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NOTES

The Striking Case of Tryptophan Provision in the Cedar Aphid *Cinara cedri*▽†

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Buchnera aphidicola **BCc has lost its symbiotic role as the tryptophan supplier to the aphid *Cinara cedri*. We report the presence of a plasmid in this endosymbiont that contains the *trpEG* genes. The remaining genes for the pathway (*trpDCBA*) are located on the chromosome of the secondary endosymbiont "*Candidatus* Serratia symbiotica." Thus, we propose that a symbiotic consortium is necessary to provide tryptophan.**

Aphids are plant sap-feeding insects that maintain endosymbiotic associations with bacteria (2). Their primary endosymbiont, *Buchnera aphidicola*, plays a role in providing nutrients, such as essential amino acids and vitamins, which are deficient in the insect diet (4). In addition to *B. aphidicola*, some aphid populations harbor facultative intracellular bacteria (3, 12). These bacteria reside in multiple host tissues, such as the gut, glands, body fluids, and cells surrounding the primary bacteriocytes, or in their own bacteriocytes (1). The cedar aphid *Cinara cedri* harbors "*Candidatus* Serratia symbitotica" as a secondary endosymbiont. Although this bacterium is a facultative symbiont in other aphid species, recent studies support the hypothesis that there has been long coevolution of the two symbionts in *C. cedri* (9). Furthermore, "*Ca*. Serratia symbiotica" SCc is always present within well-defined bacteriocytes at a density similar to that of *B. aphidicola* (7). These findings have cast doubt on the facultative status of "*Ca*. Serratia symbiotica" in *C. cedri*. Recently, the genome sequence of *B. aphidicola* BCc from *C. cedri* has been obtained (13). All the analyses performed revealed more extreme gene degradation and accelerated evolution in *B. aphidicola* BCc than in other *B. aphidicola* lineages. Furthermore, unlike other sequenced strains (14, 15, 16), *B. aphidicola* BCc has partially lost its symbiotic role, as it cannot synthesize tryptophan and riboflavin, which must come from another source not only for survival of the host but also for the survival of *B. aphidicola* BCc. Taking into account all functional, evolutionary, and microscopic data, Pérez-Brocal and coworkers (13) proposed that there may be functional replacement of *B. aphidicola* BCc by the coexisting organism "*Ca*. Serratia symbiotica" SCc, which might result in the extinction of *B. aphidicola*. In the present study, we characterized a plasmid containing the *trpEG* genes, which code for anthranilate synthase, the first enzyme of the tryptophan biosynthesis pathway. The remaining genes for the pathway (*trpDCBA*) are located on the chromosome of "*Ca*. Serratia symbiotica" SCc. By using in situ hybridization, we confirmed the physical localization of the plasmid in the bacterial consortium.

C. cedri aphids were collected from a natural population because cedar trees cannot be grown well in the laboratory. In addition, the bacteria *B. aphidicola* and "*Ca*. Serratia symbiotica" cannot be cultured. Bacteriocytes were isolated from aphids, and total bacterial DNA was obtained as previously described (5). We are currently finishing a metagenomic endosymbiont genome-sequencing project that is being carried out at 454 Sequencing Center (United States) using a Roche GS-FLX sequencer. During this project, two different contigs containing tryptophan genes were identified. One contig, which is 2,795 bp long, contains the *trpE* and *trpG* genes that code for antrhanilate synthase, and the other, which is 12,480 bp long, contains the *trpD*, *trpC*, *trpB*, and *trpA* genes. The first contig had a plasmid structure, exhibiting BLASTX search homology with all the tryptophan plasmids described previously for several *B. aphidicola* strains, and the highest scores were associated with plasmid pTrp-BCt from *Cinara tujafilina*, an aphid closely related to *C. cedri* (6, 8, 17). The new plasmid was designated pTrp-BCc. Putative Shine-Dalgarno sequences have been found upstream of each gene, and overlap between the two determinants was detected (Fig. 1A). The putative origin of replication is located downstream of *trpG* and contains 21 direct repeats, each of which has a DnaA box in the complemen-

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† Supplemental material for this article may be found at http://jb.asm.org/.

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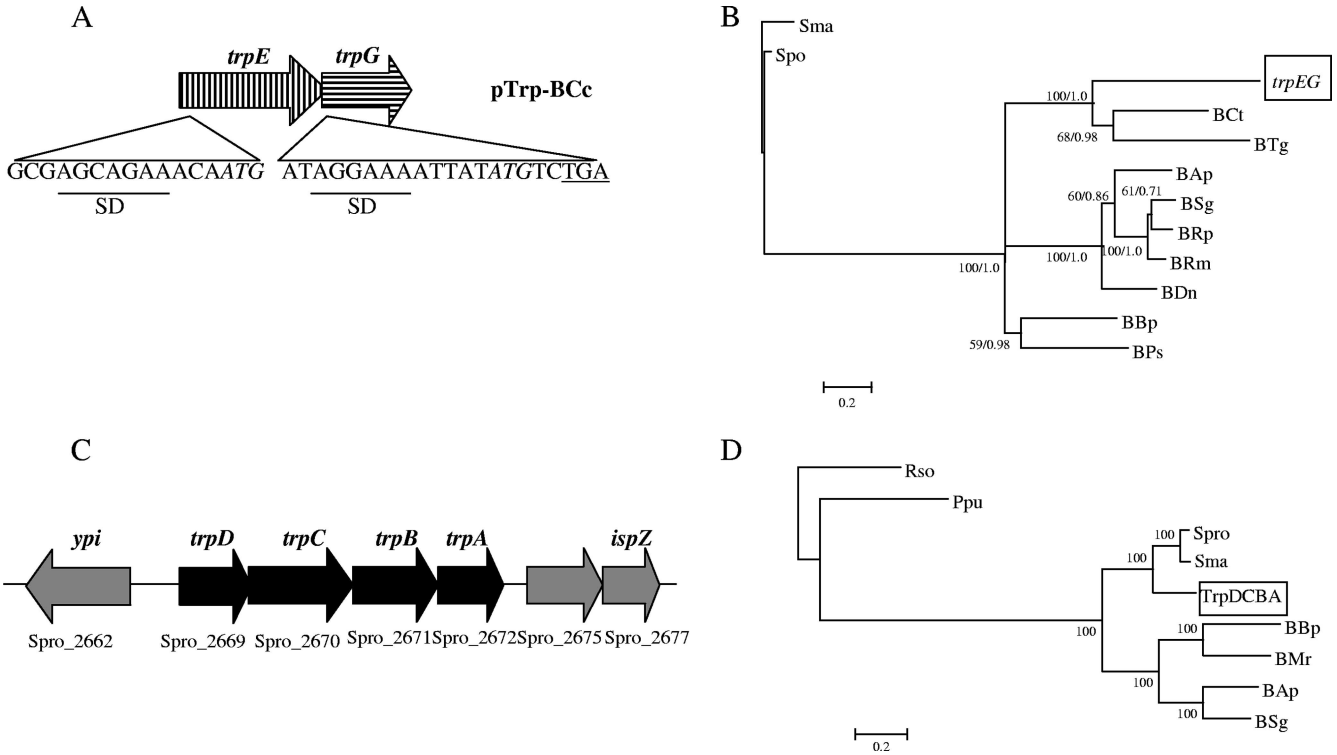

FIG. 1. (A) Physical map of pTrp-BCc. Putative Shine-Dalgarno (SD) and stop codon (TGA) sequences are underlined. Start codons (ATG) are indicated by italic type. (B) Phylogenetic reconstruction based on *trpEG* from *B. aphidicola* strains. BCt, sequence from *C. tujafilina* (accession no. AY438024); BTg, sequence from *Tuberolachnus salignus* (accession no. DQ114495); BAp, sequence from *Acyrthosiphon pisum* (accession no. AP001070); BSg, sequence from *Schizaphis graminum* (accession no. NP_778123); BRp, sequence from *Rhopalosiphum padi* (accession no. L43551); BRm, sequence from *Rhopalosiphum maidis* (accession no. L43550); BDn, sequence from *Diuraphis noxia* (accession no. L46769); BBp, sequence from *Baizongia pistaciae* (accession no. AF492591); BPs, sequence from *Pemphigus spyrothecae* (accession no. AJ012334). *S. proteamaculans* 568 (Spo) (accession no. NC_009832) and *S. marcescens* DB11 (Sma) (accession no. P00897 and P00900) were used as outgroups. (C) Physical map of the conting containing *trpDCBA*. The corresponding proteins from *S. proteamaculans* 568 are indicated. (D) Phylogenetic reconstruction using amino acid sequences obtained from *trpDCBA* genes of "*Ca*. Serratia symbiotica," *B. aphidicola* from *A. pisum* (BAp) (accession no. NP_240101, NP_240102, NP_240103, and NP_240104), *S. graminum* (BSg) (accession no. NP_777879, NP_777880, NP_777881, and NP_777882), *B. pistaciae* (BBp) (accession no. NP_660613, NP_660614, NP_660615, and NP_660616), *Melaphis rhois* (BMr) (accession no. Q9RQ33, Q44687, AAF14253, and Q9RQ35), and *S. proteamaculans* 568 (Spro) (accession no. YP_001478901, YP_001478900, YP_001478899, and YP_001478898). The nucleotide sequences of *S. marcescens* (Sma) (http://www.sanger.ac.uk/Projects/S_marcescens/) were translated to obtain the proteins. *Ralstonia solanacearum* GMI1000 (Rso) (accession no. NP_520102, NP_520104, NP_521006, and NP_521005) and *Pseudomonas putida* KT2440 (Ppu) (accession no. NP_742252, NP_742253, NP_742588, and NP_742587) were used as outgroups. The numbers on branches indicate bootstrap values. The *trpEG* and TrpDCBA described in this work are enclosed in boxes.

tary strand, which is similar to the previously described *B. aphidicola* Trp plasmids (6, 17). The *trpEG* genes described for other *B. aphidicola* strains are present either on a plasmid or on the chromosome and are always separated from the remaining genes for the pathway (10). Figure 1B shows the results of a phylogenetic analysis of the *trpEG* genes from different *B. aphidicola* strains (see the supplemental material for details). All the sequences obtained for endosymbionts of aphids belonging to the subfamily Lachninae are grouped together, supporting the BLASTX search results that showed that there were high levels of homology with the proteins of *B. aphidicola* strains from the aphids *Tuberolachnus salignus* and *C. tujafilina*. In these strains the *trpE* and *trpG* genes are located on a pTrp plasmid and a pLeu/Trp chimeric plasmid, respectively (6). In *B. aphidicola* BCc no tryptophan genes are present in the main chromosome, and no tryptophan plasmid was found during genome sequencing, probably due to the method employed to obtain an enriched fraction of the *B. aphidicola* chromosome (for details, see reference 13). The second contig contained the remaining genes for the tryptophan biosynthesis pathway flanked by three open reading frames (Fig. 1C). These genes exhibited homology to the *trpDCBA* genes, a gene coding for a hypothetical protein, the *ispZ* gene, and the *ypi* gene of the free-living bacterium *Serratia proteamaculans* 568 (http://www.ncbi.nlm.nih.gov). The tree obtained using the amino acid sequences from different endosymbionts and free-living bacteria (see the supplemental material) revealed that the TrpDCBA sequences clustered with *S. proteamaculans* 568 and *Serratia marcescens* DB11 (http://www.sanger.ac.uk/Projects/S_marcescens/), confirming that they belong to "*Ca*. Serratia symbiotica" SCc (Fig. 1D).

To further assess the plasmid location, we carried out microscopic analyses. Adult insects were fixed overnight in

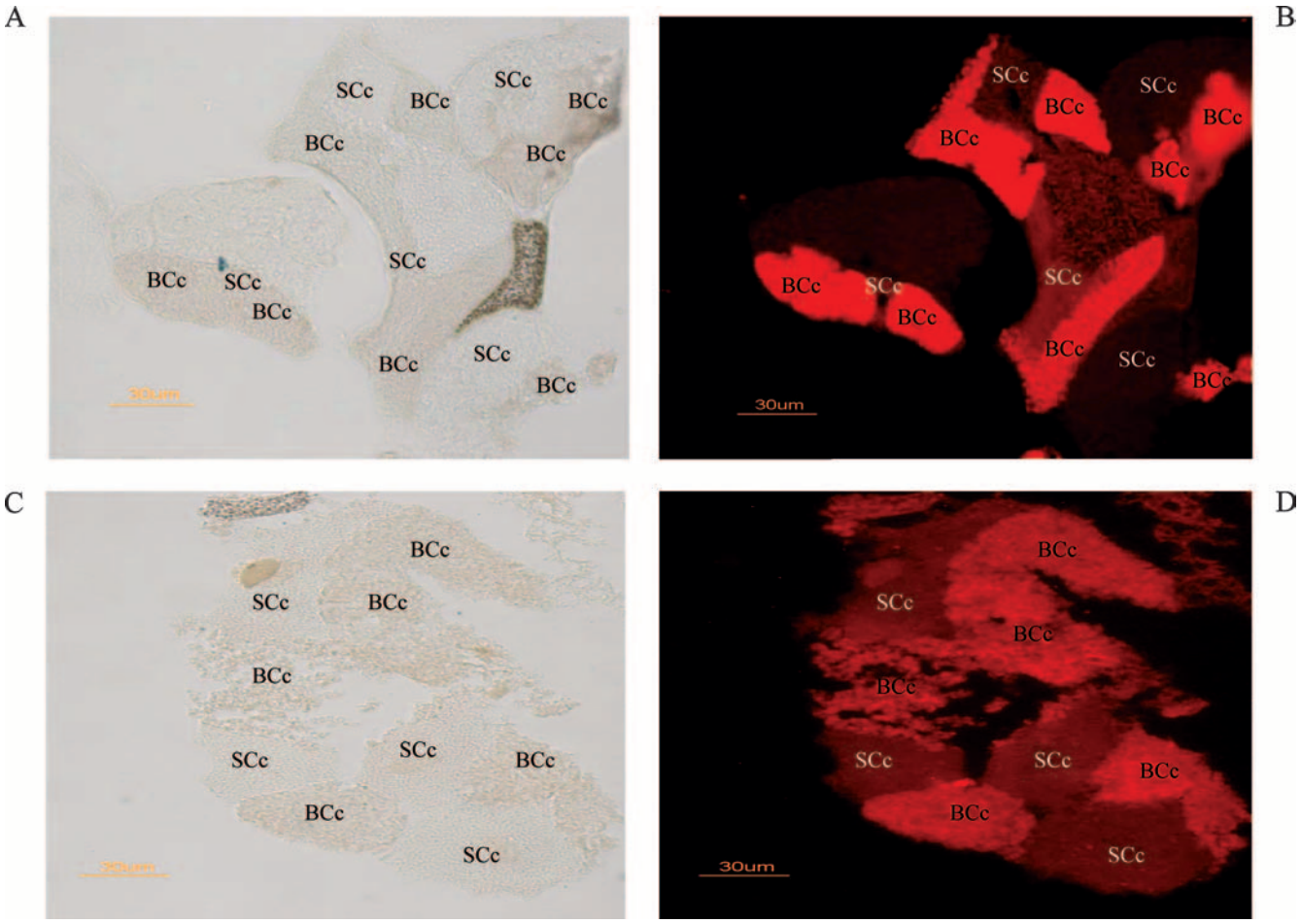

FIG. 2. (A and C) Thin tissue sections of *C. cedri*. (B abd D) In situ hybridization of tissue sections from *C. cedri* adults performed using (B) a *Buchnera*-specific probe and (D) *trpEG* probes. BCc, *Buchnera* bacteriocytes; SCc, *Serratia* bacteriocytes.

4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), embedded in paraffin, and processed to obtain tissue sections. Five-micrometer sections were mounted on poly(L-lysine)-coated microscope slides. After toluene dewaxing and rehydration, the sections were digested with 100 μg/ml proteinase K for 10 min at 37°C. Two types of bacteriocytes were identified in aphid thin tissue sections by light microscopy, which were assigned to the symbionts (7) (Fig. 2A and 2C). To confirm the previous assignment, in situ hybridization was carried out using a *Buchnera*-specific probe targeting the 16S rRNA gene (5′-Cy3-CCCGTTCGCCGCTCGCCGGCA) (Fig. 2B). A eubacterial universal probe, EUB338 (5′-Cy3-GCTGCCTCCCGTAGGAGT), was also used to detect both types of bacteriocytes (data not shown). The samples were mounted with antifading reagent (FluorSave reagent; Calbiochem) and observed with an epifluorescence microscope. In order to localize the pTrp-BCc plasmid in vivo, we used three oligonucleotides probes specific to the *trpEG* genes, TrpE1_Texas Red (TEX) (5′-TEX-GGTACACATCGCTGCATTGAGC), TrpE2_Texas Red (5′-TEX-GTTGCCGATTCCATTGTTACC), and TrpG_Texas Red (5′-TEX-TTGTTAGCGGCGCGATACCAC). The signal was clearly detected in *B. aphidicola* BCc bacteriocytes, confirming that this endosymbiont harbors the plasmid (Fig. 2D). Together, these results revealed that both endosymbionts, *B. aphidicola* and "*Ca*. Serratia symbiotica," are involved in the tryptophan biosynthesis that supplies this essential amino acid to both the host and themselves.

Complementary metabolic abilities have been described for the coresident endosymbionts of the xylem-feeding sharpshooter *Homalodisca coagulata* (11). Even more complex is the biochemical complementation between two gammaproteobacterial endosymbionts and two deltaproteobacterial endosymbionts in the segmented worm *Olavius algarvensis* (18). In this study, we showed that *B. aphidicola* BCc *trpEG* genes code for the anthranilate synthase, the first enzyme in tryptophan biosynthesis, while the other enzymes are coded on the "*Ca*. Serratia symbiotica" SCc chromosome. This complementation implies that the first metabolite, anthranilate, should enter *Serratia* bacteriocytes to obtain tryptophan, which is then supplied to *Buchnera* and the aphid (Fig. 3).

This finding revealed that there is bacterial complementation with "*Ca*. Serratia symbiotica" SCc, not *B. aphidicola* BCc replacement. The relative roles played by the two bacteria, their primary or secondary status as endosymbionts, and the

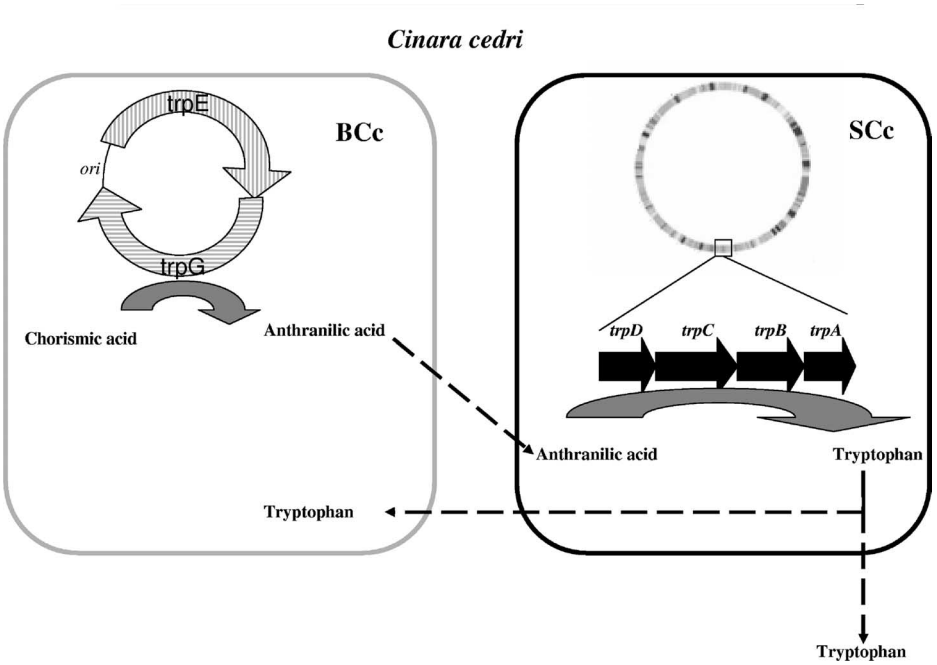

FIG. 3. Schematic representation of predicted biosynthesis pathway and tryptophan flux.

evolution toward a symbiotic consortium should be clarified when the full sequence of the "*Ca*. Serratia symbiotica" SCc genome is determined.

Nucleotide sequence accession numbers. The sequences of the *trpEG* genes of *B. aphidicola* from *C. cedri* and of the *trpDCBA* genes of "*Ca*. Serratia symbiotica" have been deposited in the GenBank database under accession numbers EU660486 and EU660487, respectively.

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