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SHORT COMMUNICATION

# Low and homogeneous copy number of plasmid-borne symbiont genes affecting host nutrition in *Buchnera aphidicola* of the aphid *Uroleucon ambrosiae*

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**Abstract**

**The bacterial endosymbiont of aphids, *Buchnera aphidicola*, often provides amino acids to its hosts. Plasmid amplification of leucine (*leuABCD*) and tryptophan (*trpEG*) biosynthesis genes may be a mechanism by which some *Buchnera* over-produce these nutrients. We used quantitative polymerase chain reaction to assess the *leuABCD*/*trpEG* copy variability within *Uroleucon ambrosiae*, an aphid with a wide diet breadth and range. Both *leuABCD* and *trpEG* abundances are: (i) similar for aphids across 15 populations, and (ii) low compared to *Buchnera* from other aphid species (particularly *trpEG*). Consequently, the plasmid location of *trpEG* combined with *Buchnera*'s chromosomal polyploidy may functionally limit, rather than increase, tryptophan production within *Uroleucon ambrosiae*.**

*Keywords*: aphid, *Buchnera aphidicola*, endosymbiont, leucine, LightCycler, tryptophan

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## Introduction

Most aphids (Hemiptera: Aphididae) feed exclusively on plant phloem sap, in which nitrogen occurs predominantly as free amino acids. Phloem typically provides insufficient concentrations of the 10 essential amino acids that insects are unable to synthesize *de novo* (Dadd 1985; Sandström & Moran 1999). Aphids receive these scarce nutrients from their bacterial endosymbiont, *Buchnera aphidicola* (Douglas & Prosser 1992; Munson & Baumann 1993; Shigenobu *et al*. 2000). In most bacterial species, the first enzyme in each biosynthetic pathway leading to the essential amino acids leucine and tryptophan (α-ketoisovalerate, *leuA*; and anthranilate synthase, *trpEG*, respectively) is regulated by feedback inhibition and is therefore rate-limited by leucine and tryptophan, respectively (Pittard 1996; Umbarger 1996). Feedback inhibition is an effective energy conservation mechanism because amino acid production is progressively curtailed as intracellular amino acid concentration increases, thus preventing excess accumulation of amino acids (Baumann *et al*. 1997b).

Interestingly, in many *Buchnera* the *trpEG* genes are localized on a plasmid, pTrp (Lai *et al*. 1994). Therefore, *Buchnera* is able to modulate the ratio of *trpEG* relative to single copy chromosomal genes (including *trpDC*(*F*)*BA*; Munson & Baumann 1993) by changing the pTrp copy number, and by changing the number of *trpEG* iterons encoded on pTrp (Lai *et al*. 1994). In addition, genes encoding the entire leucine biosynthetic pathway, *leuABCD*, are also localized on a plasmid, pLeu (Bracho *et al*. 1995). Consequently, *leuA* and *trpEG* can be amplified up to 24-fold relative to single copy chromosomal genes (Lai *et al*. 1994; Thao *et al*. 1998).

Gene amplification is a common strategy for increasing gene expression in bacteria (Romero & Palacios 1997), and this has been proposed as a mechanism by which some *Buchnera* species over-produce leucine and tryptophan for their hosts (Baumann *et al*. 1997b). However, the finding that *Buchnera* cells within the aphid *Acyrthosiphon pisum* each contain > 100 chromosomes (Komaki & Ishikawa 1999) raises the possibility that some *Buchnera* have fewer

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pLeu and pTrp plasmid copies than chromosome copies in each cell. Therefore, the plasmid location could also serve as a means of reducing leucine and tryptophan production.

The plasmid-borne *leuA* and *trpEG* genes of *Buchnera* have been quantified in only two aphids, *Schizaphis graminum* and *Diuraphis noxia*, each represented by a single aphid clone (Lai *et al*. 1994; Lai *et al*. 1996; Thao *et al*. 1998). Therefore, nothing is known about intraspecific *leuA* or *trpEG* gene copy variability within any species (apart from evidence that *trpEG* repeat number per plasmid differs among *A. pisum* clones; Baumann *et al*. 1995; Birkle & Douglas 1999). Although the relative *leuA* and *trpEG* gene quantities reported for *S. graminum* (~24 × and ~15 × amplification relative to single-copy chromosomal genes, respectively) and *D. noxia* (~1 × and ~2 × amplification, respectively) are generally considered species-level measurements, no published studies to date support the assumption that *leuA* and *trpEG* gene quantities are constant within species. On the contrary, amino acids are energetically expensive to produce (Atkinson 1977), thereby probably imposing selective pressure on aphid/*Buchnera* populations to have relative *leuA* and *trpEG* gene copies commensurate with their needs. Also, the chromosomal ploidy of *Buchnera* fluctuates within aphid lifetimes and among aphid morphs (Komaki & Ishikawa 2000), suggesting that the relative copy numbers of these plasmid-borne genes may similarly fluctuate.

Our goal was to assess intraspecific *leuA* and *trpEG* gene copy variability within a single aphid species; we chose the widely distributed North American aphid, *Uroleucon ambrosiae*. The *U. ambrosiae* populations in the eastern USA primarily inhabit one host plant (*Ambrosia trifida*) while southwestern populations inhabit ≥ 20 different hosts (Funk & Bernays 2001). *Uroleucon ambrosiae* is an ideal species within which to assess intraspecific *leuA*/*trpEG* variability because of its: (i) wide distribution, i.e. populations exposed to different environmental conditions may have different growth rates, different amino acid requirements, and different *leuA* and *trpEG* gene ratios; and (ii) wide diet; i.e. different host plants of *U. ambrosiae* probably have different phloem amino acid profiles (Sandström & Moran 1999; Bernays and Klein, personal communication), which may influence aphid amino acid requirements and consequently *leuA* and *trpEG* copy numbers.

Based on molecular markers, *U. ambrosiae* shows no evidence of genetic subdivision in North America (Funk *et al*. 2000). Therefore, if relative *leuA*/*trpEG* copy numbers are genetically mediated, they may be homogenized across this range. However, natural selection does appear strong enough to mould genetic differences between *U. ambrosiae* populations despite extensive gene flow: eastern and southwestern populations differ genetically in diet breadth (southwestern aphids have a more catholic diet; Funk & Bernays 2001) and in the size and number of antennal rhinaria chemoreceptors (southwestern aphids have smaller and fewer rhinaria; Bernays *et al*. 2000). These genetic differences probably result from differential selection linked to host plant prevalence; specifically, suitable hosts are more sporadically distributed and unpredictable in the arid southwest, thus favouring aphids that choose and utilize a variety of plants (Funk & Bernays 2001). In view of these findings, natural selection may also mould inter-population genetic differences in *U. ambrosiae*'s *Buchnera* endosymbiont (*B-Ua*), especially in characters important for the symbiosis and potentially exposed to ecological differences between populations (e.g. numbers of amino acid synthesis genes).

## Materials and methods

### *Collections*

A total of 86 *Uroleucon ambrosiae* individuals were analysed from 15 populations across the United States (Table 1; see this for population abbreviations). These aphids were from the Funk *et al*. (2001) collection (see this for a map of localities), and are composed of fourth-instar nymphs and adult female alates, as well as eggs and embryos dissected from adult female alates from the MI, AZ-5 and VA populations (Table 1). Three embryos from each of two adults ($n = 6$), and three embryos from each of three adults ($n = 9$) were sampled from the MI and AZ-5 populations, respectively (Table 1). Also, three embryos from one adult, and three eggs from each of two adults ($n = 6$) were sampled from the VA population (Table 1). All aphids collected east of the Rocky Mountains were from *Ambrosia trifida* host plants (MD, MI, MN, MS and VA populations), while those west of the Rockies (UT and all AZ populations) were collected from *A. trifida*, *Ambrosia ambrosioides*, *Ambrosia confertiflora*, an unidentified *Ambrosia* sp. and *Viguiera dentata* (Table 1).

### *Gene quantification*

Total DNA was extracted from fourth-instar and adult *U. ambrosiae* individuals using the protocol described by Bender *et al*. (1983), while DNA from eggs and embryos was extracted using the protocol described by Engels *et al*. (1990). The *trpE* and *leuB* (which is linked to *leuA*, and therefore an appropriate analogue) gene quantities were calculated relative to a single copy chromosomal gene, *trpB*, using real-time quantitative PCR on a LightCycler (Roche Molecular Biochemicals). We used the following primers to amplify each target gene: *trpE* (372 bp): TCTTGCTCGTATTTGTGAAC and ATTTCTTCTGTCGCTATTGAG, *leuB* (377 bp): CTTCCAATAGATAAGCGTCC and ACTTTTTGAACAGTTTCTCTCC, and *trpB* (370 bp):

**Table 1** Collection information for the aphids used in this study, including the location of each population, the host plant from which collections were made, and the life history stage and number of individuals analysed; see Funk *et al*. (2001) for a map of these collection locations

| Population abbreviation | Collection location | Host plant | Life history stage | *n* |
|---|---|---|---|---|
| MD | Maryland, Montgomery Co., Silver Spring | *Ambrosia trifida* | adult | 3 |
| MI | Michigan, Wayne Co., Plymouth | *Ambrosia trifida* | embryo | 6 |
| | | | 4th-instar | 3 |
| MN | Minnesota, Ramsey Co., St Paul | *Ambrosia trifida* | adult | 3 |
| MS | Mississippi, Madison Co., Jacksonville | *Ambrosia trifida* | 4th-instar | 3 |
| | | | adult | 3 |
| VA | Virginia, Albermarle Co., Charlottesville | *Ambrosia trifida* | egg | 6 |
| | | | embryo | 3 |
| | | | adult | 5 |
| AZ-1 | Arizona, Santa Cruz Co., Arivaca | *Ambrosia trifida* | adult | 6 |
| AZ-2 | Arizona, Cochise Co., St David | *Ambrosia trifida* | adult | 6 |
| AZ-3 | Arizona, Santa Cruz Co., Patagonia | *Ambrosia trifida* | adult | 6 |
| AZ-4 | Arizona, Santa Cruz Co., Tubac | *Ambrosia trifida* | adult | 3 |
| AZ-5 | Arizona, Pima Co., Tucson | *Ambrosia ambrosioides* | embryo | 9 |
| | | | adult | 3 |
| AZ-6 | Arizona, Pima Co., Catalina State Park | *Ambrosia ambrosioides* | adult | 3 |
| AZ-7 | Arizona, Cochise Co., Rt. 90, San Pedro River | *Ambrosia confertiflora* | adult | 3 |
| AZ-8 | Arizona, Santa Cruz Co., Tubac | *Ambrosia confertiflora* | adult | 6 |
| AZ-9 | Arizona, Pima Co., Tucson | *Viguiera dentata* | adult | 3 |
| UT | Utah, Millard Co., Cove | *Ambrosia* sp. | adult | 3 |

CCAGTAAAAAATGGTTCAGG and CGAAATAAATG-CCAGTTCTAC. Each 20 μL LightCycler reaction contained 10 ng template DNA, 0.5 μM of each primer, and either 3.5 mM (*trpE*), 4.0 mM (*trpB*), or 5.0 mM (*leuB*) $MgCl_2$ in 1 × LightCycler-DNA Master SYBR Green I (Roche Molecular Biochemicals). The amplification profile for all reactions was 95 °C for 1 min; 40 cycles of 95 °C for 0 s, 56 °C for 8 s, 72 °C for 21 s; followed immediately by a melting curve analysis of 95 °C for 0 s, 55 °C for 15 s, 95 °C for 0 s with a 0.2 °C/s transition rate.

External standard curves were generated for each gene to quantify gene copy number in experimental samples. Each standard was produced by transforming competent *Escherichia coli* cells with a plasmid (pGEM-T Easy vector; Promega) carrying a single copy of each respective target PCR product generated on a standard thermalcycler (Sambrook *et al*. 1989). We subsequently isolated plasmid DNA using a QIAprep Spin Miniprep kit (Qiagen), and confirmed that our plasmid minipreps carried one copy of our target amplicons by sequencing each at the Genomic Analysis and Technology Core Facility at the University of Arizona. We calculated the plasmid copy number in each miniprep by taking three replicate spectrophotometer readings (Eppendorf BioPhotometer), and converting the mean absorbance to target copy number per μg DNA. We then made two independent serial dilutions for each target gene and generated the external standard curve by performing LightCycler reactions containing templates from both dilution series. Experimental samples were calibrated to the standard curve using a $10^6$-copy standard, and the LightCycler software was used to calculate the copy number in each sample.

We were unable to consistently amplify the *leuB* amplicon in *U. ambrosiae* eggs or embryos, possibly because some component of the egg/embryo extraction buffer makes this reaction unsuitable on the LightCycler (e.g. Goerke *et al*. 2001). However, all other reactions worked consistently, and each produced only a single melting peak corresponding to the target amplicon.

We compared *trpE* : *trpB* ratios (TE; the number of *trpEG* genes for each single copy chromosomal gene) and *leuB* : *trpB* ratios (LB; the number of *leuB* genes for each single copy chromosomal gene) between eastern and southwestern populations using Kruskal–Wallis nonparametric one-way analyses of variance (ANOVAs) computed using JMP software (SAS Institute 1996).

## Results and discussion

The LBs and TEs for all individuals ranged from 0.5 to 2.8 (mean 1.6) and from 0.3 to 1.9 (mean 0.6), respectively (Fig. 1), and the mean ratios for nearly all populations were 1.0–2.0 and < 1.0, respectively. Although LB and TE both exhibited roughly six-fold differences among individuals, the absolute ratios were quite similar for all the aphids sampled compared to the range of values reported across

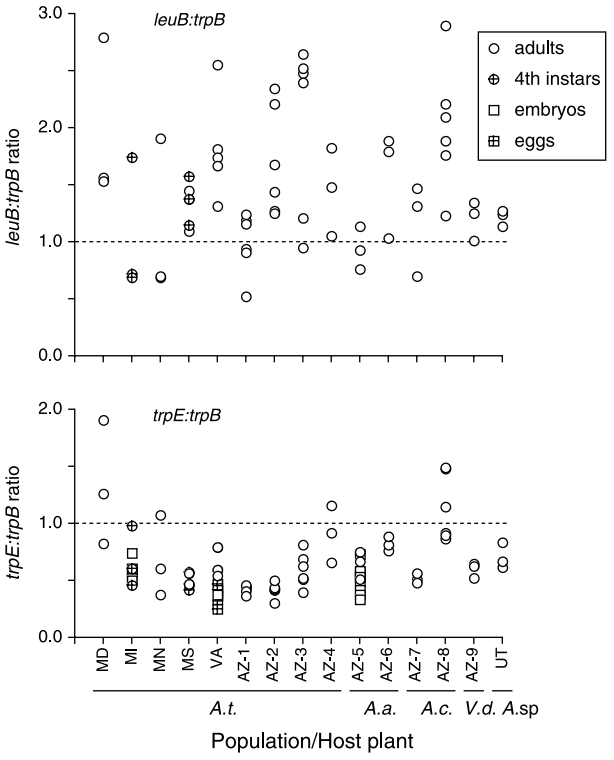


**Fig. 1** The ratios of *leuB* : *trpB* (LB) and *trpE* : *trpB* (TE) for each *Uroleucon ambrosiae* individual. The host plant for each population is also identified (host plant abbreviations: *A.t.*, *Ambrosia trifida*; *A.a.*, *A. ambrosioides*; *A.c.*, *A. confertiflora*; *V.d.*, *Viguiera dentata*; and *A.*sp., *Ambrosia* sp.). The dotted line in each graph represents the ratio when the quantities of the two respective genes are equal. The population abbreviations are as in Table 1.

**Table 2** The number of plasmid-borne *trpEG* and *leuABCD* gene copies for each single copy chromosomal gene within *Buchnera* of different aphid species

| Aphid host | *trpEG* | *leuABCD* |
|---|---|---|
| *Diuraphis noxia* | 1.8* | 0.9† |
| *Schizaphis graminum* | 14.5‡ | 23.5† |
| *Uroleucon ambrosiae* | 0.6 | 1.6 |

*Lai *et al*. (1996); †Thao *et al*. (1998); ‡Lai *et al*. (1994).

aphid species (Table 2). No eastern vs. southwestern geographical LB or TE differences existed (LB comparison: $\chi^2 = 0.027$, d.f. = 1, $P = 0.868$; TE comparison: $\chi^2 = 0.828$, d.f. = 1, $P = 0.363$). Our limited data set precludes robust comparisons among aphid life stages, host plants, or individual populations, although none of these factors exhibited consistent and unique LB or TE ratio trends (Fig. 1). Instead, the LB and TE variances largely resulted from individual differences within populations (Fig. 1). Some populations exhibited high LB and/or TE variability relative to others (e.g. MD vs. UT) (Fig. 1), which may simply result from stochasticity because of our small sample sizes, or may reflect ecological or genetic differences among populations.

The *B-Ua* TEs are the lowest yet reported for any *Buchnera* species (Table 2), with values less than 1.0 for nearly all individuals (Fig. 1). This is the first reported case of fewer pTrp plasmid copies than chromosome copies in the cell. This situation is possible because *Buchnera* displays chromosomal polyploidy (Komaki & Ishikawa 1999). Consequently, compared to many other *Buchnera* species that may elevate tryptophan production by amplifying *trpEG* on multicopy plasmids (Baumann *et al*. 1997b), *B-Ua* may limit tryptophan biosynthesis by reducing pTrp copy number below the copy number of the chromosome. This suggests that *Uroleucon ambrosiae* may not rely heavily on *Buchnera* for tryptophan provisioning, possibly because they may receive much of what they need from their host plants. Tryptophan is the most metabolically expensive amino acid to produce (Atkinson 1977), so selection may be strong within aphid/*Buchnera* populations to adjust *trpEG* ratios to tryptophan needs. Several other aphid/*Buchnera* species also have reduced numbers of functional *trpEG* relative to chromosomal genes (Lai *et al*. 1996; Baumann *et al*. 1997a; van Ham *et al*. 1999; Wernegreen *et al*. 2001). However, in these species, *trpEG* reduction occurs through pseudogene formation, with retention of only a single functional *trpEG* copy per plasmid. Similar to TE, the *B-Ua* LBs are low compared to *Buchnera–S. graminum* (Table 2), also suggesting that *U. ambrosiae* individuals may require relatively little additional leucine.

Interestingly, *trpEG* and *leuABC* genes exhibit accelerated evolutionary rates relative to two housekeeping genes (*dnaN* and *tuf*) in *Buchnera* within *Uroleucon* spp. aphids (Wernegreen *et al*. 2001). Therefore, *trpEG* and *leuABC* may experience relatively relaxed selection within these taxa, possibly because *Uroleucon* species are not as dependent on *Buchnera* for tryptophan and leucine provisioning as are other aphid species (Wernegreen *et al*. 2001).

The *Buchnera leuA* and *trpEG* locations on plasmids have been proposed as adaptations to improve amino acid provisioning to aphid hosts (Baumann *et al*. 1997b). Plasmid location allows gene amplification independent of chromosomal genes (Lai *et al*. 1994; Thao *et al*. 1998), thereby potentially increasing leucine and tryptophan production. Molecular phylogenetic studies show that both *trpEG* and *leuABCD* were transferred from the chromosome to each respective plasmid only one or a few times in ancestral aphid lineages (van Ham *et al*. 1999; Sabater-Muñoz *et al*. 2002). Ecological pressures and nutritional needs at the time of these transfers were almost certainly very different from those experienced by many modern lineages: for example, the transfer of *trpEG* likely occurred while aphids were confined to conifers as hosts (i.e. before the acquisition of

angiosperms as hosts). Extant aphid species are extremely diverse biologically and ecologically (Dixon 1985), and some may require relatively little leucine or tryptophan provisioning from their endosymbionts. *Uroleucon ambrosiae* appears to be one such species. In *B-Ua*, the plasmid location of *trpEG* combined with chromosomal polyploidy may function as a mechanism to limit, rather than increase, overall tryptophan production.

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Research in the Moran laboratory primarily focuses on the evolution and ecology of endosymbiotic bacteria and their insect hosts.

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