Parallel Evolution in the Integration of a Co-obligate Aphid Symbiosis

Highlights
- Aphids have independently evolved dependence on *Serratia symbiotica* at least 4 times
- The integration of the new co-obligate symbiont proceeds in a predictable manner
- Loss of the riboflavin and peptidoglycan pathways in *Buchnera* leads to co-dependence
- Amino acid synthesis is taken over by *Serratia* in a second phase of complementarity

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In Brief
Dependence on multiple nutrient-provisioning symbionts has evolved numerous times in insects. Monnin et al. provide evidence from the symbionts of aphids that these dependencies evolve in a predictable manner. The repeated losses of the same metabolic pathways bind the symbionts into co-dependence, and integration follows in a stepwise manner.
Parallel Evolution in the Integration of a Co-obligate Aphid Symbiosis

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SUMX

Insects evolve dependence—often extreme—on microbes for nutrition. This includes cases in which insects harbor multiple endosymbionts that function collectively as a metabolic unit [1–5]. How do these dependences originate [6], and is there a predictable sequence of events leading to the integration of new symbionts? While co-obligate symbioses, in which hosts rely on multiple nutrient-provisioning symbionts, have evolved numerous times across sap-feeding insects, there is only one known case in aphids, involving \textit{Buchnera aphidicola} and \textit{Serratia symbiotica} in the Lachninae subfamily [7–9]. Here, we identify three additional independent transitions to the same co-obligate symbiosis in different aphids. Comparing recent and ancient associations allow us to investigate intermediate stages of metabolic and anatomical integration of \textit{Serratia}. We find that these uniquely replicated evolutionary events support the idea that co-obligate associations initiate in a predictable manner—through parallel evolutionary processes. Specifically, we show how the repeated losses of the riboflavin and peptidoglycan pathways in \textit{Buchnera} lead to dependence on \textit{Serratia}. We then provide evidence of a stepwise process of symbiont integration, whereby dependence evolves first. Then, essential amino acid pathways are lost (at \textasciitilde 30–60 mya), which coincides with the increased anatomical integration of the companion symbiont. Finally, we demonstrate that dependence can evolve ahead of specialized structures (e.g., bacteriocytes), and in one case with no direct nutritional basis. More generally, our results suggest the energetic costs of synthesizing nutrients may provide a unified explanation for the sequence of gene losses that occur during the evolution of co-obligate symbiosis.

RESULTS

Independent Transitions to Co-obligate Symbiosis

Sap-feeding insects have provided elegant case studies of the evolution of co-obligate symbioses, whereby organisms harbor multiple endosymbionts that function collectively as a metabolic unit. These include species of mealybugs that depend on endosymbionts, which in turn harbor their own endosymbionts, and cicadas, in which one symbiont has fragmented into distinct but interdependent lineages [1–5]. What processes drive multiple microbial species to join into co-obligate symbioses [6], and, more generally, is there a predictable, deterministic sequence of events leading to the genomic and anatomical integration of new symbionts?

The aphids are an ideal lineage to study early-stage co-obligate symbioses. The majority of aphid species harbor a single obligate symbiont, \textit{Buchnera aphidicola}, and a second non-obligate symbiont \textit{Serratia symbiotica} (hereafter referred to as \textit{Buchnera} and \textit{Serratia}, respectively). While \textit{Serratia} is found at intermediate frequencies in numerous aphid species, the symbiont has transitioned to a co-obligate relationship with \textit{Buchnera} in the Lachninae subfamily [7–9]. Such co-obligate functioning is marked by \textit{Buchnera}’s losing metabolic capabilities, namely the ability to synthesize the essential nutrients riboflavin and, in some species, tryptophan [10]. Our aim was to determine whether (1) other cases of obligate co-dependences have arisen across the aphids, and (2) to ask whether these transitions followed predictable genomic, metabolic, and anatomical trajectories. Such patterns can provide insight into the evolutionary processes that have led to the genome structure of more ancient multi-partner symbioses [6].

Using data on the symbiont prevalence in 131 aphid species from [11], we identified species that carry \textit{Serratia} at a high frequency, and then tested aphid populations in both the United Kingdom and the Netherlands for obligate dependence on the symbiont. We defined species as having evolved obligate reliance on \textit{Serratia} if (1) all individuals within populations carry the symbiont and (2) they experience a significant fitness reduction when the symbiont is removed. Symbionts that do not meet these criteria are referred to as “facultative,” as they are not essential for host survival. We screened for the presence of \textit{Serratia} using PCR and measured dependence by “curing” individual aphids with antibiotics that selectively removed \textit{Serratia} without affecting \textit{Buchnera}, and then counted their total offspring to determine the lifetime fecundity of the aphids in the presence and absence of the symbiont.

We identified ubiquitous \textit{Serratia} symbioses in seven aphid species (Table S1), representing three independent co-obligate transitions in \textit{Microphilum camosum}, \textit{Aphis urticae}, and in the \textit{Periphyllus} genus. In the \textit{Periphyllus} genus, \textit{Serratia} was consistently present in the five species we surveyed, and we
confirmed obligate dependence via curing Serratia in both P. hirticornis and P. lyropictus. These data suggest a single transition into an obligate relationship with Serratia at the origins of the Periphyllus genus (see below). Curing had the most dramatic effect in species of the Periphyllus genus, potentially reflecting a longer-term evolutionary association with Serratia (Figure 1A). We confirmed that the antibiotic treatments had no significant effect on the fecundity of our control aphid species Acyrthosiphon pisum, which harbors Serratia as a facultative symbiont, and the uninfected Macrosiphoniella artemisiae (Figures 1A and S1; Data S1A). Likewise, we confirmed with qPCR that the antibiotic treatment reduced Serratia density (Figure 1B; Data S1B), but did not reduce Buchnera density (Figure 1C; Data S1B).

We next estimated the origins of obligate dependence on Serratia using deep coverage 16S amplicon sequencing from our field-collected populations and previous data on Serratia associations in aphids [11]. First, we found evidence of a more ancient relationship between Serratia and aphids in the Periphyllus genus: amplicon sequencing confirmed that Serratia was absent from Chaitophorus aphids, a sister lineage to the Periphyllus clade (Data S1C). This suggests that dependence on Serratia originated at the divergence of these 2 genera an estimated 63–79 mya (Figures 2 and S2). Second, we found evidence of more recent origins of Serratia obligate dependence (<30 mya) in A. urticata and M. carnosum. Specifically, Serratia was either absent or present only as a facultative infection in some individuals in the species of A. idaei and A. fabae related to A. urticata. Lack of obligate dependence was likewise confirmed in Macrosiphum euphorbiae and A. pisum, related to M. carnosum.

Genomic Basis of Buchnera-Serratia Metabolic Complementarity

We then asked whether evolving obligate dependence on Serratia was associated with a consistent genomic signature in aphids, and more specifically whether Buchnera-Serratia metabolic complementarity originates in a predictable manner across host lineages. We obtained whole-genome sequencing data for M. carnosum, A. urticatasymbiont; aphid; co-obligate;Serratia
symbiotica; Buchnera aphidicola; evolution of dependence; metabolic complementation, and three Periphyllus species. We then used previously published data from the Lachninae subfamily (Cinara cedri, C. tujafilina and Tuberolachnus salignus) to compare the gene losses in Buchnera from the four independent transitions into an obligate relationship with Serratia. This included the three new cases of co-obligate dependence identified here, and the previously identified cases in the Lachninae subfamily. Our analysis centered on the pathways and genes involved in essential nutrient provisioning to the host (Data S1D). Specifically, we focused on pathways that have experimental evidence for being essential for the aphid: riboflavin [14] and essential amino acids [15–21]. Of particular interest was the riboflavin pathway in Buchnera, as the loss of this pathway has been hypothesized to trigger the dependence on Serratia in the Lachninae aphids [10].

We found a consistent signature for the loss of the riboflavin pathway of Buchnera in both M. carnosum and aphids in the Periphyllus genus (Figure 3). In M. carnosum, Buchnera is missing one gene, part of the ribD complex, which is essential to the riboflavin pathway. In the Periphyllus genus, by contrast, the full pathway is missing, as it is in the Lachninae subfamily. Previous work in the Lachninae aphids suggests that Buchnera has also lost the capacity to synthesize the amino acid tryptophan in certain species (e.g., C. cedri and T. salignus). We find similar losses in the Periphyllus lineage. Here, the majority of genes in the tryptophan pathway have either been lost or pseudogenized, and orthologous gene copies, which are predicted to serve the same function, have been retained in the Serratia genome (Figure 3). Conversely, the tryptophan pathway has been retained in the Buchnera genomes of both M. carnosum and A. urtica, the more recent co-obligate relationships. This result suggests an advanced stage of functional losses in Buchnera of Periphyllus aphids, further supported by losses in several additional amino acid pathways that also appear to have been taken over by Serratia.

In contrast, Buchnera has retained the complete pathways to synthesize all of these essential nutrients in A. urtica. This is surprising, given the consistency of gene losses in Buchnera of M. carnosum, aphids in the Periphyllus genus, and the Lachninae aphids, all which are co-obligately dependent on Serratia. Compared to aphid lineages in which Buchnera is the sole...
obligate symbiont (A. pism, Myzus persicae, and Aphis glycines), the co-obligate association of Serratia and Buchnera in A. urticae has only six genes missing in Buchnera, in which there are orthologous gene copies in Serratia. None of the six genes has direct links to essential nutrient pathways (see Table S2 for more detail). This suggests that co-obligate dependence can arise in this system through alternative starting points, including non-nutritional pathways.

General genomic features of the different Buchnera strains likewise support the hypothesis that the co-obligate Serratia symbioses found in Lachninae and Periphyllus aphids are more ancient compared to M. carnosum and A. urticae (Figure S3). Both the genome size and GC content of Buchnera are highly reduced in the Lachninae and Periphyllus clades, which is suggestive of a more advanced degree of degradation. Gene redundancies are also indicative of the age of the co-obligate associations. In M. carnosum and A. urticae, the genomes of Serratia and Buchnera still contain a significant number of the same genes involved in synthesizing nutrients that are essential for the host aphid (72.5% and 39.2%, respectively). Conversely, in the Periphyllus lineages, both P. acercola and P. aceris have only 11.5% gene redundancy between the two symbionts. In P. lyropicuts, there is a 47.1% overlap. The higher redundancy in P. lyropicuts is likely due to Serratia being recently replaced by another Serratia strain within this aphid lineage.

As Buchnera-Serratia complementarity could also arise through pathways that are not essential for host nutrition, we investigated genes involved in additional pathways involved in translation, as it underlies essential functions in all bacteria [22] and peptidoglycan synthesis, which has been shown to be important in other symbiotic systems [23]. We also investigate the pathways to synthesize the precursors chorismate, homoserine, and vitamins B6 and B2, and lipoic acid. The additional B vitamins showed similar patterns of metabolic patchwork to riboflavin. However, genes to complete the pathways are either absent in both symbiont genomes (B6 in P. urticae and the Lachninae) or are missing where Buchnera is the sole symbiont (B6 in A. pism), so it is unclear whether these vitamins are essential for the symbiosis. Genes involved in translation, chorismate, homoserine, and lipoic acid were for the most part conserved in all of the Buchnera genomes (Data S1E). The pathway for peptidoglycan, however, was entirely lost in the ancient co-obligate Buchnera (Lachninae and Periphyllus aphids). In contrast, only one or two genes were missing in the most recent lineages of M. carnosum and A. urticae. In all cases, the missing genes to synthesize peptidoglycan in Buchnera were retracted on the Serratia genomes. This suggests that Serratia may co-obligate because of its contributing role to the peptidoglycan synthesis of Buchnera, in addition to providing nutrients to the host. The only gene consistently missing in the peptidoglycan pathway from all co-obligate Buchnera, including M. carnosum and A. urticae, was murF. However, it is unclear whether this gene is essential for Buchnera as it is pseudogenized in A. glycines, which is a species not known to host any obligate symbiont other than Buchnera.

Anatomical Integration in Co-obligate Symbiosis

Lastly, we studied the abundance and localization of symbionts within their host to look for anatomical signatures of co-obligate symbiosis. Likewise, we expected that a greater degree of metabolic reliance on Serratia in the Periphyllus aphids would correspond with greater anatomical integration—for example, through the formation of a specialized organ (bacteriome) to house Serratia. To test this idea, we performed fluorescent in...
ancient and recent associations in aphids, we find strong evidence of integration observed in ancient associations. By comparing intermediate steps leading to the extreme genomic and anatomical organization, processes that initiate dependence and provide insight into the recent associations are needed to reveal the evolutionary history. Most co-obligate symbioses are ancient. Data on numerous times in the evolution of eukaryotes. However, the vast majority of co-obligate associations have originated in the more recent past, with dependence on multiple co-obligate symbionts having originated several times in the same eukaryote lineage.

**DISCUSSION**

Dependence on multiple co-obligate symbionts has originated numerous times in the evolution of eukaryotes. However, the vast majority of co-obligate symbioses are ancient. Data on recent associations are needed to reveal the evolutionary processes that initiate dependence and provide insight into the intermediate steps leading to the extreme genomic and anatomical integration observed in ancient associations. By comparing ancient and recent associations in aphids, we find strong evidence that the mechanisms initially binding symbiotic partners in obligate relationships occur in a deterministic, predictable manner. Specifically, we find that dependence on *Serratia* originates through parallel evolutionary trajectories marked by repeated losses of the same nutrient pathways in *Buchnera* across multiple host lineages. Our genomic and FISH data show stepwise processes of symbiont integration, with the losses of essential amino acid pathways occurring between 30 and 60 million years after the co-obligate symbiosis evolves.

In contrast to high anatomical integration in the *Periphyllus* aphids, we find that both *M. carnosum* and *A. urticae* exhibit minimal integration of *Serratia* in host lineages corresponding to a greater reliance on *Serratia* (*Figure 4*). In the most extreme case, we found that the *Periphyllus* aphids evolved a large organ (bacteriome) containing numerous bacteriocytes to house *Serratia* in their abdomens (*Figure 4*).

We likewise expected *Serratia* abundance within the aphid to increase as the symbiont takes on a more metabolically demanding role. Here, we used qPCR to quantify the copies of *Serratia* genomes compared to the host aphid. In line with our predictions, we found a substantial increase in the abundance of *Serratia* that coincided with its greater metabolic role of synthesizing amino acids. Specifically, we found that the ratio of *Serratia* to host genome copies increased dramatically to an abundance ratio of 512:1 in *P. hirticornis*. This is compared to a 3:1 ratio found in the less integrated co-obligate of *M. carnosum* (*Figure 1B*).

![FISH Images of Abdomens from Six Aphid Species](image)

*Figure 4. FISH Images of Abdomens from Six Aphid Species*

*Buchnera* and *Serratia* are highlighted in green and red, respectively. The colored bar represents the degree of reliance on *Serratia*: facultative (red) or co-obligate (blue). The shades of blue represent the degree of anatomical integration with the host, from the least (*M. carnosum*) to the greatest (*Periphyllus* spp.). White arrows indicate the regions where *Serratia* is localized.

*situated* hybridization (FISH) using probes specifically targeting *Buchnera* and *Serratia*. As predicted, we found increased anatomical integration of *Serratia* in host lineages corresponding to a greater reliance on *Serratia* (*Figure 4*). In the most extreme case, we found that the *Periphyllus* aphids evolved a large organ (bacteriome) containing numerous bacteriocytes to house *Serratia* in their abdomens (*Figure 4*).

In contrast to high anatomical integration in the *Periphyllus* aphids, we found that both *M. carnosum* and *A. urticae* exhibit minimal integration of *Serratia*. In *A. urticae*, *Serratia* is localized in a small cluster of relatively large cells (~4), forming a small bacteriome surrounded by *Buchnera*-containing bacteriocytes. In *M. carnosum*, *Serratia* is the least integrated, with the symbiont being localized in sheath cells surrounding the Buchnera-containing bacteriocytes. This pattern is similar to the one found in *A. pismum* where *Serratia* maintains a consistently facultative relationship with its host.

Our results provide the first evidence that *Buchnera* has repeatedly lost the capacity to produce the essential nutrient riboflavin in multiple aphid lineages. In each case in which the pathway to synthesize riboflavin has been lost, *Serratia* has retained genes to compensate for these metabolic changes in *Buchnera*. Studies have shown that an aphid’s demand for riboflavin is relatively low compared to other nutrients, such as amino acids (14, 24, 25). This may explain why riboflavin is lost first as the modest host demand for this vitamin may be easily met by a new symbiont even at a relatively low abundance. In several species within the Lachninae sub-family, the tryptophan pathway is also missing. This suggests that once the co-obligate symbiosis with *Serratia* is established, the loss of amino acid pathways in *Buchnera* can follow (10). Our work confirms that the tryptophan pathway has likewise been lost in *Periphyllus* aphids and that the capacity to synthesize this amino acid is vulnerable to deletion. Tryptophan can be one of the most costly essential amino acids to synthesize (see Table S3) (26); it has been hypothesized that these costs may explain why its loss is associated with the presence of a second obligate symbiont (6). The second most energetically expensive amino acid synthesis pathways (phenylalanine, histidine, methionine, and isoleucine/valine) are likewise lost in the *Buchnera* of the *Periphyllus* aphid. Our finding is in line with work documenting the loss of energetically expensive amino acids and complementation by a companion symbiont in several ancient co-obligate symbioses, including *Sulcia* of some Auchenorrhyncha families (e.g., spittlebugs, cicadas).
and in Carsonella, the primary symbiont of Psyllids. In Sulcia, the amino acid pathways appear to have been lost 60 million years after co-dependence evolved, before the common ancestor of cicadas, sharpshooters, and spittlebugs [27].

We find amino acid pathways are only lost in Buchnera in the more ancient co-obligate associations of the Periphyllus and in some Lachninae species. In contrast, Buchnera has retained these functions in the more recent co-obligates of A. urticae or M. carnosum. This suggests that essential amino acids are only susceptible to deletions in the second phases of losses. This can happen once selection has been relaxed by the presence of a new obligate symbiont, 30–60 million years after dependence evolves. These results also provide strong support for the hypothesis that the energetic costs of synthesizing nutrients may provide a unified explanation for the sequence of gene losses that occur during the evolution of co-obligate symbiosis (Table S3).

In the case of A. urticae, we find that dependence on Serratia has evolved with no direct link to nutrient provisioning. Our results suggest that in this case, Buchnera may have become dependent on Serratia as a result of losing the capacity to synthesize peptidoglycan (Data S1E). Peptidoglycan is an important protective compound that is involved in bacterial cell division, shape, and integrity. The pathway to produce this polymer has also been shown to underlie the complementarity between symbiotic partners in other systems, such as in mealybugs [23]. In aphids, we find that the genes to synthesize peptidoglycan form a metabolic pathway similar to those responsible for producing essential nutrients; the entire pathway is missing from the Buchnera of ancient co-obligates of Lachninae and Periphyllus, whereas only a single gene, murF, is missing in both of the more recent associations. This suggests that the sharing of peptidoglycan biosynthesis may represent an important first step in the formation of co-obligate symbioses. However, the peptidoglycan pathway is incomplete in Buchnera of certain aphid species (e.g., A. glycines), which may reflect compensatory adaptation on the part of the host [28]; therefore, its role in aphid symbioses requires further investigation.

More generally, our data support the hypothesis that lineages recently acquired co-obligate symbionts will have cases of overlapping gene complexes. This is seen in both A. urticae and M. carnosum, in which Serratia shares many redundant genes with Buchnera in pathways for essential nutrients synthesis. While two of the three Periphyllus species showed high degrees of metabolic complementation, there was a single case (P. lyropictus) in which we did not document this pattern. This is likely due to Serratia being replaced within this lineage. Symbiont replacement is an important mechanism by which maladaptive symbionts are replaced with new functional ones [29, 30]. In the Lachninae aphids, Serratia has been replaced on multiple occasions, including by other more recently acquired Serratia strains [9, 12, 13].

The multiple independent origins of Buchnera-Serratia co-obligate symbioses also provide a unique opportunity to study the evolution of anatomical integration between hosts and their symbionts. Theory and data suggest that more ancient associations should be characterized by greater integration, both in terms of housing structures and symbiont densities within hosts [31, 32]. While our results generally support this hypothesis, we found that the evolution of specialized structures to house Serratia differed between the two most ancient aphid lineages. In the Lachninae, evidence suggests both co-obligate symbionts are located within the same bacteriome, although the arrangement of bacteriocyes differ among species. The Periphyllus aphids, by contrast, house the two symbionts in separate structures. This arrangement may have evolved de novo or through Serratia colonizing pre-existing bacteriocytes that evolved in complexity. The different configurations in the Lachninae and Periphyllus potentially represent alternative solutions to the same problem: allowing Serratia to reach densities that are high enough to efficiently perform its nutrient-provisioning role. Our curing data further support the idea that greater integration coincides with more reliance on Serratia in ancient co-obligates, as symbiont removal had the most dramatic fecundity effect in the Periphyllus genus (Figure 1A).

In the cases of A. urticae and M. carnosum, anatomical and genomic integration data suggest that Serratia is a more recently acquired co-obligate association. In A. urticae, Serratia is housed in a single small bacteriome. In M. carnosum, Serratia is not hosted in bacteriocytes at all, but rather in sheath cells that surround the large Buchnera-containing cells. This is a potential indication of its relatively recent transition to an obligate symbiont. According to this hypothesis, the lack of Serratia integration is indicative of its recent role as a facultative symbiont. These findings also suggest that at least in some cases, dependence evolves before the evolution of specialized structures to house symbionts.

Studying evolutionary transitions to obligate and co-obligate symbiosis is difficult because most events are characterized by single and ancient origins across large, diversified clades. This makes comparisons with outgroups less informative and prevents testing ideas on the relative importance of deterministic versus stochastic processes. The uniquely replicated evolutionary events of Buchnera-Serratia co-obligate symbioses differ in different aphid lineages. Buchnera-Serratia co-obligate symbioses in different aphid lineages provide a degree of temporal resolution that demonstrates that co-obligate associations can form in a relatively predictable manner. Furthermore, our findings indicate that genomic integration may occur in advance of anatomical integration. Our results provide evidence that the evolutionary forces that bind multiple organisms into single metabolic unit operate by deterministic stepwise processes. This allows us to better understand the role of symbioses in the evolution of complex organisms.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- LEAD CONTACT AND MATERIALS AVAILABILITY
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
  - Assessment of Serratia prevalence
  - Curing experiments
  - Quantiative PCR
  - Phylogeny
  - Whole genomes sequencing, assembly and analysis
  - Fluorescent in situ hybridization
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND CODE AVAILABILITY
SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2020.03.011.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES


## STAR★METHODS

### KEY RESOURCES TABLE

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Oligonucleotides

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<td>16S <em>Serratia</em> symbiotica specific primers 16sS A1:</td>
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<td>16S <em>Serratia</em> generalist primers 16sS 10F:</td>
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<td><em>Buchnera</em> dnaK primers: BHS70F2: 5'-ATGGGTAATATTGTGAT-3'; BHS70R2: 5'-ATAGGCTACATAGCAG-3',</td>
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<td><em>Serratia</em> dnaK primers: ApRF1: 5'-TGCGGATATTGCAAG-3'; ApRR1: 5'-CGGGATAGTG GTGTTTTTGG-3'</td>
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<td><em>Buchnera</em> 16S probe (<em>A. pisum, A. urticata, M. carnosum</em>): 5'-Alexa Fluor 488-CCTCTTTTGGG TAGATCC-3'</td>
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<td><em>Buchnera</em> 16S probe (<em>Periphyllus spp.</em>) probe: 5'-Alexa Fluor 488-CCTCTTTTGGG TAGATCC-3'</td>
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Software and Algorithms

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<td>mcmctree function in PAML</td>
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LEAD CONTACT AND MATERIALS AVAILABILITY

This study did not generate new unique reagents. Further information and requests for resources should be directed to the Lead Contact, Lee Henry (l.henry@qmul.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The following aphid species were used in this study: Acyrthosiphon pisum, Macrosiphoniella artemisiae, Aphis urticata, Microlophium carnosum, Chaitophorus leucomelas, C. populeti, Periphyllus acericola, P. aceris, P. coracinus, P. hirticornis, P. lyropictus and an unknown Periphyllus species (referred to as Periphyllus sp. in the main text). Clonal lines of aphids were maintained in the lab at 15°C/14°C with a 16 hours light (Sylvania Gro-Lux F36W/GRO-T8 bulb) 8h dark cycle on a leaf of their host plant: Vicia faba (A. piusm), Artemisia vulgaris (M. artemisiae), Urtica dioica (A. urticata and M. carnosum), Acer spp. (Periphyllus spp.) embedded in agar in a Petri dish to keep the leaves fresh. Leaves were changed as needed.

METHOD DETAILS

Assessment of Serratia prevalence

Collection and identification of aphid samples

Aphids were collected in the UK and the Netherlands between 2011 and 2019. They were dislodged by beating plants over a white tray or removed manually from the plant, before being placed in 100% ethanol or collected live for curing experiments. Resampling of the same aphid clones was minimized by separating collections from the same plant species by at least 10 m. Aphids were identified by barcoding based on data from [63] and morphological examination following [64]. We sequenced the COI barcoding region using standard protocols for DNA extraction, amplification and editing and performed alignments with MUSCLE in CODONCODE ALIGNER version 4.0.2 (CodonCode Corporation 2012, Centerville, MA, USA). Genomic DNA was extracted from individual specimens using DNeasy Blood and Tissue kits (QIAGEN, Venlo, Netherlands) and we amplified an approximately 700 bp DNA fragment of the cytochrome c oxidase I (COI) mitochondrial gene using Lep F (50-ATTCAACCAATCATAAAGATATTGG-30) and Lep R (50-TTAACCTTGATGCTCAAAAATCA-30) primers, which was sequenced in the forward direction. Aphids were identified to species by comparing COI sequence data to the online databases BOLD (http://www.boldsystems.org/) and GenBank using BLAST. Morphological examination was carried out by macerating individual aphids in KOH (Potassium Hydroxide) and mounting them on microscope slides.

Diagnostic screening for Serratia symbiotica

We confirm all A. urticata, M. carnosum and five species of Periphyllus aphids ubiquitously carry Serratia, in the UK and the Netherlands, by amplifying a partial region of the 16S rRNA gene using the specific primers 16sS A1 (50-AGAGTTTGATCMTGGCTCAG-30) and 16sS 2R (50-TTTGAGTTCCCGACTTTAACG-30) and sequencing the amplicons. The PCR cycling conditions were as follow: 3 minutes at 95°C followed by 30 cycles of 30 s at 95°C, 1 minute at 52°C and 1 minute at 72°C, and finally 5 minutes at 72°C. To confirm the primers were only amplifying S. symbiotica we compared sequence data to published records on GenBank using BLAST. If the specific primers failed to amplify due to primer binding specificity we used more general Serratia primers that amplify diverse Serratia species: 16sS 10F (50-AGTTTGGATCAGCGCTCAGATTG-30) and 16sS R443R (50-CTTCTGCGAGTAACGTCAATG-30),
and confirmed the presence of S. symbiotica by comparing sequences to those on GenBank using BLAST. PCR cycling conditions were as follows, 2 minutes at 94°C, followed by 10 cycles of one minute at 94°C, 1 minute at 65°C-55°C (touchdown in 1°C steps) and 2 minutes at 72°C 2:00, followed by 25 cycles of 1 minute at 94°C, 1 minute at 55°C and 2 minutes at 72°C, and finally, 6 minutes at 72°C [65].

To estimate the origins of the co-dependence in Periphyllus aphids we sampled for Serratia in the Chaitophorus genus, which is a sister taxon to Periphyllus [11]. We deep-sequenced the universal bacterial 16S rRNA gene in Chaitophorus populeti (7 samples) and C. leucomelas (1 sample) to confirm Serratia was not ubiquitously present in these aphids, indicating they had not evolved co-dependence on the symbiont or been replaced. We PCR amplified the V4 region of the bacteria 16S rRNA gene following standard protocols [65], and deep-sequenced the amplicons using the Illumina MiSeq2000 platform. 16S rRNA analysis was performed using the standard operating procedure for MOTHUR [46], including read joining filtering, and Operational taxonomic unit (OTU) selection at 99 percent. Taxonomic assignments of the reads were performed using the full length SILVA alignment [47] available from mothur.org. As a final step samples were filtered using R to only consider OTUs at a one percent relative abundance or higher in the sample. No OTU(s) corresponding to Serratia symbiotica were found in any of these samples. The total and absolute number of OTU reads for each symbiont species are presented in Data S1 C.

Curing experiments
To selectively cure aphids of Serratia while not affecting Buchnera, we used antibiotics that specifically target cell walls, which are reduced in Buchnera [66]. Curing experiments were conducted on A. urticae, M. carnosum, and the two Periphyllus species that we were able to successfully culture in the lab: P. hirticornis and P. lyropictus in both the UK and the Netherlands. We were unable to culture the remaining Periphyllus species in the lab so they were not included in the curing assays. Acyrthosiphon pisum (UK and NL) and M. artemisiae (UK only) were included to confirm the antibiotic treatments had no consistent negative fitness effects on species that harbor Serratia as a facultative symbiosis (A. pism) or were uninfected by the symbiont (M. artemisiae). The antibiotic solution was obtained by mixing 10 mg/mL of Ampicillin sodium salt, 5 mg/mL Cefotaxime sodium salt, and 5 mg/mL Gentamicin in water. All antibiotics were obtained from Sigma-Aldrich (St. Louis, MO, USA). A single leaf of the host plant was cut and placed in a 0.5 mL Eppendorf tube filled with either one of two treatments i) the antibiotic solution or ii) water (control treatment). For the curing assay we placed 3-5 one or two day old aphid nymphs on a leaf and left them to feed for five (UK experiment) or three (Dutch experiment) days on either water or antibiotic solution. At the end of the treatment, aphids were transferred to their own individual Petri dishes, each containing a single leaf in agar from their appropriate host plants. The leaves were changed each week and the lifetime fecundity of each aphid was recorded by counting the number of offspring. In the UK experiment, a sub-sample of first-generation aphids (9 control and 11 treated M. carnosum and 10 control and 10 treated P. hirticornis) were sacrificed at ~20 days old to quantify symbiont density using qPCR. The experiment was performed in the temperature and lighting conditions described previously.

Quantitative PCR
The relative density of Buchnera compared to the density of Serratia was measured by quantitative PCR on whole aphid DNA extracts. We used three single copy genes: one in the aphid nuclear genome (elongation factor 1-α) and one in each of the two symbionts (dnaK gene). The primers were as follows. Buchnera dnaK gene [43]: BHS70F2: 5’-ATGGGTAAAATTATTGGTATTG-3’; BHS70R2: 5’-ATAGCTTGACGTTTAGCAGG-3’; Serratia dnaK gene [44]: ApRF1: 5’-TGGGCGGTGATGCTGAGG-3’; ApRR1: 5’-CGGGAGTAGTGGTGGTTTGG-3’. Aphid elongation factor 1-α [43]: ApEF1-alpha 107F: 5’-CTGATTTGTGCCGTCTTATTG-3’; ApEF1-alpha 246R: 5’-TATGCTGGTTCAGTAGATCCC-3’. The quantification was performed on a CFX Connect Real-Time PCR Detection System (BioRad, Hercules, California, U.S.A.). The PCR reaction mixture included 10 μL Luna Universal qPCR Master Mix (New England BioLabs, Ipswich, Massachusetts, USA), 7 μL H2O, 0.5 μL of each primer (10 nM), and 2 μL DNA. The cycling conditions were: 15 min activation at 95°C followed by 40 cycles at 95°C for 15 s, at 60°C for 1 min, and 95°C for 15 s. The mean efficiencies were calculated using a ten-fold series of dilutions from 10^9 to 10^0 copies of purified PCR products. The efficiencies were 95.7 for the aphid gene, 96.8 for the Buchnera gene, and 97.3 for the Serratia gene. Duplicate samples were used for the determination of DNA quantities. As the deviations between the duplicates were below 0.5 cycles, the mean Cp values were used to calculate starting quantity. For each sample, the starting quantity for the Buchnera gene was divided by the starting quantity for the aphid gene to obtain the Buchnera density, and the starting quantity for the Serratia gene was divided by the starting quantity for the aphid gene to obtain the Serratia density.

Phylogeny
The aphid phylogeny was built using a concatenation of four genes: Elongation factor 1 alpha, 12S ribosomal RNA, 16S ribosomal RNA and Cytochrome oxidase subunit 1. Sequences were obtained from NCBI (Data S1G) and from the genomic data original to this study. Genes were concatenated using CLC genomic workbench 12.0 and aligned using MUSCLE [48]. Maximum Likelihood (ML) phylogeny was generated using the online PhyML server [49]. The phylogeny was bootstrapped 100 times, and rooted using sequences from Adelges japonicus, A. couleyi, Candidatus Ishikawaella capsulata and Salmonella enterica. The Adelgidae are basal to the Aphididae [67]. Candidatus Ishikawaella capsulata and Salmonella enterica are outgroups belonging to the same family as Buchnera aphidicola. The tree was visualized using FigTree v1.4.4 [50]. Chaitophorus saliniiger was included to improve node support then pruned from the phylogeny to only retain species for which Serratia-infection data are available.
We dated the phylogeny using the mcmctree function in PAML [51]. The calibrated the molecular clock using the estimated divergence (97.45-77.65 MYA) between the Lachnini (e.g., *T. salignus*) and Eulachnini (e.g., *C. cedri*) [68].

The *Serratia* phylogeny was built using GToTree v.1.4.7 under default parameters [55]. GToTree makes use of Biopython [57], HMMER3 v3.2.1 (hmmer.org), Prodigal v2.6.3 [58], TaxonKit v0.3 [59], Muscle v3.8 [60], Trimal v1.4 [61], and FastTree v2.1 [62]. Accession numbers as listed in Data S1F were provided alongside the GBK files generated by DFAST for the *Serratia* genomes we sequenced (see subsequent methods). Gammaproteobacteria was specified as the single copy gene set to use and species name information was added using the parameters -t -l -Species.

Whole genomes sequencing, assembly and analysis

We obtained whole genome sequences for all co-obligate aphid species available to us at the time: *A. urticata*, *M. carnosum*, *P. acericola*, *P. acer* and *P. lytopictus* (in addition to *P. testudinaceus* and *P. hirticornis*). DNA was extracted from individual aphids (one sample per species) and sequenced at Centre for Genomic Research (University of Liverpool). The libraries were prepared using the Nextera XT kit, and sequenced on an Illumina HiSeq 4000 (paired-end, 2x150 bp reads). Seven samples were multiplexed on one lane. Two samples were discarded at this stage (*P. testudinaceus* and *P. hirticornis*) because the coverage of the symbiont genomes was not high enough to include in the comparative analysis. Average genome coverage for the endosymbionts of interest was ~950x for *Buchnera* and ~97x for *Serratia* (Data S1H). The aphid hosts genomes were not analyzed.

Reads were trimmed for quality and Illumina adaptors were removed using Trimmomatic [52] under default settings. Reads were assembled using SPAdes v3.11.1 [53] in two stages. In the first stage and assembly was built, using assembly only mode with other parameters as default. The reads were then mapped back to this assembly using bwa mem. Contigs were partitioned into *Buchnera* and *Serratia* bins based on a DIAMOND [54] search of the contigs against the NCBI’s non-redundant Refseq protein database [69]. The reads mapping to the contigs in the *Buchnera* bin were then reassembled using SPAdes, this time using error correction and in careful mode with kmers sizes of 33, 55, 77, 99 and 127. The contigs of the resulting assembly were filtered by coverage and identity, then blasted against the NCBI’s non-redundant Refseq nucleotide database [69]. The results were manually inspected and contigs belonging to species other than *Buchnera* were removed.

Three of the five genomes, the *Buchnera* strains belonging to the *Periphyllus* species, were not able to be fully closed due to repetitive high AT content regions. These genomes were aligned against *Buchnera aphidicola* strain APS [33] and each other using MAUVE [70] and inspected using Geneious Prime 2019.06.17 (https://www.geneious.com) to ensure the contigs were oriented correctly. Using the high level of gene synteny between *Buchnera* lineages we manually inspected the gaps of all five genomes to confirm they could not contain genes relevant to provisioning of essential nutrients. In only one case, the genome of *P. acericola* contained a gap with the genes ribE and ribD, which are part of the riboflavin pathway. In this case, the remaining genes in the riboflavin pathway were confirmed to be absent followed by a comparison with the *Buchnera* of the other two *Periphyllus* species. Both species had a similar deletion that spanned the area of the gap. Finally, the assembled sequencing data from before the binning stage was inspected to ensure these genes were not overlooked due to incorrect binning. In this way we confirmed the pathway was non-functioning in this *Buchnera* lineage.

We annotated both *Serratia* and *Buchnera* genes using DFAST [71] with *Escherichia coli* K-12 genome annotations as a reference [72]. To ensure we did not miss any genes in our final assemblies, we also annotated the initial assembly prior to partitioned into *Buchnera* and *Serratia* bins, and confirmed they did not contain any additional genes involved in nutrient provision. Genes making up each pathway were determined using the Metacyc [73] pathway reference for *E. coli*. Metabolic pathways of the co-obligate *Buchnera* lineages were compared to three *Buchnera* strains where it is the sole obligate symbiont (strains APS, G002, EBg: Data S1H) to target regions that had been deleted from *Buchnera* that are in pathways involved in synthesizing essential nutrients. Where genes involved in nutrient pathways were deleted in *Buchnera*, we confirmed orthologous copies of the deleted genes had been retained in the *Serratia* genome. The presence of genes that are part of nutrient pathways in each *Buchnera* and *Serratia* genome was evaluated using Pathway Tools [74] and manual examination of the annotation files. See Data S1D for full results. We additionally evaluated the presence of genes, using the same methods, involved in the synthesis of peptidoglycan and genes involved in translation. See Data S1E for these results.

Additionally, in order to investigate what genes might be missing from the *A. urticae* strain of *Buchnera*, we used Orthofinder v2.2.7 [75] with default parameters to group orthologous genes between all of the *Buchnera* strains investigated (as listed in Data S1H).

Fluorescent in situ hybridization

Whole mount FISH was performed following a protocol adapted from [76]. Aphids were fixed overnight in Carnoy’s solution (60% ethanol, 30% chloroform, 10% glacial acetic acid). The aphids were then bleached in an alcoholic H2O2 solution (80% ethanol, 14% H2O, 6% H2O2) for 3 days, changing the solution each day. The samples were then thoroughly washed in 90% ethanol and kept at ~25°C until processed. The head of the aphids was removed to facilitate the penetration of the probes, and the samples were washed 4 times (30 minutes each) in PBS Tw (Phosphate Buffer Saline with 0.02% of Tween 20), and then 3 times (5 minutes each) in hybridization buffer (20 mM Tris-HCl at pH 8.0, 0.9 M NaCl, 0.01% sodium dodecyl sulfate and 30% formamide). The samples were then incubated overnight at room temperature in hybridization buffer (20 mM Tris-HCl at pH 8.0, 0.9 M NaCl, 0.01% sodium dodecyl sulfate and 30% formamide). The samples were then incubated overnight at room temperature in hybridization buffer supplemented with 100 mM of each 16S rRNA fluorescent probe, one targeting *Buchnera*, one targeting *Serratia*. The probes were as follows: *Buchnera* (*A. pisum, A. urticae, M. carnosum*): 5′-Alexa Fluor 488-CTCTTTTGAGTAGATCC-3′ [45], *Buchnera* (*Periphyllus* spp.): 5′-Alexa Fluor 488-CTCTTTTGGCGAGATCC-3′, *Serratia* (all species): 5′-Cy3-CCGACTTATCGCTGCC-3′ [45]. Following a washing in PBS Tw,
the samples were mounted on slides in vectashield hardset antifade mounting media with DAPI (to highlight the host body). Mounted samples were visualized using a Leica DMRA2 epi-fluorescent microscope. Monochrome pictures were obtained using a Hamamatsu Orca camera and the Velocity 6.3.1 software, and final color images were obtained using ImageJ. Probes were ordered from Eurogentec (Seraing, Belgium). All the \textit{Periphyllus} species that we were able to collect from the field at the time of the FISH experiment are shown in Figure 4. Two of those species (\textit{Periphyllus} sp. and \textit{P. coracinus}) were not previously collected by us, which is why they were not included in the other experiments.

\section*{Quantification and Statistical Analysis}

All statistical analyses were performed using R 3.6.0 \cite{56}. Lifetime fecundity was analyzed using GLMs with a quasi-Poisson distribution. Symbiont density data were analyzed using GLMs with a Gamma distribution.

\section*{Data and Code Availability}

The accession number for the data reported in this paper is Genbank: PRJNA605335. No novel software was created for this study.