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Report

Defensive Bacteriome Symbiont with a Drastically Reduced Genome

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Summary

Diverse insect species harbor symbiotic bacteria, which play important roles such as provisioning nutrients and providing defense against natural enemies [1–6]. Whereas nutritional symbioses are often indispensable for both partners, defensive symbioses tend to be of a facultative nature [1–12]. The Asian citrus psyllid *Diaphorina citri* is a notorious agricultural pest that transmits *Liberibacter* spp. (*Alphaproteobacteria*), causing the devastating citrus greening disease or Huanglongbing [13, 14]. In a symbiotic organ called the bacteriome, *D. citri* harbors two distinct intracellular symbionts: a putative nutrition provider, *Carsonella*_DC (*Gammaproteobacteria*), and an unnamed betaproteobacterium with unknown function [15], for which we propose the name "*Candidatus* Profftella armatura." Here we report that *Profftella* is a defensive symbiont presumably of an obligate nature with an extremely streamlined genome. The genomes of *Profftella* and *Carsonella*_DC were drastically reduced to 464,857 bp and 174,014 bp, respectively, suggesting their ancient and mutually indispensible association with the host. Strikingly, 15% of the small *Profftella* genome encoded horizontally acquired genes for synthesizing a novel polyketide toxin. The toxin was extracted, pharmacologically and structurally characterized, and designated diaphorin. The presence of *Profftella* and its diaphorin-biosynthetic genes was perfectly conserved in the world's *D. citri* populations.

Results and Discussion

Proposal of a Candidate Name

On account of the distinct genomic and microbiological traits reported in this study, we propose the designation "*Candidatus* Profftella armatura" for the betaproteobacterial symbiont in the syncytial bacteriome of *D. citri*. The generic name refers to the German scientist Joachim Profft, who provided the first comprehensive histological description of psyllid-microbe symbiotic associations [16]. The specific name indicates the defensive property of this bacterium (armatura is armament, the feminine form).

Genome Sequencing of the Bacteriome Symbionts of *D. citri*, *Carsonella*_DC, and *Profftella*

We dissected the bacteriomes from an isofemale strain of *D. citri* (Figure 1A), constructed shotgun libraries, and determined the complete symbiont genomes. Of 33,024 Sanger reads in total, 2,054 and 16,799 were assembled into 174,014 bp (8.3-fold coverage) and 459,399 bp (26-fold coverage) circular bacterial chromosomes, respectively (Figure 2 and Table S1 available online). Judging from the 16S ribosomal RNA (rRNA) gene sequences encoded on the chromosomes, the former represented *Carsonella*_DC, while the latter was attributed to *Profftella*. In situ hybridization targeting 16S rRNAs confirmed the previously reported in vivo localization of the symbionts: *Carsonella*_DC is found in uninucleate bacteriocytes on the surface of the bacteriome, while *Profftella* is located in syncytial cytoplasm at the center of the bacteriome (Figure 1B) [15]. In addition, 59 reads were assembled into a 5,458 bp circular plasmid (7.6-fold coverage) (Figure 2), whose in situ localization agreed with *Profftella* (Figure 1C). Transmission electron microscopy identified *Carsonella*_DC and *Profftella* as large and pleomorphic bacterial cells with distinct morphological traits (Figure 1D). The gene repertoire of *Carsonella*_DC demonstrated its nutritional role, as reported in other *Carsonella* lineages [17, 18]. More-detailed features of the genome of *Carsonella*_DC are provided in the Supplemental Results and Table S1 and Table S2.

Drastic Genome Reduction in *Profftella* Comparable to Obligate Nutritional Symbionts

In general, obligate nutritional symbionts are characterized by features such as perfect infection in host populations, specific localization to the host symbiotic organ, host-symbiont cospeciation reflecting strictly vertical symbiont transmission over evolutionary time, and drastic genome reduction down to less than 1 Mb in size [1, 19, 20]. On the other hand, facultative symbionts are typically characterized by imperfect infection frequencies in host populations, systemic infection in various

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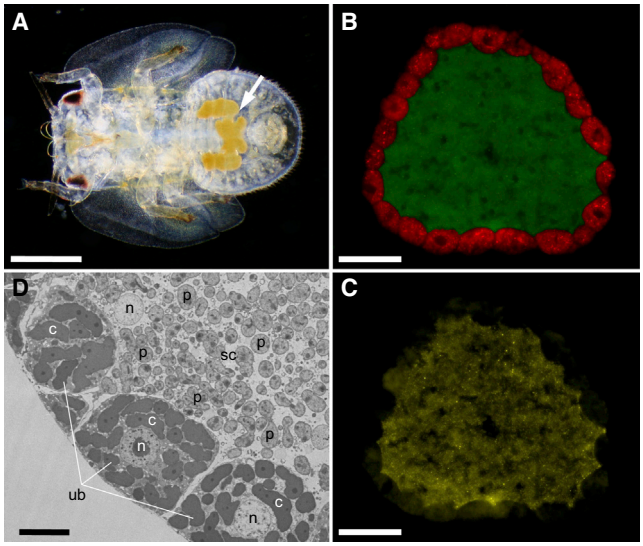

Figure 1. Structural Overview of the Dual Symbiotic System of *Diaphorina citri*

(A) Ventral view of a fifth-instar nymph of *D. citri*. The arrow indicates the yellow and U-shaped bacteriome in the abdomen. The internal tissues are visualized by fixing and clearing the insect through an acetone-ethanol-xylene series. The scale bar represents 500 μm.

(B) In situ hybridization of a cross-section of the bacteriome targeting 16S rRNA of the bacterial symbionts. The red and green signals indicate *Carsonella*_DC and *Profftella*, respectively. The scale bar represents 50 μm.

(C) In situ hybridization of a cross section of the bacteriome targeting the plasmid sequence. The yellow signals exhibit the same localization as *Profftella*. The scale bar represents 50 μm.

(D) Transmission electron microscopy of the bacteriome, in which the pleomorphic bacterial cells of *Carsonella*_DC (c, partially marked) and *Profftella* (p, partially marked) are seen. ub, uninucleate bacteriocyte; sc, syncytial cytoplasm; n, host nucleus. The scale bar represents 10 μm.

cells and tissues, no host-symbiont cospeciation due to occasional horizontal transfers, and no, or only moderate, genome reduction resulting in a size much larger than 1 Mb [1, 3]. The *Profftella* genome (464,857 bp, 24% G + C), which consists of a circular chromosome (459,399 bp) and a small plasmid (5,458 bp) (Figure 2 and Table S1 and Table S2), is much smaller in size than the genomes of facultative symbionts known to date, and, strikingly, even smaller than the genomes of many obligate nutritional symbionts (Table S3). The drastic genome reduction of *Profftella* suggests an intimate and mutually indispensable association with the host over evolutionary time.

Metabolic Complementarity between *Profftella* and *Carsonella*_DC

The metabolic genes encoded by the *Profftella* genome were found to be largely not redundant with those encoded by the *Carsonella*_DC genome (Table S2 and Figure S1A). For example, the *Profftella* genome encoded 16 genes for "coenzyme transport and metabolism (COG category H)," including those for synthesis of riboflavin (*ribA*, *ribBA*, *ribD*, and *ribH*) and biotin (*bioA*, *bioB*, *bioD*, and *birA*), whereas *Carsonella*_DC completely lacked these genes. Such patterns suggest metabolic complementarity between *Profftella* and *Carsonella*_DC, as reported in bacteriome-associated cosymbionts of other plant-sucking hemipterans like sharpshooters, cicadas, spittlebugs, and mealybugs [21–25], as well as other psyllids [18]. However, in contrast to the previously reported cosymbiont genomes of other psyllids, *Ctenarytaina eucalypti* and *Heteropsylla cubana* [18], the *Profftella* genome contained no genes for synthesis of the essential amino acids tryptophan and histidine, which are present in the *Carsonella*_DC genome but deficient in the other *Carsonella* genomes [17, 18] (see the Supplemental Results and Figure S1B). These results highlight a complementary aspect in the *Profftella-Carsonella*_DC cosymbiosis and also illustrate a relatively limited nutritional capacity of *Profftella*.

Large Portions of the *Profftella* Genome Encode Genes for Polyketide Biosynthesis

Notably, as much as 15.0% (69,678 bp/464,857 bp) of the highly reduced genome of *Profftella* was devoted to 20 genes constituting the polyketide synthase (PKS) biosynthetic gene clusters (Figure 2 and Table S2). The PKS system of *Profftella* is separated into two loci on the genome (Figure 2) and exhibited remarkable similarities to PKS clusters responsible for the biosynthesis of pederin, onnamide, and psymberin (Figure 3), which are cytotoxic metabolites produced by symbiotic bacteria of beetles and sponges [7, 26–28]. Since an especially close resemblance was found with the pederin PKS (*ped*), where Ped proteins were 42%–78% identical to their orthologs in *Profftella*, we designated the PKS loci of *Profftella* as *dip* clusters (after Di*aphorina p*ederin-like polyketide). Pederin is a defensive polyketide accumulated in the body fluid of *Paederus* rove beetles for deterring predators [7, 26, 29]. This toxin is produced by an uncultured *Pseudomonas* symbiont that resides in up to 90% of females but not in males [26, 29, 30]. The *dip* and *ped* systems differed in a divergent architecture of the multidomain PKS gene *dipO*, which resembled its ortholog *pedH* only in the upstream half (Figures 3A and 3B), and by the absence of orthologs of the methyltransferase genes *pedA* and *pedO* [31] in the *Profftella* genome (Figure 3A and Table S2).

Prediction of the Polyketide Synthetic Pathway of *Profftella*

The *dip* PKS was classified into the *trans*-acyltransferase (*trans*-AT) subgroup of PKSs [32, 33]. These PKSs are large multimodular proteins that assemble polyketide chains from starter units and several elongation units. A module contains the core chain-elongating ketosynthase (KS) domain and an acyl carrier protein (ACP) domain that serves as an anchor for the growing polyketide. In addition, various optional domains can be present to catalyze a wide range of redox and other reactions. The phylogeny of the KS domains and the overall domain architecture typically mirror the structure of the synthesized polyketide and can therefore be used to predict natural products from PKS sequences [32, 33]. On the grounds that DipP and DipT are virtually identical to PedI (42% identical to DipP) and PedF (44% identical to DipT), respectively (Figures 3A and 3B and Table S2), it was expected that the *dip* biosynthetic product should contain at least an entire pederin core structure (Figures 3B and 3C). In the case of pederin, the polyketide portion attributable to PedH is not found in the natural product purified from the insect, presumably due to a chain cleavage catalyzed by the oxygenase PedG [26]. Since the *dip* system also encodes a close ortholog of this oxygenase, DipN (78% identical to PedG) (Figures 3A and 3B and Table S2), we expected that the *dip* biosynthetic product also lacks the corresponding polyketide portion and thus the PKS DipO (45% identical to the N-terminal half of PedH) does not contribute to the metabolic prediction (Figure 3B). In addition, considering the lack of two *O*-methyltransferases

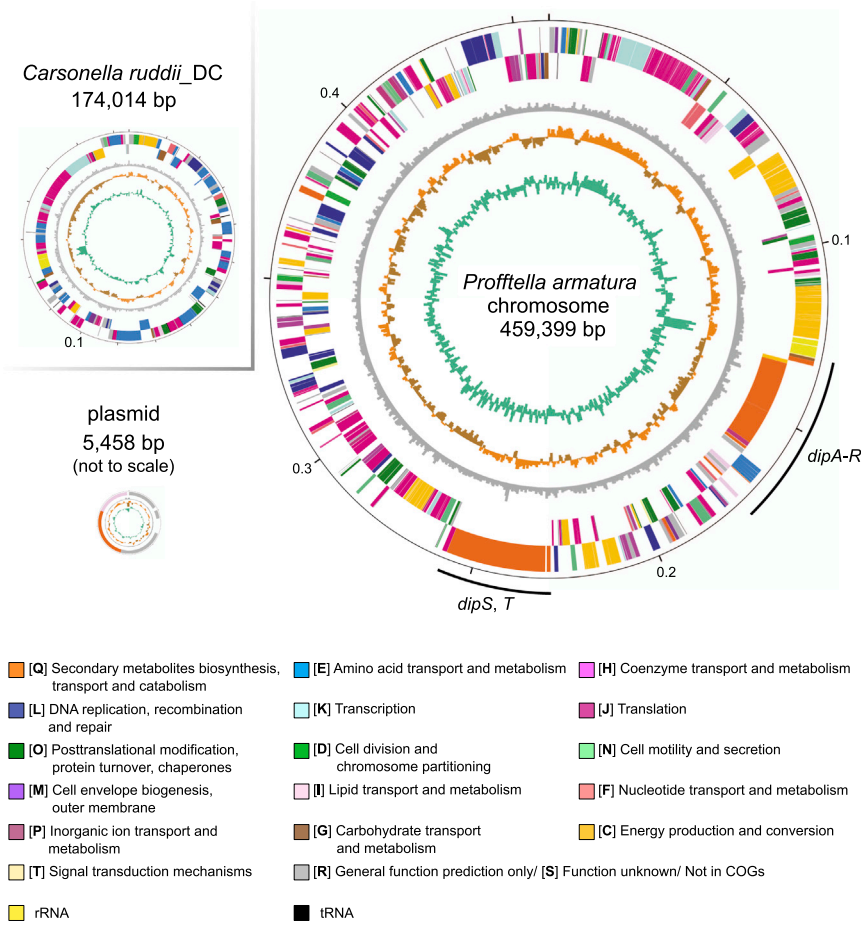

Figure 2. Circular Representation of the Genomes of *Carsonella*_DC and *Profftella*

The concentric rings denote the following features (from outside): (1) the scale in megabases, (2) forward strand genes, (3) reverse strand genes, (4) dinucleotide bias, (5) GC skew, and (6) G + C content. For calculation of (4), (5), and (6), sliding windows of 1,000 bp and a step size of 100 bp were used for the chromosomes, whereas 100 bp windows and a step size of 10 bp were used for the plasmid. The *dip* loci are indicated on the *Profftella* chromosome. See also Figures S1 and S3, and Table S1, Table S2, Table S3, and Table S5.

orthologous to PedA and PedO in the *dip* system, we hypothesized that the *dip* biosynthetic product of *Profftella* may exhibit a structure very similar to pederin but lacking at least two *O*-methyl groups (Figure 3B).

Profftella Produces Diaphorin, an Analog of Pederin

To search for the predicted compound, we extracted over 1,000 adult individuals of *D. citri* and analyzed them by liquid chromatography electrospray ionization mass spectrometry using Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) (Figures S2A and S2B). A major signal at *m/z* 484.2514 was detected, consistent with an ion of formula $[C_{22}H_{39}O_9N+Na]^+$ (theoretical *m/z* 484.2517, $\Delta m = -0.62$ ppm), which corresponds to a tridesmethylpederin. Comparison of the electron-induced dissociation tandem mass spectrometry spectra of the *D. citri* polyketide and pederin revealed similar fragmentation patterns, corroborating their common structural backbone (Figures S2C–S2J). We designated this pederin analog as "diaphorin," after the generic name of the psyllid *Diaphorina*.

Structural Characterization of Diaphorin

For unequivocal structural characterization, diaphorin was purified by reversed-phase high performance liquid chromatography (HPLC), yielding 1.3 ± 0.3 mg from the entire extract. The planar structure (Figure 3D) was solved through the interpretation of one-dimensional and two-dimensional nuclear magnetic resonance (see the Supplemental Results, Figures S2K and S2L, and Table S4), which confirmed diaphorin as a novel pederin congener lacking three *O*-methyl groups. The relative configuration of the two tetrahydropyran rings was determined by rotating Overhauser enhancement and exchange spectroscopy (ROESY) analysis and was shown to be the same as in pederin. Thus, diaphorin exhibits remarkable structural similarity to pederin and represents the first member of this compound family with a nonalkylated hemiaminal moiety.

Horizontally Acquired PKS Genes in *Profftella*

Molecular phylogenetic analyses revealed that many, if not all, *dip* genes of *Profftella* are monophyletic with corresponding *ped* genes of the *Paederus* symbiont with high levels of statistical support (Figure S3). The disparate phylogenetic affiliations of PKS-encoding bacteria (betaproteobacterial *Profftella*, gammaproteobacterial *Paederus* symbiont, and various distantly related free-living bacteria; see Figure S3) strongly suggest that the PKS genes have been horizontally transferred across the phylogenetically and ecologically divergent bacterial lineages. The horizontal transmission route and the evolutionary relationship between *dip* genes and *ped* genes are elusive, but on the basis that *Paederus* rove beetles often feed on hemipteran insects [34], it is conceivable, although speculative, that prey-predator relationship might be involved in the transfer process. The timing of horizontal gene transfer (HGT) is also currently uncertain. The GC skew values on the *Profftella* genome appeared to shift at the boundaries franking the *dip* cluster regions (Figure 2), which could hint at a relatively recent acquisition [35]. However, neither G + C content [36] (23.0% average G + C in *dip* genes versus 25.8% average G + C in other protein-coding genes; Figure 2) nor codon adaptation index (CAI) [37] of *dip* genes (average = 0.792) showed statistically significant differences from the rest of the genome ($p > 0.05$, Kolmogorov-Smirnov test).

Diaphorin Has Significant Cytotoxicity

Purified diaphorin was examined for its cytotoxicity against rat neuroblastoma B104 cells and human HeLa cells (Figure 4). Cell viability assays with a range of diaphorin concentrations revealed IC_{50} values of ~1 μM for B104 cells and ~2 μM for HeLa cells, respectively. In contrast to *Paederus* beetles, wherein only up to 90% of female insects and no male insects

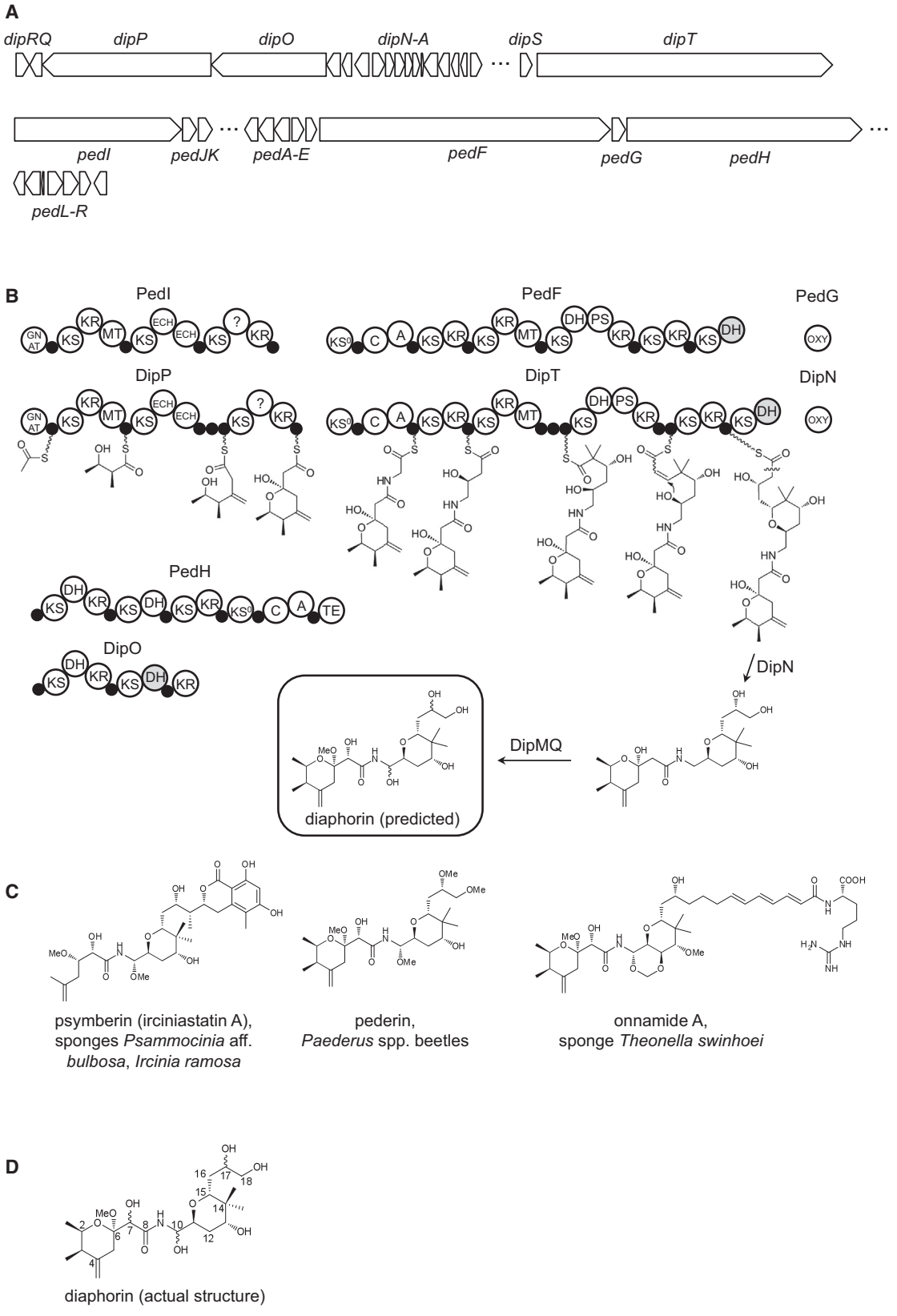

Figure 3. The *dip* Genes, Proposed Biosynthetic Pathway, and Relationship to Other Pederin-Type Metabolites

(A) Map of the *dip* PKS genes with that of *ped* genes for comparison. The dots denote regions that separate PKS loci.

(B) Architecture of the Dip and Ped PKS proteins and predicted biosynthetic pathway for diaphorin.

(C) The structures of psymberin, pederin, and onnamide A for comparison.

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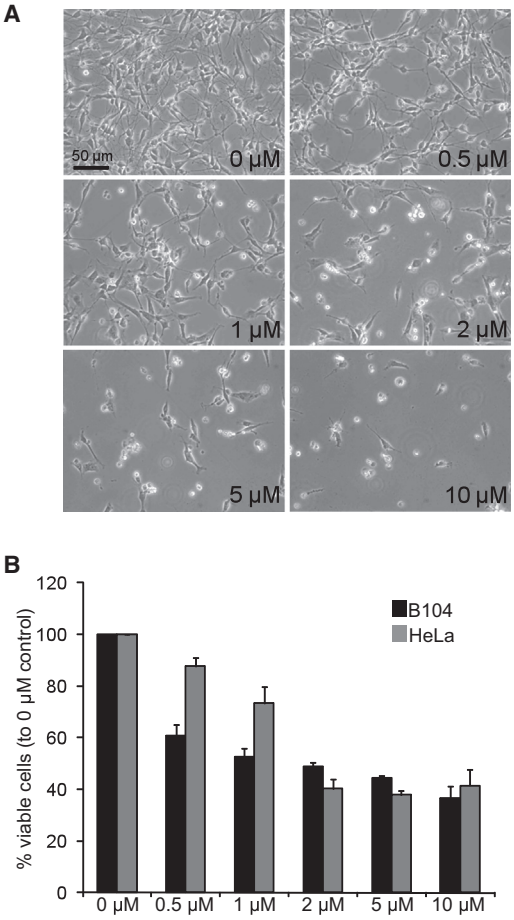

Figure 4. Cytotoxicity Assay of Diaphorin

(A) Morphology of rat B104 neuroblastoma cells after incubation with a range of diaphorin concentrations for 48 hr.

(B) Viablity of rat neuroblastoma B104 cells (black columns) and human HeLa cells (gray columns) after incubation with a range of diaphorin concentrations for 48 hr. Each column indicates the mean and SD (n = 3).

contain pederin [26, 29, 30], HPLC analysis of adult males and females of *D. citri* consistently detected diaphorin at concentrations of approximately 15 μM (~3 μg per insect). These results demonstrate that the diaphorin concentrations present in individuals of both sexes of *D. citri* are sufficient to exert a significant cytotoxicity.

Conservation of the Presence of *Profftella* and Its Polyketide Synthase Genes across World Populations of *D. citri*

In total, 806 individuals of *D. citri* representing nine populations from Japan, Taiwan, Indonesia, and the US were subjected to diagnostic PCR of 16S rRNA genes of *Profftella* and *Carsonella*_DC. Both of the symbionts exhibited 100% infection frequencies in all of the populations examined (Table S5). Furthermore, these individuals of *D. citri* were also subjected to diagnostic PCR of *Profftella*'s PKS genes, *dipP*, *dipO*, and *dipT*. All of the PKS genes exhibited 100% detection rates in all the populations examined (Table S5). No sequence variation was identified in any gene amplicons derived from nine populations. Hence, not only is *Profftella* infection highly conserved across the world's populations of *D. citri*, but so is its genetic capacity for synthesizing diaphorin. These findings suggest crucial roles of the symbionts and their secondary metabolite for the insect and present a sharp contrast to previously known defensive symbionts of other insects that exhibit imperfect infection frequencies in host populations [7–12, 26, 29, 30], while some marine invertebrates were reported to consistently harbor defensive symbionts that are specific to the host species or sibling species [38–40].

Profftella as a Defensive Symbiont with a Drastically Reduced Genome

In summary, (1) the genome size of *Profftella* is only 460 kb, which is comparable to that of obligate nutritional symbionts, (2) as much as 15% of the reduced *Profftella* genome is dedicated to defense-related genes that constitute the PKS system for synthesizing the bioactive polyketide compound, diaphorin, (3) the novel pederin-like polyketide, diaphorin, is present in *D. citri*, (4) diaphorin has cytotoxic activity, (5) the titer of diaphorin in *D. citri* is sufficient to exert a significant cytotoxicity, and (6) the presence of *Profftella* and its PKS genes is highly conserved across the world's populations of *D. citri*. These results indicate that *Profftella* is a previously unknown type of defensive symbiont.

Evolutionary History of *Profftella*

The 640 kb genome of *Profftella* metabolically complements the ancient nutritional cosymbiont and also encodes a defensive toxin. How such a compact dual-functioning symbiont genome has evolved is of interest. One plausible scenario is that an ancestral lineage of bacteriome nutritional symbiont leading to *Profftella* acquired the PKS genes via HGT and began to play a defensive role in addition to its pre-existing metabolic function. Previous studies on bacteriome symbionts have highlighted the evolutionary trends of massive and irreversible genome reduction, with the lack of novel gene acquisitions through HGT, which is a reflection of the fact that they are confined in the host symbiotic organ and sequestered from external microbial populations [1, 19, 20]. Thus, the *Profftella* genome may exemplify an unprecedented case in which a bacteriome symbiont acquired foreign functional genes via HGT. Another plausible scenario is that ancestral *Profftella* had acquired the *dip* genes before or just after it started the symbiotic association with the host. In this context, a recently reported symbiont of the marine tunicate *Lissoclinum patella* is notable [41]. This alphaproteobaterial symbiont, *Candidatus* Endolissoclinum faulkneri, has a 1.48 Mb genome that contains a 86 kb (5.8% of the genome) region encoding toxin-producing trans-AT PKS pathway genes presumably acquired through ancient HGT. While the *Endolissoclinum* genome is considerably larger than the *Profftella* genome, its coding

(D) Elucidated structure of diaphorin. A, nonribosomal peptide synthetase (NRPS) adenylation domain; C, NRPS condensation domain; DH, dehydratase; GNAT, GCN5-related *N*-acetyltransferase superfamily (usually serving as acetyltransferase in PKSs); ECH, enoyl-CoA reductase-like domain; KR, ketoreductase; KS, ketosynthase; KS^0, nonelongating KS; MT, C-methyltransferase; PS, putative pyrane synthase; OXY, oxygenase; TE, thioesterase; ?, region of unknown function. The domains in gray are predicted to be inactive due to missing active-site residues. The small black circles denote PKS and NRPS carrier protein domains.

See also Figures S2 and S3 and Tables S4 and S5.

density is as low as 57%, suggesting that *Endolissoclinum* is at an early stage of reductive genome evolution. On the other hand, *Profftella* is likely at a more advanced stage of reductive genome evolution, in which degenerative DNA regions have been purged whereas the toxin-producing PKS genes have been conserved. Genomic analyses of currently unexplored symbionts closely related to *Profftella* would provide a clue to understanding of the evolutionary process leading to this unique degenerate defensive symbiont genome.

Accession Numbers

The sequences reported in this paper have been deposited in the GenBank database under accession numbers CP003467 (*Carsonella*_DC chromosome), CP003468 (*Profftella* chromosome), and CP003469 (*Profftella* plasmid).

Supplemental Information

Supplemental Information includes Supplemental Results, Supplemental Experimental Procedures, three figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.06.027.

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