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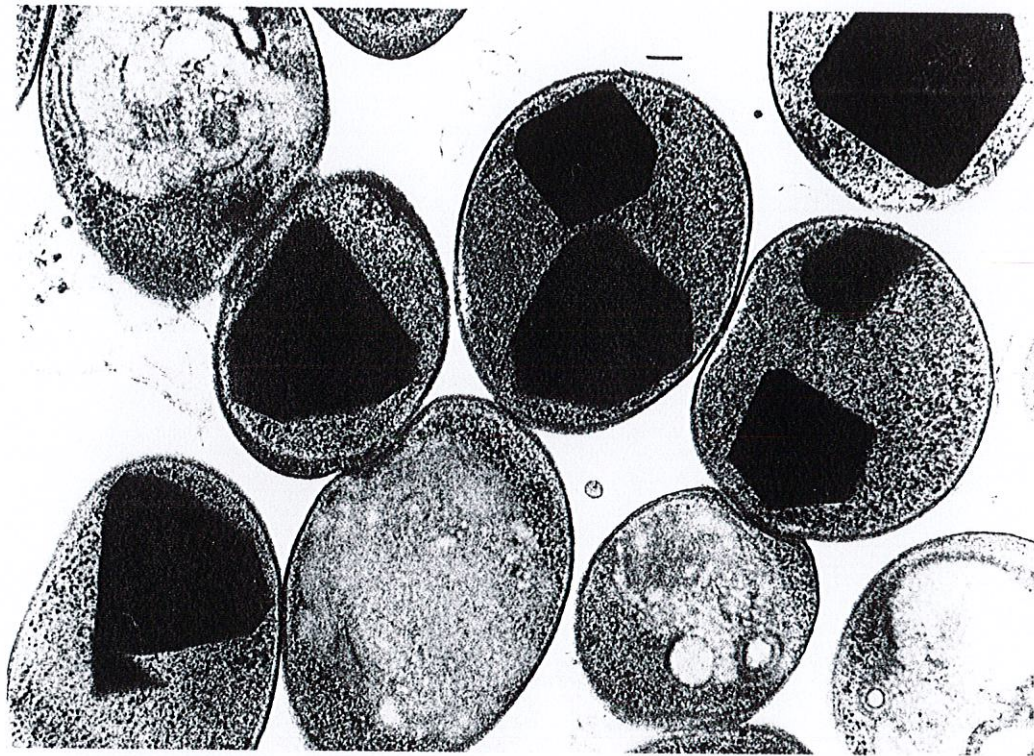


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COST 819

Entomopathogenic nematodes

**Taxonomy, phylogeny and gnotobiological studies of
entomopathogenic nematode bacterium complexes**



*European Commission
Directorate-General
Science, Research and Development*



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COMPARATIVE AND PHYLOGENETIC ASPECTS OF TWO INSECT ENDOSYMBIOSES : APHIDS AND WEEVILS

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Summary

Intracellular symbiosis is currently well documented regarding the role of the endocytobiontes on the metabolism and physiology of their host. Most insects feeding on an unbalanced diet such as phloem sap, seeds, or blood, harbor intracellular prokaryotes which supply the eukaryotic host cells with essential metabolites such as vitamins or amino acids. In the cereal weevils *Sitophilus oryzae*, these bacteria are intimately implicated in the energetic metabolism of their host by synthesizing pantothenic acid, riboflavin, and biotin; and increasing thereby mitochondrial enzymatic activities. In aphids, the intracellular bacteria *Buchnera* shows unusual molecular features allowing potentially increased synthesis of tryptophan and leucine. Some of the genes of the respective biosynthetic pathways are indeed located on plasmids.

Phylogenetic analyses based on the small subunit rDNA (16S) are routinely used to infer evolutionary relationships between bacteria. Such analyses placed both the weevil endocytobionte and *Buchnera* within the gamma group of proteobacteria. However, what remains unclear and questionable is first, the relative positions of these bacteria (and more generally intracellular bacteria) among the nearby range of extracellular bacteria and second, the precise phyletic origins of the known endocytobiontes, because intra- and extra-cellular bacteria were shown to undergo different evolutionary processes. It is for example generally assumed that the G+C content of obligate intracellular bacteria is relatively low, resembling that of cytosolic organelles like mitochondria. This particular aspect has made doubtful the precise phylogenetic positioning of intracellular bacteria, either between each other or within the extracellular bacteria, since the phylogenetic tree profiles are changing with the phylogenetic methods used for tree construction.

Introduction

Intracellular symbiosis (or endocytobiosis) represents the most sophisticated relationship between eukaryotic cells and intracellular microorganisms. This association implies the coordination between associated genomes forming the symbiocosm. The weevil symbiocosm (*Sitophilus oryzae*) involves as much as four interacting genomes: nuclear, mitochondrial, the principal endocytobionte (γ -proteobacteria), and the *Wolbachia* (α -proteobacteria), a non integrated bacterium infecting many strains of the weevils. The aphid symbiocosm is devoid of *Wolbachia*, but harbours a primary endocytobionte called *Buchnera* (Baumann et al., 1995), a secondary endocytobionte in some species and even an additional rickettsia in some populations of the pea aphid *Acyrtosiphon pisum* (Chen et al., 1997). In most insects feeding on unbalanced diets, integrated endocytobiosis often displays the differentiation of specialised host cells or bacteriocytes, packed with bacteria. Bacteriocytes themselves form a tissue (bacteriome), arranged as a packsaddle around the beginning of midgut of the weevil larvae, or localised in the haemolymph of the aphid larvae. In both

weevils and aphids, endocytobionts are transmitted to the progeny exclusively by the oocytes, and are controlled for their number and localisation by the host chromosome, as it was shown in weevils (Nardon et al., 1998). The elimination of *Buchnera* from the aphid results in a drastic decrease of the insect weight and fecundity, but adult viability is not depressed (Rahbé et al., 1993). In weevils, bacterial elimination does not kill the insects either, but impairs many physiological traits. Development time increases by 25-30%, fertility decreases by 15-25% (Nardon, 1973), and adults lose their flying abilities (Grenier et al., 1994). All these physiological disruptions were shown to be related to the metabolic role of *Sitophilus oryzae* principal endocytobiont (or SOPE), which participates for example in the energetic metabolism of its host by increasing mitochondrial enzyme activities (Heddi et al., 1993). In aphids, *Buchnera* is mainly involved in amino acid synthesis and supply to the host (Febvay et al., 1995; Liadouze et al., 1996; Douglas, 1998). This bacterium shows unusual molecular features that should allow increased synthesis of two amino acids, tryptophan and leucine (Baumann et al., 1995). Anthranilate synthase, which is the first and rate-limiting enzyme of the tryptophan pathway, is located on plasmids as multiple tandem repeats of around 3.6Kb in most aphid species (Lai et al., 1996). For leucine, the whole leucine operon is located on a 7.8Kb plasmid (Bracho et al., 1995). Table 1 summarizes the main differences between the weevil and aphid endocytobioses.

Table 1 : Compared features of two insect intracellular symbioses

	(grain) weevil symbiosis SOPE	aphid symbiosis <i>Buchnera</i>
host insect range	Coleoptera, Curculionidae unchecked outside genus <i>Sitophilus</i>	Hemiptera, Aphidomorpha all Aphidoidea, except Phylloxeridae, Adelgidae
host/ symbiont co- evolution	unknown (aged <135 My)	strictly vertical, down to the species level (aged 150-200 My)
transmission	maternal, permanent infection of germ line	maternal, cyclic reinfection of embryos (eggs) at blastod. stage
location of bacteriome	larvae: fore/midgut junction adult: gastric caeca, ovariole apex	nymph: peri-intestinal cell clusters adult: Id.+ embryos or eggs
experimental aposymbiosis	availability of permanent lines (heat-cured or antibiotic- treated)	no stable line available (antibiotic feeding)
microbial lineage	Proteobacteria, γ 3 subdivision Enterobacteriaceae	Proteobacteria, γ 3 subdivision
GC content, % (global)	54 %	ca. 30 %
GC content, % (16S gene)	54 %	50 %

	(grain) weevil symbiosis	aphid symbiosis
	SOPE	<i>Buchnera</i>
number of genes identified	3 (16S, groEL, groES)	>100
gene expression studies (bacterial genes)	overexpression of groEL protein	overexpression of groEL protein rpoH, dnaK, dnaJ, thrB, argA
extrachromosomal elements	138 kb e.c. element	leu, trp (+ 1 heat-shock) plasmids

In conclusion, the metabolic and the physiological roles of intracellular bacteria are quite well investigated not only in weevils and aphids, but also in other endocytobiotic models such as ants, cockroaches and glossine flies. However, although molecular phylogeny recently resolved the global taxonomic positioning of most non-cultivable endosymbionts, some specific features of their molecular evolution (see below) render their fine tuning positioning in bacterial systematics somehow questionable. Important questions in term of evolutionary history of such symbioses still lack unambiguous answers (are some bacterial lineages more likely to have produced insect or invertebrate endosymbionts ? what is the closest free-living relative to a given endosymbiotic microorganism ?...)

The aim of the last part of this paper is to shed light on peculiar evolutionary aspects related to the life history of intracellular bacteria that may influence their phylogenetic positioning by standard molecular methods.

Molecular characteristics of intracellular bacteria

Recent studies have shown that intracellular bacteria undergo different evolutionary processes from the free living bacteria (Moran, 1996; Heddi et al., 1998).

1- Faster Evolutionary rates

By comparing divergence of homologous regions of ribosomal DNA from aphids and *Buchnera*, substitution rates were shown to be 36 times greater for *Buchnera* than for their aphids hosts (Moran et al., 1995). Furthermore, 16S rDNA evolves about twice as fast in *Buchnera* as in related free-living bacterial lineages (Moran, 1996). One of the driving causes of such high evolutionary rates is related to their intracellular life history. Endocytobiotic bacteria are limited to their host space, a situation preventing recombination with other bacterial populations, which is a crucial phenomenon in prokaryotic evolution. Furthermore, they are submitted to a bottleneck effect during their transmission from one generation to the other, with only a small bacterial population infecting the embryo and giving rise to the new symbiotic population at each generation.

2- Mutational bias toward A+T content

It is well documented that most of intracellular bacteria are A+T biased when compared to the extracellular bacteria, as recently reviewed (Heddi et al., 1998). Figure 1 shows that genomic G+C content of intracellular bacteria ranges from 26 to 60%, while the

overall range of intra- and extracellular bacteria is about 25-77%. The mean genomic G+C content is 39.5% for the intracellular bacteria and 56.4% for the extracellular bacteria. In this context, *Buchnera* (30% G+C) and SOPE (54% G+C) have different backgrounds. We may interpret this difference as related to the respective age of these endocytobioses (200-250 My for *Buchnera* while probably less than 135 My for SOPE), but peculiar molecular evolution of SOPE can not be excluded. In free-living prokaryotes with large and/or recombining populations, mutational bias has little effect on DNA compositions because it is overridden by selection, even at sites that are close to neutral. In intracellular conditions, selection may be less effective due to environmental stability. This, combined with a Muller's ratchet in such asexual populations, may lead to a mutational bias affecting substitutions at both neutral and weakly selected sites. An analysis of the evolution of *trp* genes in *Buchnera* and *E. coli* actually confirmed one prediction to such situation, namely that elevated rates of nonsynonymous substitutions occur in *Buchnera* (Moran, 1996). AT bias seems to occur in most insect endosymbioses, with some notable exceptions such as the symbioses of weevils and mealybugs (Moran, 1996; Heddi et al., 1998).

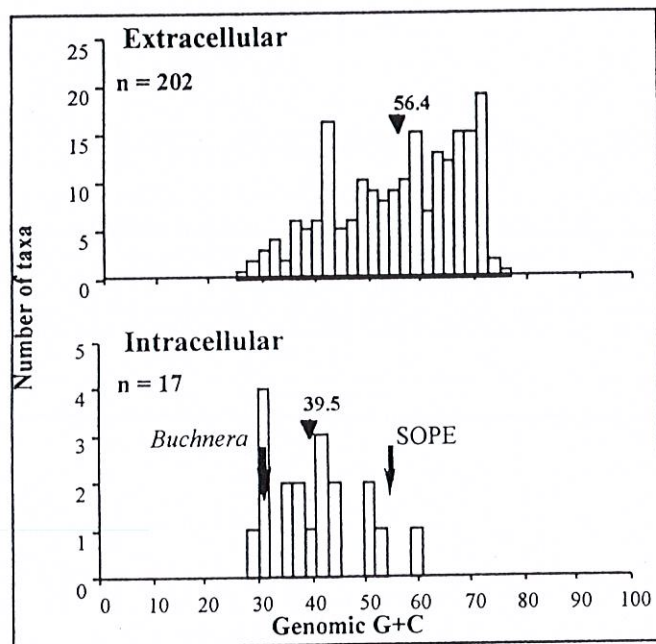


Figure 1: G + C content of genomic DNA of intra- and extracellular bacteria; from Heddi *et al.* (1998)

3- Phylogenetic consequences

Such evolutionary features, characteristic of the life history of intracellular symbioses, pose methodological problems to molecular systematics since they interfere with all basic assumptions underlying phylogeny inference¹. Biases are probably limited when analysing evolutionary relationships within groups, which may be assumed to rely on similar « rules » of molecular evolution (*e.g.* host-symbiont coevolution in aphids); however, unravelling evolutionary relationships between endocytobiontes and their free-living relatives, or between different groups of symbiotic bacteria, may be much more sensitive to such biases. An

¹ which require analogous evolution rates among taxa, among sites, absence of mutational bias...

example of such uncertainties is given by the changes in relative positions of symbiont groups within the γ -proteobacteria, comprising symbionts from nematodes, whiteflies, aphids, weevils and ants (Figure 2). In the global tree extracted from the Ribosomal Database Project (not shown), *E. coli* is given as the closest neighbour to *Buchnera* (among the selected taxa presented in Figure 2, except all ant sequences and one SOPE sequence, absent from the RDP). However, using the same alignment and this restricted set of organisms, phylogeny reconstruction by standard methods results in slightly modified relationships (Fig. 2A), where aphid and ant symbionts are clustered together; finally, choosing a distance more adapted to sequences varying in their GC content (Galtier & Gouy, 1995, 1996) results in another tree topology, which places ant symbionts close to the SOPE lineage (Fig. 2B). Although both trees contain many weak bootstrap sections, their topologies are somehow different (see also whitefly symbiont positioning), stressing the need for a specific study of methodological biases when phylogenies include intracellular bacteria. Although the 16S gene seems well adapted to the analysis of this taxonomic level, information on other sequences may also be quite helpful. Within the protein data available for *Buchnera* for example, some genes might be used for such a purpose in that the inferred phylogenies are compatible with 16S based trees (*groEL* or *ftsZ* proteins), while others show drastic divergence from the standard position (*rnpA* -ribonuclease P- gene, unpublished results). Finally, non-sequence-based information will be needed to strengthen the phylogenies resulting from such expected refined phylogenies (genome organisation, operon structures, gene clustering and gene fusion events). Only such synthetic approaches will allow deciphering the evolutionary histories which led to the establishment of pathogenesis and/or symbiosis from parental free living organisms, and the growing availability of whole genome information is a crucial step towards such a synthesis.

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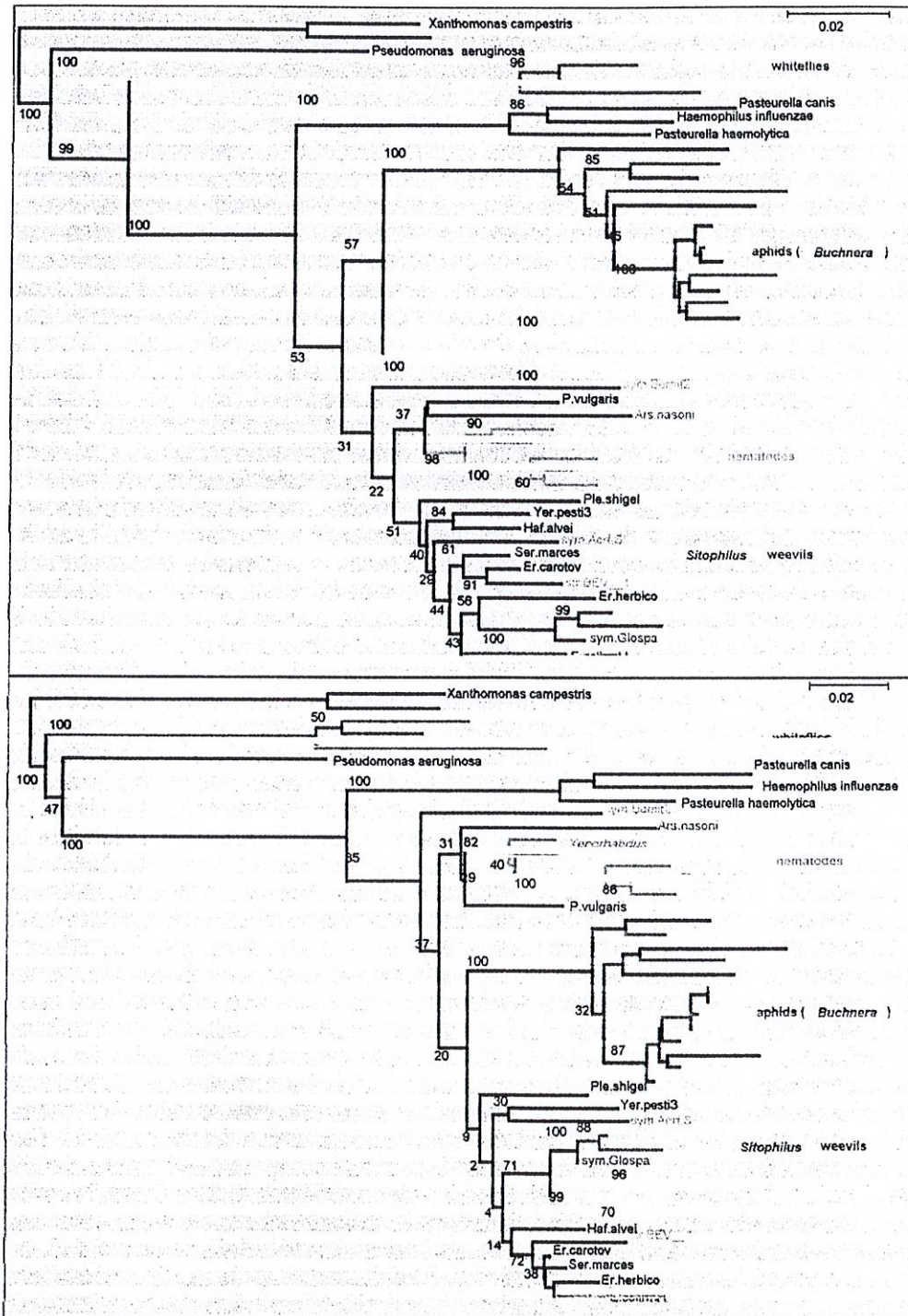


Figure 2 : Neighbour-Joining trees, constructed with 1157 sites in a 16S gene alignment extracted from the ribosomal database (Madiak et al., 1997), showing the variation in positioning of invertebrate endosymbiont taxa within the γ -Proteobacteria, when using either a Kimura distance (A: upper tree) or a Galtier & Gouy distance (B: lower tree); bootstrap values indicated ($n=500$). Sequences for ant symbionts and SOPE were added to the alignment available at the RDP web site (march 1998).

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