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## Accelerated evolution in bacterial endosymbionts of aphids

**Summary** When compared with free living bacteria, it is proposed that there are at least two endosymbiotic processes in aphids based on the A + T content as well as the increased evolutionary rate of the  $\beta$ -subunit of the F-ATPase complex in different endosymbiotic bacteria. The first well established process corresponds to the integration of *Buchnera aphidicola* more than 150 million years ago. The other is postulated to correspond to new endosymbiotic processes in which the bacteria involved contain less A + T and show a lower increase of evolutionary rates when compared with *B. aphidicola*. It is proposed, therefore, that endosymbioses are active processes in aphid evolution.

**Key words** *Buchnera aphidicola* · Endosymbiosis · Relative rate tests ·  $\beta$ -Subunit of F-ATPase complex · Aphids

### Introduction

Aphids and *Buchnera aphidicola* have a symbiotic relationship [1, 2]. Aphids are dependent on *Buchnera* for normal growth and reproduction, whereas they supply *Buchnera* with a constant intracellular environment. Aphids feed on plant phloem sap, a diet rich in carbohydrates but deficient in nitrogenous compounds, including most essential amino acids. It is proposed that *Buchnera* actively provides aphids with amino acids. Several physiological [6–9] as well as genetic studies give support to this hypothesis [3, 13–15, 19]. For instance, it has been demonstrated that *Buchnera* undergoes changes by different aphid species in the biosynthetic pathway of amino acids such as tryptophan and leucine. However, *Buchnera* has retained most of the genes that are present in free living bacteria [1–3, 5, 11].

*B. aphidicola* resides in specialized cells in the aphid hemocoel called bacteriocytes, and it is maternally inherited through controlled infection of eggs and embryos. Additionally, other bacteria have been found in some aphid species; but their location has not been well defined and whose symbiotic role has not yet been determined. They are called secondary endosymbionts.

Based on sequence data from *Buchnera* and morphological and fossil record data from aphids, it has been possible to reconstruct the evolutionary history of the symbiotic association. It is characterized by two major events: (i) the symbiosis has a single origin that took place 150–250 million years ago, and (ii) aphids and symbionts have diverged in parallel.

The objective of the present paper is to summarize some of the genetic changes that *Buchnera* has experienced since its

integration, and also to propose a model of the genetic changes that might be expected when considering ongoing endosymbiotic processes in insects.

### Aphid species, endosymbionts and sequences

Seventeen species corresponding to five aphid families were used in the present study (see Table 1). All these species contained *Buchnera aphidicola* and in some of them we had evidence (see below) based on sequence data that they also had secondary endosymbionts. Table 2 shows a list of aphid species of which we obtained partial sequences of the gene coding for the  $\beta$ -subunit of the F-ATPase complex. The sequences analyzed from 11 aphid species varied between 588 to 609 bp due to two small introns, and they had a constant exon region of 474 bp. Regarding endosymbionts, 15 out of the 18 corresponded to partial sequences of the  $\beta$ -subunit (453 bp each) of *B. aphidicola*, the primary endosymbiont. The other 3 correlated with the  $\beta$ -subunit (also 453 bp each) of secondary endosymbionts. As reference taxa we chose the  $\beta$ -subunit of *Drosophila melanogaster* for the aphid sequences and *Escherichia coli*, *Vibrio alginolyticus* and *Burkholderia (Pseudomonas) cepacia* for the endosymbionts. The accession numbers were X86015, J01594, S47656, and X76877, respectively. These four taxa were abbreviated Dme, Eco, Val and Bce, respectively. Alignments of the sequences are not shown but they are available upon request to the corresponding author.

**Table 1** Classification of the 17 aphid species used in this study according to Heie [10]

Family	Subfamily	Tribe	Species
Thelaxidae			<i>Thelaxes suberi</i>
Pemphigidae	Pemphiginae	Pemphigini	<i>Pemphigus bursarius</i>
			<i>Pemphigus spirotecae</i>
	Eriosomatinae	Eriosomatini	<i>Eriosoma lanuginosum</i>
	Fordinae	Fordini	<i>Geoica</i> sp.
Drepanosiphidae	Phylaphidinae	Phylaphidini	<i>Panaphis juglandis</i>
	Chaitophorinae	Chaitophorini	<i>Chaitophorus leucomelas</i>
Lachnidae	Lachninae	Lachnini	<i>Lachnus roboris</i> <i>Tuberolachnus salignus</i>
	Cinarinae	Cinarini	<i>Cinara pini</i>
Aphididae	Pterocommatinae	Pterocommatini	<i>Pterocomma populeum</i>
			<i>Aphis gossypii</i> <i>Rhopalosiphum padi</i> <i>Schizaphis graminum</i>
	Aphidinae	Macrosiphini	<i>Macrosiphum rosae</i> <i>Myzus persicae</i> <i>Stabicobium latifoliae</i>

## Nucleotide composition

Table 3 shows the A + T content (%) of the b-subunit corresponding to endosymbionts and free living bacteria. It shows a substantial change towards a high A + T content,

especially at third base positions. Additionally, the secondary endosymbionts had an intermediate A + T content. The average number of amino acid changes with respect to *E. coli* varied from 10 to 23, of which on average 65.8% increased their A + T content, 21.1% decreased and 13.1% did not change. On the other hand, the A + T content of the b-subunit of aphids varied between 64.2% and 67.8% with an average value of 65.9%, close to the average value for the b-subunit of *B. aphidicola*. The average A + T contents when base position is considered was also equivalent.

## Relative rate tests

In order to estimate if endosymbionts have increased their evolutionary rate when compared to free living bacteria, a relative rate test has been applied to a triad of species [18]. Prior to the application of the test it is necessary to estimate the number  $K_{ij}$  of nucleotide substitutions per site among sequences *i* and *j*. We have applied Kimura's two parameters method [12, data not shown]. Fig. 1 shows the rationale of the test. As it can be observed, there are two internal nodes or branching points denoted as *O'* and *O*. This method permits the estimation of the rate at which species 1 and 2 have evolved since their divergence (i.e.  $K_{01}$  and  $K_{02}$ ). Finally, an evaluation of whether branches leading to species 1 and 2 are statistically different has been realized [20]. Table 4 is a summary of the results obtained. As can be observed by inspecting the first fifteen relative rate tests, *B. aphidicola* evolved between five to six times faster than *E. coli* since its divergence. Moran et al. [16] and Moran [17] have

**Table 2** Available partial sequences of the b-subunit from the F-ATPase complex

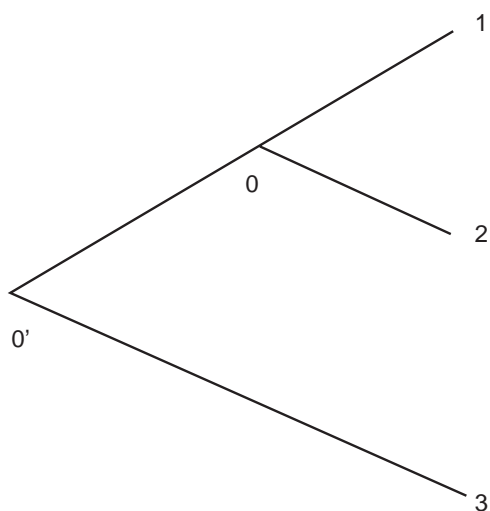
Family	Species*	b-subunit		
		Aphid	<i>Buchnera aphidicola</i>	Secondary endosymbiont
Thelaxidae	<i>Thelaxes suberi</i> (Tsu)	X	X	
	<i>Pemphigus bursarius</i> (Pbu)		X	
	<i>Pemphigus spirotecae</i> (Psp)		X	
	<i>Eriosoma lanuginosum</i> (Ela)	X	X	
	<i>Geoica</i> sp. (Geo)		X	
Drepanosiphidae	<i>Panaphis juglandis</i> (Pju)	X	X	
	<i>Chaitophorus leucomelas</i> (Cle)			X
Lachnidae	<i>Lachnus roboris</i> (Lro)	X	X	
	<i>Tuberolachnus salignus</i> (Tsa)		X	
	<i>Cinara pini</i> (Cpi)			X
Aphididae	<i>Pterocomma populeum</i> (Ppo)	X	X	
	<i>Aphis gossypii</i> (Ago)	X	X	
	<i>Rhopalosiphum padi</i> (Rpa)	X	X	
	<i>Schizaphis graminum</i> (Sgr)**	X	X	
	<i>Macrosiphum rosae</i> (Mro)	X	X	X
	<i>Myzus persicae</i> (Mpe)	X	X	
	<i>Stabicobium latifoliae</i> (Sla)	X	X	

\* The species abbreviation is indicated in parenthesis.

\*\*The sequence has been obtained from Clark and Baumann [4].

**Table 3** A + T content (%) of the  $\beta$ -subunit in *Buchnera aphidicola* of different aphid species, three secondary endosymbionts and two reference taxa

Endosymbiont	Aphid species	A + T content (%)			
		1st base	2nd base	3rd base	Total
<i>B. aphidicola</i>	Tsu	52.3	61.0	84.1	65.8
	Pbu	48.4	61.0	86.7	65.4
	Psp	49.7	60.9	87.4	66.0
	Ela	54.3	60.9	86.8	67.3
	Geo	51.7	59.1	88.9	66.5
	Pju	49.0	60.9	87.3	65.7
	Lro	49.7	61.6	90.7	67.4
	Tsa	49.7	59.6	94.0	67.8
	Ppo	49.1	58.9	88.0	65.3
	Ago	52.4	60.5	82.6	65.1
	Rpa	47.7	61.0	84.2	64.2
	Sgr	47.7	60.3	85.5	64.4
	Mro	49.0	61.6	85.4	65.3
	Mpe	49.1	61.0	86.1	65.3
	Sla	50.3	60.0	91.4	67.2
Average	50.0	60.6	87.3	65.9	
Secondary endosymbiont	Cle	43.4	59.1	62.5	54.9
	Cpi	41.7	60.3	55.6	52.6
	Mro	45.7	59.6	64.0	56.4
	Average	43.6	59.7	60.7	54.6
Outgroup	Eco	38.4	59.6	39.1	45.7
	Val	36.1	60.1	56.9	51.0
	Average	37.2	59.8	48.0	48.3

**Fig. 1** Relative rate test.  $O'$  and  $O$  are internal nodes and 1, 2, and 3 are the corresponding species. Species 3 is taken as the reference taxon. See text for explanation

reported similar results studying the genes *trpEG* and 16S rDNA from *Buchnera* of different species. The difference was lower but still significant when comparing secondary endosymbionts and *E. coli*. Relative rate tests between primary and secondary endosymbionts showed a higher and more significant evolutionary rate of *Buchnera* than secondary endosymbionts.

Finally, (data not shown) among primary endosymbionts some evolutionary rates were higher than others, and there were no significant differences among secondary endosymbionts.

### Do the non-*Buchnera* sequences really correspond to new endosymbionts?

When compared to free living bacteria, both a high A + T content and an accelerated evolutionary rate are two distinguishable features of the integration process into the intracellular life of other organisms, namely bacteria. Accordingly, the intermediate A + T content as well as the evolutionary rates of the non-*Buchnera*  $\beta$ -subunit sequences might be interpreted as active and younger endosymbiotic processes involving other bacteria. Throughout this text, we have considered these organisms as secondary endosymbionts. Contrary to *Buchnera aphidicola*, however, they do not constitute a monophyletic group, and based on different phylogenetic analyses (manuscript in preparation) we have no evidence of a single and new endosymbiotic process. In fact, within this group of non-related secondary endosymbiotic bacteria, the secondary endosymbiont from the aphid *Macrosiphum rosae* has both the highest A + T content and the highest evolutionary rate when compared to *E. coli*. We consider that this bacterium is in a more advanced stage of endosymbiotic integration than the other two endosymbionts. Secondary endosymbionts from a wider range of species are needed, however, to test the hypothesis of single versus multiple new endosymbiotic processes currently occurring in the evolution of aphids.

**Table 4** Relative rate tests to examine the evolutionary rate difference between primary and secondary endosymbionts ( $K_{O1}$ ) with respect to *E. coli* ( $K_{O2}$ )

Aphid species <sup>a</sup>	$K_{I2}$	$K_{I3}$	$K_{I2}-K_{I3}$	$K_{O1}$	$K_{O2}$	$K_{O1}/K_{O2}$ *
Tsu	0.4154	0.4740	0.2763	0.3459	0.0695	4.98
Pbu	0.3900	0.4647	0.2670	0.3285	0.0615	5.34
Psp	0.3883	0.4784	0.2806	0.3345	0.0538	6.22
Ela	0.4524	0.5087	0.3110	0.3817	0.0707	5.40
Geo	0.4081	0.4756	0.2672	0.3377	0.0704	4.80
Pju	0.4446	0.5221	0.3244	0.3845	0.0601	6.40
Lro	0.3829	0.4773	0.2796	0.3313	0.0516	6.42
Tsa	0.3715	0.4802	0.2825	0.3270	0.0445	7.35
Ppo	0.3545	0.4722	0.2745	0.3145	0.0400	7.86
Ago	0.3741	0.4530	0.2446	0.3094	0.0647	4.78
Rpa	0.3566	0.4263	0.2286	0.2926	0.0640	4.57
Sgr	0.3546	0.4353	0.2376	0.2961	0.0585	5.06
Mro	0.3620	0.4422	0.2445	0.3033	0.0587	5.17
Mpe	0.3684	0.4553	0.2576	0.3130	0.0554	5.65
Sla	0.3924	0.4833	0.2855	0.3390	0.0534	6.35
Average	0.3877	0.4699	0.2708	0.3293	0.0585	–
Cle	0.2769	0.3266	0.0939	0.1854	0.0915	2.03
Cpi	0.2247	0.3148	0.0942	0.1595	0.0652	2.45
Mro	0.3140	0.3628	0.1422	0.2281	0.0859	2.66
Average	0.2719	0.3347	0.1101	0.1910	0.0809	–

<sup>a</sup> The first 15 relative tests were carried out using *Escherichia coli* and *Burkholderia cepacia* as free living bacteria ( $K_{23} = 0.1977$ ). The last three relative rate tests with secondary endosymbionts were realized using *E. coli* and *Vibrio alginolyticus* as free living bacteria ( $K_{23} = 0.2206$ ).

\*P < 0.05.

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## References

- Baumann P, Baumann L, Lai CY, Rouhakhsh D, Moran NA, Clark MA (1995) Genetics, physiology, and evolutionary relationships of the genus *Buchnera*: intracellular symbionts of aphids. *Annu Rev Microbiol* 49:55–94
- Baumann P, Moran NA, Baumann L (1997) The evolution and genetics of aphid endosymbionts. *BioScience* 47:12–20
- Bracho AM, Martinez-Torres D, Moya A, Latorre A (1995) Discovery and molecular characterization of a plasmid localized in *Buchnera* sp. Bacterial endosymbiont of the aphid *Rhopalosiphum padi*. *J Mol Evol* 41:67–73
- Clark MA, Baumann P (1993) Aspects of energy-yielding metabolism in the aphid, *Schizaphis graminum*, and its endosymbiont: detection of gene fragments potentially coding for the ATP synthase  $\beta$ -subunit and glyceraldehyde 3-phosphate dehydrogenase. *Curr Microbiol* 26:233–237
- Clark MA, Baumann L, Baumann P (1998) Sequence analysis of a 34.7-kb DNA segment from the genome of *Buchnera aphidicola* (Endosymbiont of aphids) containing *groEL*, *dnaA*, the *atp* operon, *gidA*, and *rho*. *Curr Microbiol* 36:158–163
- Douglas AE (1993) The nutritional quality of phloem sap utilized by natural aphid populations. *Ecol Entomol* 18:31–38
- Douglas AE (1996) Reproductive failure and the amino acid pools in pea aphids (*Acyrtosiphon pisum*) lacking symbiotic bacteria. *J Insect Physiol* 42:247–255
- Douglas AE, Prosser WA (1992) Synthesis of the essential amino acid tryptophan in pea aphid (*Acyrtosiphon pisum*) symbiosis. *J Insect Physiol* 38:565–568
- Febvay G, Liadouze I, Guillaud J, Bonnot G (1995) Analysis of energetic amino acid metabolism in *Acyrtosiphon pisum* - a multidimensional approach to amino acid metabolism in aphids. *Arch Insect Biochem Physiol* 29:45–69
- Heie OE (1980) The Aphidoidea (Hemiptera) of Fennoscandia and Denmark. I. General part. The families Mindaridae, Hormaphididae, Thelaxidae, Anoeciidae, and Pemphigidae. *Fauna Entomologica Scandinavica* 9:1–236
- Ishikawa H (1987) Nucleotide composition and kinetic complexity of the genomic DNA of an intracellular symbiont of the pea aphid *Acyrtosiphon pisum*. *J Mol Evol* 24:205–211
- Kimura M (1983) *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press
- Lai C-Y, Baumann L, Baumann P (1994) Amplification of *trpEG*: adaptation of *Buchnera aphidicola* to an endosymbiotic association with aphids. *Proc Natl Acad Sci USA* 91:3819–3823
- Lai C-Y, Baumann P, Moran NA (1995) Genetics of the tryptophan biosynthetic pathway of the prokaryotic endosymbiont (*Buchnera*) of the aphid *Schlechtendalia chinensis*. *Insect Mol Biol* 4:47–59
- Lai C-Y, Baumann P, Moran NA (1996) The endosymbiont (*Buchnera* sp.) of the aphid *Diuraphis noxia* contains plasmids consisting of *trpEG* and tandem repeats of *trpEG* pseudogenes. *Appl Environ Microbiol* 62: 332–339
- Moran NA, von Dohlen CD, Baumann P (1995) Faster evolutionary rates in endosymbiotic bacteria than in cospeciating insect hosts. *J Mol Evol* 41:727–731
- Moran NA (1996) Accelerated evolution and Muller's ratchet in endosymbiotic bacteria. *Proc Natl Acad Sci USA* 93:2873–2878
- Sarich VM, Wilson AC (1973) Generation time and genomic evolution in primates. *Science* 179:1144–1147
- van Ham RCHJ, Moya A, Latorre A (1997) Putative evolutionary origin of plasmids carrying the genes involved in leucine biosynthesis in *Buchnera aphidicola* (endosymbiont of aphids). *J Bacteriol* 179:4768–4777
- Wu C-I, Li W-H (1985) Evidence of higher rates of nucleotide substitution in rodents than in man. *Proc Natl Acad Sci USA* 82:1741–1745