**Serratia symbiotica from the Aphid Cinara cedri: A Missing Link from Facultative to Obligate Insect Endosymbiont**

Araceli Lamelas¹, María José Gosalbes¹, Alejandro Manzano-Marín¹, Juli Peretó¹, Andrés Moya¹,², Amparo Latorre¹,²*  

Abstract  
The genome sequencing of *Buchnera aphidicola* BCc from the aphid *Cinara cedri*, which is the smallest known *Buchnera* genome, revealed that this bacterium had lost its symbiotic role, as it was not able to synthesize tryptophan and riboflavin. Moreover, the biosynthesis of tryptophan is shared with the endosymbiont *Serratia symbiotica* SCc, which coexists with *B. aphidicola* in this aphid. The whole-genome sequencing of *S. symbiotica* SCc reveals an endosymbiont in a stage of genome reduction that is closer to an obligate endosymbiont, such as *B. aphidicola* from *Acyrthosiphon pisum*, than to another *S. symbiotica*, which is a facultative endosymbiont in this aphid, and presents much less gene decay. The comparison between both *S. symbiotica* enables us to propose an evolutionary scenario of the transition from facultative to obligate endosymbiont. Metabolic inferences of *B. aphidicola* BCc and *S. symbiotica* SCc reveal that most of the functions carried out by *B. aphidicola* in *A. pism* are now either conserved in *B. aphidicola* BCc or taken over by *S. symbiotica*. In addition, there are several cases of metabolic complementation giving functional stability to the whole consortium and evolutionary preservation of the actors involved.

Introduction  
Symbiotic associations are widespread in insects, being particularly well studied in aphids [1,2]. They feed on phloem sap, which has unbalanced nitrogen/carbon content and is deficient in a number of nutrients that the aphids cannot synthesize and that are provided by *Buchnera aphidicola*, their primary endosymbiont [3,4]. In addition to *B. aphidicola*, some aphid populations harbor additional facultative (or secondary symbionts) that are not required for growth or reproduction [5], which are sometimes transmitted horizontally [6–8]. Three main facultative symbionts have been found in aphids, i.e. *Hamiltonella defensa*, *Regiella insecticola* and *Serratia symbiotica* [9]. Although their presence is not necessary for the maintenance of the aphid-*Buchnera* association, several studies have demonstrated that they can provide certain benefits to their hosts, such as influencing interactions with the host’s natural enemies, or defense against environmental heat stress [revised in [5]]. Most of the experimental studies on facultative symbionts in aphids have involved members of the subfamily Aphidinae, mainly in *Acyrthosiphon pisum*. In addition, the genome of the three above mentioned facultative symbionts from *A. pism* have been sequenced [10–12]. These sequences have revealed that all three bacteria have lost the ability to synthesize most of the essential amino acids, although they retain active uptake mechanisms to import them. Thus, it seems that while *B. aphidicola* does not need the help of these facultative symbionts for host survival, they are dependent on *B. aphidicola* for amino acid provision when they infect *A. pism*.

Furthermore, in the cedar aphid *Cinara cedri*, a member of the Lachninae subfamily, it was found that the co-existing endosymbiont *Serratia symbiotica* SCc was necessary for the survival of the *C. cedri* consortium. The genome of *B. aphidicola* BCc, the obligate endosymbiont of *C. cedri* with only 416 kb is the smallest *Buchnera* among all *Buchnera* genomes described, and one of the smallest bacterial genomes sequenced so far. Functional genome analysis revealed that with 362 genes, *B. aphidicola* BCc is able to support cellular life. However, its symbiotic role has been questioned because, contrary to other *Buchnera*, it was unable to fulfill some symbiotic functions [13]. Thus, it was postulated that the nutrients that *B. aphidicola* BCc cannot synthesize could be supplied by *S. symbiotica* SCc. Moreover, it was reported that this bacterium has characteristics of an obligate symbiont which differ from the other *S. symbiotica* described so far [7]. Microscopic analysis of *C. cedri* demonstrated that *S. symbiotica* SCc is confined in a second type of bacteria whose presence is as abundant and homogeneous as *B. aphidicola* BCc [14]. In addition, both
endosymbionts were found to be involved in tryptophan biosynthesis, supplying this essential amino acid to both their host and themselves [15]. Regarding the situation in the subfamily Lachninae, most members of the subfamily were found to have a massive presence of secondary symbionts, mainly S. symbiotica [7,8]. Phylogenetic studies of these symbionts in all aphids from different subfamilies whose presence was reported, showed the existence of two clades, A and B, of S. symbiotica hypothetically playing two different roles: clade A is composed of facultative endosymbionts, whereas in clade B they would be obligate endosymbionts. Interestingly, S. symbiotica from A. pisum (herein S. symbiotica SAp) whose genome has recently been sequenced [12] belongs to clade A, whereas in this work we report a S. symbiotica genome belonging to clade B.

In the present study, we have carried out the genome sequencing and metabolic analysis of S. symbiotica SCc, the third partner of the C. cedri consortium. This bacterium has suffered an important genome size reduction to become a co-obligate symbiont. The comparative genomics of S. symbiotica SCc with S. symbiotica SAp and other obligate and facultative symbiotic bacteria, as well as with free-living Servatia relatives, mainly S. proteamaculans and the genetic and metabolic information retrieved from the genome sequence of A. pisum [16] and derived studies [17–19], provide an evolutionary scenario of how a symbiotic bacterial consortium is established.

Results

Genome of S. symbiotica SCc strain

General and specific features of the S. symbiotica SCc genome (CP002295) reflect its lifestyle as a host-restricted, mutualistic symbiont that invades host cells. The moderately reduced genome consists of a 1,762,765 bp circular chromosome with average G+C content of 29.22% (Table 1 and Figure S1). This genome size represents a 67.7% reduction compared to the free-living bacterium S. proteamaculans (CP000826) and a 36.8% reduction compared to S. symbiotica SAp (AENX00000000) [12]. A total of 711 putative genes have been described, with 672 protein coding genes (CDS), 36 tRNAs, 3 rRNAs and one tmRNA. It is worth mentioning that the ribosomal genes 23S and 5S are located on a chromosomal region separated from the 16S rDNA gene, a situation already detected in other obligate endosymbions. However, S. symbiotica SAp, H. defensa HAp (CP001277.1) and S. proteamaculans have more than one copy of the ribosomal gene in an operon structure. Also, the number of genes coding for tRNAs are closer to B. aphidicola BAp (NC_011833) than to facultative or free-living bacteria. Finally, 38 readily identifiable pseudogenes were present, which is a number closer to the one observed in primary than in secondary symbionts (Table S1).

The origin of replication was located between the genes gidA and atpB. The overall coding density is 38.7%, the lowest among insect endosymbionts described so far, including facultative symbionts of aphids, like H. defensa HAp (88.8%), R. insestercola RAp (ACYF00000000) (71.0%) or S. symbiotica SAp (60.9%) [10–12]. A very interesting feature of this genome is the average length of the intergenic regions (IGRs) (1,672 bp), which is much higher than in the other selected species (Table 1). A detailed analysis of these IGRs indicates that they do not show any traces of homology to coding regions from other bacteria. Due to the fact that protein-coding regions (CDSs) were found to be more G+C rich than non-coding regions [20], we decided to analyze the GC content distribution of S. symbiotica SCc and compare it with selected bacteria. We found a striking two-peak distribution of the genome GC content in S. symbiotica SCc, instead of the one peak found in any of the other selected organism [21] (Figure S2). To analyze where this two-peak distribution could originate, we took both S. symbiotica and plotted their CDSs and IGRs GC distribution separately. In S. symbiotica SCc, the IGRs mean GC content (27%) was found to fall very far from that of CDSs (38.74%), which contrasts with the case of S. symbiotica SAp where both IGRs and CDSs mean GC content only differed by 7% (Figure S3). In addition, the great number of pseudogenes in S. symbiotica SAp (550) also gave a similar GC mean of 51%. This points towards the last stages of genomic degradation of S. symbiotica SCc by displaying no evident homology with any known gene and displaying a high A+T content, a common feature arising in many bacterial endosymbionts in advanced stages of genome reduction. Finally, this genome has lost all the insertion sequences (IS) that are characteristic of free-living bacteria and facultative symbionts, as also observed in other bacterial genomes with long-term insect host associations [1,2].

Functional analysis of the predicted protein-coding genes

The protein genes of S. symbiotica SCc were classified according to COG categories [22] and compared with those of selected symbionts and free-living bacteria (Figure S4). The most relevant result is that S. symbiotica SCc has retained genes devoted to systems for which B. aphidicola BCc (NC_008513) was especially impaired compared with other Buchnera, such as biosynthesis of nucleotides, cofactors, lipid transport and metabolism, and cell envelope biogenesis. The only category in which it is clearly underrepresented is in amino acid metabolism (1.2% and 11.8% in S. symbiotica SCc and B. aphidicola BCc, respectively), which suggests the absolute metabolic dependence of S. symbiotica SCc on B. aphidicola BCc. Accordingly, S. symbiotica SCc possess many amino acid transport systems. Additionally, it has also preserved a wide range of transporters for other metabolites.

Metabolic pathway reconstruction

S. symbiotica SCc has preserved all the steps of glycolysis as well as pentose phosphate pathway. Contrary to S. symbiotica SAp but similar to Buchnera spp, it has lost a functional TCA cycle, preserving only the genes sucC and sucD. These two genes may
Table 1. Comparison of S. symbiotica SCc genome features to those of obligate (B. aphidicola BCc and B. aphidicola BAp, from C. cedri and A. pisum, respectively) and facultative (S. symbiotica SAp, and H. defensa HAp, both from A. pisum) endosymbionts, and a free-living bacterial genome (S. proteamaculans).

|                       | B. aphidicola BCc | B. aphidicola BAp | S. symbiotica SCc | S. symbiotica SAp | H. defensa HAp | S. proteamaculans |
|-----------------------|------------------|------------------|------------------|------------------|----------------|------------------|
| Genome size (bp)      | 424,849          | 652,115          | 1,762,765        | 2,789,218        | 2,169,363      | 5,495,657        |
| Chromosome size (bp)  | 416,380          | 640,681          | 1,762,765        | 2,789,218        | 2,110,331      | 5,448,853        |
| Plasmids              | 2                | 2                | unknown          | unknown          | 1              | 1                |
| Plasmid size (bp)     | 8,849            | 11,434           | -                | -                | 59,032         | 46,804           |
| Total number of genes | 401              | 609              | 711              | 2,098            | 2,420          | 5,064            |
| CDS (chromosome+plasmids) | 357+7         | 562+9            | 672              | 2,098            | 2,094+54       | 4,891+51         |
| tRNAs (16S, 5S, 23S)  | 1, 1, 1          | 1, 1, 1          | 1, 1, 1          | 5, 5, 5          | 3, 3, 3        | 7, 8, 7          |
| tRNAs                 | 31               | 32               | 36               | 44               | 43             | 85               |
| Pseudogenes           | 3                | 12               | 58               | 550              | 187+1          | 12               |
| CDS average size (bp) | 994.00           | 984.00           | 1,019,77         | 845              | 810.41         | 972.11           |
| Coding density (%)    | 90.0             | 86.7             | 38.7             | 60.9             | 88.8           | 87.1             |
| IGRs average size (bp)| 135.80          | 126.90           | 1,672,01         | 204.27           | 240.26         | 165.67           |
| G+C content           | 20.20            | 26.24            | 29.22            | 52.00            | 40.32          | 55.07            |
| CDSs                  | 21.40            | 28.00            | 38.51            | 52.53            | 40.99          | 56.34            |
| 1st+2nd position      | 28.10            | 33.98            | 43.88            | 52.56            | 42.99          | 51.28            |
| 3rd position          | 7.77             | 14.23            | 28.46            | 52.48            | 36.99          | 66.47            |
| IGRs (%)              | 9.30             | 16.10            | 26.53            | 45.43            | 37.26          | 45.71            |
| Insertion Sequences (Is) | No             | No              | No               | No               | Yes            | Yes              |

doi:10.1371/journal.pgen.1002357.t001

have been retained to produce succinyl CoA, necessary for lysine biosynthesis. As in other endosymbionts, acetyl-CoA could be used to produce acetate and ATP via the products of the genes aca and pta, and conserve energy under oxygen-limiting conditions.

For most of the other pathways, one must postulate the involvement of one or even the two other members of the consortium, i.e. B. aphidicola BCc and the aphid (see Figure 1 for a summary of the shared metabolism). This is the case of the purine metabolism, where S. symbiotica SCc can only synthesize AIR from PRPP, but needs an external uptake of IMP to obtain AMP, GMP and XMP (Figure 2). Additionally, S. symbiotica SCc could salvage nitrogen bases from nucleotides or nucleosides that, when in excess, could in turn be transformed and eliminated as uric acid excretion by the aphid metabolism. This role is taken by B. aphidicola BAp in A. pisum [23]. On the other hand, S. symbiotica SCc possesses the complete machinery for pyrimidine biosynthesis. This is in clear contrast with the situation in S. symbiotica SAp where the purine de novo synthesis is complete, but to obtain pyrimidines it requires the nucleoside import, either from the aphid of from B. aphidicola BAp [12,23].

Most secondary endosymbionts retain the pathways for the synthesis of non-essential amino acids. However, S. symbiotica SCc has only preserved the pathways for alanine, cysteine and asparagine (Table 2). Regarding essential amino acid biosynthesis, S. symbiotica SCc has retained the ability to synthesize lysine and tryptophan provided that Buchnera metabolism supplies the respective precursors, i.e., aspartyl-4-phosphate and anthranilate (Figure 1). The latter situation (described elsewhere [15]) is similar in S. symbiotica SAp, which would also require exogenous anthranilate to synthesize tryptophan. However, in A. pisum, B. aphidicola BAp can provide the tryptophan as it possesses the complete pathway. A striking result relates the case of the non-essential amino acids serine and cysteine and the essential ones isoleucine and methionine, which, as shown in Figure 3, is necessary to postulate the metabolic complementation of all three members of the consortium to be synthesized. The aphid would produce serine from glucerase-3-p and then, S. symbiotica SCc could make cysteine. In turn, B. aphidicola BCc can provide threonine to the aphid to obtain the precursor of isoleucine. This is also similar in A. pisum and B. aphidicola BAp [17–19]. Finally, B. aphidicola BAp could synthesize methionine, isoleucine and arginine with the external supply of homocysteine, 2-oxobutanoate and ornithine, respectively. We postulate that they come from the aphid, as might be the case in B. aphidicola BAp for methionine and isoleucine biosynthesis [17,19].

With regard to cofactors and vitamins, genome sequencing has revealed that S. symbiotica SCc is capable of synthesizing the same metabolites as B. aphidicola BAp as well as vitamin B6 (Table 3) although for biotin, folate and CoA, S. symbiotica SCc would require the provision of the respective precursors from B. aphidicola BCc, i.e. pimeloyl CoA, chorismate and L-pantoate (Figure 1). Clearly, S. symbiotica SCc has taken over these functions, which have been completely lost in B. aphidicola BCc. Moreover, S. symbiotica SCc could synthesize heme group in collaboration with the aphid, which must provide the porphobilinogen. This differs hugely from S. symbiotica SAp, which has preserved only four pathways (Table 3).

Cell wall and membranes

The genome sequencing of S. symbiotica SCc revealed that it retains the ability to synthesize peptidoglycan and liposaccharides to make its well-structured and complex membranes (Figure 4). This contrasts with its B. aphidicola partner, which has lost all the genes related to these functions [13]. Although S. symbiotica SCc retains the ability to synthesize these compounds, they are macromolecules and it is unlikely that they can enter B. aphidicola BCc. However, as can be seen in Figure S5, both bacteria

Cell wall and membranes

The genome sequencing of S. symbiotica SCc revealed that it retains the ability to synthesize peptidoglycan and liposaccharides to make its well-structured and complex membranes (Figure 4). This contrasts with its B. aphidicola partner, which has lost all the genes related to these functions [13]. Although S. symbiotica SCc retains the ability to synthesize these compounds, they are macromolecules and it is unlikely that they can enter B. aphidicola BCc. However, as can be seen in Figure S5, both bacteria
maintain all three expected membranes, the two gram-negative and the external bacteriocyte-derived membrane.

Pseudogenes, absent genes, and genome degradation

To gain insight into the pseudogenization process undergone by *S. symbiotica* SCc and *S. symbiotica* SAp in their respective lineages, we have compared the state of the annotated pseudogenes in both *Serratia* and the free-living *S. proteamaculans* (see Table S1 for details). From the 58 pseudogenes found in *S. symbiotica* SCc, two (*tuf* and *bamA*) have a duplicated functional copy. From the other 56, eighteen are also inactive genes (nine pseudogenes and nine absent genes) in *S. symbiotica* SAp, whereas 38 are active copies. Regarding the 311 chosen pseudogenes in *S. symbiotica* SAp (see Materials and Methods), as expected, most are absent in *S. symbiotica* SCc, and some are also absent in *S. proteamaculans*, thus being strain specific. A very interesting result is that sixteen of the *S. symbiotica* SAp pseudogenes are putatively active genes in *S. symbiotica* SCc (Table S1), thus indicating differential degradation fates in both *Serratia* lineages. Moreover, *S. symbiotica* SCc possesses 20 CDSs that are totally absent in *S. symbiotica* SAp.

Synteny plots of *Serratia* species

In order to further compare the two intracellular *Serratia*, we performed the analysis of the synteny between both bacteria and also a comparison with free-living relatives. The results are shown in Figure 5 and clearly display the great number of rearrangements that occurred when the bacterium adopted an intracellular lifestyle, as is the case for both *S. symbiotica* compared to *S. proteamaculans*. The most interesting result is the comparison between *S. symbiotica* SCc and *S. symbiotica* SAp (panel D) where a series of rearrangements are found even in the biggest contigs, which suggest a past history of active mobile elements in *S. symbiotica* SCc, which are already unidentifiable in the current genome but still present in the *S. symbiotica* SAp.

Discussion

Symbioses involving prokaryotes living in close relationships with insects have been widely studied from the genomic perspective [1,2]. In the process towards host accommodation, symbionts experience a series of major genetic and phenotypic changes that can be detected by comparison with free-living relatives. Several scenarios could account for the evolution of symbiotic associations, from the first stages of free-living bacteria, through facultative symbiosis, to obligate symbionts. Of particular relevance is the association formed by the coexistence of several symbionts in a given host. Aphids are a good model to dissect the different stages of the integration process undertaken by the
different symbionts coexisting therein. At present, the genome of *B. aphidicola* from five aphid species, belonging to different aphid lineages have been sequenced providing information of the last steps leading to obligate endosymbiosis [13,24–27]. On the other hand, the genome of three facultative endosymbionts from the aphid *A. pisum* are also available [10–12]. They are in the early

Figure 2. Outline of the putative synthesis of nucleotides by the consortium (*C. cedri, B. aphidicola BCc, and S. symbiotica SCc*). The number of genes involved in each pathway is shown as black circles beside them. The intermediate metabolites are colored in green. Green arrows indicate the movement of intermediary metabolites. In the case of aphid, the genes are postulated.

doi:10.1371/journal.pgen.1002357.g002

Table 2. Metabolic capacity of amino acids biosynthesis by selected endosymbionts and free-living bacteria.

|                   | B. aphidicola BA| B. aphidicola BC | S. symbiotica SCc | S. symbiotica SA | H. defensa HA | R. insecticola RA | S. proteamaculans |
|-------------------|-----------------|-----------------|-------------------|-----------------|---------------|-------------------|------------------|
| Essential         |                 |                 |                   |                 |               |                   |                  |
| Histidine         | +               | +               | −                 | −               | −             | +                 |                  |
| Arginine          | +               | C-B             | −                 | +               | −             | +                 |                  |
| Lysine            | +               | +               | −                 | B-S             | +             | +                 | +                |
| Threonine         | +               | +               | −                 | −               | +             | +                 |                  |
| Methionine        | A-B             | C-B             | −                 | −               | −             | +                 |                  |
| Valine            | +              | +               | −                 | −               | −             | +                 |                  |
| Isoleucine        | A*-B           | C*-B            | −                 | −               | −             | +                 |                  |
| Phenylalanine     | +              | +               | −                 | +              | −             | −                 | +                |
| Tryptophan        | +              | B-S             | B-S              | −               | −             | −                 | +                |
| Leucine           | +              | +               | −                 | +              | −             | −                 | +                |
| Non-essential     |                 |                 |                   |                 |               |                   |                  |
| Glycine           | +              | +               | −                 | +              | +            | +                 |                  |
| Proline           | −              | −               | −                 | −              | +            | +                 |                  |
| Glutamine         | −              | −               | −                 | −              | +            | −                 | +                |
| Cysteine          | +              | −               | +                 | +              | −            | +                 |                  |
| Asparagine        | −              | −               | +                 | +              | +            | +                 | +                |
| Alanine           | +              | +               | +                 | +              | +            | +                 | +                |
| Serine            | +              | +               | −                 | +              | +            | +                 | +                |
| Tyrosine          | −              | −               | −                 | −              | −            | +                 |                  |
| Glutamate         | −              | −               | −                 | +              | +            | +                 | +                |
| Aspartate         | −              | −               | +                 | +              | +            | +                 | +                |

(+) synthesized, (−) not synthesized, (A-B) metabolic complementation between *A. pisum* and *B. aphidicola*, (C-B) postulated metabolic complementation between *C. cedri* an *B. aphidicola*, (B-S) metabolic complementation between *B. aphidicola* and *S. symbiotica*, (*) Absence of one enzyme involved in the pathway.

doi:10.1371/journal.pgen.1002357.t002
stages of transition from a free-living to symbiotic lifestyle, with $S.\ symbiotica$ SAp probably representing the earliest stage of all three [12].

Our work indicates that $S.\ symbiotica$ from $C.\ cedri$ is a good candidate for a missing link between a facultative and an obligate insect endosymbionts. For comparative purposes, the two most relevant genomes are $B.\ aphidicola$ BAp, the $Buchnera$ with the biggest genome that does not need a second symbiont for aphid survival [17,18], and $S.\ symbiotica$ SAp because it is a $Serratia$ symbiont, but in a much earlier step of the integration process [12].

Many features of the $S.\ symbiotica$ SCc genome, such as the A+T content, the number of genes, the loss of $recA$ gene, as well as the total absence of ISs or other mobile DNA still present in all the facultative symbionts analyzed so far, are indicative of an obligate endosymbiont. It is worth mentioning that in $S.\ symbiotica$ SAp, there are still a certain number of ISs, although because the genome sequence is incomplete, the exact number is not known. Moreover, transposases, plasmid-associated genes, and phage-associated genes can make up to 4% of the total number of genes [12]. On the other hand, $S.\ symbiotica$ SCc has lost all the genes involved in bacterial pathogenesis that are still retained in $S.\ symbiotica$ SAp. However, the $S.\ symbiotica$ SCc genome size (1,763 kb) is intermediate between the two $A.\ pisum$ symbionts, the obligate $B.\ aphidicola$ (641 kb) and the facultative $S.\ symbiotica$ SAp (ca. 2,789 kb), with non-coding DNA comprising a huge part.

### Table 3. Metabolic capacity of cofactor and vitamin synthesis by selected endosymbionts and free-living bacteria.

|                        | $B.\ aphidicola$ BAp | $B.\ aphidicola$ BCc | $S.\ symbiotica$ SCc | $S.\ symbiotica$ SAp | $H.\ defensa$ HAp | $R.\ insecticola$ RAp | $S.\ proteamaculans$ |
|------------------------|----------------------|----------------------|----------------------|----------------------|------------------|----------------------|----------------------|
| Biotin                 | –                    | –                    | –                    | +                    | –                | –                    | +                    |
| Lipoic acid            | +                    | –                    | –                    | –                    | –                | –                    | +                    |
| Folate                 | –                    | –                    | –                    | +                    | +                | +                    | +                    |
| Glutathione            | +                    | –                    | +                    | –                    | +                | +                    | +                    |
| Heme                   | –                    | –                    | C-S                  | –                    | +                | +                    | +                    |
| Siroheme               | +                    | –                    | +                    | –                    | +                | +                    | +                    |
| Nicotinate and nicotinamide | –                | –                    | –                    | +                    | –                | +                    | +                    |
| Pantotenate and CoA    | –                    | –                    | –                    | +                    | –                | +                    | +                    |
| Riboflavin             | +                    | –                    | +                    | +                    | +                | +                    | +                    |
| Thiamine               | –                    | –                    | –                    | +                    | –                | +                    | +                    |
| Ubiquinone             | –                    | –                    | –                    | –                    | +                | +                    | +                    |
| Menaquinone            | –                    | –                    | –                    | +                    | +                | +                    | +                    |
| Vitamin B6             | –                    | –                    | +                    | +                    | +                | +                    | +                    |

(+) synthesized and (−) not synthesized. (C-S) postulated metabolic complementation between the aphid and $S.\ symbiotica$. 

doi:10.1371/journal.pgen.1002357.003

Figure 3. Outline of the putative synthesis of the amino acids serine, cysteine, isoleucine, and methionine by the consortium ($C.\ cedri$, $B.\ aphidicola$ BCc, and $S.\ symbiotica$ SCc). Rest as in Figure 2.

doi:10.1371/journal.pgen.1002357.g003
of the genome. In fact, the coding density is extremely low (more than two times lower than that of \textit{B. aphidicola} BCc), whereas the average size of the intergenic regions is extremely high (more than seven-fold that of \textit{H. defensa}). According to our knowledge of prokaryotic genomes, these regions must correspond to ancient genes. However, in contrast with its related and recent symbiont \textit{S. symbiotica} SAp, which has around 550 pseudogenes, in \textit{S. symbiotica} SCc only 58 pseudogenes could be clearly identified \cite{12}. These data support the postulated gradual process of genome degradation of the pseudogenes, ending up in their total disappearance in obligate bacterial endosymbions \cite{28–30}. In fact, if we substitute the size of the intergenic region in \textit{S. symbiotica} SCc (1,672 bp on average) for the size of these regions in \textit{B. aphidicola} BCc (135.8 bp on average), the chromosomal length would be 771,075 bp, a reduction of 43.7% and in the range of other obligate endosymbions published so far \cite{1,2].

The functional annotation of the \textit{S. symbiotica} SCc genome indicated that its main symbiotic role would be the metabolism of cofactors, vitamins and nucleotides, whereas in \textit{B. aphidicola} BCc it would be that of amino acid provider. However, the inferred metabolism of both endosymbionts has revealed a strong interdependence and a fine tuning of different biosynthetic pathways which, in some cases, probably also involves metabolic complementation with the aphid, as shown to occur in \textit{A. pisum} \cite{16–19,23}. Overall, it seems that \textit{B. aphidicola} BCc and \textit{S. symbiotica} SCc in \textit{C. cedri} jointly perform the metabolic functions that \textit{B. aphidicola} BAp performs in \textit{A. pisum}.

Another interesting feature relates to cell morphology. When \textit{S. symbiotica} SCc was first reported, its spherical morphology at the microscopic level was surprising \cite{14}, similar to the shape cells of \textit{B. aphidicola} (Figure S5C), and different to the rod-shaped bacteria observed in \textit{S. symbiotica} SAp \cite{31} and in \textit{S. symbiotica} from \textit{C. tujafilina} \cite{7}. These last two Serrata could be present in different locations in some individuals of the population, whereas \textit{S. symbiotica} SCc are confined to their own bacteriocytes and occur in all individuals and at the same density as \textit{B. aphidicola} BCc \cite{14}. However, \textit{S. symbiotica} SCc, like \textit{S. symbiotica} SAp, has retained the genes involved in bacillary morphology (\textit{meB}, \textit{meC}, \textit{meD}, \textit{meB}). These genes have been lost in all \textit{B. aphidicola} genomes sequenced so far. At present, it is not clear whether these genes are being expressed or not, although the observed morphology is unexpected. The possible role played by the intracellular environment cannot be ruled out, possibly exerting some kind of effect on the morphology if those genes are expressed \cite{31}.

In summary, all the data presented (diversity in symbiont morphology, distribution and function) correlate with the existence of two different clades of \textit{S. symbiotica} in aphids, at least, as also indicated by the phylogenetic analyses \cite{7,8}. The analysis of the synteny between \textit{S. symbiotica} SCc and \textit{S. symbiotica} SAp and the comparison with free-living \textit{Serratia} indicate the great and different number of rearrangements undergone when the two bacteria adopted an intracellular lifestyle (Figure 5).

The comparison of the genome of all three secondary endosymbionts of \textit{A. pisum}, \textit{H. defensa}, \textit{S. symbiotica} and \textit{R. insecticola}, provides some clues to the scenario of how the \textit{C. cedri} consortium came into being. These three bacteria, despite being facultative, could be retained by the aphid because they provide certain benefits to the host under particular conditions \cite{3}. Specifically, \textit{S. symbiotica} SAp is involved in defense against environmental heat stress \cite{32–34}. Due to the inactivation of some of their biosynthetic pathways, such as those related to the essential amino acid biosynthesis, over time, these bacteria have become dependent on the presence of \textit{Buchnera}, and thus preserve active uptake mechanisms for their provision. On the other hand, as \textit{B. aphidicola} is still undergoing a genome reduction process, some symbiotic functions may be lost and taken over by the second endosymbiont. When this happens, the consortium is established. The different agents involved in tryptophan biosynthesis in \textit{A. pisum} and \textit{C. cedri} is an amazing example of evolution towards the establishment of a consortium. In all the \textit{B. aphidicola} strains, the two first genes of the tryptophan pathway, \textit{tpE} and \textit{tpG}, coding for anthranilate synthase, are either on a plasmid or in the chromosome, but always separated from the rest of the genes on the chromosome. Both \textit{S. symbiotica} have lost these two genes, but
preserve the other genes of the pathway (tpABCD), implying Buchnera dependence for anthranilate provision. The main difference between both systems involves the obligate endosymbiont. In *A. pisum*, Buchnera can make tryptophan autonomously because it possesses the complete pathway, whereas in *C. cedri*, Buchnera has lost the *trp*ABCD genes, which are present in *Serratia*. This example could be enough to seal a consortium. Another case of metabolic collaboration between the two endosymbionts is the biosynthesis of lysine from aspartate. This pathway is complete in *B. aphidicola* BAp [19] whereas in *B. aphidicola* BCc only the first step, catalyzed by aspartokinase (*thrA*), takes place, whereas the other eight steps occur in *S. symbiotica* SCc (Figure 1). Moreover, additional cases of metabolic complementation might also exist during the synthesis of biotin, folate, and CoA in *S. symbiotica* SCc. Finally, the fact that 36 active genes in *S. symbiotica* SCc are either pseudogenes, or absent genes in *S. symbiotica* SAp point towards different genome degradation processes in both *Serratia*. Such processes are context-dependent, i.e., the consequence of the different gene repertoire of the other agents when the association started, particularly the different genome composition of *B. aphidicola* in *A. pisum* or *C. cedri*.

In summary, here we report a missing link in the evolution from a facultative to an obligate endosymbiont. This is the case of *S. symbiotica* SCc when compared with *S. symbiotica* SAp, two different endosymbionts belonging to the same genus but in different stages of the integration process leading to intracellular lifestyle: *S. symbiotica* SAp, a recently acquire facultative symbiont, and *S. symbiotica* SCc a recent co-obligate endosymbiont. We also gain insights into the establishment of a bacterial consortium between two co-obligate symbionts in aphids, *B. aphidicola* BAp and *S. symbiotica* SCc.

**Materials and Methods**

**Aphid collection and total DNA extraction**

*C. cedri* aphids were collected in Valencia, Spain. An enriched fraction of bacteriocytes was obtained as in [35], and then used to extract total DNA following a CTAB (Cetyltrimethylammonium bromide) method [36].

**Genome sequencing and assembling**

The complete genome sequence of *S. symbiotica* SCc was obtained using single and paired-end shotgun reads from 454 pyrosequencing method (454 Life Science, Lifesequencing). The sequencing run generated 831,450 reads that assembled into 105,723 contigs using the GS De novo Assembler (version...
Contigs expected to belong to the \textit{S. symbiotica} genome were identified by BLASTX searches against the GenBank non-redundant database [37], and reads associated with these contigs were extracted and reassembled to generate the \textit{S. symbiotica} SCc genome. Reassembly produced 15 contigs. The order and orientation of some of the 15 contigs were predicted using the pair-ends information. All contig joins were confirmed using PCR amplification followed by Sanger sequencing. The tool Gap4.0.1 from Staden Package [38] was used for the total assembling of the Sanger sequences. This resulted in a single 1,762,765 bp contig with an average 434 (both single and paired-ends) coverage of 25.90×.

Gene annotation and pseudogene prediction

The protein coding sequences (CDSs) were identified with the GLIMMER v3.02 program [39]. The ARTEMIS [40] program was used to check for the start and stop codons. Final annotation was performed using BLASTP comparison [37]. The tRNAscan [41] program was used to predict tRNAs, as well as other small RNAs, like tmRNA, the RNA component of the RNase P. Signal Recognition Particle RNA was identified by programs like SRPscan [42], as well as consulting the Rfam database [43]. Intergenic regions (IGRs) were manually analyzed by BLASTX and BLASTN to locate pseudogenes that were not found by GLIMMER. Then they were reanalyzed with Rfam, Pfam and BLASTN to locate pseudogenes that were not found by GLIMMER. After this, the size of the genome, genes and intergenic regions was determined with ARTEMIS [40]. GC content was calculated by the on-line tool GeeCee (http://srs.nchc.org.tw/emboss-bin/emboss.pl?action=input&_app=geecce).

Genome GC difference and CDSs and IGRs GC content analysis

The nucleotide sequences from complete genomes, or from the contigs when the closed genome was unavailable, as occurred in \textit{S. symbiotica} SAp and \textit{R. insecticola}, were recovered from both \textit{S. symbiotica} (SCc and SAp), \textit{S. proteamaculans} (as a free-living symbiotica representative), \textit{H. defensa}, \textit{C. cedri}, \textit{H. defensa}, \textit{S. odorifera}, \textit{S. symbiotica}, \textit{B. aphidicola} (both aphid endosymbionts representatives), and \textit{S. proteamaculans} genomes (in both pseudogene and CDS databases for each organism). The CDSs were grouped by presence of same pseudogenes or by BLAST (using pseudogenes as query and CDSs as subjects, with an e-value cut-off of 1e-05) and checked manually for function annotation. Also, BLASTX was run using pseudogenes as query against CDSs from all three \textit{Serratia} and hits with a Bit Score Ratio >=30 were selected and manually checked. Finally, genes not detected in \textit{S. proteamaculans} (both against \textit{S. symbiotica} SCc and \textit{S. symbiotica} SAp CDSs and pseudogenes) were manually searched on the KEGG orthology database and in selected cases using BLAST with the pseudogene sequence against or (restricted to \textit{Serratia} taxonomy). The \textit{S. symbiotica} SAp pseudogenes that did not match any \textit{S. symbiotica} SCc pseudogenes in the analysis described above were matched to their CDS or pseudogene counterparts in both \textit{S. symbiotica} (SCc and SAp) and \textit{S. proteamaculans} in a similar fashion.

Synteny plots

Protein coding genes from \textit{S. symbiotica} SAp, \textit{S. odorifera} 4Rx13 (ADBX000000000) and \textit{S. proteamaculans} 568 were downloaded from Genbank. \textit{S. symbiotica} SAp CDSs that were in more than one sequence were omitted. We then used BLAST with an e-value cutoff of 1e-05 and 70% match cutoff. The results were clustered using MCL [49]. Common genes from all different \textit{Serratia} species were extracted from each nucleotide FASTA file and ordered by contigs (when not in a single one), and Promer from the Mummer package [50] was used to plot the comparisons. \textit{S. odorifera} 4Rx13 was used to exemplify the contig rearrangement algorithm due to the low number of contigs present in the genome annotation.

Supporting Information

\textbf{Figure S1} Circular map of \textit{S. symbiotica} SCc genome. From outer to inner circles: COG categories in both strands, tRNAs (grey), rRNAs (green), GC skew (red: positive skew, blue: negative skew), G+C content ( purple and orange, % value above and below average, respectively). (TIF)

\textbf{Figure S2} Distributions of GC differences in selected bacteria. The histograms show the distribution for the GC difference (see Materials and methods). The blue curves are empirical density estimates, whereas the red vertical lines represent the sample mean. (TIF)

\textbf{Figure S3} CDSs and IGRs GC content distributions in \textit{S. symbiotica} SCc (A, B) and \textit{S. symbiotica} SAp (C, D), respectively. The blue curves are empirical density estimates, whereas the red vertical lines represent the sample mean. (TIF)
**Figure S4** COG distribution of protein-coding genes in the *S. symbiotica* SAp compared with some obligate and some free living bacterial distributions. (TIF)

**Figure S5** Electron micrograph of the cell (A) *B. aphidicola* BCc, (B) *S. symbiotica* SAp and (C) bacteriocytes of *B. aphidicola* and *S. symbiotica*. mit: mitochondria, m i: inner membrane, m o: outer membrane, m v: eukaryotic vesicle membrane, n: nucleus of bacteriocyte. (TIF)

**Table S1** Pseudogene state comparison of *S. symbiotica* SAp, *S. symbiotica* SAp and *S. proteamaculans* (Spro) and *S. symbiotica* SAp missing genes. (DOC)

---

**References**

1. Moya A, Pereto J, Gil R, Latorre A (2005) Learning how to live together: genomic insights into prokaryote-animal symbioses. Nat Rev Genet 9: 219–229.

2. Moran NA, McCutcheon JP, Nakabachi A (2008) Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet 42: 163–190.

3. Baumann P (2005) Biology bacterioyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol 59: 155–189.

4. Douglas AE (1996) Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu Rev Entomol 41: 37–46.

5. Oliver KM, Degnan PH, Burke GR, Moran NA (2010) Facultative symbionts in aphids and the horizontal transfer of ecologically important traits. Annu Rev Entomol 55: 247–266.

6. Russell JA, Moran NA (2005) Horizontal transfer of bacterial symbionts: heritability and fitness effects in a novel aphid host. Appl Environ Microbiol 71: 7897–7904.

7. Lamelas A, Pérez-Brocal V, Gómez-Valero L, Gosalbes MJ, Moya A, et al. (2008) Evolution of the secondary symbiont ‘‘Candidatus Serratia symbiotica’’ in aphid species of the subfamily Lachninae. Appl Environ Microbiol 74: 4236–4240.

8. Burke GR, Normark BB, Favret C, Moran NA (2009) Evolution and diversity of facultative symbionts from the aphid subfamilly Lachninae. Appl Environ Microbiol 75: 5328–5335.

9. Moran NA, Russell JA, Koga R, Fukatsu T (2005) Evolutionary Relationships of three new species of Entrobacteriaceae living as symbionts of aphids and other insects. Appl Environ Microbiol 71: 3302–3310.

10. Degnan PH, Leonardo TE, Cass BN, Hurwitz B, Stern D, et al. (2009) Dynamics of genome evolution in facultative symbionts of aphids. Environ Microbiol 12: 2050–2069.

11. Degnan PH, Yu Y, Sintner S, Wing RA, Moran NA (2009) *Hymenodictya defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. Proc Natl Acad Sci U S A 106: 9063–9068.

12. Burke GR, Moran NA (2011) Massive genomic decay in *Serratia symbiotica*, a recently evolved symbiont of aphids. Genome Biol Evol 3: 195–208.

13. Pérez-Brocal V, Gil R, Ramos S, Lamelas A, Postigo M, et al. (2006) A small microbial genome: The long of a symbiotic relationship? Science 314: 312–313.

14. Gómez-Valero L, Soriano-Navarro M, Pérez-Brocal V, Hedí A, Moya A, et al. (2004) Coexistence of *Wobachia* with *Buchnera aphidicola* and a secondary symbiont in the Aphid *Cimex lectularius*. J Bacteriol 186: 6626–6633.

15. Gosalbes MJ, Lamelas A, Moya A, Latorre A (2008) The striking case of tryptophan provision in the cedar aphid *Caenis cedri*. J Bacteriol 190: 6026–6029.

16. Consortium TiAg (2010) Genome sequence of the pea aphid *Acyrthosiphon pisum*. Nature 462: 938–939.

17. Lamelas A, Gosalbes MJ, Moya A, Latorre A (2001) New clues about the evolutionary history of metabolic losses in bacterial endosymbionts, provided by the genome of *Buchnera aphidicola* from the aphid *Caena typhonii*. Appl Environ Microbiol 77: 4446–4454.

18. Silva EF, Latorre A, Moya A (2001) Genome size reduction through multiple events of gene disintegration in *Buchnera APS*. Trends Genet 17: 615–618.

19. Belda E, Moya A, Bentley S, Silva EF (2007) Mobile genetic element proliferation and gene inactivation impact over the genome structure and metabolic capabilities of *Saddles glossinidius*, the secondary endosymbiont of tsetse flies. BMC Genomics 11: 149.

20. Cole ST, Eagonliek K, Parkhill J, Thomas KD, et al. (2001) Massive gene decay in the leprous bacillus. Nature 409: 1007–1011.

21. Fukatsu T, Nikoh N, Kayai K, Koga R (2000) The secondary endosymbiotic bacterium of the pea aphid *Acyrthosiphon pisum* (Insecta: homoptera). Appl Environ Microbiol 66: 2748–2758.

22. Chen D-Q, Moullier CB, Porell AH (2000) Fitness effects of two facultative endosymbiotic bacteria on the pea aphid, *Acyrthosiphon pisum*, and the blue alfalfa aphid, *A. kondoi*. Entomol Exp Appl 95: 315–323.

23. Moullier CB, Maxmen A, Porell AH (2002) Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecol Entomol 27: 189–195.

24. Russell JA, Moran NA (2006) Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proc Biol Sci 273: 603–610.

25. Gil R, Silva EF, Zientz E, Delmotte F, Gonzalez-Candelas F, et al. (2003) The genome sequence of *Buchnera aphidicola* and a secondary symbiont of tsetse flies. Nucleic Acids Res 31: D131–D141, 2003.

26. Regalia M, Rosenblad MA, Samuelsson T (2002) Prediction of signal reagents/materials/analysis tools: J Pereto´, A Manzano-Marı´n. Wrote the paper: A Latorre, A Moya, MJ Gosalbes, J Pereto´.

---

**Acknowledgments**

We would like to acknowledge Dr. M. Pignatelli and Dr. G. D’Auria for their helpful assistance with the sequencing assembly. We thank S. Ramos for technical help.

**Author Contributions**

Conceived and designed the experiments: A Lamelas, MJ Gosalbes, A Latorre. Performed the experiments: A Lamelas, MJ Gosalbes, A Latorre. Analyzed the data: A Lamelas, A Latorre, MJ Gosalbes. Contributed reagents/materials/analysis tools: J Pereto, A Manzano-Marin. Wrote the paper: A Latorre, A Moya, MJ Gosalbes, J Peretó.
Transition from Facultative to Obligate Symbiont

44. Bateman A, Coin L, Durbin R, Finn RD, Hollich V, et al. (2004) The Pfam protein families database. Nucleic Acids Res 32: D138–D141.
45. Moriya Y, Itoh M, Okuda S, Yoshizawa AG, Kanehisa M (2007) KAAS: an automatic genome annotation and pathway reconstruction server. Nucleic Acids Res 35: W182–185.
46. Chang A, Scheer M, Grote A, Schomburg I, Schomburg D (2009) BRENDA, AMENDA and FREnda the enzyme information system: new content and tools in 2009. Nucleic Acids Res 37: D588–592.
47. Caspi R, Foerster H, Falcher CA, Kaipa P, Krummenacker M, et al. (2008) The MetaCyc Database of metabolic pathways and enzymes and the BioCyc collection of Pathway/Genome Databases. Nucleic Acids Res 36: D623–631.
48. RDC-Team (2010) R: A language for statistical computing. R Foundation For Statistical Computing, Vienna, Austria, 2.11.1.
49. Van Dongen S (2000) Graph clustering by flow simulation [PhD Thesis]. Utrecht, The Netherlands: University of Utrecht.
50. Kurtz S, Phillippy A, Delcher AL, Smoot M, Shumway M, et al. (2004) Versatile and open software for comparing large genomes. Genome Biol 5: R12.