Cultivation-Assisted Genome of *Candidatus* Fukatsuia symbiotica; the Enigmatic "X-Type" Symbiont of Aphids

Vilas Patel[1,†], Germain Chevignon[2,†], Alejandro Manzano-Marín[3], Jayce W. Brandt[1], Michael R. Strand[1], Jacob A. Russell[4], and Kerry M. Oliver [1,*]

[1]Department of Entomology, University of Georgia

[2]Montpellier Cell Biology Research Center, Montpellier, France

[3]CBGP, INRA, CIRAD, IRD, Montpellier SupAgro, University of Montpellier, France

[4]Department of Biology, Drexel University

†These authors contributed equally to this work.

*Corresponding author: E-mail: kmoliver@uga.edu.

Accepted: November 13, 2019

Data deposition: This project has been deposited at GenBank under the accessions CP021659-CP021662.

Abstract

Heritable symbionts are common in terrestrial arthropods and often provide beneficial services to hosts. Unlike obligate, nutritional symbionts that largely persist under strict host control within specialized host cells, heritable facultative symbionts exhibit large variation in within-host lifestyles and services rendered with many retaining the capacity to transition among roles. One enigmatic symbiont, *Candidatus* Fukatsuia symbiotica, frequently infects aphids with reported roles ranging from pathogen, defensive symbiont, mutualism exploiter, and nutritional co-obligate symbiont. Here, we used an in vitro culture-assisted protocol to sequence the genome of a facultative strain of *Fukatsuia* from pea aphids (*Acyrthosiphon pisum*). Phylogenetic and genomic comparisons indicate that *Fukatsuia* is an aerobic heterotroph, which together with *Regiella insecticola* and *Hamiltonella defensa* form a clade of heritable facultative symbionts within the Yersiniaceae (Enterobacteriales). These three heritable facultative symbionts largely share overlapping inventories of genes associated with housekeeping functions, metabolism, and nutrient acquisition, while varying in complements of mobile DNA. One unusual feature of *Fukatsuia* is its strong tendency to occur as a coinfection with *H. defensa*. However, the overall similarity of gene inventories among aphid heritable facultative symbionts suggests that metabolic complementarity is not the basis for coinfection, unless playing out on a *H. defensa* strain-specific basis. We also compared the pea aphid *Fukatsuia* with a strain from the aphid *Cinara confinis* (Lachninae) where it is reported to have transitioned to co-obligate status to support decaying *Buchnera* function. Overall, the two genomes are very similar with no clear genomic signatures consistent with such a transition, which suggests co-obligate status in *C. confinis* was a recent event.

Key words: heritable symbiont, bacteria, comparative genomics, insect, evolutionary transition.

Introduction

Insects frequently harbor maternally transmitted symbionts that facilitate host resource acquisition, impact dietary breadth, or contribute defensive services (Moran et al. 2008; Oliver and Martinez 2014). Many associations are ancient (>100 Ma), and by augmenting the nutritional profiles of feeding substrates they have allowed insects to occupy and radiate on a variety of feeding niches, including plant phloem and xylem (Moran and Telang 1998; Baumann 2005; Wilson and Duncan 2015). These "obligate" symbionts are often sequestered in specific host cells called bacteriocytes, and exhibit very small (0.1–1 Mb), static genomes that contain few if any mobile genetic elements (McCutcheon and Moran 2012). More widespread are heritable facultative symbionts, which vary widely in characterized roles across a spectrum from parasitic to conditional mutualist. Heritable facultative symbionts show large variation in age of host-restriction, tissue tropism, and distributions within and among host species (Duron et al. 2008; Oliver et al. 2010; Feldhaar 2011; Guo et al. 2017).

© The Author(s) 2019. Published by Oxford University Press on behalf of the Society for Molecular Biology and Evolution.
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/4.0/), which permits non-commercial re-use, distribution, and reproduction in any medium, provided the original work is properly cited. For commercial re-use, please contact journals.permissions@oup.com

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

Some heritable facultative symbiont-containing lineages, including *Serratia* and *Sodalis*, also contain species of "free-living" bacteria that exist in soil, on plants or are known community members in the guts of different insects (Lo et al. 2016). Examples have also been identified where species have transitioned from facultative to obligate status by substituting for decaying functions in obligate nutritional symbionts that are present in the same host (Meseguer et al. 2017). Heritable facultative symbiont genomes are often intermediate in size (2–4 Mb) between "free-living" bacteria and obligate symbionts, but more similar to "free-living" species, often retain an abundance of mobile genetic elements and pathogenicity-associated factors (Moran et al. 2008; Lo et al. 2016).

Aphids are a group of phloem-feeding insects, including important pests, which are associated with diverse heritable facultative symbiont species (van Emden and Harrington 2007; Oliver et al. 2010; Guo et al. 2017). Individual aphids require infection with an obligate symbiont, usually *Buchnera aphidicola*, which supplements a N-poor phloem diet (Douglas 1998; Gunduz and Douglas 2009). Aphids may also be infected with one or more heritable facultative symbionts, which have functions that range from dietary breadth expansion to conferring protection against biotic and abiotic threats, and aiding decaying *Buchnera* function (Oliver et al. 2014; Vorburger 2014; Meseguer et al. 2017). For instance, the pea aphid, *Acyrthosiphon pisum* (Aphidinae) is variably infected with seven facultative symbionts (Ferrari et al. 2012; Russell, Weldon, et al. 2013; Rock et al. 2018) each with reported conditional benefits in defense against fungal pathogens (Scarborough et al. 2005; Łukasik et al. 2013), parasitic wasps (Oliver et al. 2003; McLean and Godfray 2015; Martinez et al. 2016) and/or thermal stress (Montllor et al. 2002; Russell and Moran 2006; Heyworth and Ferrari 2015).

Sequenced genomes are an important component to understanding the potential effects symbionts have on their hosts. One or more complete genomes are available for some pea aphid facultative symbionts, including *Hamiltonella defensa*, *Regiella insecticola*, *Rickettsiella viridis*, and *Serratia symbiotica* (Degnan et al. 2009, 2010; Burke and Moran 2011; Hansen et al. 2012; Chevignon et al. 2018; Nikoh et al. 2018), but not others, including any strain of *Candidatus* Fukatsuia symbiotica (Manzano-Marin et al. 2017), henceforth *Fukatsuia*, which have been studied under the provisional labels "X-type" or "PAXS" (Guay et al. 2009; Heyworth and Ferrari 2015, 2016; Doremus and Oliver 2017; Doremus et al. 2018). This symbiont also occurs in some Lachninae aphids where it has recently transitioned to a co-obligate symbiont, bolstering a declining *Buchnera* (Meseguer et al. 2017; Russell et al. 2017). In contrast, *Fukatsuia* is clearly a facultative symbiont in pea aphids given that it occurs at intermediate infection frequencies (0–70%) in field populations. Intriguingly, while most pea aphid facultative symbionts are found naturally in both single (i.e., occurs with only *Buchnera*) and coinfection (i.e., occurs with *Buchnera* plus other facultative symbionts) contexts (Ferrari et al. 2012; Russell, Weldon, et al. 2013; Henry et al. 2015; Smith et al. 2015; Rock et al. 2018), *Fukatsuia* typically occurs as a coinfection suggesting other symbionts may be required to sustain its maintenance within host populations. For example, a survey conducted over several years in Wisconsin, found that *Fukatsuia* occurred as a coinfection in 93% ($N = 485$) of infected clones, with a large majority associating with *H. defensa* (Doremus and Oliver 2017). Based on multilocus typing, strain diversity of *Fukatsuia* in pea aphids also appears limited compared with other facultative symbionts (Henry et al. 2013; Doremus and Oliver 2017).

As a coinfection in pea aphids, *Fukatsuia* has been reported to be a "jack-of-all trades" symbiont, providing protection (or enhancing that of its coinfecting symbionts) against thermal stress, fungal pathogens, and parasitoid wasps (Heyworth and Ferrari 2015, 2016; Donald et al. 2016). However, studies that controlled for symbiont and aphid genotypes, found that the prevailing North American strain of *Fukatsuia* provided none of these benefits. Instead, single *Fukatsuia* infections carried high fitness costs, which were partially ameliorated in coinfections (Doremus and Oliver 2017; Doremus et al. 2018). These findings led to the not-mutually exclusive hypotheses that *Fukatsuia* persists in field populations by exploiting the defensive benefits provided by the mutualist *H. defensa* or by enhancing the stability of beneficial *H. defensa* infections through improved transmission rates (Doremus and Oliver 2017; Rock et al. 2018).

Recently, we developed protocols for cultivating *H. defensa* to high abundance in vitro (Brandt et al. 2017) which enabled us to generate sequencing templates that were free of aphid, *Buchnera* or other bacterial DNA, and through long read sequencing produced fully assembled genomes for multiple strains of this symbiont (Chevignon et al. 2018). Here, we used a similar approach to culture a strain of *Fukatsuia* from pea aphids. Having a culture comprised of only *Fukatsuia* enabled us to greatly increase the proportion of sequencing reads that map to this species and to fully assemble its genome using PacBio single molecule real-time (SMRT) sequencing. We then compared this *Fukatsuia* genome to other pea aphid protective symbionts and a *Fukatsuia* strain from a Lachinae aphid, *Cinara confinis*, to identify factors that potentially contribute to the unusual biology of this symbiont.

Materials and Methods

In Vitro Cultivation of the *Fukatsuia* Symbiont

Pea aphid clonal line 5D, naturally infected with *Fukatsuia* and *H. defensa*, was collected on alfalfa (*Medicago sativa*) in 2012 from Dane County, WI. A selective antibiotic cocktail was used to create a subline (i.e., same aphid genotype) carrying

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

only *Fukatsuia* that was used in prior experimental studies to characterize phenotypic effects of infection (Doremus and Oliver 2017; Doremus et al. 2018). This strain of *Fukatsuia* was named Ap5D (= *A. pisum* clone 5D). We next established *Fukatsuia* strain Ap5D in culture following Brandt et al. (2017). Individual aphids were surface sterilized by washing with 5% bleach, 1% Tween 20, and 0.1% ROCCAL-D (Pfizer) in water for three minutes, followed by a 70% ethanol wash (15 s), and then rinsed in sterile, deionized water (30 s). Using sterile forceps, aphids were pierced in the abdomen to release a small amount of *Fukatsuia*-containing hemolymph into a droplet of a commercially available culture medium, TC100 (Sigma), that was supplemented by adding 10% fetal bovine serum (FBS) and TN5 (BTI-TN-5B1-4) cells derived from the lepidopteran *Trichoplusia ni*. The droplet was then transferred into individual culture wells that each contained 1 ml of medium and held at 22–23 °C. We conducted diagnostic PCR with primers that amplify the *hrpA* gene specific for *Fukatsuia* (Doremus and Oliver 2017) to verify cultivation of the expected symbiont. Cultures of *Fukatsuia* strain Ap5D were passaged weekly and ~20 times prior to sequencing.

To estimate the abundance of *Fukatsuia* and *H. defensa* cells in culture, we performed "absolute" quantitative real-time PCR of the single-copy gene *dnaK* using a standard curve with primers specific to each facultative symbiont. We collected 1 ml of medium at 24, 48, and 120 h at 22 °C after passaging a culture and pelleting the bacteria present by centrifugation at 8,000×g for 10 min at 4 °C. We then decanted the medium and extracted DNA from the pellet of bacteria as previously described (Weldon et al. 2013). We also estimated *Fukatsuia* abundance in fourth instar pea aphid nymphs ($N = 6$) using "whole aphid" DNA extractions and correcting for extraction efficiency using the aphid gene ef1α (as in Weldon et al. 2013). Ten microliter reactions, with three technical replicates per sample, were carried out with PerfeCTa SYBR Green Master Mix (Quantabio) using a qTower^3 cycler (Analytik Jena). Primers and reaction concentrations and conditions can be found in Doremus and Oliver (2017).

Genome Sequencing and Assembly

To isolate *Fukatsuia* DNA for sequencing, nonadherent TN5 cells were removed via centrifugation at 500×g; supernatant was then collected and centrifuged at 10,000×g for 15 m to produce a bacterial pellet. DNA extraction was performed using the DNeasy Blood and Tissue kit (Qiagen) followed by titration using a NanoDrop (Thermo Scientific) and visualization of aliquots on a 1.5% agarose gel stained with ethidium bromide to verify DNA integrity. We also Sanger sequenced the above-mentioned diagnostic PCR amplicons as well as a ~1,200 bp 16S rRNA amplicon produced by "universal" bacterial primers (primers and reactions conditions in Doremus and Oliver 2017) which were a 100% matches to prior NCBI GenBank submissions for pea aphid-associated *Fukatsuia* (accession numbers KY271023; KY271016). Samples were then sent to the Drexel University College of Medicine Genome Core Facility for SMRTBell fragment library construction using Long-Insert Genomic DNA followed by SMRT sequencing. Data were collected on a SMRTCell and the number of reads was 110,204 with mean read size of 11,342 (supplementary table S1, Supplementary Material online). *De novo* assemblies were performed with the Hierarchical Genome Assembly Process (HGAP.2) algorithm in the SMRT Portal (version 2.3.0) using default parameters.

We also reassembled and reannotated the genome of a strain of *Fukatsuia* from the Lachninae aphid *C. confinis* (hereafter strain Ci) for improved comparison with the pea aphid Ap5D strain. We used reads from a prior study (Meseguer et al. 2017) combined with an additional 250 bp paired-end library generated from the original source material using the Illumina HiSeq2500 platform. Preassembly treatment of reads and assembly were conducted as described in Meseguer et al. (2017). Briefly, reads were right-tail clipped (minimum quality threshold = 20) using FASTX-Toolkit v0.0.14 (http://hannonlab.cshl.edu/fastx_toolkit/ last accessed August 10, 2018) dropping reads shorter than 75 bp. We also used PRINSEQ v0.20.4 (Schmieder and Edwards 2011) to remove unpaired reads and those containing undefined nucleotides. The remaining reads were assembled using SPAdes v3.10.1 (Bankevich et al. 2012) using the options only-assembler and k-mer sizes of 33, 55, 77, 99, and 127 and contigs shorter than 200 bp dropped. The remaining contigs were binned using results from a BlastX (Altschul et al. 1997) search (best hit per contig) against a database consisting of the pea aphid's proteome and a selection of aphid symbiotic bacteria proteomes (see table S4 at https://doi.org/10.1093/gbe/evy173; Manzano-Marín et al. 2018), including our newly assembled Ap5D *Fukatsuia*. Contigs assigned to *Fukatsuia* were manually confirmed using a BlastX search of the nr database. We also ran a BlastN search against the Ap5D *Fukatsuia* genome recovering the 16S and 23S rRNA-containing contigs. The resulting contigs were then used as a reference for read mapping and genome assembly using SPAdes with read error correction.

Genome Annotation

The assembled genome for the *Fukatsuia* Ap5D strain and the reassembled *Fukatsuia* Ci strain were submitted to the National Center for Biotechnology Information (NCBI) Prokaryotic Genomes Annotation Pipeline (PGAP), Rapid Annotation using the Subsystem Technology tool kit (RASTtk) (Brettin et al. 2015). Predictions from NCBI and RAST annotation were merged and compared with *B*acterial g*E*nome *A*nnotation *C*omparis*ON* (BEACON) (Kalkatawi et al. 2015) and manually curated using Geneious (www.geneious.com). Predictions for ribosomal (r), transfer (t), and transfer-messenger (tm) RNAs were retained from the PGAP annotation. ORFs that had >80% truncation or fragmented coding

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

sequences (CDSs) were designated pseudogenes in the final annotation. PacBio sequencing technology can generate a deletion bias in homopolymeric runs, especially in GC-rich regions (Ross et al. 2013) inflating pseudogene calls. After verifying that all homologous loci in the Ci strain were intact, we manually corrected all deletions in the Ap5D strain. Noncoding RNAs (ncRNA) were predicted using Infernal cmscan (www.ebi.ac.uk/Tools/rna/infernal_cmscan/; last accessed June 2018) (Nawrocki and Eddy 2013). Functional information of genomes was retrieved from PGAP and RASTtk. Pfam domains were retrieved with hmmscan (www.ebi.ac.uk/Tools/hmmer/search/hmmscan; last accessed December 2018) and protein homology with phmmer (www.ebi.ac.uk/Tools/hmmer/search/phmmer; last accessed December 2018) and Joint Genome Institute IMG/MER (Markowitz et al. 2012). KEGG Orthology (KO) was assessed by the automatic annotation servers BlastKOALA (KEGG Orthology And Links Annotation) (Kanehisa et al. 2016). Clusters of Orthologous Groups (COGs) and Gene Ontology (GOs) were retrieved with EggNOG 4.5.1 (Huerta-Cepas et al. 2016). Transposable element predictions were made using the ISfinder (Siguier et al. 2006) and ISsaga (Insertion Sequence semiautomatic genome annotation) (Varani et al. 2011). Phage island predictions were made using PHASTER (Arndt et al. 2016) and identification and classification of secretion systems was performed with TXSScan (Abby 2016). Plasmid islands were identified manually using BLAST (Altschul et al. 1990).

Comparative Genomics

The 77 contigs identified in the original sequencing of the *Fukatsuia* Ci strain (Manzano-Marin et al. 2017) along with the 381 contigs identified in the reassembly of this genome during the current study were aligned to the complete genome generated for the *Fukatsuia* Ap5D strain using nucmer from the MUMmer package (Kurtz et al. 2004). Alignments were plotted using Circos (Krzywinski et al. 2009). We then used the tool "annotation from" in Geneious v10.2 (www.geneious.com) to map our annotation for the *Fukatsuia* Ap5D strain onto the *Fukatsuia* Ci strain followed by extensive manual curation. This allowed us to identify *Fukatsuia* Ap5D CDSs not present in *Fukatsuia* Ci and fragmented CDSs in *Fukatsuia* Ci which were usually positioned at contig ends. Finally, we conducted pairwise alignment of the 2,340 CDSs shared by both *Fukatsuia* strains using MAFFT (Katoh and Standley 2016). Whole genome alignments were conducted using both the ProgressiveMauve and Mauve Contig Mover algorithms in Geneious Prime 2019.2.1. (Rissman et al. 2009; Darling et al. 2010).

Phylogenetic Reconstruction

Fragments of seven single-copy orthologous genes (*accD, dnaA, gyrB, murE, ptsI, recJ*, and *rpoS*) were used for reconstructing phylogenetic relationships of *Fukatsuia* and other enterobacterial relatives. Partial sequences for these loci from 26 species were downloaded from NCBI (supplementary table S2, Supplementary Material online). Protein alignments were conducted using the Geneious aligner v10.2 with Blosum-62 cost matrix and aligned orthologs were manually trimmed and concatenated into a supermatrix (Henikoff and Henikoff 1992; Kearse et al. 2012). We used ModelFinder (Kalyaanamoorthy et al. 2017) to identify the best models of sequence evolution for each locus separately (supplementary table S2, Supplementary Material online). We then used IQ-TREE v1.6 (Nguyen et al. 2015) to estimate evolutionary relationships using maximum likelihood with partitioned models (Chernomor et al. 2016) and branch support approximated using UFBoot2 with 1,000 replicates (Hoang et al. 2018). The phylogenetic tree was edited with TreeGraph2 v2.14.0-771beta (Stover and Muller 2010).

Base Modification

Base modification analysis and motif detection were performed using the RS_Modification_and_Motif_Analysis.1 algorithm in the SMRT Portal with standard settings (minimum-modification QV of 30). To reduce false positive motif detection due to high level base coverage, we reanalyzed the data output with specific scripts provided in Base Modification Tools accessible on the Pacific Biosciences GitHub pages (https://github.com/PacificBiosciences/Bioinformatics-Training/ wiki/BaseModification-Tools) using a minimum-modification QV of 100 for base modification and motif prediction. Motifs were then manually curated per PacBio guidelines.

Results and Discussion

Candidatus Fukatsuia symbiotica Can Be Cultured In Vitro to Produce an Isolated Template for Genomics

The in vitro culture of *Fukatsuia* strain Ap5D allowed us to generate DNA template for genome sequencing that was free of contaminating aphid, *Buchnera* or other bacterial DNA with the large majority (85%) of PacBio SMRT sequencing reads (N50 read length = 27,056) mapping to *Fukatsuia* and providing 291× coverage. See supplementary table S1, Supplementary Material online, for a summary of sequencing metrics. Cultures of *Fukatsuia* Ap5D were routinely passaged each week and maintained at an abundance per ml that was within an order of magnitude across examined time points (2.1, 1.9, and 1.6×10^8 per ml at 24, 48, and 120 h after passage, respectively) of that observed in fourth instar (6 day old) aphid nymphs (= 6.8×10^8 per aphid). We also separately cultured *Fukatsuia* and *H. defensa* at the same time to confirm that both facultative symbionts could be cultured under identical conditions. Although *H. defensa* abundance and growth rates were higher in culture (1.9, 2.5, and

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

3.3×10^9 per ml for 24, 28, and 120 h, respectively) compared with *Fukatsuia*, they were also substantially higher in aphids (Brandt et al. 2017; Doremus and Oliver 2017). The ability to maintain *Fukatsuia* and *H. defensa* at relatively high titers under similar conditions provides opportunities to study function when biology observed in vivo can be recapitulated in vitro.

Fukatsuia Forms a Clade with the Protective Aphid Symbionts *H. defensa* and *R. insecticola*

The order Enterobacteriales (class Gammaproteobacteria) traditionally contained the single large family Enterobacteriaceae. However, recent analyses propose seven families, including the Yersiniaceae, which contains the well-known genera *Yersinia* and *Serratia* (Adeolu et al. 2016). Our maximum likelihood phylogeny indicates that *Fukatsuia* resides in the Yersiniaceae and is the sister group to *Regiella* with 100% bootstrap support (fig. 1). *Fukatsuia*, *R. insecticola*, and *H. defensa* further form a highly supported clade (99% bootstrap support) of insect heritable facultative symbionts that are primarily associated with aphids although *H. defensa* is also found in other sternorrhynchan Hemiptera, including whiteflies, and possibly a coccinellid predator (Clark et al. 1992; Russell et al. 2003; Majerus and Majerus 2010). The sister-relationship between *Regiella* and *Fukatsuia* contrasts with previously reported phylogenies using fewer loci that instead showed *Fukatsuia* as basal to a clade comprised of *H. defensa* and *R. insecticola* (Doremus and Oliver 2017; Manzano-Marin et al. 2017).

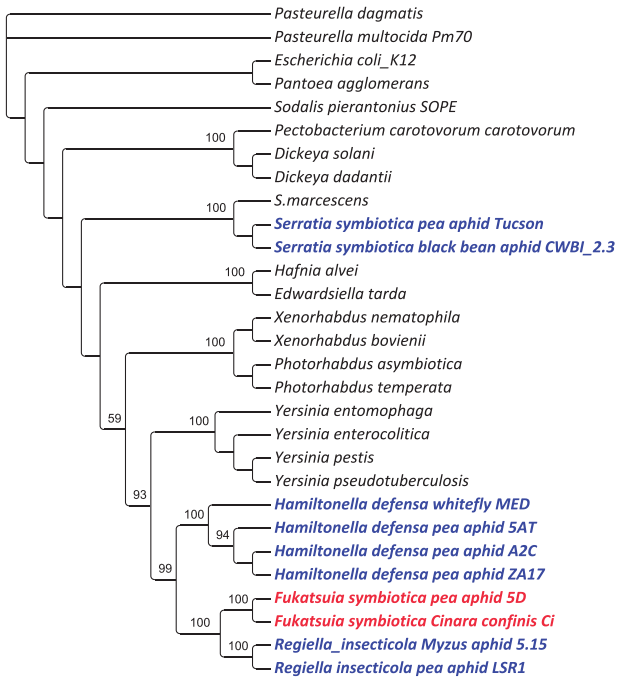

Fig. 1.—Maximum likelihood chronogram based on amino acid sequences of fragments of seven single-copy orthologs from 26 bacterial species/strains. The colored taxon label indicates our focal species, *Fukatsuia symbiotica* (red) as well as related symbionts of hemipteran insects (blue). The numbers indicate percent support based on 1,000 ultrafast bootstrap replicates.

Overview of the *Fukatsuia* Genome Compared with Related Facultative Symbionts

Long-read sequencing of the *Fukatsuia* Ap5D strain yielded a fully assembled genome that consisted of a circularized main chromosome of 2,824,275 bp and three plasmids named pFS5D.1 (148,330 bp), pFS5D.2 (91,928 bp), and pFS5D.3 (67,451 bp) (supplementary table S1, Supplementary Material online). As is the case for other aphid facultative symbionts, genome-based inferences of central metabolism indicated that *Fukatsuia* is a host-dependent, aerobic heterotroph with a total genome size (3.1 Mb) intermediate between the obligate nutritional symbiont, *Buchnera aphidicola* (640 kb) and the related free-living pathogen *Yersinia pestis* (4.7 Mb) (fig. 2 and table 1). GC content (43.5%) was nested within the range of other aphid facultative symbionts (40–52%) while a total of 2,607 CDSs were identified in the main chromosome, including 160 pseudogenes. Gene content was also overall similar to other aphid facultative symbionts (*S. symbiotica*, *H. defensa*, and *R. insecticola*) with large overlap in genes involved in central metabolism, transport, energy production as well as lipid and cell wall synthesis (see fig. 3 for metabolic reconstruction; supplementary table S3, Supplementary Material online, for specific gene comparisons). The *Fukatsuia* Ap5D genome further contained a relatively larger repertoire of genes with predicted functions in replication, recombination and repair, cell motility, inorganic ion transport and metabolism, secondary metabolism, and signal transduction. The proportion of the genome containing mobile genetic elements was also similar to that found in *H. defensa* and *R. insecticola* (see supplementary table S4, Supplementary Material online, for complete annotation).

Annotation of the main chromosome for the *Fukatsuia* Ap5D strain indicated that the pathways required for biosynthesis of nonessential AAs, cofactors (coenzyme A, isoprenoids, ubiquinone), and B vitamins (B1, 2, 3, 6, 7, and 9) are intact, which is similar to related symbionts (*H. defensa* and *R. insecticola*) that reside in the same clade (supplementary table S3, Supplementary Material online). In addition, similar to related symbionts, Ap5D lacked genes in the pathways required for biosynthesis of some essential amino acids (EAAs) (supplementary table S3, Supplementary Material online). However, the presence of EAA transporter genes, suggests *Fukatsuia* Ap5D likely acquires these products from the aphid host and *Buchnera* and persists, similar to other facultative symbionts, as a nutritional parasite. In contrast, *Fukatsuia* Ap5D differed from all known strains of *H. defensa* or *R. insecticola* in that it encodes all genes required for synthesis of branched-chain amino acids (BCAAs)

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

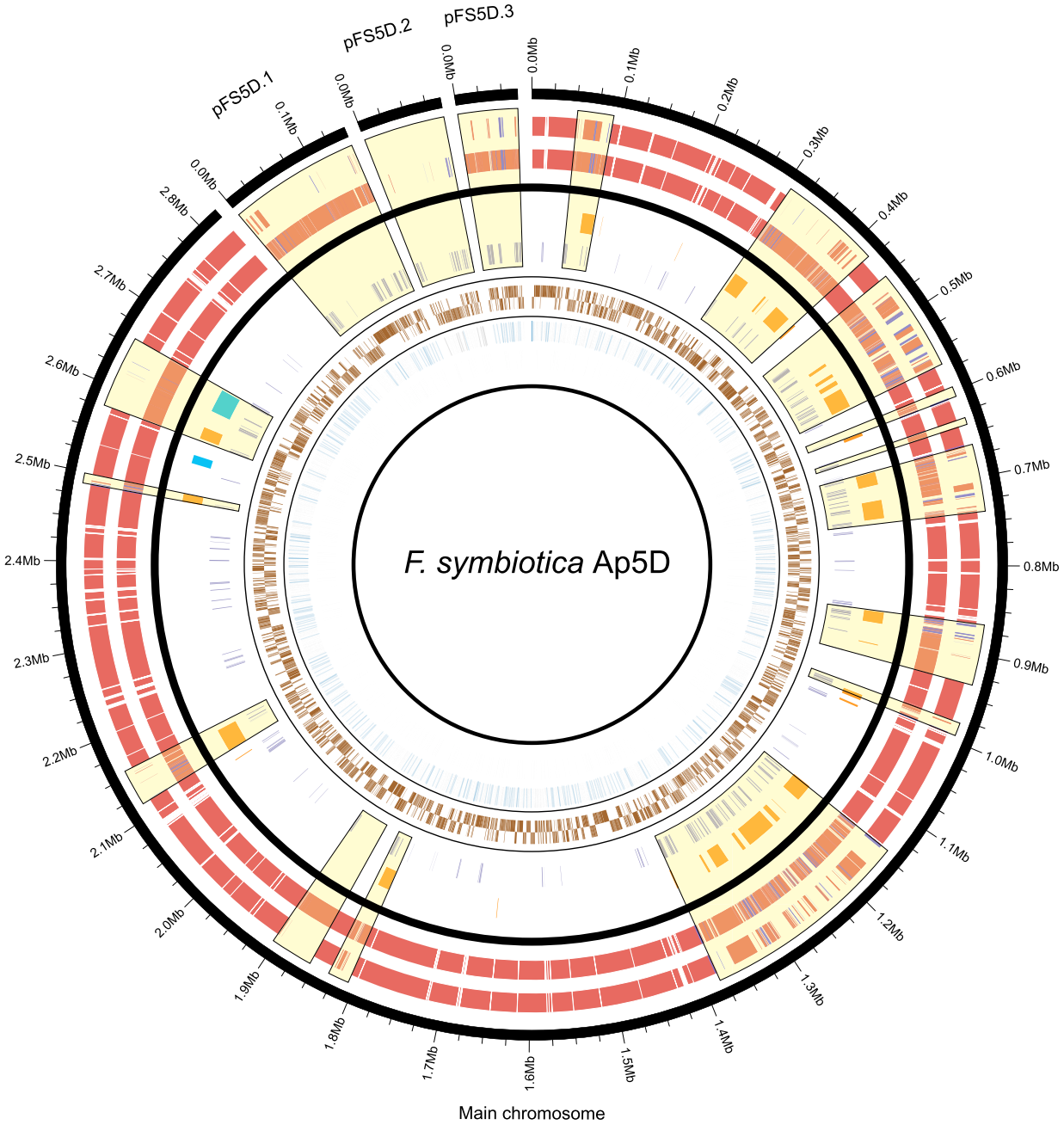

Fig. 2.—Circos plot highlighting the genomic features of *Fukatsuia* Ap5D and its relationships with *Fukatsuia* Ci. Circles from outermost radius to innermost: nucmer alignment of 77 contigs from the first version of *Fukatsuia* Ci genome (Manzano-Marin et al. 2017), red = > 95% identity, blue <95% identity; nucmer alignment of 381 contigs from the updated version of *Fukatsuia* Ci genome (this study); genomic islands on the main chromosome of *Fukatsuia* Ap5D, orange = prophage islands, light-blue = plasmid islands; Transposable elements TEs; sense and antisense CDSs; Methylated G^{m6}ATC motifs, blue = modified motifs, gray = unmodified motifs; Methylated TGGCC^{m6}A motifs, green = modified motifs, gray = unmodified motifs. Light-yellow squares highlight most the divergent regions between *Fukatsuia* strains Ap5D and Ci.

such as valine, leucine, and isoleucine. Although the nutritional symbiont *Buchnera* lacks the terminal genes in the BCAA pathways, the presence and expression of these genes in aphids enables coordinated BCAA biosynthesis (Shigenobu et al. 2000; Wilson et al. 2010; Russell, Bouvaine, et al. 2013). Whether an intact pathway for BCAA synthesis by *Fukatsuia* Ap5D affects the biology of aphids, symbiont phenotypes, or interactions with other bacterial symbionts is currently unclear.

Annotation of the three plasmids in the Ap5D strain indicated that pFS5D.1 contains 134 CDSs including 17 pseudogenes, 35 TEs, partial components of T1SS (TolC, HylD) and

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

Table 1

General Features of the Genome of *Candidatus* Fukatsuia symbiotica Strain Ap5D Relative to Other Pea Aphid Symbionts

	Buchnera aphidicola APS	*Hamiltonella defensa* 5AT	*Regiella insecticola* LSR1	*Serratia symbiotica* Tucson	*Fukatsuia symbiotica* Ap5D
Chromosome (bp)	640,681	2,110,331	2,035,106	2,789,218	2,824,275
Extrachromosomal elements	2	1	1	unknown	3
Total G+C (%)	26.2	40.3	42.4	52.0	43.5
Total predicted CDSs	571	2,243	1,761	2,098	2,929
Total pseudogenes (including plasmids)	13	203	214	550	189
rRNA operons	2	3	4	5	5
tRNAs	32	42	36	44	48
Lifestyle	Obligate	Facultative	Facultative	Facultative	Facultative

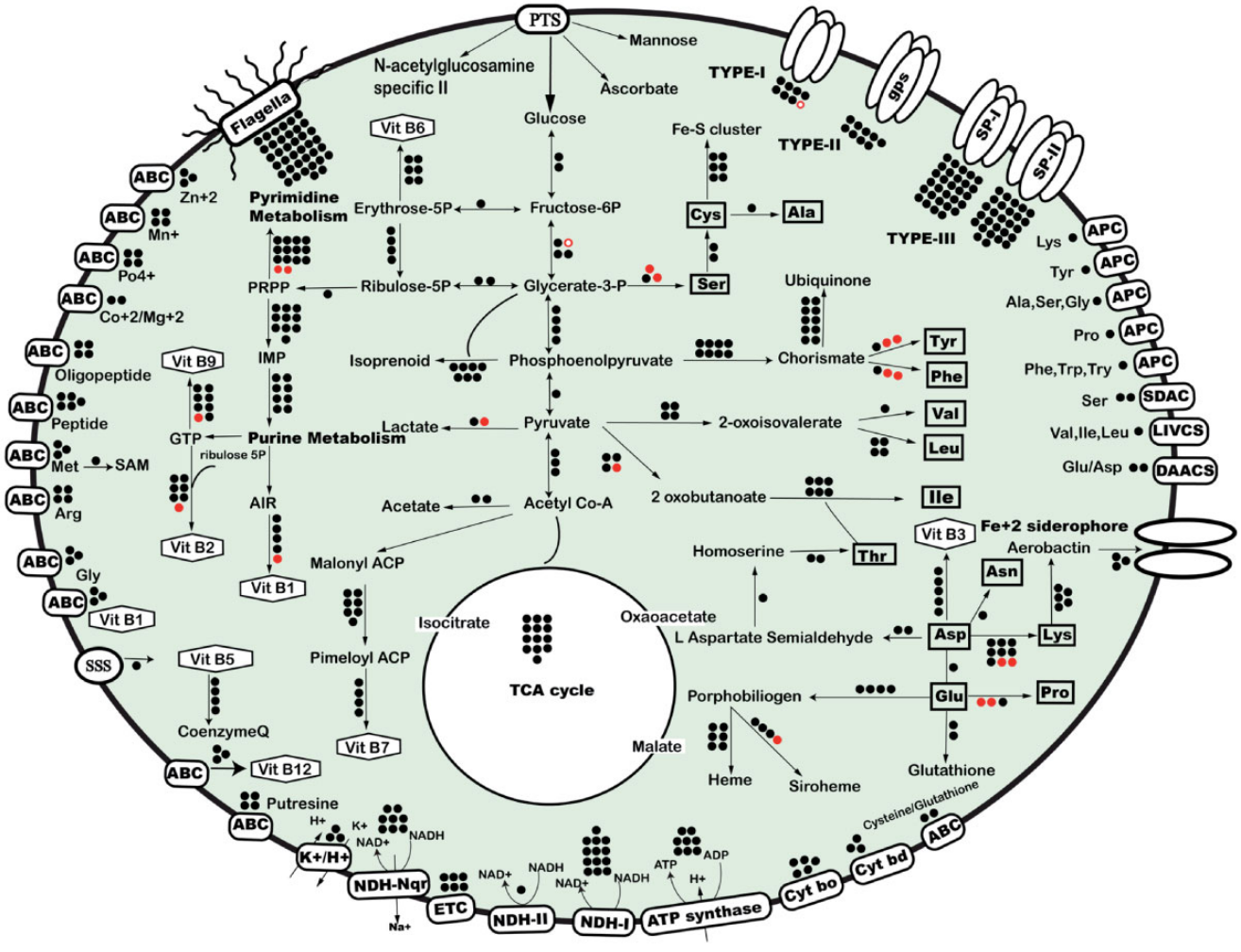

Fig. 3.—Metabolic map of *Fukatsuia symbiotica* strain Ap5D constructed using BLASTKOALA (Kanehisa et al. 2016) and IMG-ER (Markowitz et al. 2012). Amino acid pathways are shown in boxes; vitamins as hexagons. Solid black dots represent intact genes, solid red circles depict absent genes and open red circles indicate pseudogenes.

T4SS (*tra* and *pil* genes), and toxin–antitoxin systems. Ten genes comprising 34,136 bp (spanning two loci) encode a nonribosomal peptide synthase (NRPS) module with homology to one found in betaproteobacterial *Chromobacterium* species. NRPS are ribosome-independent units often involved in the production of bio-active molecules (Izore and Cryle 2018), which together with its presence on a plasmid, warrants further investigation. pFS5D.2 contained 121 CDSs

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

(21 repeats, 9 pseudogenes), including genes associated with T4SS (*virB*), toxin–antitoxin genes (*higA/higB* and *vapBC*), and transcription regulator genes, while pFS5D.3 contained 70 CDSs (23 repeats, 4 pseudogenes) and was also very similar in gene content to the plasmid p5DHAT in *H. defensa*.

Overall, the ability to culture *Fukatsuia* Ap5D using conditions identical to those used for *H. defensa* is consistent with the high similarity in genome content among this related clade of facultative symbionts, and suggests *R. insecticola* may be similarly cultivated.

Diverging Paths for *Fukatsuia*?

Our reassembly and -annotation of previously generated short-read sequence data yielded a genome for the *Fukatsuia* Ci strain that was comprised of 381 contigs (up from 77) and had an estimated size of 2.79 Mb (up from 2.03) (supplementary table S1, Supplementary Material online). We also identified two of the three plasmids (pFS5D.1 and pFS5D.3) present in the Ap5D strain and numerous fragmented CDSs. As earlier noted, the *Fukatsuia* Ci strain was previously hypothesized to support the loss of biosynthetic capability in *C. confinis*-associated *Buchnera* for riboflavin (B2) and biotin (B7), and thus has potentially transitioned from facultative to co-obligate status (Meseguer et al. 2017). These pathways are intact in the *Fukatsuia* Ci strain, but are also present in the Ap5D strain as well as other common heritable facultative symbionts (supplementary table S3, Supplementary Material online).

One expectation for a facultative symbiont transitioning toward co-obligacy would be a further reduction in genome size compared with facultative strains. The estimated size of the genome for the Ci strain is 337,000 bp smaller than for Ap5D (total CDS of main chromosome + plasmid = 2,800 vs. 2,932, respectively). However, we note that a large proportion of this difference is due to the apparent absence of pFS5D.2 in the Ci strain, while most other "missing" genes in the Ci strain are associated with mobile genetic element islands rather than single copy orthologs. Thus, while the Ci strain is a subset of the Ap5D strain (fig. 4 and supplementary table S3, Supplementary Material online), which is consistent with genome reductions following a transition to co-obligacy, the smaller estimated genome size for the Ci strain more likely reflects an incomplete assembly owing to the short read Illumina data generated to sequence this strain.

A recent transition to co-obligate status might also lead to differences in the number of pseudogenes present between strains. Proliferation of mobile elements and pseudogenes is often observed after transition to a host-restricted lifestyle (Moran and Plague 2004). We identified more pseudogenes in the Ap5D ($N = 189$) than Ci strain ($N = 126$) (supplementary table S1, Supplementary Material online) but also noted that about half (46) of the pseudogenes found only in the Ap5D strain occur as fragments at contig ends in the Ci strain, which prevented us from classifying them as intact. A total of 23 pseudogenes in the Ap5D strain had no homolog (intact or pseudogenized) in the Ci strain, whereas no pseudogenes in the Ci strain were missing homologs in the Ap5d strain. This pattern could reflect intensified genome degradation associated with transition to co-obligacy by the Ci strain, since a similar pattern was observed for a range of *S. symbiotica* strains that vary from having a facultative to obligate association with their hosts (Manzano-Marín and Latorre 2016). However, it is more likely the differences observed between the Ci and Ap5D strains reflect the differential quality of the genomes we were able to assemble. When considering only pairwise comparisons (i.e., genes present in both genomes; fig. 4), 117 pseudogenes are shared by both strains, 8 pseudogenes in the Ci strain are intact in Ap5D, and 10 pseudogenes in the Ap5d strain are intact in Ci. Most of these pseudogenes are also in mobile regions of the genomes.

Altogether, the most striking feature in our comparison of the Ci and Ap5d genomes is just how similar they are overall: 92% of complete pairwise CDSs share 100% nucleotide identity and 98% share ≥99% similarity, with what little variation exists occurring in genes associated with mobile genetic elements. Although we cannot order the 381 contigs from the Ci strain and hence determine long range rearrangements, synteny within larger contigs is similar to the Ap5D strain (supplementary fig. S1, Supplementary Material online). Thus, despite an abundance of mobile genetic elements (see below), that are often associated with genome rearrangements (Wu et al. 2004; Degnan et al. 2010; Chevignon et al. 2018), gene order to the extent we can compare it, appears conserved between the Ap5D and Ci strains. This similarity becomes more impressive given the very different impacts posited for the Ci and Ap5D strains in their respective aphid hosts (Doremus and Oliver 2017; Meseguer et al. 2017), which belong to distantly related subfamilies (Ortiz-Rivas and Martínez-Torres 2010). Such similarity may result from *Fukatsuia* persisting as a low genetic diversity heritable symbiont with a widespread distribution or this similarity might reflect a relatively recent horizontal transfer event between these distantly related aphid hosts. The overall similarity between the two strains also confirms that the transition to co-obligate status in Lachninae aphids was likely a recent event as proposed by Meseguer et al. (2017). In Lachninae aphids several other facultative symbionts have likewise transitioned to co-obligate status, and exhibit repeated turnover, which may prevent the accumulation of genomic changes seen with more established co-obligate symbionts (Manzano-Marín and Latorre 2016).

Why is *Fukatsuia* Frequently Associated with *H. defensa*?

Many groups of sap-feeding insects harbor multiple obligate nutritional symbionts in their bacteriomes that exhibit metabolic complementation between species (Wu et al. 2006;

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

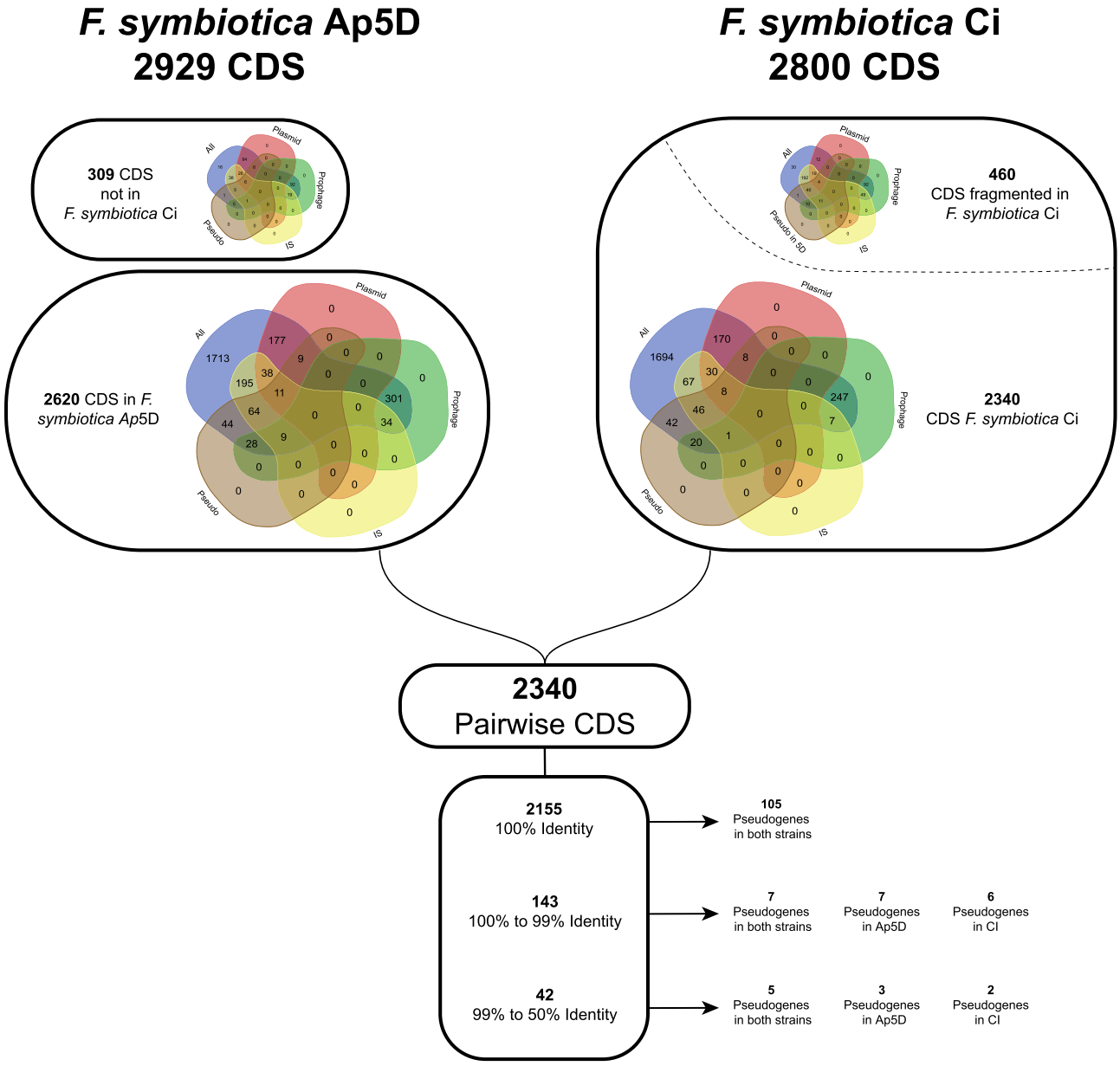

Fig. 4.—Venn diagrams comparing the classification of CDSs between *Fukatsuia* strains. CDSs were classified as belonging to prophages, plasmids, insertion sequences (ISs), pseudogenes (Pseudo), or regular CDSs. In *Fukatsuia* Ap5D, 2,929 CDSs, were divided into those with (2,620) and without (309) homology to those in *Fukatsuia* Ci. For *Fukatsuia* strain Ci, 2,340 CDSs show homology to those in strain Ap5D, while 460 are fragmented (mostly due to IS-associated assembly issues). The 2,340 shared homologs were pairwise aligned and percentage similarity and pseudogene content were compared between strains.

McCutcheon et al. 2009). Metabolic interdependence is also the basis for the transition of facultative symbionts into co-obligate status when the obligate symbiont falters due to genome degradation (Lamelas et al. 2011). Prior screening indicated that *Fukatsuia* has a strong tendency to co-occur with specific *H. defensa* strains (Doremus and Oliver 2017). Metabolic complementation is obviously one possible explanation for the frequent co-occurrence of *Fukatsuia* and *H. defensa* in pea aphids. However, results from this study indicate the nonmobilome components of the *Fukatsuia* genome are very similar to the *H. defensa* genome as well as the genomes of other aphid facultative symbionts. There are a few genes missing or pseudogenized in Ap5D that are present in *H. defensa*, but these genes are either present or also missing in other aphid facultative symbiont species (supplementary table S3, Supplementary Material online), and so does not

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

explain *Fukatsuia* preferentially co-occurring with *H. defensa*. Thus, if metabolic complementation is the basis for coinfection, it must play out on an *H. defensa* strain-specific basis. Alternative explanations for *Fukatsuia* and *H. defensa* coinfections include improved competitive abilities against third party heritable facultative symbionts (Rock et al. 2018), or tamed *Fukatsuia* virulence toward aphid hosts under coinfection. Interestingly, *Fukatsuia* encodes siderophores which are molecules involved in iron-chelation and uptake and are often important pathogenicity factors (Buckling et al. 2007). Since these are secreted molecules that can be utilized by coinfecting bacterial symbionts, this potentially selects for reduced siderophore production under coinfection resulting in lower virulence toward the insect host (Vorburger and Perlman 2018). Consistent with this hypothesis, we have observed reductions in infection costs when *Fukatsuia* coinhabits aphids with *H. defensa* relative to infection with *Fukatsuia* alone (Doremus and Oliver 2017).

Mobile DNA in *Fukatsuia*

Similar to other aphid facultative symbionts (Plague et al. 2008; Degnan et al. 2009, 2010; Burke and Moran 2011; Manzano-Marín and Latorre 2016), a substantial portion of the *Fukatsuia* main chromosome is comprised of mobile elements, including prophage and prophage-like elements, plasmid elements, and transposable elements (TEs). Of the 2,607 total CDSs of the main chromosome, 833 CDS (32%) occurred in mobile elements (fig. 2 and supplementary tables S5 and S6, Supplementary Material online). We identified TEs from 20 families, with IS630 being the most abundant (supplementary table S7, Supplementary Material online). We identified 33 prophage elements that varied in size from 291 bp (1 CDS) to 43 kb (59 CDSs) that shared homology with known siphovirus- or myovirus-derived elements in other Enterobacteriales including other aphid facultative symbionts (supplementary table S6, Supplementary Material online). Nearly all of these phage islands, however, were not intact prophages because they lacked one or more essential domains. However, prophage island 31 (32.6 kb) showed homology to *Salmonella enteritidis* lysogenic phage S (ELPhiS) (Myoviridae) and did appear to be intact. The APSE bacteriophages found in *H. defensa* that are associated with defense against parasitoids (Oliver et al. 2009) were not present in *Fukatsuia*. Two regions encoding plasmid-associated elements were also present in the main chromosome of *Fukatsuia*. One (32,886 bp) encodes transfer (*tra*) genes and toxin homologs and is most similar to aeromonad plasmids, while the second (12,032 bp) is similar to plasmids found in the plant pathogen *Xylella fastidiosa* and encodes *trb* and *recA* homologs. Together, the diverse mobile genetic elements associated with *Fukatsuia* and related heritable facultative symbionts maintain genomic dynamism that underlies functional roles and the capacity for lifestyle transitions.

Putative Toxins and Virulence Factors Associated with *Fukatsuia*

The Ap5D strain of *Fukatsuia* is costly to aphid fitness when occurring as a single infection, while other strains of *Fukatsuia* are reported to have diverse protective effects if additional facultative symbiont species are present (Heyworth and Ferrari 2015, 2016; Doremus and Oliver 2017). *Fukatsuia* Ap5D carries numerous genes with homology to Type 1–4 bacterial secretion systems (supplementary table S8, Supplementary Material online) that are dispersed across the main chromosome and a plasmid. The T2SS and T3SS (SPI-1 and 2) *Fukatsuia* Ap5D were further noted to have a twin arginine translocation (Tat) pathway, which transports unfolded proteins and is involved in bacterial pathogenesis. All sequenced strains of *R. insecticola* but not *H. defensa*, also have a similar twin Tat pathway.

The most common toxins found in *Fukatsuia* are those from the repeats-in-toxin RTX superfamily, including virulence factors with homology to hemolysin (*hlyA*) and MARTX (*rtxA*). RTX toxins occur in diverse gram negative bacterial species and possess aspartic acid/glycine repeats with a variety of proteins typically secreted by T1SS (Linhartova et al. 2010). All of the 30+ RTX variants present in *Fukatsuia* also occur in *R. insecticola* and/or *H. defensa*, suggesting these may have diversified in the ancestor to these symbionts with subsequent inactivation of specific copies in different lineages. This same pattern of similar inventory but variable inactivation also occurs between the two *Fukatsuia* strains (supplementary table S9, Supplementary Material online), suggesting these toxins may change rapidly when transferred to new hosts. Other putative insecticidal toxins are present, including tcc and tcd toxins typically associated with the nematode symbionts *Xenorhabdus nematophila* and *Photorhabdus luminescens*, and pathogens including *Y. pestis* and *Serratia entomophila*, but not reported in *H. defensa* or *R. insecticola*. We also identified nine *mcf* (*make caterpillars floppy*) homologs which are associated with insecticidal activity and also occur in *Photorhabdus* and *R. insecticola* (ffrench-Constant et al. 2007; Hansen et al. 2012). Other putative virulence factors were identified, such as cytolethal distending toxin subunit B (*cdtB*), which is located adjacent to a plasmid island. A *cdtB* gene is present in two APSE haplotypes (APSE2 and APSE8) that persistently infect particular strains of *H. defensa*, which have antiparasitoid functions in aphids (Moran et al. 2005; Degnan and Moran 2008; Martinez et al. 2014), but as noted above APSEs are absent in *Fukatsuia*. Furthermore, the *cdtB* encoded by *Fukatsuia* is more similar to *cdtB* genes present in certain enteric pathogens than APSEs, arguing against recent exchange among common aphid endosymbionts. Homologs of additional pathogenicity factors can be found in supplementary table S10, Supplementary Material online.

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

Restriction Modification System

Bacteria commonly harbor genes coding for DNA methyltransferases (MTases) and restriction endonucleases (REases). Together these form restriction modification (RM) systems involved in protection against foreign DNA, which also function in the regulation of various cellular processes (Vasu and Nagaraja 2013). Through PacBio SMRT sequencing, we were able to identify two putative MTase/REase recognition motifs in *Fukatsuia* strain Ap5D: 1) G^{m6}ATC motifs present in 26,436 copies and for which 95% were detected as modified, 2) TGGCC^{m6}A motifs present in 1,186 copies and for which 90% were detected as modified (supplementary table S11, Supplementary Material online). Furthermore, submission of the *F. symbiotica* strain Ap5D genome to the restriction enzyme database (REBASE: http://rebase.neb.com/rebase/rebase.html) identified two putative genes involved in the methylation pattern observed in *F. symbiotica* strain Ap5D. The gene CCS41_11020 codes for an orphan DAM dependent N6-adenine DNA MTas, which recognizes the GATC sequence motif. Gene CCS41_07300 codes for a fused type IIG REase/MTase recognizing TGGCCA motif. Whereas the orphan MTase modifying the GATC motif is probably involved in cell cycle regulation (Murphy et al. 2013), the fused REase/MTase encodes the two enzymatic activities and is therefore very likely involved in protection against foreign DNA infection.

Conclusions

This study shows that a culture-assisted protocol used to generate quality DNA template for whole genome sequencing of the heritable symbiont *H. defensa* (Brandt et al. 2017; Chevignon et al. 2018) can be used for other aphid facultative symbionts. These tools, along with other recent successes in culturing arthropod-associated bacteria (Masson et al. 2018), not only provide techniques for improved genomics, but also new avenues for functional studies in historically intractable systems. Our results also show that a facultative strain of *Fukatsuia* from the pea aphid is strikingly similar to a strain from Lachninae aphids supporting the conclusion this transition to a new role occurred in the recent past. Along with the closely related *H. defensa* and *R. insecticola*, a substantial portion of the *Fukatsuia* genome is comprised of mobile DNA encoding diverse toxins and pathogenicity factors. Together these properties equip this clade of symbionts with a diverse arsenal of factors allowing for heritable persistence via a variety of mechanisms in diverse hemipteran hosts.

Supplementary Material

Supplementary data are available at *Genome Biology and Evolution* online.

Acknowledgments

This work was supported by the US National Science Foundation awards to K.M.O. and M.R.S. (IOS 1256974) and K.M.O. and J.A.R. (IOS 1754302) Also, the Marie-Curie AgreenSkills+ fellowship program cofunded by the European 5 Union's Seventh and a Fondation pour la Recherche Médicale award (ARF201809006914) to G.C. Framework Program (FP7-609938) supported A.M.M.

Literature Cited

Abby SS, et al. 2016. Identification of protein secretion systems in bacterial genomes. Sci Rep. 6:23080.

Adeolu M, Alnajar S, Naushad S, Gupta RS. 2016. Genome-based phylogeny and taxonomy of the 'Enterobacteriales': proposal for Enterobacterales ord. nov divided into the families Enterobacteriaceae, Erwiniaceae fam. nov., Pectobacteriaceae fam. nov., Yersiniaceae fam. nov., Hafniaceae fam. nov., Morganellaceae fam. nov., and Budviciaceae fam. Nov. Int J Syst Evol Microbiol. 66(12):5575–5599.

Altschul SF, et al. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25(17):3389–3402.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. J Mol Biol. 215(3):403–410.

Arndt D, et al. 2016. PHASTER: a better, faster version of the PHAST phage search tool. Nucleic Acids Res. 44(W1):W16–W21.

Bankevich A, et al. 2012. SPAdes: a new genome assembly algorithm and its applications to single-cell sequencing. J Comput Biol. 19(5):455–477.

Baumann P. 2005. Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol. 59(1):155–189.

Brandt JW, Chevignon G, Oliver KM, Strand MR. 2017. Culture of an aphid heritable symbiont demonstrates its direct role in defence against parasitoids. Proc R Soc B. 284. doi: https://doi.org/10.1098/rspb.2017.1925.

Brettin T, et al. 2015. RASTtk: a modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Sci Rep. 5:8365.

Buckling A, et al. 2007. Siderophore-mediated cooperation and virulence in *Pseudomonas aeruginosa*. FEMS Microbiol Ecol. 62(2):135–141.

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

Chernomor O, von Haeseler A, Minh BQ. 2016. Terrace aware data structure for phylogenomic inference from supermatrices. Syst Biol. 65(6):997–1008.

Chevignon G, Boyd BM, Brandt JW, Oliver KM, Strand MR. 2018. Culture-facilitated comparative genomics of the facultative symbiont *Hamiltonella defensa*. Genome Biol Evol. 10(3):786–802.

Clark MA, et al. 1992. The eubacterial endosymbionts of whiteflies (Homoptera, Aleyrodoidea) constitute a lineage distinct from the endosymbionts of aphids and mealybugs. Curr Microbiol. 25(2):119–123.

Darling AE, Mau B, Perna NT. 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PLoS One 5(6):e11147.

Degnan PH, et al. 2010. Dynamics of genome evolution in facultative symbionts of aphids. Environ Microbiol. 12:2060–2069.

Degnan PH, Moran NA. 2008. Diverse phage-encoded toxins in a protective insect endosymbiont. Appl Environ Microbiol. 74(21):6782–6791.

Degnan PH, Yu Y, Sisneros N, Wing RA, Moran NA. 2009. *Hamiltonella defensa*, genome evolution of protective bacterial endosymbiont from pathogenic ancestors. Proc Natl Acad Sci U S A. 106(22):9063–9068.

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

Donald KJ, et al. 2016. Protection of pea aphids associated with coinfecting bacterial symbionts persists during superparasitism by a Braconid wasp. Microb Ecol. 71(1):1–4.
Doremus MR, et al. 2018. Breakdown of a defensive symbiosis, but not endogenous defences, at elevated temperatures. Mol Ecol. 27(8):2138–2151.
Doremus MR, Oliver KM. 2017. Aphid heritable symbiont exploits defensive mutualism. Appl Environ Microbiol. 83:e03276–16.
Douglas A. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria *Buchnera*. Annu Rev Entomol. 43(1):17–37.
Duron O, et al. 2008. The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biol. 6(1). doi: 10.1186/1741-7007-6-27
Feldhaar H. 2011. Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol Entomol. 36(5):533–543.
Ferrari J, West JA, Via S, Godfray HCJ. 2012. Population genetic structure and secondary symbionts in host-associated populations of the pea aphid complex. Evolution 66(2):375–390.
ffrench-Constant RH, Dowling A, Waterfield NR. 2007. Insecticidal toxins from *Photorhabdus* bacteria and their potential use in agriculture. Toxicon 49(4):436–451.
Guay J-F, Boudreault S, Michaud D, Cloutier C. 2009. Impact of environmental stress on aphid clonal resistance to parasitoids: role of *Hamiltonella defensa* bacterial symbiosis in association with a new facultative symbiont of the pea aphid. J Insect Physiol. 55(10):919–926.
Gunduz EA, Douglas AE. 2009. Symbiotic bacteria enable insect to use a nutritionally inadequate diet. Proc R Soc B. 276:987–991.
Guo JQ, et al. 2017. Nine facultative endosymbionts in aphids. A review. J Asia-Pac Entomol. 20(3):794–801.
Hansen AK, Vorburger C, Moran NA. 2012. Genomic basis of endosymbiont-conferred protection against an insect parasitoid. Genome Res. 22(1):106–114.
Henikoff S, Henikoff JG. 1992. Amino-acid substitution matrices from protein blocks. Proc Natl Acad Sci U S A. 89(22):10915–10919.
Henry LM, et al. 2013. Horizontally transmitted symbionts and host colonization of ecological niches. Curr Biol. 23(17):1713–1717.
Henry LM, Maiden MCJ, Ferrari J, Godfray HCJ. 2015. Insect life history and the evolution of bacterial mutualism. Ecol Lett. 18(6):516–525.
Heyworth E, Ferrari J. 2015. A facultative endosymbiont in aphids can provide diverse ecological benefits. J Evol Biol. 28(10):1753–1760.
Heyworth ER, Ferrari J. 2016. Heat stress affects facultative symbiont-mediated protection from a parasitoid wasp. PLoS One 11(11):e0167180.
Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS. 2018. Ufboot2: improving the ultrafast bootstrap approximation. Mol Biol Evol. 35(2):518–522.
Huerta-Cepas J, et al. 2016. eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences. Nucleic Acids Res. 44(D1):D286–D293.
Izore T, Cryle MJ. 2018. The many faces and important roles of protein-protein interactions during non-ribosomal peptide synthesis. Nat Prod Rep. 35:1120–1139.
Kalkatawi M, Alam I, Bajic VB. 2015. BEACON: automated tool for Bacterial GEnome Annotation ComparisON. BMC Genomics 16:616.
Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. 2017. ModelFinder: fast model selection for accurate phylogenetic estimates. Nat Methods. 14(6):587.
Kanehisa M, Sato Y, Morishima K. 2016. BlastKOALA and GhostKOALA: kEGG Tools for functional characterization of genome and metagenome sequences. J Mol Biol. 428(4):726–731.
Katoh K, Standley DM. 2016. A simple method to control over-alignment in the MAFFT multiple sequence alignment program. Bioinformatics 32(13):1933–1942.
Kearse M, et al. 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28(12):1647–1649.
Krzywinski M, et al. 2009. Circos: an information aesthetic for comparative genomics. Genome Res. 19(9):1639–1645.
Kurtz S, et al. 2004. Versatile and open software for comparing large genomes. Genome Biol. 5(2):R12.
Lamelas A, et al. 2011. *Serratia symbiotica* from the aphid *Cinara cedri*: a missing link from facultative to obligate insect endosymbiont. PLoS Genet. 7(11):e1002357.
Linhartova I, et al. 2010. RTX proteins: a highly diverse family secreted by a common mechanism. FEMS Microbiol Rev. 34:1076–1112.
Lo WS, Huang YY, Kuo CH. 2016. Winding paths to simplicity: genome evolution in facultative insect symbionts. FEMS Microbiol Rev. 40(6):855–874.
Łukasik P, van Asch M, Guo H, Ferrari J, Godfray CJ. 2013. Unrelated facultative endosymbionts protect aphids against a fungal pathogen. Ecol Lett. 16(2):214–218.
Majerus TMO, Majerus MEN. 2010. Intergenomic arms races: detection of a nuclear rescue gene of male-killing in a ladybird. PLoS Pathog. 6(7):e1000987.
Manzano-Marín A, et al. 2018. A freeloader? The highly eroded yet large genome of the *Serratia symbiotica* symbiont of *Cinara strobi*. Genome Biol Evol. 10(9):2178–2189.
Manzano-Marín A, Latorre A. 2016. Snapshots of a shrinking partner: genome reduction in *Serratia symbiotica*. Sci Rep. 6(1):32590.
Manzano-Marin A, Szabo G, Simon JC, Horn M, Latorre A. 2017. Happens in the best of subfamilies: establishment and repeated replacements of co-obligate secondary endosymbionts within Lachninae aphids. Environ Microbiol. 19:393–408.
Markowitz VM, et al. 2012. IMG: the integrated microbial genomes database and comparative analysis system. Nucleic Acids Res. 40(D1):D115–D122.
Martinez AJ, Kim KL, Harmon JP, Oliver KM. 2016. Specificity of multimodal aphid defenses against two rival parasitoids. PLoS One 11(5):e0154670.
Martinez AJ, Weldon SR, Oliver KM. 2014. Effects of parasitism on aphid nutritional and protective symbioses. Mol Ecol. 23(6):1594–1607.
Masson F, Calderon Copete S, Schüpfer F, Garcia-Arraez G, Lemaitre B. 2018. In vitro culture of the insect endosymbiont *Spiroplasma poulsonii* highlights bacterial genes involved in host-symbiont interaction. Mbio 9(2). doi: 10.1128/mBio.00024-18.
McCutcheon JP, McDonald BR, Moran NA. 2009. Convergent evolution of metabolic roles in bacterial co-symbionts of insects. Proc Natl Acad Sci U S A. 106(36):15394–15399.
McCutcheon JP, Moran NA. 2012. Extreme genome reduction in symbiotic bacteria. Nat Rev Microbiol. 10(1):13–26.
McLean AHC, Godfray HCJ. 2015. Evidence for specificity in symbiont-conferred protection against parasitoids. Proc R Soc B. 282. doi: 10.1098/rspb.2015.0977.
Meseguer AS, et al. 2017. *Buchnera* has changed flatmate but the repeated replacement of co-obligate symbionts is not associated with the ecological expansions of their aphid hosts. Mol Ecol. 26(8):2363–2378.
Montllor CB, Maxmen A, Purcell AH. 2002. Facultative bacterial endosymbionts benefit pea aphids Acyrthosiphon pisum under heat stress. Ecol Entomol. 27(2):189–195.
Moran NA, Degnan PH, Santos SR, Dunbar HE, Ochman H. 2005. The players in a mutualistic symbiosis: insects, bacteria, viruses, and

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019

virulence genes. Proc Natl Acad Sci U S A. 102(47): 16919–16926.
Moran NA, McCutcheon JP, Nakabachi A. 2008. Genomics and evolution of heritable bacterial symbionts. Annu Rev Genet. 42(1):165–190.
Moran NA, Plague GR. 2004. Genomic changes following host restriction in bacteria. Curr Opin Genet Dev. 14:627–633.
Moran NA, Telang A. 1998. Bacteriocyte-associated symbionts of insects – a variety of insect groups harbor ancient prokaryotic endosymbionts. Bioscience 48(4):295–304.
Murphy J, Mahony J, Ainsworth S, Nauta A, van Sinderen D. 2013. Bacteriophage orphan DNA methyltransferases: insights from their bacterial origin, function, and occurrence. Appl Environ Microbiol. 79(24):7547–7555.
Nawrocki EP, Eddy SR. 2013. Infernal 1.1: 100-fold faster RNA homology searches. Bioinformatics 29(22):2933–2935.
Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. 2015. Iq-tree: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 32(1):268–274.
Nikoh N, et al. 2018. Genomic insight into symbiosis-induced insect color change by a facultative bacterial endosymbiont, "*Candidatus* Rickettsiella viridis". Mbio 9. doi: 10.1128/mBio.00890.18.
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(1):247–266.
Oliver KM, Degnan PH, Hunter MS, Moran NA. 2009. Bacteriophages encode factors required for protection in a symbiotic mutualism. Science 325(5943):992–994.
Oliver KM, Martinez AJ. 2014. How resident microbes modulate ecologically-important traits of insects. Curr Opin Insect Sci. 4:1–7.
Oliver KM, Russell JA, Moran NA, Hunter MS. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc Natl Acad Sci U S A. 100(4):1803–1807.
Oliver KM, Smith AH, Russell JA. 2014. Defensive symbiosis in the real world–advancing ecological studies of heritable, protective bacteria in aphids and beyond. Funct Ecol. 28(2):341–355.
Ortiz-Rivas B, Martínez-Torres D. 2010. Combination of molecular data support the existence of three main lineages in the phylogeny of aphids (Hemiptera: Aphididae) and the basal position of the subfamily Lachninae. Mol Phylogenet Evol. 55(1):305–317.
Plague GR, Dunbar HE, Tran PL, Moran NA. 2008. Extensive proliferation of transposable elements in heritable bacterial symbionts. J Bacteriol. 190(2):777–779.
Rissman AI, et al. 2009. Reordering contigs of draft genomes using the Mauve Aligner. Bioinformatics 25(16):2071–2073.
Rock DI, et al. 2018. Context-dependent vertical transmission shapes strong endosymbiont community structure in the pea aphid, *Acyrthosiphon pisum*. Mol Ecol. 27(8):2039–2056.
Ross MG, et al. 2013. Characterizing and measuring bias in sequence data. Genome Biol. 14(5):R51.
Russell CW, Bouvaine S, Newell PD, Douglas AE. 2013. Shared metabolic pathways in a coevolved insect-bacterial symbiosis. Appl Environ Microbiol. 79(19):6117–6123.
Russell JA, Weldon S, et al. 2013. Uncovering symbiont-driven genetic diversity across North American pea aphids. Mol Ecol. 22(7):2045–2059.
Russell JA, Latorre A, Sabater-Munoz B, Moya A, Moran NA. 2003. Side-stepping secondary symbionts: widespread horizontal transfer across and beyond the Aphidoidea. Mol Ecol. 12(4):1061–1075.
Russell JA, Moran NA. 2006. Costs and benefits of symbiont infection in aphids: variation among symbionts and across temperatures. Proc R Soc B. 273(1586):603–610.
Russell JA, Oliver KM, Hansen AK. 2017. Band-aids for *Buchnera* and B vitamins for all. Mol Ecol. 26(8):2199–2203.
Scarborough CL, Ferrari J, Godfray H. 2005. Aphid protected from pathogen by endosymbiont. Science 310(5755):1781–1781.
Schmieder R, Edwards R. 2011. Quality control and preprocessing of metagenomic datasets. Bioinformatics 27(6):863–864.
Shigenobu S, Watanabe H, Hattori M, Sakaki Y, Ishikawa H. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera* sp. APS. Nature 407(6800):81–86.
Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. 2006. ISfinder: the reference centre for bacterial insertion sequences. Nucleic Acids Res. 34(90001):D32–D36.
Smith AH, et al. 2015. Patterns, causes and consequences of defensive microbiome dynamics across multiple scales. Mol Ecol. 24(5):1135–1149.
Stover BC, Muller KF. 2010. TreeGraph 2: combining and visualizing evidence from different phylogenetic analyses. BMC Bioinformatics. 11. doi: 10.1186/1471-2105-11-7
van Emden HF, Harrington R. 2007. Aphids as crop pests. Wallingford: CABI.
Varani AM, Siguier P, Gourbeyre E, Charneau V, Chandler M. 2011. ISsaga is an ensemble of web-based methods for high throughput identification and semi-automatic annotation of insertion sequences in prokaryotic genomes. Genome Biol. 12(3):r30.
Vasu K, Nagaraja V. 2013. Diverse functions of restriction-modification systems in addition to cellular defense. Microbiol Mol Biol Rev. 77(1):53–72.
Vorburger C. 2014. The evolutionary ecology of symbiont-conferred resistance to parasitoids in aphids. Insect Sci. 21(3):251–264.
Vorburger C, Perlman SJ. 2018. The role of defensive symbionts in host–parasite coevolution. Biol Rev. 93(4):1747–1764.
Weldon SR, Strand MR, Oliver KM. 2013. Phage loss and the breakdown of a defensive symbiosis in aphids. Proc R Soc B. 280. doi: 10.1098/rspb.2012.2103.
Wilson ACC, Duncan RP. 2015. Signatures of host/symbiont genome coevolution in insect nutritional endosymbioses. Proc Natl Acad Sci U S A. 112(33):10255–10261.
Wilson ACC, et al. 2010. Genomic insight into the amino acid relations of the pea aphid, *Acyrthosiphon pisum*, with its symbiotic bacterium *Buchnera aphidicola*. Insect Mol Biol. 19:249–258.
Wu D, et al. 2006. Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. PLoS Biol. 4(6):e188.
Wu M, et al. 2004. Phylogenomics of the reproductive parasite *Wolbachia pipientis* wMel: a streamlined genome overrun by mobile genetic elements. PLoS Biol. 2(3):e69.

Associate editor: Nancy Moran

Downloaded from https://academic.oup.com/gbe/article-abstract/11/12/3510/5625627 by guest on 27 December 2019