Peptidoglycan Production by an Insect-Bacterial Mosaic

Graphical Abstract

Highlights

- Mealybugs have two bacterial endosymbionts; one symbiont lives inside the other
- The mealybug genome has acquired some bacterial peptidoglycan (PG)-related genes
- This insect-symbiont mosaic pathway produces a PG layer at the innermost symbiont
- Endosymbionts and organelles have evolved similar levels of biochemical integration

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In Brief

A functional biosynthetic pathway formed from a combination of genes encoded by a bacterial endosymbiont and its insect host exhibits strong parallels to organelle evolution.
Peptidoglycan Production by an Insect-Bacterial Mosaic

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SUMMARY

Peptidoglycan (PG) is a defining feature of bacteria, involved in cell division, shape, and integrity. We previously reported that several genes related to PG biosynthesis were horizontally transferred from bacteria to the nuclear genome of mealybugs. Mealybugs are notable for containing a nested bacteriawithin-bacterium endosymbiotic structure in specialized insect cells, where one bacterium, Moranella, lives in the cytoplasm of another bacterium, Tremblaya. Here we show that horizontally transferred genes on the mealybug genome work together with genes retained on the Moranella genome to produce a PG layer exclusively at the Moranella cell periphery. Furthermore, we show that an insect protein encoded by a horizontally transferred gene of bacterial origin is transported into the Moranella cytoplasm. These results provide a striking parallel to the genetic and biochemical mosaicism found in organelles, and prove that multiple horizontally transferred genes can become integrated into a functional pathway distributed between animal and bacterial endosymbiont genomes.

INTRODUCTION

Horizontal gene transfer (HGT) occurs when a gene is moved from the genome of one organism to another outside of the normal processes of vertical inheritance. HGT can, in principle, occur between any two DNA-based life-forms, but most often involves either movement of genes between microorganisms (Koonin et al., 2001; Ochman et al., 2000; Richards et al., 2011) or from microorganisms to larger eukaryotic hosts (Dunning Hotopp, 2011; Husnik and McCutcheon, 2018). The process of HGT in the evolution of the cellular organelles derived from bacteria—the mitochondrion and the plastid—is not disputed and is often referred to as endosymbiotic gene transfer (EGT) when the transferred genes seem to originate from the ancestral organelle genome (Keeling and Palmer, 2008; Martin et al., 2002; Timmis et al., 2004). The role that HGT (that is, transfer from sources other than ancestral organelle genomes) has played in the evolution of organelles is less clear, but numerous examples of HGTs from bacteria unrelated to the mitochondrial or plastid ancestor are found in eukaryotic genomes (Gray, 2015; Ku et al., 2015). No matter their genome of origin, the proteins that are produced from these EGTs and HGTs, and that function in organelles are transported there by specific multiprotein complexes (Neupert and Herrmann, 2007; Schleif and Soll, 2000). This history of gene loss on organelle genomes and gene gain on nuclear genomes has led to complex mosaic biochemical pathways in organelles, where genes of diverse taxonomic origin reside on different genomes, and the protein products of these genes are shuttled to different parts of the cell without strict deference to their taxonomic origins (Ouchéne et al., 2005; Gabaldón, 2018; Koreny et al., 2013).

Eukaryotic genome sequencing has led to the discovery of many potential HGT candidates unrelated to organelle function, most often originating from bacterial and fungal sources (Dunning Hotopp and Estes, 2014; Milner et al., 2019; Moran and Jarvik, 2010; Schönknecht et al., 2013; Slot and Rokas, 2011). The roles of these HGTs are diverse, but most often include nutrition or protection from predators, pathogens, and environmental stress (Husnik and McCutcheon, 2018). The function of some of these HGTs has been verified (Chou et al., 2015; Dean et al., 2018; Kominek et al., 2019; Metcalf et al., 2014; Milner et al., 2019; Moran and Jarvik, 2010; Stairs et al., 2018), but these examples all involve single-step biochemical processes or functions gained through the transfer of multiple genes linked by residence on the same fragment of transferred DNA. These functionally verified HGT events serve as important milestones in HGT research, but none approach the complex cellular and biochemical mosaicism observed in some organelle biochemical pathways that result from EGT and HGT.

Genomic work on sap-feeding insects and their nutritional endosymbiotic bacteria has revealed a few cases where the complexity of bacterial integration into host cells seems to approach that of organelles (McCutcheon, 2016; McCutcheon and Moran, 2011; Moran and Bennett, 2014). These bacteria
provide essential nutrients to their hosts and are thus required for normal host biology and survival (Akman Gündüz and Douglas, 2009; Baumann, 2005). Many of these endosymbionts are also long-term associates of their hosts, often living exclusively in special insect cells for tens or hundreds of millions of years (Moran et al., 2005). Like organelles, they are also faithfully transmitted from one host generation to the next by maternal transmission (Koga et al., 2012). This strict host association has resulted in extreme levels of gene loss and genome reduction in some endosymbionts, leading to bacterial genomes that are similar to organelle genomes in terms of gene number and genome size (McCutcheon and Moran, 2011; Moran and Bennett, 2014). In a final parallel to organelle evolution, some of the insects harboring endosymbionts with tiny genomes appear to use both native host genes and genes acquired from bacterial HGTs to fill gaps in pathways formed by endosymbiont gene loss (Husnik et al., 2013; Luan et al., 2015; Nakabachi et al., 2014; Nikoh and Nakabachi, 2009; Sloan et al., 2014). However, none of these putatively mosaic host-symbiont pathways have been functionally verified.

One of the most complex of these insect-endosymbiont relationships is found in Planococcus citri, an insect in a group commonly referred to as mealybugs. The P. citri symbiosis exists in an unusual structure (Figure 1A), where a gammaproteobacterium (Candidatus Moranella endobia; hereafter Moranella) resides in the cytoplasm of a betaproteobacterium (Candidatus Tremblaya princeps; hereafter Tremblaya), which together exist in specialized insect cells called bacteriocytes (von Dohlen et al., 2001). The Tremblaya genome is extremely small and maintains only ~120 protein coding genes, whereas the Moranella genome is comparably larger but still encodes only ~400 protein coding genes (López-Madrigal et al., 2013; McCutcheon and von Dohlen, 2011). Complementary patterns of gene loss and retention on the Tremblaya and Moranella genomes suggest that these two endosymbionts work together to make the essential nutrients required by their host insect (McCutcheon and von Dohlen, 2011). Some of the genes missing on the endosymbiont genomes are found on the insect nuclear genome, the result of numerous HGTs from various bacteria unrelated to either Tremblaya or Moranella (Husnik et al., 2013).

One potential mosaic biochemical pathway in P. citri is that for peptidoglycan (PG) biosynthesis. A PG layer is an almost universal feature of bacteria and is critical for bacterial cell division, shape, and integrity (Errington, 2013; Otten et al., 2018; Typas et al., 2011). Because PG is not produced by eukaryotes but is a nearly ubiquitous feature of bacteria, it is a potent activator of the eukaryotic innate immune response, and the highly conserved PG biosynthetic pathway is the target of many antibiotics (Dziarski, 2003; Otten et al., 2018; Typas et al., 2011). The presence of numerous HGTs involved in PG synthesis on the nuclear genome of an insect was therefore surprising. Here we test whether mealybugs can use these PG-related
RESULTS

A Complete PG Pathway Is Predicted by Genomics

The synthesis of a complete PG layer in Gram-negative bacteria can be divided into three classes of reactions based on enzyme location (Figure 1B). The first set of reactions involves the cytoplasmic synthesis of the PG precursor, a β-1,4-linked N-acetylglucosamine (GlcNAc)-N-acetylmuramic acid (MurNAc) disaccharide linked to an L-alanine (L-Ala)-D-glutamic acid (D-Glu)-meso-diaminopimelic acid (mDap)-D-alanine (D-Ala)-D-Ala pentapeptide. The second set of reactions involves inner membrane-associated enzymes that flip this PG precursor into the bacterial periplasm, and the third set of reactions involves the cross-linking of precursor molecules into the growing PG matrix, as well as PG modifications such as trimming the pentapeptide to an L-Ala-D-Glu-mDap-D-Ala tetrapeptide. Our original annotation of the PG biosynthetic pathway of *P. citri* focused on the first two sets of reactions because the *Moranella* genome encoded some of the enzymes required to flip PG into its periplasm, and because the bacterial-to-insect HGTs mostly involved the first set of reactions (Husnik et al., 2013). But our first annotation was not complete; there were unresolved holes in the cytoplasmic part of the pathway, and we paid little attention to the third part of PG synthesis.

In an effort to better understand the functional potential of this mosaic PG biosynthetic pathway, we performed a detailed reannotation of the PG-related genes in this system (Figure 1B; Table S1). Our expanded annotation indicates that a PG layer, if produced, should be comprised of the canonical crosslinked β-1,4-linked GlcNAc-MurNAc disaccharide with an L-Ala-D-Glu-mDap-D-Ala tetrapeptide (Figure 1B; Table S1). Because no genes related to PG biosynthesis are found in the *Tremblaya* genome, we predict that *Tremblaya* should not possess a PG layer.

PG Constituent Parts Are Present in Whole Insect Preparations

We used liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS; Bern et al., 2017) to detect the presence of PG in samples prepared from ~1,500 pooled *P. citri* mealybugs. Ions matching the expected *m/z* values for GlcNAc and MurNAc monomers and dimers, some bound to a tetrapeptide chain (L-Ala-D-Glu-mDap-D-Ala), were identified in the first stage of MS, and the structure of these molecules was confirmed by tandem MS (Figure 2). To rule out the presence of contaminating bacteria in mealybugs as a significant source of PG signal, we performed five replicate 16S rRNA gene amplicon sequencing runs on age-matched insects collected from the same mealybug colony used for LC-MS/MS and a sixth sample taken from a lyse stock used for MS analysis. On average ~0.61% (range of 0.0604%–4.29%) of all amplicon reads were from bacteria other than Tremblaya (76.1% of all reads) and *Moranella* (19.7% of all reads) (Table S2). These results suggest that bacterial contamination is an unlikely source of significant PG signal in our LC-MS/MS experiments. Although these data allow us to conclude that PG is present in the *P. citri*-Tremblaya-Moranella symbiosis, they provide no information on the spatial localization of this putative PG layer.

The PG-Specific Molecule D-Ala Is Specifically Localized at the Moranella Periphery

We next attempted to visualize incorporation of D-Ala, a PG-specific molecule, in this symbiosis. Inspired by recent work using localization of fluorescently labeled D-Ala as a proxy for PG biosynthesis (Kuru et al., 2012; Liechti et al., 2014; van Teeseling et al., 2015), we developed a similar approach based on nanometer-scale resolution secondary ion mass spectrometry (nanoSIMS) (Dekas et al., 2016; Dekas and Orphan, 2011). We soaked and injected a 15N-labeled D-Ala solution onto sprouting potatoes on which the mealybugs fed (Figure S1, related to Figure 3). After a week of feeding on labeled potatoes, mealybugs were sacrificed, and the bacteriome tissue was removed and injected into *P. citri*. We then subjected to nanoSIMS, where we observed strong, specific rings of enriched 15N signal exclusively at or near the periphery of all *Moranella* cells (Figures 3B and 3C–3E). In contrast, mealybugs grown on 15N-labeled L-Ala showed uniform label incorporation throughout the bacteriome tissue, as expected.
because L-Ala should be incorporated without bias into all proteins in the symbiosis (Figures 3F and 3G). We verified a Moranella periphery-specific pattern of D-Ala enrichment using bioorthogonal, Cu-catalyzed click-chemistry and fluorescence microscopy (Figure 3H; Kuru et al., 2012; Liechti et al., 2014; van Teeseling et al., 2015). The localization of enriched D-Ala signal at the cell periphery of Moranella, but not Tremblaya or the insect tissue, in both nanoSIMS and fluorescent microscopy is consistent with our genomic prediction that PG production should be specifically localized to the Moranella periphery, but should not exist in Tremblaya (Figure 1B). Similar patterns of D-Ala enrichment are routinely interpreted as strong evidence for PG biosynthesis in bacteria (Kuru et al., 2012; Liechti et al., 2014; van Teeseling et al., 2015).

A PG-Targeting Antibiotic Specifically Affects the Moranella Cell Envelope

Our nanoSIMS and click fluorescence data show that the P. citri PG layer is specifically located at the Moranella periphery, but both nanoSIMS and light microscopy lack the spatial resolution to place this PG signal precisely in the periplasm of Moranella. To provide additional evidence that the PG layer we observe is located in the periplasm of Moranella, we fed age-matched mealybugs on diets with and without the antibiotic cefsulodin, which specifically targets the periplasmic-localized penicillin-binding protein, Pbp1B. The gene for Pbp1B is encoded on the Moranella genome (Figure 1B; Table S1). Pbp1B functions as a glycosyltransferase and transpeptidase, joining new GlcNAc-MurNAc-pentapeptide precursors to the nascent PG matrix in the periplasm (Cho et al., 2014; Typas et al., 2011). A common phenotype for bacterial cells grown in hypotonic media in the presence of antibiotics is membrane blebbing and cell lysis, although the exact responses vary considerably in different bacteria (Chung et al., 2009; Yao et al., 2012). Given that Moranella lives exclusively in the cytoplasm of another bacterium, and therefore likely in an isotonic environment, we suspected that any antibiotic-related phenotype we might observe would be subtle. Using
transmission electron microscopy (TEM), we found that the periplasmic space of Moranella was specifically enlarged in the presence of cefsulodin versus control animals (Figures 4A and 4C). Because Tremblaya has three membranes, we also measured both the distance between the inner two and from the innermost to the outermost membranes as a control. No significant changes in membrane spacing were observed in Tremblaya between control and cefsulodin treatment (Figures 4A and 4B), indicating that this membrane spacing phenotype is specific to Moranella and not simply due to a general disruption of mealybug health in the presence of antibiotics.

A PG-Related HGT of Alphaproteobacterial Origin Is Localized to the Moranella Cytoplasm

Because most of the genes involved in the cytoplasmic portion of PG synthesis exist as HGTs on the insect genome (Figure 1B), it is formally possible that the GlcNAC-MurNAc-pentapeptide PG precursor is produced in the insect cytoplasm and then transported into Moranella. We suspected, however, that it was more likely that these HGTs were first translated in the insect tissue and then transported as proteins into the Moranella cytoplasm to produce the PG precursor molecule. This suspicion is primarily based on previous results from another related insect, where the protein product of an HGT on the pea aphid genome was shown to be transported into the cytoplasm of its bacterial endosymbiont, Buchnera aphidicola (Nakabachi et al., 2014). We note that if the PG layer we report here is located in the Moranella periplasm, and if it is constructed in the Moranella cytoplasm as we predict in Figure 1B, the proteins that result from HGT to the insect genome and that function in the Moranella cytoplasm must cross five lipid bilayers to get there: Tremblaya has three lipid bilayers, and Moranella has two (von Dohlen et al., 2001) (Figure 1A).

In order to differentiate between these two scenarios for PG precursor production, we produced a polyclonal antibody to a predicted protein encoded by a bacteria-to-insect HGT and observed its localization to test whether it was found in the Moranella cytoplasm. The antibody was generated against a peptide fragment of MurF, a ligase that normally functions in the bacterial cytoplasm to join D-Ala-D-Ala to the MurNAc-linked

Figure 4. A PG-Targeting Antibiotic Specifically Affects the Moranella Cell Envelope

(A) Quantification of the distance between the inner- and outer-most membranes of Tremblaya (n = 200) and Moranella (n = 400) and the inner two membranes of Tremblaya (n = 200) from control and cefsulodin-treated insects; mean ± SEM with a jitter plot of all data points. All data points were collected from random sections from two independent biological replicates. There is a significant difference only in the periplasmic space of Moranella, 11.5 versus 26.1 nm (Student’s t test) with an effect size of 2.3. The effect sizes for Tremblaya’s inner-only and inner-to-outer measurements were 0.0016 and 0.034, respectively. (B and C) Representative TEM images of control (B) and 100 μg/mL cefsulodin-treated (C) insects with Tremblaya (blue arrow) and Moranella (red arrow) membranes visible. See also Table S3.
Figure 5. MurF, a PG-Related HGT of Alphaproteobacterial Origin, Is Localized to the Moranella Cytoplasm.

Representative confocal image of a sectioned bacteriome stained with an anti-MurF antibody (red). Insect nuclei are stained with Hoechst (blue). Signal is detected inside of the Moranella cells and insect tissue, but not Tremblaya. Scale bar, 10 μm.

tripeptide to produce the pentapeptide (Otten et al., 2018; Typas et al., 2011). In the P. citri symbiosis, the murF gene exists only as an HGT that was transferred from an alphaproteobacterium to the host insect nuclear genome (Figure 1B; Husnik et al., 2013). Immunohistochemistry on paraffin-embedded insect tissue showed that MurF is found uniformly throughout the cytoplasm of Moranella and in portions of the insect tissue, but is completely missing from the Tremblaya cytoplasm (Figure 5).

The restriction of signal to the insect and Moranella cytoplasm is consistent with the hypothesis that the insect makes MurF and transports it into Moranella as a protein but does not appreciably accumulate in Tremblaya. This localization pattern suggests that genes resulting from HGTs on the P. citri nuclear genome can be made into proteins by host machinery and can be transported across the three lipid bilayers of Tremblaya and the two lipid bilayers of Moranella to end up in the Moranella cytoplasm. We note it is formally possible that the murF mRNA, not protein, is transported into Moranella and then translated by Moranella ribosomes. The data in Figure 5 also suggest that PG is made entirely in Moranella and that the alternative scenario, where PG precursors are built in the insect cytoplasm and transported into Moranella, is unlikely.

Collectively, our data from LC-MS/MS experiments (Figure 2), nanoSIMS (Figures 3B–3E and 3G), fluorescence microscopy (Figure 3H), antibiotic treatments (Figure 4), and immunohistochemistry (Figure 5) allow us to conclude that the genetic mosaic depicted in Figure 1B is functional and produces a PG layer that is likely built in the Moranella cytoplasm and located in the Moranella periplasm.

DISCUSSION

A PG-based cell wall is an ancient and defining feature of bacteria. Not surprisingly, the highly conserved set of genes that encode PG biosynthesis is normally exclusively found on bacterial genomes. Until now, there were two known exceptions to this pattern, both related to cyanobacterial-derived endosymbionts of photosynthetic eukaryotes. The first example of PG-related HGT comes from the “chromatophore” of the rhizarian protist Paulinella chromatophora. EM suggests that the Paulinella chromatophore has a PG layer (Kies, 1974), which is encoded primarily on the chromatophore genome with the exception of one bacterial HGT to the host protist genome (Nowack et al., 2016). The second example comes from the group of photosynthetic eukaryotes whose ancestor formed the original endosymbiosis with the cyanobacterium that became the chloroplast. This group, called the Archaeplastida, includes land plants, red algae, green algae, and glaucophyte algae (Lane and Archibald, 2008; McFadden, 2001). Many archaeplastidal nuclear genomes encode some PG-related EGT and HGTs (van Baren et al., 2016; Sato and Takano, 2017), but these genes do not always seem to work together to form a functional PG layer at the chloroplast periphery. A chloroplast-localized PG layer has been verified using fluorescently labeled D-Ala in the moss Physcomitrella patens (Hirano et al., 2016), and possible chloroplast PG layers have been observed by EM in glaucophytes (Schenk, 1970). But in the land plant Arabidopsis thaliana, which retains some PG-related genes on its nuclear genome, although fewer than in the moss P. patens, no PG layer exists at the chloroplast periphery and at least one PG-related enzyme has been coopted for a different function (Garcia et al., 2008). These results serve as a cautionary note about inferring function from the presence of HGTs alone: gene presence is not a reliable predictor of biological function (Doolittle, 2013).

Exactly how the Moranella PG layer is built, its function, and its precise cellular location remain unknown. One important remaining question is the source of D-Ala and D-Glu in Moranella’s PG. Our original annotation left the activities of Air (alanine racemase) and Muri (glutamate racemase) unaccounted for: they did not exist as HGTs on the insect genome, and they were not present on the Moranella genome (Husnik et al., 2013). Our new annotation confirms that homologs of these genes are missing in the P. citri symbiosis. Interestingly, GlyA and MetC have been shown to moonlight as alanine racemases in Chlamydia trachomatis and Escherichia coli, respectively (De Benedetti et al., 2014; Kang et al., 2011; Otten et al., 2018), and we find eukaryotic homologs for these genes on the mealybug genome (Table S1). Similarly, DapF has been shown to moonlight as a glutamate racemase in C. trachomatis (Liechti et al., 2018), and this gene exists as an HGT of alphaproteobacterial origin on the P. citri genome (Husnik et al., 2013). These data suggest that the loss of air in Moranella may be compensated by a moonlighting insect enzyme, and that the loss of dapF on the Moranella genome may be compensated for by a moonlighting alphaproteobacterial dapF HGT (Table S1). But it is also possible that the source of D-Ala and D-Glu is not from these putatively moonlighting enzymes at all, but rather from either the plant sap diet of the insect or from D-amino acids in P. citri produced from normal insect biochemistry. Although not studied extensively, D-amino acids have been found in both plants (Robinson, 1976) and insects (Auclair and Patton, 1950; Corrigan and Srinivasan, 1966; Corrigan,
although to our knowledge the levels of these compounds have not been measured in *P. citri*. The source(s) of D-Ala and D-Glu in Moranella could therefore be from the diet of the insect, the insect’s native amino acid biochemistry, moonlighting enzymes of various origins, or from some combination of all of these sources.

Our MurF immunohistochemistry localization data show that the protein products of HGTs on the insect genome can be specifically targeted to the Moranella cytoplasm (Figure 5). Import of enzymes (or mRNA) to the Moranella cytoplasm for precursor production rather than producing the GlcNAc-MurNAc-pentapeptide precursor in the insect cytoplasm may limit the risk to the insect host of triggering a PG-based immune response. Other insects with long-term endosymbionts devote resources to scavenging PG fragments in order to prevent continuous immune activation (Maire et al., 2019). By apparently sequestering PG production to inside of Moranella, *P. citri* may avoid the need for such contingency pathways, at least until Moranella cells are recycled near the end of the mealybug’s life (Kono et al., 2008).

It is notable that most of the proteins made from PG-related HGTs on the insect genome are predicted to function in the cytoplasmic part of PG synthesis, whereas the PG-related genes retained by Moranella all code for inner membrane- or periplasm-associated proteins (Figure 1B). These patterns of gene loss, gene retention, and HGT suggest that genes encoding proteins that function in the Moranella cytoplasm are more likely to be successfully transferred to the host insect nucleus. It is tempting to speculate that these HGT and protein localization patterns reflect the (currently unknown) mechanism used by the symbiosis to traffic proteins or RNAs made from the host genome to the correct subcellular compartment. It may be that this trafficking mechanism can transport soluble proteins and RNA, but is unable to transfer membrane-associated proteins. Whatever the trafficking mechanism, our data show that these proteins of foreign genomic origin, now encoded on the insect genome, work together with proteins still encoded on the Moranella genome to produce a genuine PG layer at the Moranella periphery. Future work will be required to definitively locate the Moranella PG layer because our current experiments lack the resolution to place this PG layer precisely in the Moranella periplasm.

Although the exact role that this PG layer plays in the *P. citri* symbiosis remains unknown, work from plastids suggests that host takeover of endosymbiotic PG production can be an important step in the regulation of endosymbiotic cell division and potentially further integration with the host organism (de Vries and Gould, 2018). In moss, knocking out a PG-related HGT on the nuclear genome results in enlarged chloroplasts (Machida et al., 2006), and treatment with various PG-targeting antibiotics results in fewer and larger chloroplasts per host cell (Katayama et al., 2003). Together these data suggest that the movement of PG-related genes from organelle genome to the host is a way for hosts to regulate organelle division (de Vries and Gould, 2018; Katayama et al., 2003; Machida et al., 2006). In *P. citri* mealybugs, *Tremblaya* was acquired before Moranella (Hardy et al., 2008; Thao et al., 2002), and so the host insect must have found a way of controlling Tremblaya as the sole endosymbiont prior to the acquisition of Moranella. Because the patterns of HGT and protein targetting we observe here are strongly convergent with moss chloroplasts (Garcia et al., 2008; Katayama et al., 2003; Machida et al., 2006), it is tempting to speculate that the function is also convergent; that is, PG-related HGTs have been retained on the insect genome as a way of controlling the cell division of a bacterium that lives inside of another bacterium inside of insect cells.

The frequency and importance of bacteria-to-eukaryote HGT are a matter of debate (Husnik and McCutcheon, 2018; Martin, 2017; Martin and Herrmann, 1998). Although in our view numerous studies using genomic and transcriptomic data strongly support the idea that bacteria-to-eukaryote HGT is common in some groups of eukaryotes and is likely to be biologically significant (Husnik and McCutcheon, 2018), functional validation of most of these HGTs is lacking. In some cases, HGTs involving single-step (or single operon) biochemical functions have been experimentally validated by *in vitro* protein expression, enzymatic assays, and/or *in situ* RNAi (Chou et al., 2015; Dean et al., 2018; Kominek et al., 2019; Metcalf et al., 2014; Milner et al., 2019; Moran and Jarvik, 2010; Stairs et al., 2018). Although these examples serve as important milestones in HGT research, none approaches the genetic complexity we describe here, which is more akin to the host-organelle genetic mosaic used by eukaryotes to build proto-heme (Koreny et al., 2013; Oborník and Green, 2005). Our data show that multi-gene, multi-genome, and multi-cellular compartment conglomerations are not unique to organelles (Booth and Doolittle, 2015a; McCutcheon and Keeling, 2014; but see Lane and Martin, 2015 and Booth and Doolittle, 2015b), and that cell biological, genetic, and biochemical mosaics can become functional in examples outside of the mitochondrion and plastid.
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AUTHOR CONTRIBUTIONS

D.C.B.: conceptualization, investigation, analysis, methodology, validation, visualization, and writing; G.L.C. and J.S.M.: investigation, methodology, analysis, validation, visualization, and writing; K.M.S.: conceptualization, analysis, methodology, investigation, resources, and writing; S.M.: analysis, methodology, resources, software, and writing; D.M.B.: investigation, visualization; P.J.B.: methodology, resources, and administration; V.J.O.: conceptualization, funding acquisition, resources, and administration; J.P.M.: conceptualization, funding acquisition, administration, resources, visualization, and writing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Rabbit polyclonal anti-MurF | This paper; ProSci Incorporated | N/A |
| Goat polyclonal anti-rabbit IgG (H+L) Alexa Fluor 594 | Abcam | Cat# 150084 RRID: AB_2734147 |
| Normal goat serum | Vector Laboratories | Cat# S-1000 RRID: AB_2336615 |
| Rabbit pre-immune serum | This paper; ProSci Incorporated | N/A |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| Azide Alexa Fluor 488 | Thermo Fisher Scientific | Cat# A10270 |
| Cefsulodin | Research Products International | Cat# C52000 |
| D-propargylglycine | Alfa Aesar | Cat# 15402639 |
| Epon-Araldite resin kit | Electron Microscopy Sciences | Cat# 13940 |
| Lysozyme | Thermo Fisher Scientific | Cat# 90082 |
| MurF peptide (FVKSLENDYQKTKE) | This paper; ProSci Incorporated | N/A |
| Mutanolysin | Sigma-Aldrich | Cat# M9901 |
| 15N D-alanine | Sigma-Aldrich | Cat# 618527 |
| 15N L-alanine | Sigma-Aldrich | Cat# 332127 |
| Paraplast Extra | Leica Biosystems | Cat# 39603002 |
| Technovit 8100 resin kit | Electron Microscopy Sciences | Cat# 14654 |
| **Critical Commercial Assays** | | |
| Click-IT Reaction buffer kit | Thermo Fisher Scientific | Cat# C10269 |
| DNeasy Blood and Tissue kit | Qiagen | Cat# 69504 |
| MiSeq Reagent Kit v2 | Illumina | Cat# MS-102-2003 |
| Nextera XT DNA library preparation kit | Illumina | Cat# FC-131-1096 |
| Nextera XT indices | Illumina | Cat# FC-131-1002 |
| **Deposited Data** | | |
| Planococcus citri protein and nucleotide sequences | Priyam et al., 2015 | https://blast.mealybug.org/ |
| Trembiaya princeps protein and nucleotide sequences | NCBI | GenBank: CP002244 |
| Moranella endobia protein and nucleotide sequences | NCBI | GenBank: CP002243 |
| 16S rRNA amplicon sequences | This paper | NCBI BioProject: PRJNA546070 |
| SILVA rRNA database | Quast et al., 2013 | https://www.arb-silva.de/ |
| **Experimental Models: Organisms/Strains** | | |
| Planococcus citri (Insecta: Hemiptera: Pseudococcidae) | University of Montana | N/A |
| **Oligonucleotides** | | |
| 536F (Prokaryotic 16S rDNA) (TCGTCGGGACGCTCA GATGTGTAAGAGACGACGACGACGACGAGCTTAAATWC) | Holben et al., 2002 | N/A |
| 907R (Prokaryotic 16S rDNA) (GTCTCGTGGGCTCGGA GATGTGTAAGAGACGACGACGACGAGCTTAAATTTAGTTT) | Holben et al., 2002 | N/A |
| Eub 338 (GCTGCCCTCCGTAGGAGT) | Fuchs et al., 2007 | N/A |
| Gam42a (GCTTCCCCACATCGGT) | Fuchs et al., 2007 | N/A |
| **Software and Algorithms** | | |
| Adobe Illustrator CC 2019 | Adobe | https://www.adobe.com/ |
| Byonic 3.3.9 | Protein Metrics | https://www.proteinmetrics.com/products/byonic/ |
| Effect Size Calculator for t test | Social Science Statistics | https://www.socscistattest.com/effectsize/default3.aspx |

(Continued on next page)
LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, John McCutcheon (john.mccutcheon@umontana.edu). The reagents generated in this study are available from the Lead Contact with the following possible restrictions. Mealybug distribution may be restricted based on U.S.D.A. import/export regulations.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mealybugs
Planococcus citri were reared on squash or sprouted potatoes at 25°C, 77% relative humidity, and 12 h light/dark cycles in a Percival incubator until use (Figure S1). Where bacteriomes were dissected, only second and third instar female insects were used. For the LC-MS/MS experiments, whole insects of all instars and both sex were used except winged fourth instar males as these no longer contain bacteriomes.

METHOD DETAILS

Gene annotation
A list of known PG-related genes was generated using published descriptions of the system (Husnik and McCutcheon, 2018; Husnik et al., 2013) (Table S1) along with more up-to-date literature on the PG pathways (primarily: Otten et al., 2018; Typas et al., 2011). One to six protein sequences for each gene were collected from different bacterial species on Uniprot and blasted against the genomes of Candidatus Tremblaya princeps (GenBank: CP002244), Candidatus Moranella endobia (GenBank: CP002243), and Planococcus citri (https://blast.mealybug.org; Planococcus citri Pcitri v1 proteins database) (Priyam et al., 2015). Top hits for any unannotated proteins were blasted using the NCBI non-redundant protein sequence database to confirm similarity to known versions of those proteins. Sequences in Table S1 for P. citri are from https://blast.mealybug.org.

Mealybug feeding and dissection
For detection of 15N-labeled compounds by nanoSIMS, second and third instar mealybugs were moved to sprouted potatoes (Figure S1) that had been covered in 1 mL of 3.57 mg/mL of 15N D-alanine or 15N L-alanine (Sigma-Aldrich). The D-amino acids were dissolved in water and filtered through a 0.22 µM syringe filter. For fluorescent detection of D-alanine via click-chemistry and fluorescence microscopy, 3.57 mg/mL of D-propargylglycine (Alfa Aesar) was prepared and applied in the same manner as above. For both nanoSIMS and click chemistry experiments, the labeled compound treatments were reapplied every day for a
week with a 27-gauge needle and syringe and distributed gently with a paintbrush across the potato, focusing on sprouts or any locations near feeding mealybugs. The same process was used for the TEM antibiotic experiments using 100 μg/mL 0.22 μM-filtered cefsulodin (Research Products International) in water. One mL of water was added in the same manner as described above for all control animals. After seven days, the bacteriomes were dissected from *P. citri* in *Drosophila* Ringer’s Solution (3 mM CaCl$_2$·2H$_2$O, 182 mM KCl, 46 mM NaCl, 10 mM Tris base; adjusted to pH 7.2) and fixed as described in the following sections.

**LC-MS/MS**

Approximately 1500 *P. citri* individuals were collected whole and frozen at –80°C until use. The animals were suspended in 500 μL of chilled, filtered water, flash frozen in liquid nitrogen, and lysed using a 3 mm tungsten carbide bead in a Tissuelyser II (QIAGEN) for 3 min at 25 Hz, flipping the holders once and repeating. The lysates were dripped into 50 mL of boiling 6% SDS with constant stirring and boiled for 1 h; filtered water was added as it boiled off. The lysate was centrifuged at 130,000 x g for 1 h at room temperature (RT). The supernatant was decanted and the pellet resuspended with 50 mL filtered water and centrifuged as above for a total of 6 washes. The SDS-free pellet was resuspended in 25 mM NaH$_2$PO$_4$ (pH 5.5) and digested with 100 U of mutanolysin (Sigma-Aldrich) overnight at 37°C. The sample was dried under vacuum and resuspended in 50 μL 0.25 M Na$_2$B$_4$O$_7$ (pH 9.0). Terminal sugars were reduced by addition of NaBH$_4$ to a final concentration of 5 mg/mL and incubated at RT for 30 min. Reduction was terminated by addition of 15 μL H$_3$PO$_4$ (0.05% v/v). Reduced muropeptides were desalted by reverse-phase HPLC using water-formic acid 0.1% (v/v) and one-step elution with 25% acetonitrile (v/v)-formic acid 0.1% (v/v) gradient and analyzed by LC-MS/MS (Bern et al., 2017). Byonic version 3.3.9 was used to search LC-MS/MS data to identify PG monomers (Bern et al., 2017).

**16S rRNA sequencing**

Total DNA was extracted with the DNeasy Blood and Tissue Kit (QIAGEN) from five pools of five *P. citri* (second and third instars) and from 30 μL of the total lysate (all instars) used in LC-MS/MS analysis. The V4 and V5 regions of the 16S rRNA gene were amplified from each pool of DNA using the primers 536F and 907R (Holben et al., 2002) and “Amplicon with overhang” cycle conditions (below).

| Primer Name | Sequence |
|-------------|----------|
| 536F        | TCGTCGGCAGCGTCAGATGTGATATAAGACAGCAGCGCGGTAATWC |
| 907R        | GTCTCGTGAGCTCGAGATGTGATATAAGACAGCAGCGGTAATWC |

**Amplicon with overhang PCR conditions**

| Cycle Temp | Cycle Duration | Cycle Rounds |
|------------|----------------|--------------|
| 98°C       | 1 min          | 1            |
| 98°C       | 30 s           | 15           |
| 55°C       | 30 s           | 15           |
| 72°C       | 30 s           | 15           |
| 72°C       | 5 min          | 1            |

The PCR products were cleaned with AMPure XP beads (Beckman Coulter) and indexed using the Nextera XT Indicies DNA Library Preparation Kit (Illumina) per manufacturer’s instructions and amplified with the “Index PCR” conditions (below).

| Index PCR conditions |
|----------------------|
| Cycle Temp | Cycle Duration | Cycle Rounds |
| 95°C       | 3 min          | 1            |
| 95°C       | 30 s           | 12           |
| 55°C       | 30 s           | 12           |
| 72°C       | 30 s           | 12           |
| 72°C       | 5 min          | 1            |
The indexed amplicons were bead-cleaned as before and sequenced using a MiSeq v2 and related reagent kit (Illumina; University of Montana Genomics Core). MiSeq sequence data were quality-trimmed using Trimmomatic v.0.27 (ILLUMINACLIP:TruSeq3-PE.fa:2:30:10 LEADING:3 TRAILING:3 SLIDINGWINDOW:4:15 MINLEN:98) (Bolger et al., 2014), then processed using mothur v.41.2 (Schloss et al., 2009), following mothur’s standard operating procedures for Illumina MiSeq data (Kozich et al., 2013). Briefly: Paired-end reads were combined into contigs, those with ambiguous base calls or lengths shorter than 410 bp were removed. Contigs were then aligned to the SILVA database (Release 132) (Quast et al., 2013; Yarza et al., 2008) of reference SSU rRNA genes. Sequences in poor alignments, as well as those containing homopolymers longer than 8 bp were removed. Sequences were then pre-clustered using a minimum bootstrap threshold of 50%. Sequences were then classified by comparing them to the SILVA database (release 132), using BLASTn (minimum query coverage: 90%) (Altschul et al., 1990). Summary data can be found in Table S2, reads are deposited at NCBI under BioProject ID: PRJNA546070.

**FISH-nanoSIMS**

Bacteriomes from 15N-fed insects were fixed overnight at 4 °C in 2% EMS-grade paraformaldehyde (PFA) and embedded in Technovit 8100 (Electron Microscopy Sciences) following the manufacturer’s protocol. Bacteriomes were located in blocks under a stereo-dissecting microscope and semi-thin sections between 1-2 μm were cut dry using an ultramicrotome with a glass knife. Sections were dehydrated in a series of ethanol washes before being float mounted in 100% (40 °C) and 90% (50 °C) ethanol. The sections were floated onto a glass slide support and then dried at room temperature for 1 h. The sections were then air-dried and cover-slipped with Permount (Fisher), N,N-dimethylformamide (DMF) and a glass coverslip. Samples were imaged using a Zeiss Elyra S.1 system.

**Cu-click chemistry**

Whole bacteriomes were fixed in 2% PFA in 0.1 M cacodylate and 5% sucrose solution (cacodylate buffer) then rehydrated with PBS for 15 min at RT followed by permeabilization with 0.5% Triton X-100 in PBS for 15 min at RT. The bacteriomes were washed with 2% BSA in PBS (pH 7.35) and then resuspended in 500 μL of Click-IT reaction cocktail (Thermo Fisher) according to the manufacturer’s protocol with 2.5 μM Alexafluor 488-azide (Thermo Fisher) and incubated for 1 h at RT protected from light followed by a 5 min wash in 2% BSA in PBS and a final resuspension in ProLong Gold Antifade mountant with DAPI (Invitrogen). The tissue was incubated at 4 °C overnight, protected from light after which the bacteriomes were pipetted onto a clean glass slide and a glass coverslip was carefully placed on top. Thirty to fifty Z stacks were acquired for each bacteriome using a Zeiss 880 laser scanning confocal microscope at 63X magnification (63X objective, type: Plan-Apochromat, aperture: 1.4, immersion: oil) and ZEN Black software (Zeiss). Z stacks were created by manually stitching together tiled analyses. Box trace data were generated by exporting raw data as images and using the Plot Profile function in ImageJ to calculate average fractional abundance values along transects across the Moranella cell wall.

**Note:** *Tremblaya* 23S rRNA has significant mismatches with the traditional betaproteobacterial probe Bet42a, so *Tremblaya* cytoplasm was identified as the presence of Eub 338 mix signal, and lack of Gam42a signal. After the FISH imaging, coverslips were removed gently in a Petri dish filled with deionized water. Deionized water was gently poured over the slide for 2 min to remove mounting medium, with care taken to not dislodge sections by direct flow. Slides were air-dried, scored with a diamond scriber, and broken and filed to fit into nanoSIMS sample holders. Fragments were gold coated with a Cressington sputter coater (40 nm gold thickness). NanoSIMS analyses were carried out using a Cameca NanoSIMS 50L (Cameca). Fluorescence-microscopy mapped sample regions were identified using the nanoSIMS CCD camera. Samples were pre-sputtered with the Cs+ ion beam (D1 = 1, 1 nA) until the 12C15N– ion counts stabilized. Bacteriomes were imaged using a ~1 pA primary Cs+ ion beam current (D1 = 3), a raster size of 10 x 10 μm at 256 x 256 pixel resolution or 20 x 20 μm at 512 x 512 pixel resolution. Masses (12C14N–, 15C15N–, 31P, 32S) were collected in parallel using electron multipliers with a dwell time of 12-48 ms/pixel. Mass...
analyzed using ImageJ (Version 2.0.0). Background was subtracted from all channels using a 50-pixel rolling ball radius; a representative image was created using the Z-project and Max Intensity options for four consecutive slices of a single Z stack.

**TEM and dual-axis tomography**

Bacteriomes used for TEM were fixed in 3% EM-grade glutaraldehyde and 1% EM-grade PFA in cacodylate buffer on ice and shipped overnight where they were immediately rinsed with fresh cacodylate buffer and placed into Type A brass planchettes (Ted Pella, Inc.) prefilled with 10% Ficol in cacodylate buffer. Nodes were covered with the flat side of a Type B brass planchette and the samples were rapidly frozen with a HPM-010 high-pressure freezing machine (Leica Microsystems). The vitrified samples were transferred under liquid nitrogen to cryotubes containing a frozen solution of 2.5% osmium tetroxide, 0.05% uranyl acetate in acetone. Tubes were loaded into an AFS-2 freeze-substitution machine (Leica Microsystems) and processed at −90°C for 72 h, warmed over 12 h to −20°C, held at that temperature for 8 h, then warmed to 4°C for 1 h. The fixative was removed and the samples rinsed 4X with cold acetone following which they were infiltrated with Epon-Araldite resin (Electron Microscopy Sciences) over 48 h. Bacteriomes were embedded on Teflon-coated glass microscope slides using Secure-Seal imaging spacers (Sigma-Aldrich) and covered with ThermaNox coverslips (Electron Microscopy Sciences). Resin was polymerized at 60°C for 48 h. Embedded bacteriomes from age-matched insects were observed with a stereo-dissecting microscope to ascertain and select well-preserved samples. Bacteriomes were extracted with a microsurgical scalpel and glued to the tips of plastic sectioning stubs. Semi-thick (400 nm) serial sections were cut with a UC6 ultramicrotome (Leica Microsystems) using a diamond knife (Diatome, Ltd.). Sections were placed on Formvar-coated copper-rhodium slot grids (Electron Microscopy Sciences) and stained with 3% uranyl acetate and lead citrate. Ten nm gold beads were placed on both surfaces of the grid to serve as fiducial markers for subsequent image alignment. Sections were placed in a dual-axis tomography holder (Model 2040, E.A. Fischione Instruments) and imaged with a Tecnai TF30ST-FEG transmission electron microscope (300 KeV; Thermo Fisher) equipped with a 2k x 2k CCD camera (XP1000; Gatan, Inc.). Tomographic tilt-series and large-area montaged overviews were acquired automatically using the SerialEM software package (Mastronarde, 2005). For tomography, samples were tilted ± 64° and images collected at 1° intervals. The grid was then rotated 90° and a similar series taken about the orthogonal axis. Tomographic data were calculated and analyzed using the IMOD software package (Kremer et al., 1996).

**Immunohistochemistry**

Whole third instar insects were gently perforated with a dissection pin and fixed in 4% PFA in PBS for 1.5 h at RT, washed in PBS 3X for 10 min, and transferred to 30% EtOH for 10 min followed by fresh 30% EtOH overnight at 4°C. The following day, samples were dehydrated through a series of EtOH: 50%, 70%, 80%, 90%, 95%, (2X 10 min each at RT). Samples were transferred to 100% EtOH (1 h at RT), methyl salicylate (3X 20 min at RT) and Paraplast Extra (2X 20 min, 1X 45 min at 56°C; Leica Biosystems) and then embedded in paraffin. Tissue was sectioned at 5 μm and the location of bacteriome was mapped by hematoxylin (Cancer Diagnostics) and eosin (Cancer Diagnostics) staining in a subset of sections. A custom polyclonal antibody was generated (ProSci Incorporated) to a peptide sequence (FVKSLENDYQKTKE) from the P. citri murF gene (accession: AGR65718) that was predicted to be surface exposed. Antibody response to the immunizing peptide was confirmed by ELISA by ProSci Incorporated. For staining, sections were deparaffinized and rehydrated in distilled water. The PG membrane was permeabilized by incubating sections in 2 mg/mL lysozyme (Thermo Fisher Scientific) in dH2O for 30 min at 37°C followed by two PBS washes, and then incubated in 0.1% Triton X-100 in PBS for 5 min at RT (Cimino et al., 2006). Sections were washed 2X in PBS and then treated for heat-induced antigen retrieval by boiling for 10 min in 0.01 M citrate (pH 6.0) and returned to RT for 30 min. The tissue was then washed in PBS, blocked in 4% normal goat serum (Vector Laboratories) for 20 min at RT, and incubated in a 1:50 dilution of primary antibody in 0.05% BSA in PBS for 1 h at RT then overnight at 4°C. Negative controls were incubated with either no primary antibody or with pre-immune serum from the rabbit used to produce the primary antibody (ProSci Incorporated). The following day, sections were washed 3X in PBS. Primary antibody was detected by incubation with Alexa Fluor 594 Goat anti-rabbit IgG secondary antibody (1:400 in PBS; Abcam) for 1.5 h at RT. DNA was labeled with 40 μg/mL Hoechst 33342 Solution (Sigma-Aldrich) in PBS for 10 min at RT. Sections were washed 3X in PBS and then mounted using FluorSave reagent (EMD Millipore). All imaging was done using identical settings as described for Cu-click chemistry. Linear contrast stretching was applied equally to antibody stained tissue and matching controls using ImageJ.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Membrane measurements**

Five TEM images from the bacteriomes of each of four, age-matched, P. citri (two control and two cefsulodin-treated) for a total of 20 images were analyzed for differences in the periplasm width of Tremblaya and Moranella. Twenty measurements of the distance between the outside of the outermost membrane and the inside of the innermost membrane for one to three Tremblaya (n = 200) and Moranella (n = 400) cells as well as the for the innermost and middle membrane of Tremblaya (n = 200) in each image were taken using the IMOD software package (Kremer et al., 1996) (Table S3, related to Figure 4). Data were visualized using ggplot2 (Wickham, 2009).
in RStudio. The boxplots (Figure 4A) show the mean ± SEM (also listed in the Results) with overlaid jitter plot of all data points; p values are reported above each comparison and total n are listed here as well as in the legend. The control and cefsulodin-treated animals were compared using a student’s two-sided t test in RStudio. Effect size calculations were performed using the Effect Size Calculator for t-Test (https://www.socscistatistics.com/effectsize/default3.aspx).

DATA AND CODE AVAILABILITY

16S rRNA amplicon sequences have been deposited in NCBI under BioProject ID: PRJNA546070. All membrane measurements presented in Figure 4 are included in this manuscript as Table S3.
Figure S1. Representative Setup for Rearing and Feeding Experiments Involving *P. citri*, Related to Figures 2, 3, 4, and 5
Mealybugs (yellow arrows) were allowed to feed and reproduce on sprouted potatoes. Where treatments were applied, potatoes (often focusing on the sprouts) were covered and/or injected with 1 mL of the given treatment (modified D- or L-ala, cefsulodin, or water). Mealybugs were carefully placed on the potato with a paintbrush and allowed to eat *ad libitum*. Treatments were repeated each day for one week.