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Review article.

Sitophilus oryzae L.: A model for intracellular symbiosis in the Dryophthoridae weevils (Coleoptera)

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Abstract

Bacterial intracellular symbiosis in the weevil *Sitophilus* spp. was discovered in 1927 and since then has been studied by several authors through many approaches. This has led to a deep characterization of variety of symbiotic aspects in relation with morphology, histology, genetic, physiology, as well as host-symbiont molecular and cellular biology. This paper reviews the basic findings from several teams and analyzes them with respect to current knowledge on weevil symbiosis evolution and cell-to-cell molecular interactions. *Sitophilus* spp. intracellular symbiosis appears as a model appropriate for studying and elucidating molecular and genetic mechanisms and evolutionary features involved in the early steps of insect intracellular symbiosis establishment.

Keywords: *Sitophilus* weevils, Dryophthoridae, symbiosis, evolution, innate immunity

1. Introduction

Intracellular symbiosis, or endosymbiosis (Nardon and Charles, 2001), was discovered in the weevil *Sitophilus oryzae* by Pierantoni (1927). Since then, several authors and teams have embarked upon the study of this endosymbiosis: in Italy (Tarsia in Curia, 1933), in Egypt (Mansour, 1930), in the United States (Murray and Tiegs, 1935; Baker, 1975), in Canada (Musgrave and Miller, 1956), in Germany (Scheinert, 1933; Schneider, 1956) and in France (Nardon, 1971; Heddi, 2003). These insects destroy up to 40% of cereal stocks (Grenier et al., 1986). Two other symbiotic species are also cereal pests, including *S. zeamais*, the sibling species of *S. oryzae* (Grenier et al., 2000, Nardon and Nardon, 2002), and *S. granarius*, a closely related species to *S. oryzae*. We believe that intracellular symbiosis in *Sitophilus* spp. contributes significantly to the weevil's performance and invasive power (Heddi, 2003). Recently, *Sitophilus* spp. has been classified among the Dryophthoridae insect family (Alonzo-Zarazaga and Lyal, 1999).

The Dryophthoridae family comprises about 140 genera and is of particular interest as regards intracellular symbiosis evolution in that the members of this family, contrary to most symbiotic insects, thrive on monocot angiosperms and feed on a large variety of plant tissue, such as leaves, stipes and roots. Some known exceptions include *Sipalinus* and *Trigonotarsus*, which feed on decaying wood, and *Sitophilus*, which feeds on seeds including dicots (Delobel and Grenier, 1993). To examine how intracellular symbiosis has evolved across the wide ecological spread of the Dryophthoridae insects, we have recently collected and analyzed several Dryophthoridae species worldwide (Nardon et al., 2002; Lefèvre et al., 2004).

This work has provided new perspectives on the role of endosymbiosis in insect adaptation and diversification, as well as how established endosymbionts enter into competition with invasive secondary endosymbionts and how symbiont replacement can occur during the life history of an insect.

In this review, we will focus only on *S. oryzae* because this species has been extensively investigated during the last three decades. Nevertheless, the other species studied (i.e. *S. zeamais* and *S. granarius*) exhibit a very similar organization, except for some known variability in the endosymbiont morphology (Nardon et al., 2003). We will review the basic data obtained by several authors in the past and analyze them with respect to current knowledge on the biology of weevil associations. The analysis of intracellular symbiosis raises several questions, including: what is the structure of the symbiotic cells and organs? How is hereditary symbiosis maintained? What are the consequences

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of a host/symbiont interaction, particularly the role of the endosymbionts on growth, reproduction and behavior? And, what are the consequences of symbiosis on the co-evolution of the host and the endosymbionts?

2. Insights into Symbiotic Structures

Endosymbiont features

Cells, organs and organisms are called "symbiotic" when they harbor symbiotic microorganisms, generally represented by bacteria within the insect world. In *S. oryzae* most insects harbor only one endosymbiotic bacterial species, called SOPE for "*Sitophilus oryzae* Primary Endosymbiont" (Charles et al., 1997; Heddi et al., 1998).

SOPE is a Gram-negative, non-sporulating, and non-ciliated bacterium (Grinyer and Musgrave, 1966; Nardon, 1971). A detailed description can be found in Nardon and Wicker (1981) and in Dasch et al. (1984). Briefly, SOPE is bacilliform, with a size varying from 1 to 30 μm, depending on certain physiological factors and the geographical origin of the insect strains under study (Nardon et al., 2003).

This pleomorphism is probably a consequence of genetic polymorphism and/or host/bacterium interaction (Heddi, 2003). SOPE is closely related to the free-living bacterium *Escherichia coli*, with 95% homology on the 16S rDNA gene, and also to the primary endosymbiont of *S. zeamais* (97.8% identity) (Heddi et al., 1998), despite the fact that the latter is morphologically quite different from SOPE (Nardon et al., 2003). SOPE was classified within the enterobacteriaceae family, belonging to the γ3 proteobacterial group (Lefèvre et al., 2004).

The GC content in the 16S rDNA gene is 54%, which is the highest value observed for an intracellular bacterium, excluding *Mycobacterium leprae* (Heddi et al., 1998). The genome size of SOPE is slightly reduced (3.0 Mb compared to the 4.7–5.4 Mb of the closely related free-living bacterium *E. coli*) (Charles et al., 1997), but not as much as in the other insect endosymbiont genomes (between 450 kB to 750 kB).

Such a difference in genome size is thought to be the consequence of intracellular evolutionary constraints that drive bacterial genome reduction (Moran, 1996; Itoh et al., 2002; van Ham et al., 2003).

In addition to SOPE, some wild strains of *S. oryzae* harbor a second endosymbiont (Heddi et al., 1999), which is an α-proteobacterium that belongs to the B-group of *Wolbachia*, according to the classification of Werren et al. (1995).

In contrast to SOPE, *Wolbachia* is disseminated throughout the whole body of the insect, with a notably high density in the male and female germ cells, where this bacterium has been shown to induce nucleo-cytoplasmic incompatibility (Heddi et al., 1999).

Insect symbiotic structures

Although a *Wolbachia* infection is not tissue-specific and does not induce any structural change in the cells (except germ cells DNA), SOPE has been shown to interact with the host embryogenesis as an epigenetic factor, and this interaction culminates in the "differentiation" of bacteria-bearing host cells, called bacteriocytes, grouped together as an organ, the bacteriome. Bacteriomes are located at the apex of ovarioles in the nymph and in the mature female, attached to the intestine during the four larval stages, and to the apex of anterior mesenteric caeca in the young male and female adults (Nardon and Wicker, 1981). The genetic program involved in bacteriocyte cell differentiation and evolution throughout the host development is not currently understood, but it may involve a molecular dialogue between the bacteria and the host early in embryogenesis (Heddi et al., 2005). Host-bacterial communications are currently being investigated to unravel the genes and pathways of interest.

In the germ cells (oocytes and trophocytes), SOPE grows and multiplies but it does not induce any cytological modifications, as can be deduced from comparison with oogenesis in aposymbiotic strains. During vitellogenesis, endosymbionts actively divide and concentrate at the posterior pole of the oocytes, where they remain in close contact with the oosome (Nardon, 1971). This is also the case in the other Dryophthoridae weevils studied, such as *Metamasius hemipterus* (Nardon and Nardon, 1998).

In the apical bacteriome of ovarioles, and in the larval bacteriome, SOPE interacts with the cell nucleus and induces cell polyploidization. The bacteriocytes become huge, reaching up to 30 μm in size, and their cytoplasms are completely packed with endosymbionts (Nardon, 1971). A thin membrane surrounds the larval bacteriome organ, which contains an important tracheal network (Nardon et al., 2003). Although the larval bacteriome attaches as a U-shape form to the junction of the fore-gut/mid-gut, no obvious communication was seen between these tissues.

Moreover, in addition to the bacteriocytes, the polyploid cells harboring the endosymbionts, small flat cells forming the interstitial tissue can also be distinguished. These interstitial cells are also present in the apical bacteriome of ovarioles, in the other studied species of the Dryophthoridae. However, their role with regard to symbiosis remains unclear.

Interestingly, in contrast to most insect endosymbionts, SOPE is not surrounded by a third membrane, called M3 (or a symbiosome vacuole, according to Ahn et al. (1990)). The other Dryophthoridae endosymbionts studied also lack the M3 membrane (Nardon et al., 2002, 2003). The bacteria lie free in the cytoplasm of the bacteriocytes and germ cells (Nardon, 1971; Nardon and Grenier, 1989). Whereas this feature may facilitate bacterial trafficking in the cytoplasm, no data are available on the process used by the bacteria to escape the insect hydrolytic activities, such as the action of

lysozymes or antimicrobial molecules. Ongoing projects may help to tackle this problem and to understand how SOPE interact with the host innate immune system.

Nevertheless, previous genetic studies have demonstrated that the weevil is able to regulate the symbiont density through an as yet unknown mechanism (Nardon et al., 1998). One possibility may involve lytic factors, as suggested by observation of the apical bacteriome where clusters of degenerating bacteria are always seen (Musgrave and Grinyer, 1968).

Moreover, some species, like *S. granarius*, secrete a mucus-like material that draws a halo around the bacteria and may play a crucial role in insect cell protection and/or the control of endosymbiont invasion. Such a halo has also been identified, though very rarely, in some geographical strains of *S. oryzae*, such as "Bouriz" (Nardon et al., 2003).

Transmission and control of SOPE

The larval bacteriome and its formation in the egg

The precise means of SOPE transmission from one generation to the other remains questionable. Since the bacteria have never been seen in the testes, it is firmly established that only females transmit the endosymbiont (Musgrave and Miller, 1956; Nardon et al., 1998). Endosymbionts are present permanently in ovaries, where they multiply during oogenesis and infect the eggs, with about 18,600 bacteria per egg for the SFr strain of *S. oryzae* (Nardon and Grenier, 1988). Several authors have confirmed this characteristic, including Pierantoni (1927), Scheinert (1933), Tiegs and Murray (1938), and Schneider (1956). The fate of SOPE during embryogenesis is not completely understood in detail. Authors have expressed different opinions; we report here what we consider to be the most likely situation from our own observations.

According to Scheinert (1933), the larval bacteriome is differentiated in both sexes from the stomodeum. Tiegs and Murray (1938) have suggested that the mid gut cells are also involved, since some of them migrate in the ooplasm and differentiate into bacteriocytes later. Subsequently, most of them disrupt and release the bacteria in the yolk, where new migrating cells will capture them, and so the cycle continues. As a result, the bacteriome is formed during embryogenesis by the accumulation of intact bacteriocytes around the blind end of the stomodeum.

However, our knowledge is limited concerning the process used by SOPE to penetrate the gut cells, although the recent data published by Dale et al. (2002) suggest that SOPE uses the function of a type three secretion system to achieve this. The bacteriocytes divide and enlarge during the four larval stages whilst the bacteriome remains as a compact organ throughout this period (Nardon, 1973; Nardon and Wicker, 1981).

Dissociation of the larval bacteriome

During nymphal metamorphosis, the bacteriome dissociates (probably under the influence of hormonal program changes), and the bacteriocytes "slip" back along the intestine to become incorporated into the anterior midgut caeca (Pierantoni, 1927; Tarsia in Curia, 1933; Murray and Tiegs, 1935; Nardon and Nardon, 1998). All these authors agree about the migration of bacteriocytes, with only a few variations not reported here. The phenomenon begins in the pre-nymph and ends in the nymph, after the formation of the mesenteric caeca bacteriomes. In adults, bacteria are progressively eliminated over a period of about three weeks following the imago emergence from cereal seeds. After the bacteriocyte's disintegration, bacteria are released into the gut lumen (Pierantoni, 1927) and proliferate in the posterior part (Mansour, 1930). This observation is highly questionable, as SOPE remains uncultivable. Moreover, Mansour (1930) described the presence of cocci as possible transformed symbiotic bacteria. Since then this observation has not been confirmed and we do not know how many insect samples have been examined in this context.

Contamination of the germ line

SOPE is permanently present in eggs and young oocytes (Mansour, 1930). It divides actively during oogenesis (Nardon, unpublished data), and, as reported above, it exhibits a high density at the posterior pole in close association with the oosome, the germinal determinant (see Büning, 1994). According to Scheinert (1933), the primordial germ cells are formed at the very beginning of embryogenesis by protrusion at the posterior pole of the egg. Therefore, these cells include both the oosome and the endosymbionts. They are the first differentiated cells and they appear as permanent organelles of the germ line, just like mitochondria. For unknown reasons, the bacteria are rapidly eliminated from the male germ line and the rudiments of testes never contain any endosymbionts. Cytological data have been confirmed by genetic experiments, demonstrating an exclusive maternal heredity of endosymbionts (Nardon et al., 1998). During metamorphosis, the three cell types of the female germ line (i.e. oocytes, trophocytes and apical bacteriocytes) differentiate. It is noteworthy that SOPE does not modify the cytology of oocytes and trophocytes, which suggests that bacteriocyte inducing factors are not expressed in the female germ line.

Another interesting point is that transmission forms are obviously not required as the endosymbionts are permanent in the female germ line. In this regard, we are suspicious about the interpretation of Pierantoni (1927) who thought that bacteria released in the lumen of the gut infect the oocytes. To do so, the bacteria would have to cross many barriers, such as the intestine and the vagina, to reach the micropyles of mature oocytes. According to Mansour (1930), the infection of the oocytes is achieved by the bacteria belonging to the apical bacteriome of ovaries. Scheinert (1933) considered the possibility of a double

infection by the primordial germ cells and by the nutritive cords. This last means of symbiont transmission has not been proven yet and, in the absence of any new relevant observations, we have chosen to adopt the theory of a unique contamination of the primordial germ cells in the embryo. It is actually the simplest and the best-illustrated hypothesis.

Endosymbiont control by the host

Endosymbionts are not a transitory infection of the insect cells, and long-term interactions between partners must take place to favor symbiotic maintenance. To our knowledge, a complete demonstration of this phenomenon was made for the first time on *S. oryzae* (Nardon et al., 1998). In fact, we have selected a weevil line with a high density of endosymbionts (110,000±4,000) and another one with a low bacterial density (41,200±3,000). By crossing and backcrossing these two lines, we have provided strong evidence of the existence of a chromosomal factor controlling the number of bacteria per ovary, per egg, and per larval bacteriome. Furthermore, we have conducted irradiation experiments (with X rays) to cause a depressive effect on males, and have shown that the effect was transmissible to the females even though males do not transmit the endosymbiont. Moreover, when oocytes are destroyed, endosymbionts proliferate in the ovarioles, suggesting that the control mechanism is probably exerted in the germ cells.

3. Morphological and Biochemical Interactions

Aposymbiotic strain selection

Among the original features of the *Sitophilus* species is the possibility of obtaining insects devoid of endosymbionts, and it is possible to maintain them as aposymbiotic lines in the laboratory (Nardon, 1973). A treatment of 3 to 4 weeks at 35°C and 90% relative humidity removes the bacteria and some female weevils are resistant to the treatment and lay a small number of sterile eggs. The use of sorghum cereals (instead of wheat) improves the aposymbiotic larval development. It should be noted, however, that a few naturally asymbiotic strains have been found in the field (Mansour, 1930; Schneider, 1956).

We have explored this aspect by comparing the performance of symbiotic and aposymbiotic strains, and have assessed the role of endosymbionts on the insect physiology and development (Nardon and Wicker, 1981; Gasnier et al., 1984; Nardon and Grenier, 1988; Heddi et al., 1993).

Symbiosis and morphology

Currently, it is well documented that endosymbionts modify insect morphology and behavior (Nardon and Nardon, 1998). In *S. oryzae*, two main morpho-genetic changes that have been described are attributed to SOPE absence/presence: the embryonic differentiation of apical bacteriomes and larval bacteriome, and the subsequent bacteriocyte's nucleus polyploidy (Nardon, 1973), and the soft body and light cuticle color of the aposymbiotic adults, particularly in the young weevils. Whereas the former aspect may result from molecular interactions between SOPE and the host nucleus, the latter is likely to result from metabolic interactions.

Effects on nutrition, growth and fertility

Although symbiotic and aposymbiotic weevils consume the same amount of cereals, symbiotic insects grow much better than aposymbiotic insects (Grenier et al., 1986). An efficiency factor of the conversion of digested food (ECD) was defined and was shown to be much higher in symbiotic (17.09±0.20) than in aposymbiotic insects (15.95±0.26). The reason was shown to be mainly the consequence of the supply of growth factors by SOPE to the host. Indeed, investigations using artificial diets have demonstrated that bacteria supply the weevil with as many as five vitamins: pantothenic acid, biotin and riboflavin, provided in sufficient quantities to promote growth, and pyridoxin and folic acid provided in smaller quantities (Wicker, 1983).

Moreover, vitamin supplementation by SOPE was shown to result in a decreased larval development time, 27 days for symbiotic insects versus 44 days for aposymbiotic insects reared at 27°C and 75% relative humidity. Larval development time was also shown to be correlated with SOPE density; the more numerous the symbionts, the faster the development is (Nardon and Grenier, 1988). As an example, the RR strain (with 2.8×10^6 SOPE per larval bacteriome) grows in 29 days, the Sfr strain (with 1.4×10^6 of SOPE) grows in 32 days, and the LL strain (with 9.5×10^5 of SOPE) grows in 35 days. In the absence of endosymbionts, the development time is lengthened by 55% during the first five generations, and about 30% afterwards. Moreover, the elimination of SOPE reduces insect fertility by about 30%, probably because of folic acid deficiency (King and Sang, 1959).

Metabolic interactions

The endosymbionts not only supply the host with some necessary nutrients, but they also interfere with several cellular pathways. In the following sections, we will review three kinds of interactions identified in *S. oryzae* using different approaches.

Amino acid metabolism: Methionine catabolism (Fig. 1)

The elimination of SOPE in *S. oryzae* changes dramatically the amino acid profiles of the whole insect body (Gasnier et al., 1984; Gasnier-Fauchet and Nardon,

Table 1. Genes and functions upregulated in the bacteriocyte cells of *Sitophilus zeamais* weevils.

	Gene description	Cellular function
Biosynthesis and catabolism	Aspartyl-tRNA synthase	Amino acid synthesis
	Ribosomal proteins (60S, S18)	Protein synthesis
	Transcriptional coactivator kohtalo (RNA polymerase II transcription mediator)	
	Eukaryotic translation initiation factor 3, subunit 4	
	Proteasome subunits (26S, Beta)	Protein turnover
	Ubiquitin carboxyl-terminal hydrolase 16	
	Ubiquitin-protein ligase	
	Chymotrypsin B	
	Triacylglycerol lipase, FABP	Lipid metabolism
	Glutamine-dependent NAD+ synthetase	Cofactor synthesis
	Porphobilinogen synthase	
	Malate dehydrogenase, Cytochrome c oxidase II	Mitochondrial metabolism
	Alpha amylases, Beta-glucosidase	Carbohydrate metabolism
	Aldose reductases	
Transport	Sugar transporters	Carbohydrate transport
	Fatty acid binding protein	Lipid transport
	Phosphatidylethanolamine-binding protein	
	Cellular retinaldehyde-binding protein	
Cellular stress	Glutathione S-transferases	Anti stress enzymes
	Lactoylglutathione lyase	
	Glutathione peroxidase	
	Thioredoxin peroxidase	
	Cytochrome P450	
Cell structure and signaling	Alpha-catenin	Cytoskeleton proteins
	Fasciclin	
	Actinin	
	Tubulin	
	Calpain	
	Programmed cell death protein 6 (ALG-2)	Cellular control
	Translationally controlled tumor protein	
	Ras small GTP-ase, Rab type	Vesicle synthesis
	Vacuolar ATPase	
	Peptidoglycan-recognition protein	Innate immunity

These data were obtained from a RT-PCR subtraction approach using the bacteriome tissue RNA as a driver and the whole aposymbiotic larva RNA as a tester (From Heddi et al., 2005).

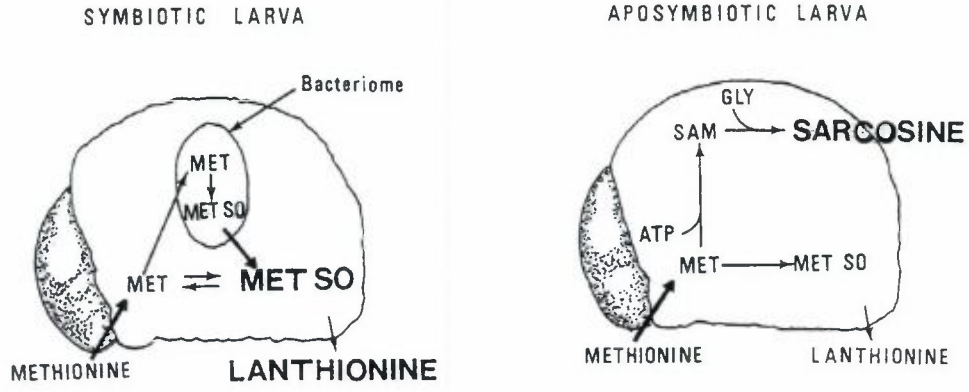

Figure 1. Methionine metabolism pathways in symbiotic and aposymbiotic strains of *Sitophilus oryzae*.

1986, 1987). The most striking differences between symbiotic and aposymbiotic weevil larvae are the level of methionine sulfoxide, which is always present in higher amounts in symbiotic insects, and the level of sarcosine, the concentration of which increases during the last instar in aposymbiotic larvae (43.5 nmoles per aposymbiotic larva, versus 3.8 nmoles per symbiotic larva) (Gasnier-Fauchet and Nardon, 1986).

The reason is related to the separate pathways that catabolise methionine, an amino acid found in excess in wheat, in symbiotic and aposymbiotic larvae. Methionine is transformed into methionine sulfoxide in the bacteriome of symbiotic insects, then eliminated as lanthionine, while aposymbiotic insects fail to carry out this energy-free reaction and instead transform methionine into sarcosine, an ATP-consuming reaction (Fig. 1).

Molecular interactions: Symbiotic and aposymbiotic protein profiles

The first evidence of a SOPE-host molecular interaction in *Sitophilus* was provided by two-dimensional electrophoresis experiments (Charles et al., 1997). This work revealed four differentially expressed proteins, including α, β, γ, and δ. The α is a 30 kD protein that is expressed in the symbiotic strain only. Its origin and function are still unknown; it is either expressed by the weevil genome in response to SOPE, or expressed in SOPE and exported to the host cells. The β protein (33 kD) was visualized specifically in aposymbiotic insects.

The two other proteins (i.e. γ and δ) are encoded by the bacteria. The δ protein was shown to be a chaperonin (GroEL) (Charles et al., 1995), and its expression can represent up to 40% of the total neo-synthesized symbiont proteins.

Although the expression of the GroEL is also up-regulated in other insect symbiotic models, its precise function with respect to symbiosis has not been determined yet. It may help in buffering the effects of deleterious mutations on symbiotic intracellular bacterial genes (Fares et al., 2002).

Mitochondrial energy metabolism interaction

The fact that SOPE interacts with the physiology of the insect, particularly with the larval development time, and that it provides the host with several vitamins involved in cofactor synthesis, has suggested that symbiosis may improve mitochondrial energy metabolism. We have tested this hypothesis by examining and comparing some mitochondrial activities in symbiotic and aposymbiotic insects. As a result, mitochondria isolated from symbiotic insects exhibited high levels of respiratory control ratios (RCR) when compared with aposymbiotic insects (Heddi et al., 1991).

This finding was confirmed by measuring six mitochondrial enzymatic activities, three belonging to the oxidative phosphorylation chain and three others from the Krebs cycle (Heddi et al., 1993). Symbiotic weevils always showed higher specific enzymatic activities, regardless of the insect stage. However, the differences between symbiotic and aposymbiotic weevils are attenuated in the adult phase, at which stage endosymbiont density decreases progressively with age (see above).

Moreover, experiments based on the vitamin complementation of cereals show clearly that differences between symbiotic and aposymbiotic strains are attenuated, or became even non- significant, when insects are reared on vitamin-supplemented pellets (pantothenic acid and riboflavin) (Heddi et al., 1999). These findings led to the conclusion that SOPE improves mitochondrial oxidative phosphorylation and ATP production via nutrition. SOPE interacts with the insect metabolic pathway involved in the biosynthesis of coenzyme A and NAD/FAD, which are required cofactors for mitochondrial enzyme function. This assumption was recently strongly supported by the work of Rio et al. (2003) that has identified, using micro-array technology, several genes encoding vitamin pathways in the SOPE genome.

One interesting consequence of mitochondrial energy metabolism improvement in *Sitophilus* populations is the high flight performance of symbiotic strains. Grenier et al. (1994) have indeed demonstrated that aposymbiotic insects are not able to fly.

However, when aposymbiotic insects are allowed to feed on vitamin-supplemented pellets flight ability is partly restored. These findings highlight the potential impact of symbiosis on insect behavioral traits and indicate that intracellular bacteria could influence the invasive power of insect populations through their interaction with energy metabolism (Heddi, 2003).

4. Genetic and Evolutionary Features in *Sitophilus* Symbiosis

Molecular aspects of the bacteriocytes

While several interacting pathways have been identified in plant symbioses during the last decade, only relatively few data have emerged from insect symbioses. Among the still unknown mechanisms are those involved in bacteriome tissue differentiation and homeostasis. To gain an insight into this aspect, we have explored the molecular dialogue between the symbiont and the host through the identification (with RT-PCR subtraction) of up-regulated genes in the bacteriome of *S. zeamais*, the sibling species of *S. oryzae* (Heddi et al., 2005). Many cellular pathways are induced in response to intracellular bacteria, including metabolism-transport-stress (MTS), cell signalling and trafficking, growth and apoptosis, as well as innate immunity (Table 1). Here, we will focus on two specific aspects that deal with metabolic balance and the role of the innate immunity.

Polyol pathway induction in response to increased sugar transport

During the last two decades, many investigations have been devoted to describing how the association, and in particular the insect, benefits from the bacteria, and often the notion of mutualism was considered from this point of view. However, only few studies have been aimed at analyzing the advantages to the bacteria from the host. The investigation of up-regulated genes in the weevil bacteriocytes has provided clear ideas about the pathways engaged by the host to supply the bacterial metabolism and physiology.

Around 30% of the bacteriocyte subtracted ESTs (expressed sequence tags) were shown to encode genes related to metabolism, transport and stress (MTS) (Table 1).

Among these, a concerted induction of 29 genes has revealed a high carbohydrate metabolism and import/export through the bacteriocyte membranes, including sugar transporters, amylases, glucosidases and aldose reductases. The intimate attachment of the bacteriome tissue to the larval foregut and the high abundance of carbohydrates in cereal grains (70% starch), in which the weevil larvae develop, suggest that the up-regulation of MTS genes may induce an increased sugar flow in the bacteriocytes.

We have subsequently performed biochemical analyses on the bacteriome carbohydrate contents and have confirmed this hypothesis mainly through the accumulation of polyols, such as sorbitol (Heddi et al., 2005).

In mammalian tissues, and particularly diabetic cells, sugar accumulation also induces the polyol pathway that reduces excess glucose to sorbitol via aldose reductases. However, as sorbitol is weakly transported across the membranes, its intracellular accumulation alters the cellular redox potential and promotes cell swelling. In weevils, microscopic examinations did not show any obvious cell abnormality, which raises an interesting question on how the bacteriocyte deals with the increased polyol levels, and the subsequent generated hyper-osmotic stress.

The insect bacteriocyte system may have been selected to promote high sugar uptake regulation to avoid mammalian-like-diseases caused by sorbitol and to maintain cellular homeostasis. The analysis of genes encoded by the endosymbiont has indeed provided evidence that this regulation involves the bacterial metabolism, since SOPE was shown to encode polyol transporters such as the mannitol carrier (Rio et al., 2003).

Interestingly, this phenomenon seems to be common to insects feeding on sugar-rich diets, such as aphids. The genome of the aphid endosymbiont *Buchnera*, which was drastically reduced, also encodes a polyol transporter among the rare transporters retained by this bacterium (Shigenobu, 2000). If polyol synthesis and accumulation also exist in the aphid bacteriocytes this would offer a new approach in understanding the specific cellular traits that help symbiosis to maintain homeostasis in such a particular stress situation.

Permanent induction of the innate immunity in Sitophilus *bacteriocytes*

As opposed to all the other insect endosymbionts sequenced so far, the *Sitophilus* primary endosymbiont possesses two particular characteristics: its genome is not much reduced (Charles et al., 1997), and it encodes functional genes that promote cell invasion (Dale et al., 2002). However, no data are available on the mechanisms used by the insect to perceive the endosymbiont and to control its invasion.

The study on the weevil bacteriocyte cDNA subtraction has revealed for the first time, in insect symbiosis, that innate immunity is involved in the control of intracellular bacteria (Heddi et al., 2005). Several genes related to innate immunity are up-regulated in the bacteriocyte, including a peptido-glycan recognition protein (*PGRP*) gene.

The PGRP proteins are known to trigger the innate immune defense system via the NF-kappa B (NF-κB) pathway. This pathway consists of the activation of proteolytic cascades ending with the induction of synthesis of antibacterial molecules (Hoffmann and Reichhart, 2002). One pathway, called immune deficiency (Imd) (Lemaitre et al., 1996), is currently described in *Drosophila* as being induced in response to injury with Gram-negative bacteria.

In weevils, the high expression of the *PGRP* gene in the bacteriocyte is interpreted as a specific insect immune system response to SOPE, eventually via an Imd-like pathway and antimicrobial molecule production. Nevertheless, one must emphasize that antibacterial molecule synthesis is not in accordance with bacterial survival in the bacteriocytes, unless the molecules produced through the weevil *PGRP* are molecules that control the growth of the endosymbiont instead of being toxic to it.

In addition to the Imd pathway, some *PGRP* genes, such as *PGRP-LB*, are known to interact enzymatically with the bacterial wall. The amidase activity of the PGRP-LB protein has been confirmed (Kim et al., 2003) and has been shown to rely on the conservation of five amino acid residues H^{17}, Y^{46}, H^{122}, K^{128} (or T) and C^{130} (T7 lysozyme numbering). Interestingly, a phylogenetic analysis has indicated that the weevil *PGRP* is orthologous to the *Drosophila PGRP-LB* gene, and its protein sequence contains the above five amino acid residues.

These results suggest that the weevil *PGRP* gene might interact with the endosymbiont growth and control, either through an Imd-like pathway or by acting directly on the bacterial cell wall through the expression of the amidase activity.

5. Phylogeny and Evolution of *Sitophilus* Symbiosis within the Dryophthoridae Weevils

To get an insight into how intracellular bacteria have evolved in the Dryophthoridae weevils, a phylogenetic analysis based on symbiont 16S rDNA sequences was

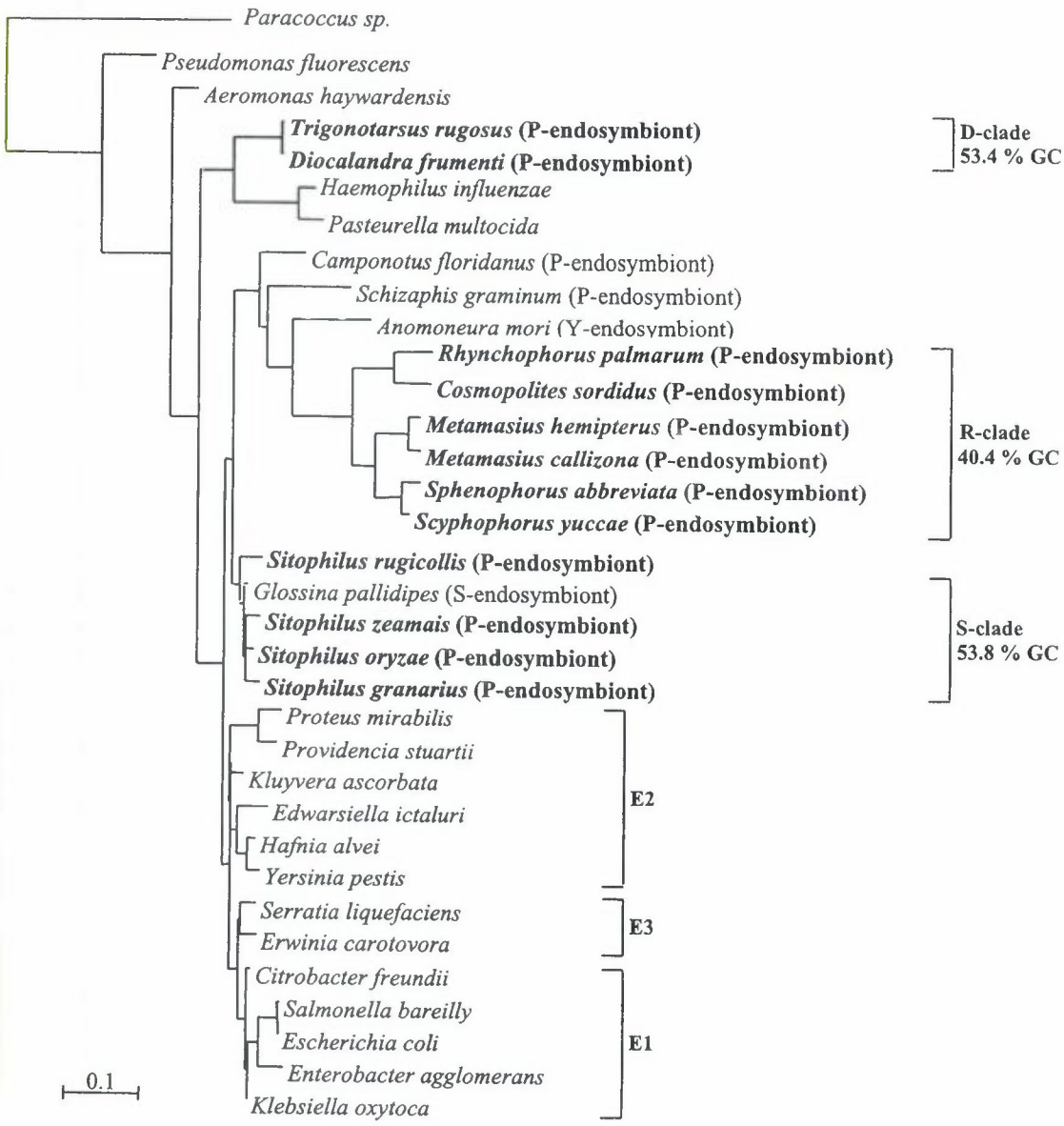

Figure 2. Phylogenetic tree of Dryophthorinae endosymbionts. Maximum likelihood phylogeny, based on the 16S rDNA gene sequence of Dryophthorinae and others insect endosymbionts within the γ-Proteobacteria. E1, E2 and E3: Enterocluster 1, 2 and 3 defined by Ahmad et al. (1990); P-endosymbiont: Principal endosymbiont; S-endosymbiont: Secondary endosymbiont. (From Lefèvre et al., 2004).

performed. Three clades of endosymbionts (named R, S, and D) were separated regardless of the phylogenetic method being employed (Lefèvre et al., 2004), which ensures that the Dryophthoridae endosymbionts are derived from distinct bacterial species established in three ancestral Dryophthoridae species.

The D-clade includes endosymbionts of *Diocalandra frumenti* and *Trigonotarsus rugosus*, the S-clade comprises endosymbionts of *Sitophilus rugicollis*, *S. granarius*, *S. zeamais*, *S. oryzae*, and the R-clade is represented by the endosymbionts of *Rhynchophorus palmarum*, *Cosmopolites sordidus*, *Sphenophorus abbreviata*, *Scyphophorus yuccae*, *Metamasius hemipterus* and *Metamasius callizona* (Fig. 2).

The R-clade comprises 6 out of the 9 Dryophthoridae genera studied and its bacteria exhibit evolutionary features that support a deeply seated symbiosis. R-endosymbionts show much higher relative rates of substitution when compared to both the Enterobacteriaceae and the other insect endosymbionts, and their genomes are AT bias (40.5% GC on the 16S rDNA) and contain AT rich insertions. According to insect fossil data (O'Meara and Farrell personal communication), symbiosis in the R-clade was apparently established 100 million years ago. *Candidatus* Nardonella

was proposed as a name for these putative Dryophthoridae ancestral endosymbionts (Lefèvre et al., 2004).

The S- and the D-endosymbionts have become associated more recently, around 20 million years ago, probably by endosymbiont competition and displacement. Recently, the hypothesis of endosymbiont replacement has gained particular interest in some insect models. Authors have suggested a potential interplay between old-primary and recent-secondary endosymbionts and have noted a beneficial role of secondary endosymbionts for the host fitness in some aphid species (Sabater et al., 2001; Chen et al., 2000), and glossine species (Montllor et al., 2002).

More recently, an interesting finding was described in *Cinara cedri* aphids that harbor *Buchnera* with the smallest and "exhausted" genome (450 MB only). In this aphid species, living on conifers, the secondary endosymbiont was shown to occupy a larger bacteriocyte space than the *Buchnera* (Gomez-Valero et al., 2004), thus supporting the process of serial symbiont acquisition and replacement in the insect world.

The D-clade is closely related to free-living parasitic bacteria, such as *Haemophilus* and *Pasteurella*. This finding suggests that D-clade endosymbionts may have evolved from an ancestor with a parasitic intracellular life cycle. Accordingly, recent investigations on *Sodalis glossinidius*, the secondary endosymbiont of the tsetse flies (Dale et al., 2001), and *S. zeamais* endosymbionts (Dale et al., 2002) demonstrate the presence of functional genes of the type-three-secretion system, closely related to those of the pathogenic bacteria *Salmonella* and *Shigella*. Secretion systems are used for protein pathogen export and toxin delivery to the host cells. Therefore, these results suggest that *Sodalis* spp. and the S-clade endosymbionts share functional infection faculties. Since both bacteria have only recently established their symbiotic relationships with their respective insects, we suggest here that early mechanisms in symbiogenesis may involve pathogenic-like pathways and genes recognized for bacterial invasion and virulence.

6. Conclusion

In the Curculionide beetles, 9 different types of symbiosis have been described (Nardon and Grenier, 1989). In the Dryophthoridae family, most species are symbiotic and *S. oryzae* is considered as a model for studying symbiosis establishment and evolution. Among the original traits of *Sitophilus* spp. is the possibility to select aposymbiotic insects and hence to compare their physiological and cellular performances with symbiotic strains. This particular aspect has largely contributed, in the past, to the understanding of symbiotic interactions in insects and their impact on animal evolution. The presence of SOPE greatly enhances the metabolism performances and changes the behavior of insect populations and their invasive power, particularly through the improvement of energy metabolism (Heddi et al., 1993, 1999; Heddi, 2003). SOPE-weevil interactions and their "incomes" to the association could be viewed as adaptive traits for insect dispersal and diversification (Grenier et al., 1986; Heddi, 2003).

From an evolutionary point of view, endosymbionts could be considered as a "kit of genes" acquired by the host, and then (at least for some of them), domesticated after natural selection (Nardon and Grenier, 1991). This process ends in the creation of a new unit where exhibited phenotypes are not only the result of insect and bacterial genes together, but also the expression of innovative traits from the interaction of a prokaryote-eukaryote (Heddi et al., 2001). The term "symbiocosm" was proposed as a name for the new biological entity created by symbiosis (Nardon, 1999).

Moreover, the phylogenetic position of SOPE and its gene content are also interesting features for studying the mechanisms of symbiosis establishment and evolution. This bacterium has entered into symbiosis with *Sitophilus* spp. only recently (20 MY), and represents the unique insect primary endosymbiont (so far described) to use the type-three-secretion system for cell invasion. Therefore, the consequent question is to consider how these symbiotic associations occur and evolve between bacteria that are potentially pathogenic and insects that are potentially defensive? This is what we are currently aiming to understand through the study of innate immunity-virulence interplay in *Sitophilus* weevils.

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