasmidless *C. burnettii* strains that contain chromosomally integrated plasmid-homologous DNA sequences, also encoding for essential housekeeping information [35]. Since plasmid sequences have been conserved in all *Coxiella* strains examined, even in plasmidless isolates, it is feasible to propose that the cryptic plasmids may carry essential functions that benefit their bacterial hosts. In this respect, the so-called 'cryptic plasmids' found in strict intracellular bacteria or endosymbionts may be, in fact, chromosomes. Similarly, the anthranilate synthase (*trp*EG) genes found in 'plasmids' of some bacterial endosymbionts (gamma subclass) of aphids seem to have originated from chromosomal genes of bacteria endosymbionts of the same lineage [36]. The anthranilate synthase (TrpEG) is the first, as well as the rate-limiting enzyme in the tryptophan biosynthetic pathway. The amplification of TrpEG on plasmids may result in an increase of this essential amino acid, which is required by the aphid host. A similar phenomenon has been described on plasmid genes involved in leucine biosynthesis in these aphid bacterial symbionts [37]. Congruence of phylogenetic trees based on sequences from aphid mitochondrial and bacterial genes [37,38], supports the exclusively vertical transmission of the endosymbionts within aphid lineages, limiting in this manner, the exchange of 'plasmids' among new lineages of bacterial endosymbionts of different species of aphids. Therefore, it seems that the plasmids-like bacterial genomes of the aphids' endosymbionts are elements containing housekeeping genes [39] and in this sense they may be considered as permanent replicons. The reason for not recognising these elements as chromosomes lays mainly in the fact that they do not confer an obvious phenotype or do not possess characteristic chromosomal markers, such as ribosomal RNA genes or heat shock proteins [40]. The definition of a plasmid in many cases may simply rest in the uncritical acceptance that smaller additional genomic structures are dispensable elements, when in reality they could be essential replicons which must be defined as chromosomes [41].

The genomic differences among the phototrophic, the plant-associated, the opportunistic and the animal pathogenic *Proteobacteria* of the alpha subclass are consistent with their local adaptations. The metabolic versatility of photosynthetic bacteria, such as

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Fig. 1. Phylogenetic tree showing the relationship among representative plant-associated, free living, opportunistic and animal cell-associated *Proteobacteria* of the alpha subclass. The genomic characteristics, and the preferred host(s) for each bacterium are indicated in the table. The tree was constructed by the neighbour-joining method [159] from Knuc values derived from 16S rDNA sequences available in EMBL, GenBank and DDBJ Nucleotide Sequence Database. The presence of essential genes has not been confirmed (a); may behave as animal-opportunistic bacteria (b); linear chromosome (c); non-curable essential replicon (d) megaplasmid may be a chromosome (e). (References cited in figure, but not in text: [150–158].)

Rhodobacter species enables them to grow heterotrophically or autotrophically, both in the dark or in the light and either in the presence or absence of oxygen. Moreover, these bacteria perform nitrogen fixation under all these growth conditions [42]. In contrast to *Rhizobium* spp., the nitrogen fixation genes of phototrophic bacteria are chromosomal [43–45]. Phototrophs conserve their plasmids, because several genes carried by them provide selective advantages for local growth in the open environment, such as soil, water, manure, gut systems and external surfaces of plants and animals [42,43,45,46]. A similar event is observed with the bacteriochlorophyll *a* containing *Bradyrhizobium* spp. and the nodule forming *B. japonicum* spp., whose nodulating and nitrogen fixation genes are located in the chromosome rather than in the plasmids [47,48]. Therefore, it is anticipated that free living phototrophic bacteria population subjected to constant changes would benefit from the horizontal transfer of genes coding for different local adaptations [20].

Members of the genera *Agrobacterium*, *Rhizobium*, *Sinorhizobium* and *Phyllobacterium* have been defined by their ability to induce plant tumours or root nodulation [49,50]. However, many non-tumorigenic agrobacteria and non-nodulating rhizobiae, possessing plasmids, have been isolated from soil [51–54]. In agrobacteria and rhizobiae, the genes responsible for neoplastic transformation (*onc*, and *vir*) are present in the *Ti* and *Ri* plasmids and those responsible for root nodules (*nod*) and nitrogen fixation (*fix* and *nif*) are in the *Sym* megaplasmids, respectively [49,50,55]. The phylogeny of these plant-associated bacteria, based on ribosomal DNA sequences and supported by many genotypic and phenotypic characteristics [14,56,57], shows that the species of these genera are intertwined with each other as well as with opportunistic bacteria and animal pathogens (Fig. 1). Since *Sym*, *Ti* or *Ri* sequences are also intermixed with the different bacterial species, it is possible that horizontal flux of these genes among the plant-associated bacteria has occurred several times during evolution [58]. Also, it is probable that many of these genes located in plasmids or chromosomes have a common origin. This is further supported by the fact that *Sym*, *Ti* and *Ri* plasmids share sequences among themselves as well as with genes located in the chromosomes [47,53,59–62].

Similar to their phototrophic counterparts, the constant changes of the plant-associated bacteria from soil to plants would exert selective forces on the versatility of the genome, which would be better achieved by non-essential genes carried in plasmids than in chromosomes [20]. Selection directed towards the maintenance of synchrony between genes in the resident plasmids and the host chromosome, is expected to be necessary to achieve complex functions, such as replication, tumour induction or bacteroid formation, among others. Support for these ideas is based on: (1) the preferential associations between resident plasmids and bacterial hosts in particular soil populations [52,63]; (2) the different abilities of various plasmid bacteria strains to survive in different soil and plant types [51,52,66]; and (3) the occurrence of homologous chromosomal genes (e.g. *chv*A and *chv*B homologous to *A. tumefaciens* genes) for the early phases during plant colonisation in the different plant-associated bacteria [50]. Two of the remarkable characteristics of plant-associated bacteria are their high genetic diversity found world-wide and their clonal population structure encountered in specific ecosystems [52,56,64,65].

Within the plant-associated alpha *Proteobacteria* there are some genes, such as those required for tumour induction, nodulation or nitrogen fixation, that are non-essential for subsistence in soil. The absence of one of these genes is commensurate with the absence of the specific function, but not with survival, as is demonstrated by the existence of agrobacteria and rhizobiae devoid of *Ti*, *Ri* or *Sym* plasmids [50,51,54,66]. In contrast, the movement of housekeeping genes from the chromosome to a plasmid is expected to have deleterious consequences if the function of the gene is not correctly expressed in the recombinant plasmid or if the plasmid is segregated from the bacterium [44,67,68]. However, if the basic need originally supplied by the lost housekeeping gene is provided by the local environment, then the bacteria would survive and clonally expand [69,70]. The price paid for survival would be the restriction of the emerging bacterial clone to its new 'nursing' milieu. The dynamics of these events provide a framework for explaining the generation of animal

alpha *Proteobacteria* without plasmids and with reduced genomes.

3. Plasmid-free alpha *Proteobacteria*

Although under suitable conditions some of the animal pathogens of the alpha subclass can survive in open environments, there is no evidence that these organisms replicate to a significant extent in soil, water or manure. Thus, these bacteria behave essentially as strict parasites and rely for their survival, reproduction and persistence in nature on transmission between animal hosts and vectors. For instance, *Brucella* spp. are naturally transmitted between vertebrate hosts, whereas *Bartonella*, *Ehrlichia*, *Rickettsia* and *Anaplasma* organisms are naturally transmitted from invertebrates to vertebrates and seldom from soil or inert sources to animals [71–75]. This picture differs from that which occurs in their plasmid-containing human opportunistic (*Ochrobactrum*, *Afipia* and *Agrobacterium* species) and plant-associated (*Rhizobium*, *Sinorhizobium*, *Phyllobacterium*, *Bradyrhizobium* and *Agrobacterium* species) relatives, which constantly shift from external environments to their immunocompromised or plant hosts, respectively [49,52,76–78]. Under natural circumstances, the animal pathogenic alpha *Proteobacteria* do not have to confront adverse local conditions found in external surroundings, such as antibiotics, heavy metals, bacterial competition and rapid environmental changes. Therefore, the advantage of plasmids carrying genes to cope with these conditions would be trivial. Alternatively, the host defence mechanisms are one of the major selective forces operating to shape bacterial pathogens [79]. In consequence, it could be argued that plasmids carrying genes (e.g. for adhesion/invasion and antiphagocytic proteins) necessary to deal with variable local conditions, such as the host immune response would be maintained by positive selection [20]. At first glance, this statement seems to contradict the Local Adaptation Hypothesis, which explains the existence of animal parasitic alpha *Proteobacteria* devoid of plasmids. Consequently, a further explanation is required.

The immune response is itself flexible and tends to eliminate or restrict pathogen replication. Therefore, successful bacterial invaders must be able to avoid or adapt to evolving host defences. The escape mechanisms of pathogenic bacteria can be attributed to three general principles: evasion of cytotoxic activities, modulation of the immune response and survival within cells [23,79,80]. Among these, intracellular survival confronts the bacteria with two phenomena: on one hand, the cellular milieu that protects the bacteria against intermittent variable defences, such as antibodies and complement; on the other hand, the cellular digestive mechanisms activated to destroy the invader. Cell associated microorganisms are capable of evading the digestive mechanisms of cells by diverse strategies [34,81]. For instance, among the alpha *Proteobacteria*, *Brucella abortus*, *Bartonella henselae* and *Afipia felis* downregulate the digestive mechanisms of phagocytic cells [82–85]; *Bartonella bacilliformis*, *Bartonella talpae*, *Bartonella henselae* and *Anaplasma marginale* elude digestion by invading erythrocytes [72,73,86]; whereas *Rickettsia* species escape from the phagocytic vacuole to the cytoplasmic milieu [75,87,88]. In all these cases, even after bacterial invasion, the eukaryotic cellular environment is relatively constant concerning temperature, acidity, oxygen tension, availability of substrates and presence of bactericidal molecules. Moreover, the physiology of the invaders must match that of their hosts, in that the bacteria must be able to salvage the metabolites they need [87,88]. Since under natural conditions several of the obligate animal pathogens of the alpha subclass do not harbour plasmids or lysogenic phages, it is in these bacteria that the housekeeping genes are directly responsible for dealing with the complementary functions for transmission and parasitism. In this case, the vertical chromosomal heritage of such characters is favoured over horizontal gene flux.

The absence of plasmids in the obligate animal cell-associated alpha *Proteobacteria* is not caused by the impossibility of harbouring them. For instance, the experimental inclusion of transposons and plasmids has been achieved in *Brucella*, *Bartonella* and *Rickettsia* organisms, although with lower efficiency than with other bacteria [89–92]. However, as mentioned above, without positive selection imposed by external or immune local forces, the plasmid-bearing cells would be at a slight disadvantage (in comparison with plasmid-free bacteria which would reproduce more rapidly). It is known that

unpaired segregation and loss of plasmids are likely to occur when plasmid carriage reduces bacterial fitness in the absence of selection for specific functions [68]. The periodic invasion by parasitic bacteria of higher fitness (arising from one or very few individuals without transmissible plasmids) would clonally expand. Horizontal transmission of the bacteria to a naive host would generate a founder effect and the diversity of the 'new parasite' would be restricted to chromosomal changes [93]. Insertion sequences and retrons could remain as the only mobile elements participating in the diversity of these bacteria [18].

Direct and indirect evidence supporting these ideas has been derived from microbiological and epidemiological studies, as follows: (1) no plasmids or temperate bacteriophages have been detected in any animal cell-associated *Proteobacteria* of the alpha subclass in spite of numerous attempts to find them in some of the strains or during chromosomal studies; (2) the metabolic and antibiotic susceptibility patterns are maintained constant throughout space and time [71,72,89–91,94–99]. Moreover, (3) as a rule the animal pathogens are isolated as pure cultures, indicating that these bacteria are in less intimate contact with other species of bacteria; (4) infections by the animal pathogens are initiated by a few bacteria that are clonally expanded in the host; (5) multiple infections by different biovars seldom occur in the same host; (6) striking similarity among isolates of the same species obtained from different localities during different periods of time has been demonstrated; and (7) bacteria diversity (e.g. number of species) is commensurate with host and vector diversity [18,34,71–75,80,95,98–102].

Human opportunistic *Ochrobactrum*, *Afipia* and *Agrobacterium* alpha *Proteobacteria* species harbour plasmids [40,49,50], which are probably needed to deal with the external surroundings which they commonly confront rather than for parasitic functions. This is suggested by the broad antibiotic resistance expressed by these organisms under different circumstances [76–78,103,104] and by the absence of plasmids in some strains isolated from immunocompromised hosts [77]. The intermixed phylogeny of opportunistic and primary animal pathogens within the alpha *Proteobacteria*, besides demonstrating the coexistence of a great diversity of metabolisms among closely related microorganisms, also reveals the existence of common motives (e.g. shared membrane features) for adaptation to eukaryotic cellular environments [83,105]. Although the capacity to invade may be a prerequisite retained by the opportunistic bacteria, it is clear that pathogenicity requires more than just an organism with the potential to do harm. Manifestations of harmfulness demand a singular host unable to respond in the normal fashion to microbial activities at specific times and in particular environments. Therefore, both the host and the circumstances for future transmissions are the major determinants for the outcome of the clinical manifestations in infectious diseases and reveal the selective forces that have shaped the evolutionary pathways followed by the new invaders. The presence of plasmids in most of the opportunistic bacteria, their absence in some of the opportunistic strains and their absence in the animal pathogenic close relatives, may illustrate only one of these transitional events that have moulded the bacterial genome of the alpha *Proteobacteria*.

4. Small and large genomes

In contrast to their plasmid-containing counterparts, the animal pathogenic *Proteobacteria* of the alpha subclass have narrower metabolic alternatives for the utilisation of carbon components, a reduced number of metabolic pathways, slower generation time and more restricted temperature range for growth [71–73,75,94,95,97]. These characteristics correlate well with the smaller total genome size (1200–3200 kb contained in one or two chromosomes) of the animal pathogenic bacteria, in relation to the larger genomes (3800–9500 kb contained in one or more chromosomes plus plasmids) of free living, opportunistic, and plant-associated bacteria. Another important difference between these two groups is the lower percentage of guanine plus cytosine (G+C) in the genomes of the animal pathogenic bacteria compared to the higher content in the free and plant living bacteria (Fig. 1). With these organisms at least, autotrophy seems to correlate well with higher G+C, and heterotrophy correlates with lower G+C content. For instance, the photoautotrophic *Rhodobacter* and *Bradyrhizobium* species contain the highest G+C proportion, whereas the most het-

erotrophic non-cultivable *Rickettsia* organisms contain the lowest G+C percentage among the alpha subclass members. The comparison of nucleotide sequences in bacteria reveals that codon composition correlates well with overall genomic composition and that differences in codon arrangement occur to a greater extent in the third base position [3,106], a trend associated with the redundancy of the genetic code. Thus, variations in the G+C content observed between phylogenetically close bacteria could result in differences in the inherent mutational pressure and probably also in the structure of the translational machinery [106]. In this respect, the lower G+C content of the animal cell-associated *Proteobacteria* of the alpha subclass is not due to a remote phylogeny with their photoautotrophic and chemoautotrophic relatives, but rather to their atypical mode of evolution. The analysis of several systems with low genomic G+C content (including some members of the alpha subclass, such as mitochondria) has revealed non-universal codon usage [106]. However, this has not been investigated in *Bartonella*, *Ehrlichia* or *Rickettsia* species, all bacteria with low G+C content.

In general terms, for a given bacterial genome, the G+C content of the ribosomal RNA genes is biased in the same direction as the entire genome [7,106]. However, in several intracellular alpha *Proteobacteria* and in endosymbionts (for rRNA/rDNA sequences and accession numbers see Fig. 1 and [37]), the proportion of G+C in rRNA genes may show dramatic deviation from the value found in the total bacterial genome. The reason for this discrepancy can be understood as the consequence of the selective constrains that have been exerted to eliminate alternative chromosomal sequences in these bacteria in contrast to rDNA genes. Since most parts of the rDNA genes are important for biological function and their transcripts are non-translatable, they are subjected to less variation than other genes. This is one of the reasons why rDNA genes are good molecular chronometers [6,7].

The selecting forces that persisted to fix these events during the evolution of small genomes are unknown. The evolutionary history of pathogens is expected to be host determined [5]. In this respect, the relationship of cell associated bacteria with reservoirs and victims lead to complex selection pressures that have moulded the genome of pathogens and symbionts [100]. Most evolutionists believe that selection favours reproductive success. When tested in vitro, the animal pathogenic bacteria have a slower generation time than their opportunistic, free living or plant-associated relatives. This characteristic which correlates to the number of *rrn* operons in the genome (see below), may be the consequence of the heterotrophic metabolism required for growth rather than the time needed for replicating the smaller genome of these bacteria. However, once installed in the host, the intracellular pathogens can reproduce successfully, persist and reach high densities; in this manner, the invader bacteria compensate for their slower generation time [21,49,71–75,94]. Therefore, the reproductive success of these bacteria inside host cells seemed to correspond with a reduction in genome size and heterotrophic metabolism. It is reasonable to propose that the smaller genome of pathogenic alpha *Proteobacteria* evolved as a consequence of fine metabolic adjustments that allowed the bacteria to extract cellular 'ready-made' substrates available for the generation of energy and anabolic reactions [87,88]. A life cycle in association with animal cells would render unnecessary many of the genes needed to deal with an open environment or for interaction with plant cells. For instance, putative genes highly similar to *S. meliloti* and *B. bacilliformis* flagellin are present in non-motile *Brucella* spp. (GenBank accession number AFO 19251). Over time, without positive selection, non-essential genes tend to disappear, favouring in this manner, smaller genomes. As result of reduction and gene rearrangements, highly derived genomes with respect to the ancestral bacteria may result during evolution of intracellular bacteria [107,108]. These arguments could also apply to other cell associated bacteria possessing small genomes such as chlamydiae, coxiellae, mycoplasmae and endosymbionts [3,34,39].

Bradhyrhizobium spp. genomes represent a different perspective within the alpha subclass of *Proteobacteria* [47,48,109]. *B. japonicum* contains a very large circular chromosome (8700 kb) as well as plasmids (from 200 to 800 kb). The chromosome of this bacterium is larger than the sum of the three megareplicons of S. *meliloti* (6500 kb). In addition to housekeeping genes, the *B. japonicum* chromosome possesses all the genes required for nodulation, bac-

teroid formation and nitrogen fixation. Genes on *B. japonicum* plasmids are not devoted to nodulation and nitrogen fixation functions, although it is possible that they are involved in the adaptations of this bacterium to local soil conditions. Likewise, there are laboratory type strains without plasmids that successfully nodulate and fix nitrogen [47]. As with other rhizobiae, *Bradyrhizobium* species are known to alternate their life cycle between soil and plant roots, and to be exposed to similar environmental. However, *B. japonicum* strains differ from their faster growing rhizobiae relatives in their extensive metabolic diversity, ample capabilities to cope with different oxygen concentrations, host ranges, broad resistance to antibiotics, preference for acidic soil and slower growth [49]. Although the slower generation time of the chemoautotrophic or photoautotrophic *Bradyrhizobium* spp. (in comparison to *Rhizobium* spp.) may be related to the time needed to replicate the larger genome of these bacteria rather than to their synthetic metabolism, the true is that there is not strict correlation between genome size and generation time. For instance, *B. japonicum* (genome of >9000 kb), *B. henselae* (genome of 1400 kb) and *R. rickettsii* (genome of 1300 kb) are slow growers. On the contrary, it appears that there is a negative correlation between generation time and *rrn* copy number. For instance, *rrn* loci are commonly repeated up to ten times within genomes of fast replicating bacteria, while in slow growers, such as *Rickettsia*, *Mycoplasma*, pathogenic *Mycobacterium* and *Bradyrizobium* species, there is only one *rrn* locus [15,108,110]. In conclusion, it appears that bacteria with higher copy of rRNA genes tend to grow faster than bacteria with a single copy. Unfortunately the reasoning for explaining this phenomenon is at the moment speculative [110].

The evolution of the *B. japonicum* chromosome could have taken various possible routes. It may be that the chromosome increased in size through the duplication of the entire genome [109]. Following this, the irreversible integration of large plasmids into the chromosome and the exclusion of photosynthetic information could have proceeded. Data favouring chromosome duplication include the occurrence of several cytochrome oxidases, heat shock proteins, cytochromes, and various genes for growth that, besides being dispersed around the chromosome, are located on opposite sides of the circular map, as expected after chromosome duplication [47]. The location of *nif* and *nod* clustered in a section of about 400 kb argues in favour of a later integration of a symbiotic plasmid that lost its former condition. The position and orientation of highly conserved chromosomal genes and integrase genes derived from accessory elements such as plasmids or bacteriophages may favour fusion and excision events among genes and chromosomes as well as between large replicons in alpha *Proteobacteria* [25,47,111, 112]. Finally, the close phylogeny of *B. japonicum* with the bacteriochlorophyll *a* containing *Bradhyrhizobium* species [65], supports the idea that essential genetic information needed to conduct photosynthesis was lost in many *Bradyrhizobium* strains. The rational for this is that photosynthesis is too complex to be acquired independently at different branching points during bacterial evolution [5–7]. Moreover, the mobilisation of chromosomal photosynthetic genes by plasmids has been shown in phototrophic bacteria [44].

In conclusion, it seems that the smaller genomes of the animal cell-associated bacteria evolved through subsequent reductions of the larger genomes of opportunistic alpha *Proteobacteria*. This genome reduction may have occurred, among several alternatives, by loss of accessory plasmids, plasmid sequences and chromosome rearrangements (e.g. mediated by recombination of homologous sequences, followed by truncation or mobilisation of genes). During this event, the genomes of the animal cell-associated alpha *Proteobacteria* became rich in adenosine and thymidine as compared with the genomes of their ancestors. For a heterotrophic animal cell-associated bacterium, the eukaryotic cellular environment seems to be an alternative for supplying metabolic substrates which are not codified by the bacterial chromosome. On the contrary, the extreme and diluted conditions prevailing in terrestrial and aquatic environments are expected to select more flexible and larger genomes as occurs in the free-living, plant-associated and opportunistic bacteria. Since all these bacteria are phylogenetically intertwined (Fig. 1), it thus appears that these trends proceeded independently with each lineage.

5. Bacteria with more than one chromosome

One of the most striking features found in some members of the alpha subclass is the presence of more than one different chromosome (Fig. 1) a phenomenon that raises several questions [3,40,41]. Bacteria possessing one chromosome (with and without plasmids) are phylogenetically entwined with bacteria bearing two chromosomes (with and without plasmids), as well as with bacteria containing permanent megaplasmids. For instance, *R. sphaeroides* contains two chromosomes and close to five plasmids, while *R. capsulatus* and other closely related phototrophs contain only one chromosome plus plasmids. Similarly, *A. tumefaciens*, *S. meliloti*, most *Rhizobium* species, *Ochrobactrum intermedium* and most *Brucella* species all harbour two chromosomes, while *Phyllobacterium* spp., *Mycoplana dimorpha*, *Bartonella* spp., and *Rickettsia* spp., their close relatives, carry only one chromosome.

The generation of bacteria with more than one different chromosomes could have arisen through chromosomal excision into different replicons, unequal chromosomal division, independent mutation of multicopy chromosomes, horizontal transfer, or transformation of a plasmid into a chromosome, among several alternatives. Once the two chromosomes were present in the bacterium, the differentiation of each one could have been continued independently. Based on recent evidence, the option of plasmid transformation into a second chromosome through the acquisition of housekeeping genes from the parent chromosome, deserves attention [3,41]. This hypothesis is supported by the coexistence of chromosomes and non-curable essential megareplicons in *Rhizobium* and *Sinorhizobium* species, and by features of both plasmid and chromosomal replication origins in the *Sym* plasmid [113]. Moreover, replication without the incompatibility problems of *Sym* and *Ti* megaplasmids in several members of the Rhizobiaceae suggests similar segregational systems [50,52,113]. Finally, the *A. tumefaciens* arginase and ornithine cyclodeaminase genes, present in the virulence *Ti* plasmid necessary for inducing tumours in plants, display high similarity with homologous genes located in the same operon and are in the same order in one of the *Brucella* chromosomes [59]. These *Brucella* chromosome genes present in a single copy are regulated in a similar fashion to the plant pathogen plasmid genes. Therefore, it seems plausible to hypothesise that the second *Brucella* chromosome derived from ancestral megaplasmids containing these sequences rather than excision of a larger chromosome into two megareplicons as proposed by some investigators [111]. Further genomic derivations are suggested by the coexistence of plasmids together with two chromosomes as shown in *R. sphaeroides*, *O. intermedium* chromosome *A. tumefaciens* (possessing one circular and one linear), and by the existence of plasmid free bacteria harbouring one or two chromosomes as in *Brucella* members. This last genus deserves special attention since it has been found that within the same species, there are strains with two chromosomes and others with one.

Up to now, the genus *Brucella* has comprised six species [71,97], despite the fact that the genus is considered monophyletic [92]. For instance, the phylogenetic analysis based on conserved molecules such as 16S rRNA similarity and DNA relatedness values indicate figures above 99 and 98%, respectively (Fig. 1 and [92]). The reason for maintaining the concept of separated *Brucella* species is not only because they may be differentiated on the basis of DNA restriction patterns [114–118], phylogenetic analysis of idiosyncratic sequences [116], as well as of phenotypic, chemotypic and antigenic characteristics [83,103], but because of the distinct biological behaviour of each of the species [71,97]. All studied *Brucella* species, except some strains of *B. suis*, possess two chromosomes of about 2.1 and 1.15 Mb [111]. Among the exceptions, one strain of *B. suis* (biovar 2) harbours two chromosomes of 1.85 and 1.35 Mb and another strain, *B. suis* (biovar 3), carries only one chromosome with a size of 3.1 Mb. The differences in chromosome size and number have been explained by rearrangements at the chromosomal regions by recombination at homologous sites [111] and by small insertions and deletions ranging from 1 to 34 kb [118]. Two contrasting explanations for the generation of heterogeneity in chromosomal number within this monophyletic genus can be proposed: one suggests that the two chromosome-containing strains emerged by homologous recombination at *rrn* sites followed by excision of the megareplicon from an hypothetical *Brucella* ancestor with one single chromosome [111]; the other, that the recombination of

the two chromosomes at homologous sites (e.g. *rrn* genes) could have resulted in a generation of strains (e.g. *B. suis* biovar 3) with only one chromosome. Although the former alternative has been favoured by the authors of this important finding [111], phylogenetic evidence supports the hypothesis of a *Brucella* ancestor with two chromosomes. For instance, dendrograms constructed on the basis of phenic characters [83,97,103,104] and phylogenetic trees constructed by comparing 16S rRNA/rDNA sequences (Fig. 1, [57,103]) suggest that the *B. suis* strains are not located in the deep branches of the dendrograms, but rather clustered together with *B. canis*, *B. melitensis* and *B. abortus* in the more neoteric branches. Since *B. ovis* and *B. neotomae*, lying in the deepest branches of the tree, possess two chromosomes, it is feasible to propose that the *Brucella* ancestor also possessed two chromosomes. Moreover, *O. intermedium*, the closest relative of *Brucella* spp. (Fig. 1), also possesses two chromosomes and plasmids [40] suggesting that the ancestor of these two genera exhibited two chromosomes and plasmids.

The effective number of megareplicons per cell in alpha *Proteobacteria* is not known. Equally unknown is the way in which two or more different chromosomes co-ordinate their replication and how they segregate properly during cell division. When several different megareplicons per cell are present there must exist at least one different replication origin for each megareplicon. If segregation occurs randomly, one of the two daughters may not receive the different megareplicons, resulting in non-viable bacteria and in very inefficient division. As pointed out previously, in eukaryotes, effective division is achieved by means of the mitotic apparatus. Evidence for the existence of a dynamic mitotic-like apparatus responsible for equal chromosome segregation during bacterial cell division has been suggested in several bacteria [119–121]. In *E. coli*, the biochemical properties of the FtsZ and FtsA proteins suggest that they are similar to the eukaryotic tubulin and actin, respectively [119]. In the case of multichromosomal bacteria, it seems plausible that co-ordinated segregation mediated by a mitotic-like apparatus may occur rather than the random segregation of chromosomes [113].

I have proposed in previous paragraphs that the selective forces working for maintenance or exclusion of plasmids in the cell-associated bacteria of the alpha subclass, depend upon local conditions which favour or restrict the horizontal transference of genes. Similarly, the selective forces working for the maintenance of large genomes or reduction of the same depend upon local conditions which may favour the utilisation of a diverse set of substrates in open environments or the availability of already made molecules within the more restricted cellular milieu. More difficult is the question concerning the evolutionary advantages that possessing more than one chromosome provides to some bacteria lineages. The need for a larger genome size cannot explain the presence of more than one chromosome, since the *Bradyrhizobium* species possess single circular chromosomes close to 9000 kb in addition to plasmids. Analysis of the 16S rDNA phylogenetic tree of the cell associated alpha *Proteobacteria* (Fig. 1) clearly indicates that closely related bacteria, displaying very similar biological adaptations and functions, may display one or two chromosomes. Furthermore, the fact that within the same bacterial genus or species some display two different chromosomes while others exhibit only one chromosome, strongly suggests that there is not a sine qua non evolutionary advantage in having two chromosomes or only one chromosome comprising the information of both megareplicons. It seems that the true evolutionary advantage is to engrave important genes in replicons, which then become essential and make the transition from non-permanent elements to a housekeeping genetic structures. Some of these genes may be fixed in chromosomes while others in alternative replicons. However, non-curable replicons carrying essential genes deserve the designation of secondary chromosomes [3,41]. Since segregation of chromosomes and non-essential replicons was necessarily pre-established by a mitotic-like apparatus in the ancestor bacteria, there is no need to engineer a new mechanism for separating the new generated chromosomes. Subsequent changes in genome size may proceed independently in each chromosome, according to the selective forces working in particular local conditions. Succeeding fusions between megareplicons may be the consequence of recombination at homologous sites, without obvious deleterious expression of the essential genetic information, as demonstrated by the different chromosome number and sizes with-

in species of the monophyletic *Brucella* genus and *Rhodobacter* species. As previously stated, infections by the animal pathogens are initiated by a few bacteria that are clonally expanded in the host. Therefore, as long as the essential genetic information is correctly expressed, the fixation of different genomic alternatives within very similar bacterial strains, such as *Brucella* organisms, may be the mere result of contingency during the infection processes. In this respect, there is no need for explaining a specific selective force working for the maintenance of either chromosomal type. In conclusion, it seems feasible to propose that the common ancestor of all the extant species of alpha *Proteobacteria* shown in Fig. 1 had one circular large chromosome, megaplasmids and small- to medium-sized plasmids. The transition to bacteria having small genome that resulted from reduction in the number of genes (e.g. *Rickettsiae*, *Anaplama* and *Bartonella*), having more than one chromosome (e.g. *A. tumefaciens, S. meliloti, O. intermedium, B. abortus* and *R. sphaeroides*), linearisation of one chromosome (e.g. *A. tumefaciens*) or possessing a single chromosome that originated from the fusion between different megareplicons (as suggested in *B. suis* biotype 3, *B. japonicum* and *R. capsulatus*), occurred independently at different branches during evolution.

6. Clonal and reticulate genomic bacterial populations

A major problem in understanding the processes occurring during genome evolution is knowing whether the bacteria are clonal or reticulate evolutionary units. In the first case, the inherence is vertically transmitted as a result of binary fission and clonal expansion. This may be the case of some animal cell associated bacteria and endosymbionts in which recombination appears to be precluded or exceptional. In the second case, adaptive changes occurring within an individual can be horizontally transferred to many or all members of the group. This seems to be the condition of many intestinal and plant-associated bacteria. Genetic drift and speciation in clonal bacteria will depend almost exclusively on mutation and internal genetic rearrangement processes, whereas speciation in reticulate bacteria will depend not only on these processes, but also on their genetic interactions with other bacterial strains.

For some time it was believed that recombination among bacteria occurred at such low frequency, relative to mutation, that it was considered evolutionary insignificant. However, recently it has been demonstrated that recombination is the dominant force driving the clonal divergence of some enterobacterial species [122], thus it must be considered a significant factor in the structuring of bacterial populations capable of horizontal gene transfer. Among enterobacterial species, recombination seems to take place more often between closely related bacteria than across species. The genetic relationships between closely related individuals could be regarded as tokogenetic [123], in contrast to the phylogenetic links that interconnect the different species. Tokogenetic recombinations are expected to homogenise the gene pool among the interacting organisms, thereby, restricting the network structure of the species to a limited level. In the case of some *E. coli* strains, the divergence from a common ancestor was traced as recently as 2400 years ago [122]. In comparison, recombination between different species [1] may result in genetic diversification promoting not only the expansion of the network structure of the species, but also favouring a fast and severe genetic drift which may eventually cause speciation [124] in the manner proposed by the punctuated equilibrium hypothesis [125]. A similar recombinational phenomenon occurred between chromosomes and plasmids and between homologous sequences within chromosomes [47,53,59–62,112] may explain the difficulties in recognising distinct genus and species among certain groups of soil and plant-associated bacteria, such as *Agrobacterium*, *Phyllobacterium*, *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium* and *Rhodobacter* species [126,127].

Another important example that may sustain the network structure hypothesis of some species is the horizontal transfer of protein genes between soil/plant bacteria. It has been found that a phylogenetic tree constructed on the basis of different protein genes has unexpected assemblage with respect to phylogenetic trees constructed on the basis of ribosomal genes [11,15,128]. The incongruity seen between the various protein gene trees and the rRNA trees suggests cases for horizontal gene transfer be-

tween these parties. Finally, several studies have demonstrated discrepancies between phylogenetic trees based on 16S rRNA and dendrograms based on numerical taxonomy [64,126,128–130]. These findings support the notion that within this group of bacteria, the horizontal transfer of genes, rather than convergence or parallel evolution, has influenced the evolution of the various species [124].

As mentioned previously, there are a considerable number of intracellular bacteria such as *Brucella*, *Bartonella*, *Rickettsia*, and *Anaplasma* species as well as some endosymbionts without plasmids which must be contemplated within the context of clonality rather than within a reticulate structure. Since clonality is maintained by the vertical, asexual transmission of genetic material from parent to offspring, all evolutionary change through time must be considered the result of a mutational process or the autogenous rearrangement of genes mediated by insertion sequences, retrons or transposons or homologous recombinations [107,111,131]. The relative contribution of mutation and autogenous recombination to divergence is not known, mainly due to problems associated with the collection of accurate figures during internal recombinational processes. However, in some plasmid-free bacteria, such as *Brucella* spp., with an upper limit DNA–DNA reassociation values, the relative contribution between these two processes could be estimated by comparing the homologous gene sequences in two different species with the position of the genes in the chromosomes. This approach has been successfully applied to different isolates of *R. capsulatus,* revealing a significant genome mosaic structure among them [112]. Differences in the number and location of insertion sequences, chromosome size and chromosome number among different *Brucella* species [18,118] suggest that autogenous recombination in addition to mutation could be a driving force of genome variation in bacteria devoid of heterologous recombination promoted by plasmids.

7. Chromosomal genes and association to eukaryotic cells

A search in the data banks revealed that the different members of the cell associated alpha-2 *Proteobacteria* share several chromosomal systems that seem necessary for the invasion of cells and intracellular survival. For instance *Brucella*, *Bartonella*, *Agrobacterium* and *Rhizobium* species share extensive amino acid identity on the stress–response protein HtrA. Modification in the expression of this protein renders cell associated bacteria avirulent or incapable of endosymbiosis in restricted systems [132,133]. The *rop*A and *rop*A2 coding for outer membrane proteins repressed during symbiosis of *R. leguminosarum* are closely related to two genes found in *Brucella* genome [134]. The CcrM DNA methyltransferase, which is essential for viability, has been found in *Rhizobium*, *Sinorhizobium*, *Agrobacterium* and *Brucella* species [135]. The molecular chaperone DnaK, proposed as one of *Brucella* virulence proteins, is highly similar to those proteins present in *Ochrobactrum*, *Phyllobacterium*, *Rhizobium* and *Agrobacterium* [136]. The GroEL and the Hsp60 chaperone proteins, which may be important for virulence [137], are highly similar among *Proteobacteria* of the alpha subclass, including mitochondria [138,139]. Virulence arginase and ornithine cyclodeaminase genes present in plant pathogens are also found in animal pathogens of the alpha-2 *Proteobacteria* [59]. Two chromosomal loci associated with the synthesis and transport of cyclic β-(1,2)-glucans have been identified in *A. tumefaciens* (*chv*A and *chv*B) and in *S. meliloti* (*ndv*A and *ndv*B) strains (for review see [140]). Cyclic glucans in plant pathogenic bacteria have been proposed as essential factors for nodule formation and parasitism [140]. Since chemically identical cyclic glucans have been identified in *Brucella* organisms and in *O. intermedium* [141,142], it is possible that the *chv*A/*chv*B and *ndv*A/*ndv*B genes have their counterpart in chromosomes of these two genera.

Recent work has demonstrated that *Brucella* virulence genes (*bvr*S and *bvr*R) of a two regulatory system showed a high similarity with chromosomally encoded virulence systems present in *S. meliloti* and *A. tumefaciens* [85]. The similarity of this genetic region is further accentuated by the contiguous presence of a phosphoenolpyruvate carboxykinase gene in these three species [143,144]. This group of highly related systems seems to be of critical importance for the establishment of a relationship between these bacteria and their hosts, no matter whether the latter

are animal or plant cells. *S. meliloti exo*S is involved in regulating the production of succinoglycan, which plays a crucial role in the establishment of the symbiosis between *Sinorhizobium* and its host plant [145]. Similarly, insertion mutations in either *chv*G (the sensor histidine protein kinase) and *chv*I (the response regulator) render *A. tumefaciens* unable to elicit tumour formation in susceptible plants [146]. Furthermore, although the genes regulated by ChvI–ChvG are unknown, it is significant that the *chv*I and *chv*G and the *Brucella bvr*R and *bvr*S mutants show an increased sensitivity to surfactants [85,143,146]. Thus, it is likely that these regulatory elements control the synthesis and/or assembly of outer membrane components essential in the interaction with eukaryotic cells [146]. This does not mean that the sensor proteins (ChvG, ExoS and BvrS) should respond to the same environmental stimulus.

The above similarities have a phylogenetic significance which supports the notion that the establishment of pericellular and intracellular relationships with eukaryotic cells, no matter whether they are animal or plant, is an evolutionary trend in the alpha *Proteobacteria* [6,105]. In other unrelated cell-associated bacteria, such as *Salmonella* and *Yersinia*, several of these sequences were 'recently' acquired horizontally as pathogenicity islands [24,27], a situation different from the ChvI–ChvG, ChvI–ExoS and BvrR–BvrS systems of alpha *Proteobacteria* [85]. This is mainly deduced not only by the high similarity displayed by the proteins of these systems, but by the similar G+C contents of these regions which reflect the overall G+C content of the chromosomes. The complete amelioration of these sequences suggests that some of these systems, necessary for survival in association to eukaryotic cells, were already present in chromosomes before the divergence of the *Agrobacterium–Rhizobium–Sinorhizobium–Mycoplana –Phyllobacterium–Bartonella–Brucella* cluster, about 300 million years [5,58].

8. Concluding remarks

Intracellular parasitism is not a rare event since it has evolved several times in different phylogenetic lineages, from nucleic acid viroids to multicellular metazoans, such as triquinella worms. In general terms, it seems that symbiosis and intracellular parasitism are connected to reduction of functions and genome size, while the discovery of terrestrial or aquatic environments is probably not. The common characteristic among all different cell associated parasites is the evasion of destructive cellular mechanisms. However, there are various solutions to live in association with eukaryotic cells and to confront their destructive intracellular processes. In the soil, opportunistic, and plant-associated alpha *Proteobacteria*, some of these solutions are coded in plasmids while others have been fixed in chromosomes. In the animal cell-associated *Proteobacteria* of the alpha subclass without plasmids, the relevant genes for survival in association to eukaryotic cells are necessarily chromosomal coded and vertically inherited.

It seems that the animal cell-associated lineages of the alpha subclass with limited metabolic abilities and without plasmids evolved from a chemoautotrophic soil and plant-associated bacterial ancestor with more than one megareplicon (e.g. one or two chromosomes and megaplasmids) and plasmids. The corresponding agrobacteria spp. that move from soil into plants and produce tumours by the acquisition of plasmids can invade animals and produce tuberculosis-like diseases or abortion in humans [78,147], revealing the potential of the lineage for adapting to different living conditions. However, it is important to understand that fitness of a bacterium to a certain habitat is not necessarily commensurate with its phylogenetic position, but rather with subtle changes in functionally important chromosomal genes as well as the acquisition of genes by horizontal transfer of genetic elements. The functional 'new' genes may lead to conspicuous phenotypic changes that may characterize each bacterial lineage [5,7]. For instance, the periplasmic domain (involved in environmental sensing) of the sensory proteins (ChvG, ExoS and BvrS) of the two regulatory system necessary for bacterial parasitism of *Agrobacterium*, *Sinorhizobium* and *Brucella* shows less similarity than other protein domains, implying that they were derived for sensing a different stimulus [85]. However, it is remarkable that the two intracellular bacteria (*S. meliloti* and *B. abortus*) are more similar in this region than the pericellular one (*A. tumefaciens*).

Among certain groups, lateral gene transfer mediated by plasmids or lysogenic phages seems to play

an important role in building bacterial communities with relevant ecophysiological functions. In other organisms, such as the animal pathogens of the alpha subclass, horizontal transfer between bacteria seems to be an irrelevant phenomenon. If bacteria behave as independent evolutionary units, adaptive genomic changes may expand clonally resulting in a distinctive variety. On the contrary, if they behave as reticulate evolutionary units, genomic adaptive changes occurring within a single bacterium are horizontally transferred to many or all members of the species. Consequently, the species gene pool will be homogenised, even as it creates different genotypes and diversifies clones [122]. Recombination between different genomic types may result in unbounded divergence and speciation. Depending on local circumstances, bacteria may alternate from one form to the other; that is, either propagate clonally for long periods of time, or shift into a network structure as a result of recombination. If given enough time, gene flux between the parasitic bacteria and the host chromosome may be an important mechanism for establishing endosymbiotic interactions, as has been suggested for mitochondria [148]. In this case, genome reduction has already attained critical levels and the integration between the prokaryotic–eukaryotic living systems is almost complete. However, the evolutionary affiliation of mitochondria within the alpha subclass of *Proteobacteria* can still be established thanks to conserved molecules [149].

Acknowledgments

I thank William Eberhard (Smithsonian Tropical Research Institute, Escuela de Biología, Universidad de Costa Rica, San José, Costa Rica), Ignacio Moriyón (Departamento de Microbiología, Universidad de Navarra, Pamplona, Spain), an anonymous reviewer and Gregg Pancoast (Programa de Investigación en Enfermedades Tropicales) for their critical review of the manuscript.

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