Intracellular symbiotic bacteria within insects

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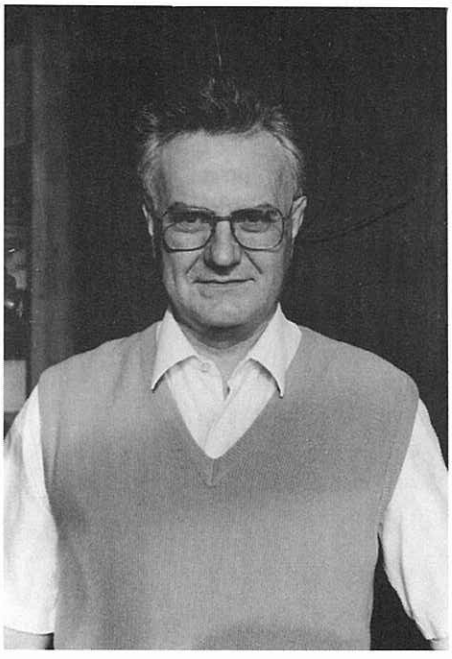

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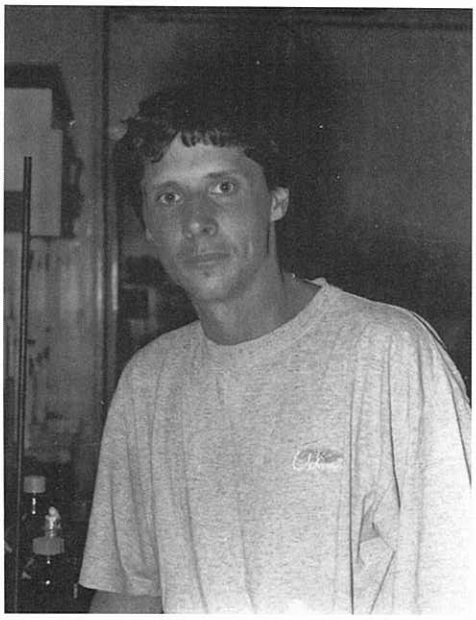

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INTRACELLULAR SYMBIOTIC BACTERIA WITHIN INSECTS

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1. Introduction

Symbiosis is a permanent and hereditary association between two or more partners specifically distinct and, most of the time, very different from a phylogenetic point of view. A very common type of symbiosis involves bacteria, called endocytobiotes, living inside eukaryotic cells. Our investigation was limited to the endocytobiotes of insects. Most of the time they are Gram negative and non sporulating bacteria (Dash *et al.*, 1989). Such a habitat is very particular or extreme for at least three reasons: first, the bacteria, associated with their host, constitute a new biological unit, the symbiocosm, which is submitted to natural selection (Nardon and Grenier, 1993). Second, in this symbiocosm, the bacteria entirely depend on the host for their nutrition, and third, the growth of bacteria is strictly controlled by the insect (Nardon *et al.*, 1998).

In most intracellular symbiosis models, endocytobiotes are surrounded by a membrane of host origin, probably resulting from a phagocytosis event (cockroaches, aphids...). This structure, of bacteria inside vacuoles, is called a symbiosome. However in some insects, like the weevil *Sitophilus oryzae*, the bacteria lie free in the cytoplasm of the host cell, which indicates a high degree of intimacy between the two partners (Nardon and Grenier, 1989). Important problems that arise are the modalities of exchange between symbiotes and the host cell, and the mechanism of recognition. Unfortunately, very little is known about this relationship, but the possibility of transferring bacteria by microinjection from one strain to another, allows for an investigation into the specificity of the association, at least for the *Wolbachia* endocytobiotes (Chang and Wade, 1994 and laboratory results).

In insects, we must distinguish two different types of endocytobiosis (Nardon and Grenier, 1993). In "primitive symbiosis", bacteria are not present in all the insect population but, when present, they are found in numerous tissues and genital organs. However the host cells are apparently not modified. In contrast, in "integrated symbiosis", the bacteria are always present and limited to specialized cells, the bacteriocytes, and the ovaries. These bacteriocytes are generally giant and polyploid cells, transformed by the presence of bacteria (Nardon, 1988). They may be disseminated in the fat body where they divide, as in cockroaches (Brooks and Richards, 1955), or

grouped to form one or several symbiotic organs, or bacteriomes, as in aphids (Ishikawa, 1989a), leafhoppers (Tiivel, 1989), or weevils (Nardon and Grenier, 1989). These traits are specific and the number, the location and the morphology of bacteriomes are perfectly controlled (Buchner, 1965; Nardon, 1988).

2. Phylogenetic position and molecular characterization

A wide variety of intracellular prokaryotes are found in association with insects; some of them are lethal for their host (*e.g.*, *Rickettsiella*) but most are engaged in symbiotic relationships. Because the endocytobiotes cannot be cultivated outside their host cells, their taxonomy has not been precisely defined. Nevertheless, recent advances in molecular biology, and especially the use of rDNA sequences for phylogenetic studies, have greatly improved our knowledge of these bacteria. At this time, insect endocytobiotes can be divided into four major groups of bacteria: (1) the flavobacteria-bacteroides group which includes the cockroach endocytobiotes; (2) the α-proteobacteria, a very homogeneous monophyletic group (2% 16S rDNA sequence divergence) corresponding to the genus *Wolbachia* (Werren, 1997); (3) the β-proteobacteria (mealybug endocytobiotes) and (4) the highly diversified group of γ-proteobacteria. Phylogenetic studies also facilitate the reconstitution of the evolutionary history of symbiosis. Hence, Bandi *et al.* (1995) showed that endocytobiotes of *Mastotermes darwiniensis*, a primitive Australian termite, are very close to the genus *Blattabacterium* (cockroach endocytobiotes). Since termites and cockoaches probably evolved from a common ancestor by the early Carboniferous period, it has been suggested that the endocytobiote integration occurred at least 300 million years ago in the primitive host insect. Concerning the α-proteobacteria group, based on sequence divergence of *ftsZ* genes, Werren *et al.* (1995) have suggested that the common ancestor of all examined *Wolbachia* lived about 50 million years ago. Finally, in the γ-proteobacteria group, the symbiotic association of *Buchnera* (aphid endocytobiotes) was estimated to be about 250 million years old, while the weevil *Sitophilus* endocytobiotes were probably integrated later (100 million years ago). These last two endocytobiotes are closely related to *Erwinia herbicola*, a free leaving bacteria associated with plant leaves, suggesting that they may be derived from an inhabitant of the plant on which host insects feed (Heddi, personal communication).

The genomic G+C content of insect endocytobiotes is generally low, regardless of their phylogenetic position (*e.g.*, *Blattabacterium*, 26%; aphid endocytobiotes, 30%; ant *Formica* endocytobiotes, 41%; leafhopper *Euscelidius* endocytobiotes, 31%). *Sitophilus* endocytobiotes are an exception with 54% C+G. It has been suggested that A+T accumulation occurred during intracellular evolution, but the molecular mechanism involved is not yet understood.

The genome of intracellular bacteria is reduced compared with that of extracellular bacteria (Herdman, 1985). This could be explained by gene deletions (or transfers to the nucleus) which may have occurred during intracellular evolution. Deleted genes may be those that became useless in the protected and stable intracellular environment. Hence, the genome size of the endocytobiotes of the weevil *Sitophilus oryzae* was estimated at 3.0Mb by pulsed field gel electrophoresis, which corresponds to about 60% of the genome size of the closest free living bacteria (Charles *et al.*, 1997).

Furthermore, endocytobiotes generally contain a single or a few rDNA operons in their genome while extracellular bacteria most often carry multiple operons (*e.g.*, seven in the Enterobacteriaceae family). *Buchnera*, as well as the primary endocytobiote of tsetse fly, possess only one copy of the rRNA operon in their genome, while *S. oryzae* endocytobiotes possess two copies of the same operon (Unterman *et al.*, 1989; Aksoy, 1995; Charles *et al.*, 1997). Such a low rDNA copy number would explain the slow growth rate of these bacteria in their intracellular environment.

3. Role of endocytobiotes

In primitive endocytobioses, particularly in insect / *Wolbachia* symbioses, the bacteria generally appear to have a weak influence on the fitness of the host (see Nardon and Grenier, 1993). Exceptions include the *Wolbachia* of *Trichogramma Bourarachae* whose presence in a laboratory strain enhances the fecundity (Girin and Bouletreau, 1995). Increased male fertility has also been reported in a strain of *Tribolium confusum* beetles following infection with *Wolbachia* (Wade and Chang, 1995). In contrast, it has been shown in *Drosophila simulans* that *Wolbachia* do not enhance productivity in infected strains but do not negatively affect the insect either (Poinsot and Merçot, 1997). However, in the same insect, as in numerous others (Werren, 1997), the presence of *Wolbachia* induces cytoplasmic incompatibility (CI) when an infected male is crossed with an uninfected female, whereas the reverse cross is normal. This incompatibility leads to high embryonic mortality. In some other strains it is bidirectional, when the male and the female harbour two different *Wolbachia* (Rousset and Solignac, 1995). Especially in the case of bidirectional CI, this incompatibility is thought to promote rapid speciation between populations which become reproductively isolated when infected by *Wolbachia* (see Werren, 1997 for extensive discussion). Another spectacular effect of *Wolbachia* is the thelytoky (production of females only) induced in *Trichogramma* (Louis *et al.*, 1993; Stouthamer *et al.*, 1993). The mechanism involved and the consequences on populations is exhaustively discussed in Werren (1997).

In specific integrated symbiosis (Nardon and Grenier, 1993), the endocytobiotes play different roles, which have been reviewed by several authors: Nardon and Grenier (1989, 1991, 1993), Douglas (1989), Ishikawa (1989a). To summarize, we can distinguish three essential distinct roles for the integrated endocytobiotes of insects.

Morphogenetic action. Generally the bacteria induce gigantism and polyploidy in host cells, except for germ cells. For instance, in the banana weevil *Metamasius*, the bacteriocytes may be more than 100 µm in diameter (Nardon *et al.*, 1985). In cockroaches, growth and division of bacteriocytes seems to be controlled by the symbiotes (Richards and Brooks, 1958). In the same way, the bacteriomes of the weevil *Sitophilus oryzae* disappear in the absence of bacteria (Nardon, 1973). In the cockroach *Blattella germanica*, extensive structural changes are also visible after partial or total elimination of symbiotes (Grigolo *et al.*, 1987).

Symbiotes as metabolite suppliers. Integrated endocytobiotes play a major role in the biology of their hosts, which are supplied with nutrients or other substances not synthesized by eukaryotic genomes. As a consequence, the presence of these bacteria greatly enhances the insect fitness through more rapid growth and increased fertility (Ishikawa, 1989a; Nardon and Grenier, 1989, 1993). Deprived of their symbiotes, aphids or cockroaches are no longer capable of reproducing. Aposymbiotic strains of *Sitophilus* can be maintained in the laboratory but with a reduced fitness. This example shows that the degree of integration of the bacteria is variable, probably as a function of the age of symbiosis, this being more recent for weevils than for aphids.

Among growth factors synthesized by endocytobiotes are vitamins supplied, for instance, to *Blattella germanica*: Pantothenic acid, riboflavin, pyridoxine and thiamine (Pant and Frankel, 1954). In *Sitophilus oryzae*, pantothenic acid, biotin and riboflavin are supplied in sufficient quantities to promote growth and reproduction. Pyridoxine and folic acid are also supplied, but at concentrations too low to enable the development of more than one generation (Wicker, 1983). The limited quantity of B vitamins in cereals may explain why aposymbiotic insects present a decreased fitness.

The supply of amino acids to the host by endocytobiotes is another good example of the role of symbiotic bacteria in adaptation to the environment. The diet of aphids is phloem sap, which is very poor from a nutritional point of view, and aphids can only survive and reproduce thanks to complementation of this diet by symbiotes (Douglas, 1996). They synthesize several essential amino acids: tryptophan (Douglas and Prosser, 1992), threonine, isoleucine, lysine (Febvay *et al.*, 1995) and methionine (Douglas, 1988a). Glutamate is the only amino acid supplied by the insect to the symbiotes (Febvay *et al.*, 1995). Concerning lipids, the role of endocytobiotes seems to be less important. Nevertheless, in aphids, it seems highly probable that sterols are synthesized by the bacteria, at least in *Myzus persicae* (Douglas, 1988b).

Interactions of symbiotes with host metabolism. This phenomenon has been principally studied in our laboratory on *Sitophilus oryzae* (see in Nardon and Grenier, 1989). Its natural food (wheat) contains too much methionine. In symbiotic weevils, the excess methionine is converted into methionine sulfoxide, whereas in aposymbiotic ones it is demethylated into the amino acid sarcosine which accumulates in the haemolymph. This second way consumes ATP. Therefore, it is clear that the symbiotic bacteria allow their

host to conserve ATP and avoid sarcosine accumulation, a function normally carried out by mitochondria.

Finally, we have demonstrated an interaction with the mitochondrial energetic metabolism. The activities of six enzymatic complexes have been compared in symbiotic and aposymbiotic insects. They are always higher in the presence of bacteria and this must be related to the supply of B vitamins, particularly pantothenic acid and riboflavin (Heddi *et al.*, 1993).

4. Intracellular living conditions of endocytobiotes

4.1. HOST CONTROL OF THE ENDOCYTOBIOTE POPULATION

The establishment of endocytobiotic relationships requires a very high host-symbiote compatibility. On the one hand, the bacteria have to avoid the defensive reaction of the insect. On the other hand, the host has to prevent bacterial invasion by controlling both the location and the density of the endocytobiotes. Among factors involved in the insect defensive system, lysosomal enzymes, hemaglutinins and antibacterial peptides have been described (Tiivel, 1993).

In amoebae symbiosis, Jeon (1995) showed that the endocytobiotes produce one protein of 96 kDa and lipopolysaccharides that could prevent symbiosomes fusing with lysosomes. As previously mentioned, the endocytobiotes of *Sitophilus oryzae* are lying free in the bacteriocyte cytosol. This supposes a full immunological compatibility between the two partners, and should hence protect the endocytobiotes from lysosomal fusion. Nevertheless, bacterial lysis occurs in the bacteriocytes of the weevil ovaries (Nardon, 1971), and the number of symbiotes is stable for a given strain, controlled by chromosomal factors not yet identified at the molecular level (Nardon *et al.*, 1998). Other factors, such the nature of the diet, can also influence the symbiote density (Nardon and Grenier, 1989). We are in the presence of what we call a microecosystem, the equilibrium of which is regulated by complex interaction factors.

4.2. STRESS PROTEIN PRODUCTION BY THE ENDOCYTOBIOTES

Stress proteins have been observed in numerous intracellular parasitic bacteria (Van der Vies and Georgopoulos, 1996). In these bacteria, the most predominant protein over-expressed in response to the intracellular environment is the chaperonin protein Hsp60 (60 kDa heat shock protein). In free living bacteria, this protein is essential for cell viability at all temperatures (Fayet *et al.*, 1989). It is known to be involved in the folding of nascent polypeptides, the assembly of oligomeric protein complexes and protein export from bacteria (Zeilstra-Ryalls *et al.*, 1991). In the case of parasite

survival inside host cytosol, it has been suggested that Hsp60 could be implicated in the maintenance of essential bacterial proteins and/or the excretion of virulence factors .

The over-expression of chaperonins has also been reported in non-pathogenic bacteria, such as the endocytobiotes of tsetse flies *Glossina* (Aksoy, 1995), *Sitophilus* weevils (Charles *et al.*, 1995) and aphids (Ishikawa, 1989b). In *Sitophilus oryzae,* protein labelling experiments revealed that chaperonin represents about 40% of the total neosynthesized proteins of the endocytobiotes *in vivo*. In the aphid *Schizaphis graminum*, Baumann *et al.* (1996) showed that the amount of Hsp60 inside the endocytobiotes corresponds approximately to that found in *Escherichia coli* grown at 46°C (close to its maximal growth temperature). Finally, Morioka *et al.* (1993) reported that the Hsp60 protein of another aphid *Acyrthosiphon pisum,* is autocatalytically phosphorylated *in vitro* at elevated temperatures and can transfer radioactive phosphate from ATP to GTP. These results suggested that the Hsp60 protein could function in the symbiocosm not only as a molecular chaperone but also as an energy-coupling protein.

Very little is known about the regulation of *hsp60* gene expression in the insect endocytobiotes. In *Buchnera*, the *symSL* operon (containing the *hsp10* and *hsp60* genes) seems to be under the control of a heat shock promoter that is functional in *E. coli*. Nevertheless, the operon is not heat inducible *in vivo* and seems to be constitutively expressed thanks to an A- and T- rich region around the heat shock promoter that might facilitate transcription (Sato and Ishikawa, 1997). The common characteristic of all intracellular bacteria studied (parasite or mutualist) is that, whereas a stoechiometric ratio of 2:1 (Hsp60:Hsp10) is theoretically required in the cell, a large quantity of Hsp60 protein relative to Hsp10 was observed in the bacteria located in the host cytosol. Thus, the strict over-expression of Hsp60 may reflect physiological needs, specific for bacteria living in an intracellular environment.

From a general point of view, it is noteworthy that the comparison of stress protein expression between free living and intracellular symbiotic bacteria led several authors to assume that endocytobiosis conditions generate stress for symbiotes (Jeon, 1995). In our opinion, it is not really surprising to observe an Hsp60 protein in all intracellular bacteria. This protein is indeed essential for bacterial survival in all environmental conditions and one wonders what could represent intracellular stress for a bacterium that has been living in the host cytoplasm for a couple of hundred million years? The conservation of the Hsp60 over-expression mechanism during intracellular bacterial evolution (from the parasite to the integrated endocytobiotes and probably to the cytosolic organelle) rather reveals an essential function of the chaperonin in maintaining the equilibrium and/or the protein import/export occurring in the symbiocosm. In this sense the term symbionin, proposed by Ishikawa (1982) to qualify the chaperonin produced by the aphid endocytobiotes, seems to be particularly accurate.

4.3. INTRACELLULAR EVOLUTION OF ENDOCYTOBIOTES

Endocytobiote populations are very different from those of free living bacteria, both in terms of dynamics and evolution. They are indeed small, asexual and pass through a bottle-neck at each host generation when progeny are inoculated. They are hence clonal since no recombination can occur between lineages sequestered in different hosts and since horizontal transfers between hosts appear to be relatively rare. Moran (1996) showed that endocytobiote population structures are characterized by, first, a faster substitution rate relative to the free living organisms, second an accumulation of deleterious mutations in non neutral sites and, third, as selective constraints are reduced in the protected intracellular environment there is a greater effect of mutational bias (A-T pressure) on the genomic DNA composition (*i.e.*, on neutral and non neutral sites). Regarding this peculiar evolution, the following question comes: "why have asexual endocytobiotes not decayed to extinction?" (Hurst and McVean, 1996). Compensatory responses must be involved to explain the persistence of endocytobiotes inside their hosts. To decrease the accumulation of mutations, in the absence of recombination, the per genome mutation rate must be somehow reduced. This could be achieved by a reduction in the number of genes in the genome. Genes could be deleted (if their function in the intracellular environment becomes unnecessary) or transferred to the host nucleus. A second compensatory process may involve the Hsp60 protein that could prevent misfolding of proteins, whose tertiary or quaternary structures have been modified by an accumulation of mutations. Finally, intracellular living conditions seem to be particularly suitable for such a compensatory mechanism because (1) the host can supply the bacteria with excess enzymes or nutrients to compensate for some of the endocytobiotes failing and (2) the host can select (by elimination of the deleterious bacteriocytes) its most competitive endocytobiote populations.

As natural selection will occur both inside and outside the symbiotic association, the formation and maintenance of the symbiocosm require, first, the co-adaptation of the two partners (inside selection) and, second, the adaptation of the symbiocosm itself to the environmental conditions (outside selection). By this means, symbiosis leads to the modification of both partners and to the formation of a new biological unit (Nardon and Grenier, 1991, 1993). It is not yet clear whether intracellular bacterial populations take advantage of these associations since the integration process seems to be very costly for the bacteria. Moreover, bacterial populations might never reach equilibrium until they are totally integrated in the host cell, as has been suggested in the amitochondrial protozoa *Trichomonas vaginalis* (Horner *et al.*, 1996). Future molecular studies on these peculiar bacteria promise to elucidate this integration process, one of the most fascinating aspects of Eukaryote cell evolution. The different types of intracellular symbiosis currently observed probably represent different steps of integration of symbiotes, which finally behave as cell organelles. Such observations are in agreement with the endosymbiotic theory of the formation of the Eukaryote cell (Margulis, 1993).

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