Functional Convergence in Reduced Genomes of Bacterial Symbionts Spanning 200 My of Evolution

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Abstract

The main genomic changes in the evolution of host-restricted microbial symbionts are ongoing inactivation and loss of genes combined with rapid sequence evolution and extreme structural stability; these changes reflect high levels of genetic drift due to small population sizes and strict clonality. This genomic erosion includes irreversible loss of genes in many functional categories and can include genes that underlie the nutritional contributions to hosts that are the basis of the symbiotic association. Candidatus Sulcia muelleri is an ancient symbiont of sap-feeding insects and is typically coresident with another bacterial symbiont that varies among host subclades. Previously sequenced Sulcia genomes retain pathways for the same eight essential amino acids, whereas coresident symbionts synthesize the remaining two. Here, we describe a dual symbiotic system consisting of Sulcia and a novel species of Betaproteobacteria, Candidatus Zinderia insecticola, both living in the spittlebug Clastoptera arizonana. This Sulcia has completely lost the pathway for the biosynthesis of tryptophan and, therefore, retains the ability to make only 7 of the 10 essential amino acids. Zinderia has a tiny genome (208 kb) and the most extreme nucleotide base composition (13.5% G + C) reported to date, yet retains the ability to make the remaining three essential amino acids, perfectly complementing capabilities of the coresident Sulcia. Combined with the results from related symbiotic systems with complete genomes, these data demonstrate the critical role that bacterial symbionts play in the host insect’s biology and reveal one outcome following the loss of a critical metabolic activity through genome reduction.

Key words: genome reduction, minimal genome, genome sequencing, Bacteroidetes, nutritional symbioses.

Introduction

Bacteria that have developed obligate symbioses with multicellular hosts often have smaller genomes than those of their free-living relatives (Andersson and Kurland 1998; Andersson and Andersson 1999b; Moran 2002; Moran and Plague 2004; Moran et al. 2008; Moya et al. 2008). Genome reduction is thought to be the result of a combination of factors, including small population sizes, frequent population bottlenecks (due to their strict cytoplasmic inheritance), asexuality that limits the efficacy of selection (Moran 1996; Andersson and Kurland 1998), an unusually stable and metabolically rich growth environment, and the general bacterial mutational bias favoring deletions over insertions (Andersson and Andersson 1999a; Nilsson et al. 2005; Moran et al. 2009). Genes from nearly every cellular process are lost in the genomes of obligate intracellular symbionts, including genes involved in DNA recombination, repair, and uptake (Dale et al. 2003; Silva et al. 2003). The loss of recombinogenic activities results in genomes that are unusually stable, and several examples of symbiont genome pairs diverged by 20–200 My show complete colinearity among shared genes (Tamas et al. 2002; et al. 2005; McCutcheon et al. 2009a), a level of genome stability that is unique in bacteria. It appears that once a bacterial lineage becomes obligately associated with a host cytoplasm and its genome shrinks beyond a certain size threshold (around 1-Mb pairs),
Insects that have nutritionally unbalanced food sources often have acquired intracellular symbiotic microorganisms to supplement their diet (Buchner 1965; Douglas 1989). For example, insects that feed exclusively on plant sap—which can have high levels of carbohydrates but extremely low levels of essential amino acids—possess stably associated (coevolving) endocellular bacteria that provision the host insect with essential amino acids and/or vitamin cofactors (Shigenobu et al. 2000; Baumann 2005; Nakabachi et al. 2006; Wu et al. 2006; McCutcheon and Moran 2007; McCutcheon et al. 2009a, 2009b). Insects in the suborder Auchenorrhyncha, which includes sharpshooters (leafhoppers), cicadas, planthoppers, and spittlebugs (froghoppers), have formed particularly elaborate symbioses with diverse groups of microorganisms (Buchner 1965; Moran 2007). These insects typically have multiple—often 2, but sometimes up to 6—diverse nutritional symbionts living in specialized tissues called bacteriomes (Buchner 1965). The most ancient and widely distributed is the Bacterioideetes Candidatus Sulcia muelleri (hereby referred to as Sulcia for simplicity) (Moran et al. 2005). Complete genomes for Sulcia and its coresident symbiont have been sequenced from three insect species: the glassy-winged sharpshooter Homalodisa vitripennis (Sulcia-GWSS) (McCutcheon and Moran 2007), the green sharpshooter Draeculacephala minerva (Sulcia-DMIN) (Woyke et al. 2010) and the cicada Diceroprocta seminicta (Sulcia-DSEM) (McCutcheon et al. 2009b). (The Sulcia-GWSS and Sulcia-DMIN genomes are functionally identical from a nutritional perspective and so only Sulcia-GWSS will be discussed further.) Although Sulcia-GWSS diverged from Sulcia-DSEM at least 200 Ma based on host fossils (Schcherbakov and Popov 2002), the genomes are completely collinear and almost identical in gene content; importantly, they both retain identical and near-complete gene sets for the production of 8 of the 10 amino acids (leucine, isoleucine, valine, threonine, lysine, arginine, phenylalanine, and tryptophan) (McCutcheon et al. 2009a). In both the GWSS and DSEM systems, the remaining 2 essential amino acids (methionine and histidine) are made by Sulcia’s cosymbiont, which in GWSS is the Gammaproteobacteria Candidatus Baumannia cicadellinicola and in DSEM is the Alphaproteobacteria Candidatus Hodgkinia cicadica (hereby referred to as Hodgkinia for simplicity) (Wu et al. 2006; McCutcheon et al. 2009a).

In all studied cases for bacteriome-associated symbionts in sap-feeding insects, the relationship is mutually obligate: the host and its symbionts are completely dependent on each other to survive (Douglas 1989; Nakabachi and Ishikawa 1999; Moran et al. 2008; Moya et al. 2008). This presents a seemingly tenuous situation, where the host is dependent on its symbiotic bacteria to survive, but these same bacteria have irrevocably lost the genomic dynamism typical of bacteria and are incapable of restoring genes that are lost through genome reduction. Here, we show one possible outcome of the complete loss of an essential amino acid pathway in dual symbiotic system containing Sulcia and a coresident symbiont: the lost pathway in Sulcia is completely retained in the cosymbiont, precisely conserving the collective production of all ten essential amino acids by the bacterial partners.

### Materials and Methods

#### DNA Preparation

Spittlebugs were collected from Rosmarinus officinalis (rosemary) bushes on the University of Arizona campus. Red (containing Sulcia) and yellow (containing Zinderia) portions of the bacteriomes were dissected from 4 to 5 insects in phosphate-buffered saline. The volume was reduced to 10 µl, and the bacteriome tissue was lysed in 10 µl of cell lysis buffer (400 mM KOH, 10 mM ethylenediaminetetraacetic acid, 100 mM dithiothreitol) on ice for 10 min, followed by the addition of 10 µl of neutralization buffer (400 mM HCl, 600 mM Tris–HCl, pH 7.5). Five separate whole-genome amplification reactions were performed using 3 µl of the neutralized sample as template following the instructions in the Amersham GenomiPhi V2 kit. This procedure was repeated several times over the course of 2 years to generate DNA for Roche 454 FLX, Roche 454 FLX Titanium, and Illumina sequencing.

#### Genome Sequencing

The Sulcia genome was assembled into 10 contigs (274,670 nts) from a Roche 454 FLX run of 457,666 reads totaling 113,860,631 bases in Newbler version 1.1.02.15 and closed by polymerase chain reaction (PCR) and Sanger sequencing. The Zinderia genome was highly fragmented (50+ contigs) and of poor quality at this stage, but it was clear that the guanine + cytosine (GC) content was extremely low.

Due to the low GC content of the Zinderia genome, an amplification-free Illumina protocol was used, as this was reported to prevent biasing the sequencing library toward GC-rich sequences (Kozarewa et al. 2009). A paired-end run on an Illumina Genome Analyzer IIx generated 4,653,772 fifty-nine nt reads totaling 274,572,548 nts. The Illumina data were used to 1) correct homopolymer errors in the Sulcia genome generated by 454 FLX (by mapping the Illumina reads to the 454 genome with BlastN; parameters: -G 2 -e 1 -F F -e 1e-15 -W 7 -b 1 -v 1) and 2) attempt a de novo assembly of the Zinderia genome. After removing the 355,976 Illumina reads that mapped to the Sulcia genome, the remaining 4,297,796 reads were assembled using velvet (Zerbino and Birney 2008) (velveth parameters: k = 41 –shortPaired;
velvetg parameters: –ins_length 200 –cov_cutoff 20 –exp_cov 255). The Illumina/Velvet Zinderia assembly had 8 contigs totaling 212,107 nts with an average GC content of 13.4%, and these contigs formed the core of the Zinderia genome.

A 454 FLX Titanium run was done in parallel to the Illumina sequencing, which produced an additional 52,484 reads with an average length of 378 nts. These reads were assembled with Newbler version 2.0.00.22, resulting in 34 Zinderia genome contigs with an average GC content of 13.7%. The eight Illumina/Velvet contigs were assembled with the thirty-four 454 FLX Titanium contigs in phrap (Felsenstein 1989), and the results were hand checked for consistency. A few minor Velvet misassemblies were corrected, and some contigs from each separate assembly were joined, resulting in four supercontigs. These four supercontigs were closed by PCR and Sanger sequencing. Both genomes were annotated as described previously (McCutcheon and Moran 2007), except that Infernal (Nawrocki et al. 2009) was used to predict the boundaries of the ribosomal RNA genes.

Transmission Electron Microscopy

Yellow portions of the bacteriome, which exclusively contains Zinderia, were dissected from spittlebugs and fixed in room temperature 2.5% glutaraldehyde in 0.1 M 1,4-piperazinediethane-sulfonic acid (PIPES) buffer pH 7.4 for 1 h. The bacteriomes were washed in 0.1 M PIPES, postfixed in 1% osmium tetroxide, and washed for 10 min in deionized water. The sample was dehydrated by 5–10 min washes in 50, 70, 90, and 100% ethanol. Microwave resin infiltration was done using a 1:1 mix of Spurr’s resin to ethanol at 250 W at 20 °C for 3 min in a vacuum; two additional infiltrations were done in pure Spurr’s resin, 25 W at 20 °C for 3 min in a vacuum. The samples were then left in Spurr’s resin for 30 min at room temperature and finally embedded with an overnight incubation at 60 °C. Sixty nm sections were cut onto uncoated copper mesh grids, stained with 2% uranyl acetate for 20 min followed by 2% lead acetate for 2 min. Sections were viewed in an FEI CM12 transmission electron microscope operated at 80 kV.

Phylogenetics

Individual protein alignments (FusA, RplB, TufA, MnmG, and RpoB) for the protein-based tree were produced using the linsi module of MAFFT (Katoh et al. 2005), hand-edited to remove poorly aligned regions, and concatenated. The resulting data set had 73 species and 3,255 columns. Maximum likelihood trees were generated in RAxML (relevant parameters: -d -m PROTGAMMAJTT -x 12345 -# 200 -f a). A list of species (and GenBank accession numbers) used in the generation of the protein tree can be found in the supplemental materials (Supplementary Material online).

Results

Spittlebugs Have Two Long-term Symbionts

Previous work showed that the spittlebug Clastoptera arizonana contains Sulcia as a symbiont (hereby referred to as Sulcia-CARI) (Moran et al. 2005). We identified a second symbiont by universal 16S rDNA PCR (data not shown) and transmission electron microscopy (fig. 1). Together, these experiments revealed that the second symbiont was a novel member of the Betaproteobacteria with large amorphous cells. This cell shape often indicates a bacterium that has undergone substantial genome reduction (Moran et al. 2005; Nakabachi et al. 2006; McCutcheon et al. 2009b). Therefore, in an ongoing effort to understand genome reduction in multiple bacterial lineages and to further document complex symbioses containing diverse bacterial partners, we sequenced the genomes from Sulcia-CARI and the novel Betaproteobacteria for which we propose the name Candidatus Zinderia insecticola (and will refer to as Zinderia for simplicity).
**Sulcia-CARI Does Not Encode the Tryptophan Biosynthetic Pathway**

The *Sulcia*-CARI genome is 276,511 nts in length, has a 21.1% GC content, and codes for 246 protein-coding genes, 29 tRNAs, and one copy each of tmRNA, RNase P RNA, 5S, 16S, and 23S rDNA. A three-way alignment of the *Sulcia* genomes from GWSS, DSEM, and CARI shows that the only gross genomic changes between the lineages are differential losses of various genes; there is complete co-linearity among retained genes (fig. 2a). This striking pattern of conservation was also observed in previous whole-genome alignments between *Sulcia*-GWSS and *Sulcia*-DSEM (McCutcheon et al. 2009a), as well as in previous comparisons of other highly reduced bacterial symbiont genomes (Tamas et al. 2002; Degnan et al. 2005), and this result provides further confirmation of genome stability as the prevailing pattern in reduced-genome symbionts. More surprising was the complete loss of all six genes in the tryptophan biosynthetic operon in *Sulcia*-CARI (fig. 2b), as this is one of the eight essential amino acids produced by *Sulcia*-GWSS and *Sulcia*-DSEM. All other genes involved in the production of the remaining seven essential amino acids (leucine, isoleucine, valine, threonine, lysine, arginine, and phenylalanine) are conserved among all three *Sulcia* genomes (fig. 2a).

**Zinderia: A Betaproteobacterial Symbiont with Extreme Genomic Features**

The *Zinderia* genome is 208,564 nts and codes for 202 protein-coding genes, 25 tRNAs, and one copy each of tmRNA, RNase P RNA, 5S, 16S, and 23S rDNA. Similar to other insect symbionts with highly reduced genomes, *Zinderia* does not code for genes involved in making a cell membrane, peptidoglycan, nucleotides, or vitamin cofactors. *Zinderia* is not capable of doing most reactions involved in carbohydrate metabolism—including glycolysis and the citric acid cycle—and seems incapable of making adenosine triphosphate (ATP), as no F1F0 ATPase is present and no obvious pathway for substrate-level phosphorylation exists. The *Zinderia* genome does however encode homologs for all 14 genes of the minimal NADH:ubiquinone oxidoreductase I proton translocation machinery (nuoABCDEFGHIJKLMN), all four genes in the cytochrome bo terminal oxidase complex (cyoABC|

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**FIG. 2.**—Whole-genome alignment for three *Sulcia* species. Each complete *Sulcia* genome is shown in a linear representation where each gene is represented by a box. Boxes for genes involved in the synthesis of essential amino acids are colored red; all others are represented by white boxes except for selected genes flanking the tryptophan biosynthetic pathway region. (a) The histogram above the linear genome schematic indicates the level of conservation, where a higher bar represents greater sequence identity. Regions that are shared between all three genomes are colored orange; those that are shared between *Sulcia*-CARI and *Sulcia*-DSEM are green; those shared between *Sulcia*-CARI and *Sulcia*-GWSS are blue; and those shared between *Sulcia*-DSEM and *Sulcia*-GWSS are purple. The region flanking the tryptophan biosynthetic pathway is shaded in gray. (b) Zooming in on the tryptophan biosynthesis region shows the precise nature of genome reduction, as no fragment of any gene in the tryptophan pathway remains.

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FIG. 3.—Phylogenetic analysis indicates that Zinderia is a Betaproteobacteria in the family Oxalobacteraceae. Select bootstrap values greater than 70 are shown on each maximum likelihood tree. The tree in (a) was calculated from a concatenated alignment of several protein sequences and that in (b) was calculated from the 16S rDNA sequence alone.
reduced symbiont genomes (McCutcheon 2010), the Zinderia genome contains a minimal set of gene orthologs involved in genome replication, transcription, and translation (supplementary fig. S1, Supplementary Material online). Phylogenetic analyses with concatenated protein (fig. 3a) and 16S rDNA (fig. 3b) sequences clearly define Zinderia as member of the Betaproteobacteria and suggest that genera in the Oxalobacteraceae (e.g., Oxalobacter, Herminiimonas, and Janthinobacterium) are its closest free-living relatives, although this should be interpreted with some caution as the branch lengths on the Zinderia lineage are extremely long (fig. 3a and b).

The genome sequence strongly suggests that Zinderia uses an alternative genetic code, in which UGA codes for tryptophan instead of stop. This code change has been reported in certain lineages of Mollicutes, such as Mycoplasma (Yamao et al. 1985), the Alphaproteobacteria Candidatus Hodgkinia cicadica (McCutcheon et al. 2009b), some ciliate nuclear genomes (Lozupone et al. 2001), and in several mitochondrial lineages (Knight et al. 2001). Evidence for the mapping of UGA to tryptophan in Zinderia comes from multiple sequence alignments of proteins, which show tryptophan occurring in several highly conserved positions in other Proteobacteria that are coded for by UGA in Zinderia (fig. 4). If UGA is assumed to be reassigned, 360 of 374 (96%) of the putative tryptophans encoded in Zinderia open-reading frames use the UGA codon, whereas only 14 use the standard tryptophan codon UGG. This usage pattern is consistent with that of other degenerate codon families in Zinderia, where the more AT-rich codon is always used preferentially over the more GC-rich codon (supplementary table S1, Supplementary Material online).

The genomic GC content of Zinderia is 13.5%, the lowest yet observed in any cellular genome. This is also lower than any reported viral genome (at 17.8%, the Entomopoxvirus Amsacta moorei [Bawden et al. 2000] has the lowest GC content of 13.5%, the lowest yet observed in any cellular genome. This is also lower than any reported viral genome (at 17.8%, the Entomopoxvirus Amsacta moorei [Bawden et al. 2000] has the lowest GC content of all 3,573 viral genomes listed in the GenBank Viral Genomes Resource, http://www.ncbi.nlm.nih.gov/genomes/VIRUSES/viruses.html) and lower than the vast majority of organelar genomes (only 4 out of the 3,472 genomes listed in the GObASE Organelle Genome Database [O’Brien et al. 2009] have a lower GC content; the lowest is 10.9% GC in the mitochondrion of the yeast Kluveromyces bacilliforpus [Bouchier et al. 2009]). Although the GC content of the protein-coding regions is 13.2% overall, the GC content of first, second, and third codon positions are 17.5, 18.8, and 3.3%, respectively. This pattern suggests a strong mutational bias toward AT in the genome, with purifying selection acting on the first and second codon position to maintain amino acid residues, similar to what has been observed in other reduced genomes with strong compositional bias (Moran and Wernegreen 2000). As expected from previous work (Moran 1996; Clark et al. 1999), this extreme GC bias has a profound effect on the amino acid profile of the Zinderia proteome; 36.1% of all amino acid pairs use the isoleucine (58.9% ATG, 40.6% ATT, 0.5% ATC) or lysine (97.9% AAA, 2.1% AAG), and amino acids with GC-rich codons are greatly underrepresented compared with organisms with more balanced genomic GC contents (fig. 5 and supplementary table S1, Supplementary Material online).

Zinderia Is Able to Produce Three Essential Amino Acids

It is thought that all animals have lost the ability to make ten amino acids (the essential amino acids) and that these compounds are not present in high levels in xylem sap (Redak et al. 2004). Therefore, because the tryptophan biosynthetic pathway was lost in Sulcia-CARI and the previously studied cosymbiont of Sulcia in GWSS and DSEM produced methionine and histidine (Wu et al. 2006; McCutcheon et al. 2009a), it was of interest to see if Zinderia had gene homologs for the production of tryptophan, methionine, and histidine. Genome analysis indicates that this is the case—Zinderia makes exactly this set of amino acids (fig. 6). In the production of methionine, Zinderia uses the direct sulfhydration pathway (metXY), which is unique in insect nutritional symbionts; all previous examples use the
transsulfuration pathway (metABC) (Hacham et al. 2003). In the production of both histidine and tryptophan, Zinderia has gene homologs for all the standard reactions, although shikimate is needed as a precursor in the production of tryptophan, as homologs of genes for the conversion of phosphoenolpyruvate (PEP) to shikimate (aroABDE) are missing in Zinderia. Sulcia-CARI has retained all genes for the conversion of PEP to phenylalanine, including aroABDE, and may be the source of shikimate for tryptophan production in Zinderia.

**Discussion**

**The Tryptophan Operon Is Precisely Excised in Sulcia-CARI**

Bacterial mutation exhibits an inherent deletional bias (Andersson and Andersson 1999a, 2001; Mira et al. 2001), and this bias has two important consequences. The first is that intergenic regions are typically small, resulting in a coding density that is stable (about 1 gene every 1,000 bases) across the entire two orders of magnitude range of bacterial genome size (Bentley and Parkhill 2004; Ochman and Davalos 2006). The second is that a gene that no longer provides a selective advantage in a certain environment does not persist over long periods of time; when selection is no longer strong enough to maintain a gene it is pseudogenized and eventually the DNA is removed from the genome (Andersson et al. 1998; Mira et al. 2001; Ochman and Davalos 2006). Because obligate endosymbionts have small effective population sizes (which limits the efficacy of selection) and live exclusively in a stable and metabolically rich environment, they lose many genes that are retained in free-living bacterial genomes, even genes that are slightly beneficial (Andersson and Andersson 1999a, 2001).
FIG. 6.—The essential amino acid metabolisms of three Sulcia-containing dual symbiont systems. Complete pathways for the production of essential amino acids (red font) or related cofactor compounds (blue font; Hodgkinia uses the B12-dependent version of methionine synthase in the last step of methionine production [McCutcheon et al. 2009a]) are shown. Missing genes are represented in a light gray font. Note the three different pathways taken in the production of methionine in Sulcia’s cosymbionts.
The process of genome reduction in obligate endosymbionts seems to have two phases, where early in the symbiosis large blocks of sequence corresponding to many unrelated genes can be lost (Moran and Mira 2001; Nilsson et al. 2005), followed by a later phase of DNA loss in smaller blocks of sequence (Moran et al. 2009). The pattern of loss seen in the region of the Sulcia-CARI genome that codes for the tryptophan pathway in the other Sulcia genomes demonstrates the precision of the later phase of genome reduction (fig. 2b). No remnant of any gene in the tryptophan pathway remains in the Sulcia-CARI genome, and intergenic regions between the flanking genes that are conserved in all three Sulcia genomes (tilS and proS) are nonexistent, as the coding region of remaining intervening gene in Sulcia-CARI (tal) overlaps the coding region of both flanking genes.

Evolutionary Origins of the Observed Genomic Patterns

Given the long time periods involved and the lack of known intermediate steps, it is hard to delineate a model that describes the evolutionary processes required to arrive at the genome structures observed in the three Sulcia-containing insect systems sequenced to date. A few facts are known, however, that fix some points along the paths taken to arrive at these structures: 1) based on the insect fossil record and phylogenetic reconstructions, the initial Sulcia infection was acquired by the auchenorrhynchan ancestor at least 260 Ma (Moran et al. 2005), 2) again based on the insect fossil record, the split between cicadas, spittlebugs, and sharpshooters was at least 200 Ma (Shcherbakov and Popov 2002), and 3) the extreme size and features of the Zinderia genome suggest that it is an ancient association not a recent symbiont acquisition. Therefore, by 200 Ma, the genomic structure of Sulcia was likely similar to what is observed in Sulcia-GWSS and Sulcia-SDEM, with Sulcia producing 8 of the 10 essential amino acids. This is consistent with extensive gene loss in the Sulcia lineage early in the symbiosis and suggests that Sulcia had lost the ability to produce methionine and histidine early in its association with insects, prior to the common ancestor of cicadas, sharpshooters, and spittlebugs, at least 200 Ma. Furthermore, this means that Sulcia has likely had a cosymbiont for at least 200 Ma because all extant examples of Sulcia-containing symbioses collectively make all ten essential amino acids, which implies that all ten are required.

Ten Amino Acids by Any Means Necessary: Sulcia and Its Cosymbionts in Three Insect Systems

Whatever the timing and mechanism of genome reduction that lead to the current metabolic contributions schematized in figure 6, the myriad mechanisms that Sulcia’s diverse cosymbionts have evolved to complement the respective Sulcia genome are remarkable. The three distinct paths taken by Zinderia, Baumannia, and Hodgkinia in the production of methionine exemplify this point. Zinderia uses the direct sulfhydrylation pathway (metXY) in the production of methionine, whereas Baumannia and Hodgkinia use the transulfuration pathway (metABC). Despite using the same pathway in the production of homocysteine, Baumannia and Hodgkinia use different enzymes for its conversion to methionine; Baumannia uses the cobalamin (vitamin B12)-independent version of methionine synthase (MetE), whereas Hodgkinia uses the cobalamin-dependent version (MetH). Hodgkinia is therefore obliged to retain a large number of genes responsible for the production of the complex vitamin cofactor cobalamin (cobAJMLHSTNOQDP), a gene complement that corresponds to about 7% of its proteome (McCutcheon et al. 2009a). It is sometimes unclear whether these differences reflect the phylogenetic origin of the symbiont or the random nature of genome reduction (McCutcheon et al. 2009a), but in the case of Zinderia’s use of the direct sulfhydrylation pathway in the production of methionine, it seems largely a phylogenetic signal, as Hemminonas arsenicoxidans and Janthinobacterium sp. Marseille, Zinderia’s closest free-living relatives, both encode copies of metX and metY but not metA or metB (both however do encode metC, which can also be used in cysteine degradation) (Audic et al. 2007; Muller et al. 2007).

When examined at the level of essential amino acid production, the three systems shown in figure 6 present a striking case of convergent evolution in the context of a common selection pressure to retain the capacity for a full complement of amino acids. In all three pairs of symbionts, no genes involved exclusively in essential amino acid production overlap in a genome pair, with the exception of arokAC in Sulcia-CARI and Zinderia (a few genes that function in two or more pathways, such as carAB, which have roles in amino acid and nucleotide production, are present in both Sulcia-GWSS and Baumannia). Our new findings for Zinderia reveal the first reported case of extreme genome reduction for a member of the Betaproteobacteria. (As members of the Alpha-, Beta-, and Gammaproteobacteria, Hodgkinia, Zinderia, and Baumannia are estimated to have diverged from each other at least 2 billion years ago [Battistuzzi et al. 2004]). Each cosymbiont developed a separate symbiosis with the insect host and Sulcia, underwent massive amounts of genome reduction, and converged on a gene set that perfectly complements their Sulcia partner; this finding highlights the critical role that these symbionts play in the biology of their insect hosts.

These results are similar to what has been observed in the pea aphid Cinara cedri, where part of the tryptophan pathway is retained on a plasmid of the endocellular symbiont Buchnera, and the remaining genes are present in the co-residing symbiont Candidatus Serratia symbiotica (Gosalbes...
et al. 2008). In this case, however, the level of complementarity is not precisely known because the *Serratia* genome is not yet complete.

**Candidatus Zinderia Insecticola, a Novel Symbiont of Spittlebugs**

We propose the name *Candidatus Zinderia* insecticola for the Betaproteobacterial symbiont of spittlebugs described here. The genus refers to the geneticist Norton D. Zinder (born 1928), and the species name refers to *Zinderia*’s exclusive presence in insect hosts. Distinctive features include large amorphous cells, an existence restricted to the host cell cytoplasm, a low GC content, a reocoding of UGA from stop to tryptophan, and the unique 16S rDNA sequence CTAGTTATATAAATGTTTAAG (positions 826–858, *Escherichia coli* numbering).

**Supplementary Material**

Supplementary figure S1 and table S1 are available at *Genome Biology and Evolution* online (http://www.gbe.oxfordjournals.org/).

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