Adaptive horizontal transfer of a bacterial gene to an invasive insect pest of coffee

Ricardo Acuña[a,1], Beatriz E. Padilla[a,1], Claudia P. Flórez-Ramos[a], José D. Rubio[a], Juan C. Herrera[a], Pablo Benavides[a], Sang-Jik Lee[b,c], Trevor H. Yeats[b], Ashley N. Egan[b,d], Jeffrey J. Doyle[b], and Jocelyn K. C. Rose[b,2]

[a]Plant Breeding, Biotechnology, and Entomology Departments, Cenicafé, A.A. 2427 Manizales, Colombia; [b]Department of Plant Biology, Cornell University, Ithaca, NY 14853; [c]Biotechnology Institute, Nongwoo Bio Co., Ltd., Gyeonoggi 469-885, Korea; and [d]Department of Biology, East Carolina University, Greenville, NC 27858

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Horizontal gene transfer (HGT) involves the nonsexual transmission of genetic material across species boundaries. Although often detected in prokaryotes, examples of HGT involving animals are relatively rare, and any evolutionary advantage conferred to the recipient is typically obscure. We identified a gene (*HhMAN1*) from the coffee berry borer beetle, *Hypothenemus hampei*, a devastating pest of coffee, which shows clear evidence of HGT from bacteria. *HhMAN1* encodes a mannanase, representing a class of glycosyl hydrolases that has not previously been reported in insects. Recombinant HhMAN1 protein hydrolyzes coffee berry galactomannan, the major storage polysaccharide in this species and the presumed food of *H. hampei*. *HhMAN1* was found to be widespread in a broad biogeographic survey of *H. hampei* accessions, indicating that the HGT event occurred before radiation of the insect from West Africa to Asia and South America. However, the gene was not detected in the closely related species *H. obscurus* (the tropical nut borer or "false berry borer"), which does not colonize coffee beans. Thus, HGT of *HhMAN1* from bacteria represents a likely adaptation to a specific ecological niche and may have been promoted by intensive agricultural practices.

lateral gene transfer | transposable element | transposon

Horizontal gene transfer (HGT), or lateral gene transfer, the process whereby genes move across species boundaries by nonsexual means, is accepted as a significant evolutionary force among prokaryotes, and there are many examples of its importance as a mechanism of ecological adaptation in this domain of life (1, 2). In contrast, other than ancient massive HGT associated with the acquisition of endosymbiotic organelles, there are far fewer reported examples of HGT involving eukaryotes, either between eukaryotic species or from prokaryote to eukaryote (3). Examples of HGT involving animals are even rarer, presumably because the germline is isolated from the somatic cells, and so is less likely to come into contact with foreign DNA (4). Indeed, most of the documented cases have been limited to invertebrates as the recipient and involve endosymbionts, where the association between the bacteria and host cells is particularly intimate and can occur in germ cells (5–8). However, there are reported examples of HGT where the gene donor is not an endosymbiont (3), such as the transfer of genes allowing the synthesis of carotenoids from fungi to aphids (9) and the spider mite *Tetranychus urticae* (10), and large scale transfer of genes to bdelloid rotifers that appear to be derived from bacteria, fungi, and plants (11). Additionally, some cases of close symbiotic associations, such as that seen in coral, do not show evidence of HGT (12), underling its rarity.

There is considerable debate as to whether the apparent relative infrequency of HGT to animals is a reflection of fundamental differences in biology, or whether such events are being overlooked in animals, in part, because of the strategies used for genome sequencing, data filtering, and gene annotation (3, 13). Moreover, there has been considerable controversy regarding the lack of functional validation of genes that are putatively assigned as being present following HGT, and the underlying evolutionary pressures and biological significance of such genes has often been unclear (14). In prokaryotes, HGT has repeatedly been associated with adaptation to new environments (2), and this trend is also beginning to emerge in eukaryotes. For example, the adoption of a plant–parasite lifestyle in several genera of nematodes was enabled by multiple HGT events (15), and a similar case of HGT facilitating plant parasitism was suggested by genome-scale analyses of oomycetes of the genus *Phytophthora*, which revealed that a substantial portion of the secreted proteomes was a consequence of HGT from fungi (16).

In this study, we obtained clear evidence for functionally important HGT involving an animal through studies of *Hypothenemus hampei*, commonly known as the coffee berry borer. This beetle is a specific pest of coffee (*Coffea* spp.) and is a major threat to coffee production worldwide, causing losses estimated at >500 million US dollars per year and affecting >20 million rural farming families (17). We describe the identification of a mannanase gene in the *H. hampei* genome that is likely to have originated in gut bacteria, as well as features of the associated genomic region that suggest a potential mechanism of gene transfer to the insect. There is a growing awareness that the metabolism of bacterial symbionts can confer important evolutionary advantages to insects (18), whether as endo- or ectosymbionts. This current study further suggests that intestinal bacteria may also provide a source of genetic material that allows rapid adaptations to new ecological niches, in this case, in the context of intensive agricultural production.

Results

Identification of *HhMAN1*. The coffee berry borer (Fig. 1*A*) reproduces and feeds exclusively inside coffee berries (Fig. 1*B*), which are primarily composed of carbohydrates (60%), proteins (10%), lipids (12%), organic acids, and caffeine (1%) (19). The feeding habits of *H. hampei* larvae suggest that they possess a hydrolytic system that is capable of enzymatically digesting the polysaccharides of coffee seeds (commonly referred to as coffee beans), which, in turn, are absorbed by the insect midgut during feeding. However, this process is poorly understood. To identify proteins that are secreted into the *H. hampei* midgut that may be involved in coffee berry infestation and digestion, we conducted a secretome functional screen using a yeast-secretion trap (20) with cDNA derived from *H. hampei* midgut. Among the cDNAs

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[1]R.A. and B.E.P. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: jr286@cornell.edu.

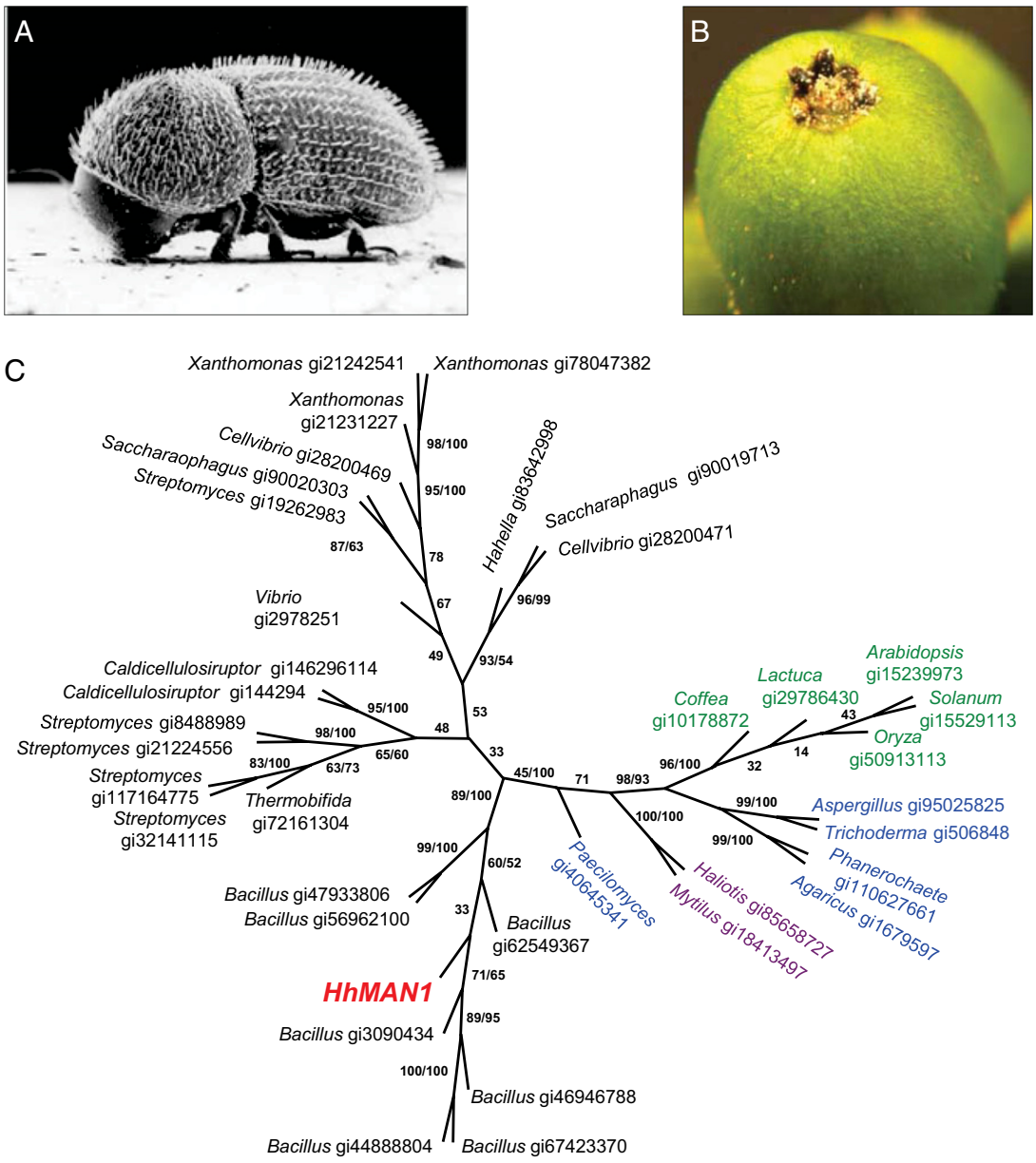

Fig. 1. The mannanase gene *HhMAN1* from the coffee berry borer (*Hypothenemus hampei*). (*A*) An adult *H. hampei* burrows into and lays its eggs in coffee berries (*B*). (*C*) Best maximum likelihood (ML) phylogenetic analysis of *HhMAN1* gene (red) with homologs from fungi (blue), bacteria (black), animals (purple), and plants (green). GenBank accession numbers are shown adjacent to each gene and bootstrap values are indicated on key branches. Where relevant, bootstrap values are notated with maximum likelihood followed by maximum parsimony (MP). Nodes with only one bootstrap value are from ML analyses only and were not supported in the MP bootstrap analysis.

recovered, we identified one (*HhMAN1*) that is predicted to encode a secreted protein with homology to mannanase enzymes of glycosyl hydrolase family 5 (Carbohydrate-Active enZYmes Database; www.cazy.org). This gene was selected for further characterization not only because galactomannan is the major storage polysaccharide of coffee beans (21) but also because there was no precedent for an insect mannanase in sequence databases.

We subsequently determined the full-length sequence by 3′ RACE and performed phylogenetic analyses of taxonomically diverse mannanase genes, including examples from both prokaryotes and eukaryotes (Fig. 1*C*). Whereas mannanases from plants, fungi, and animals formed a distinct eukaryotic clade, *HhMAN1* was most closely related to prokaryotic mannanases. Phylogenetic analyses strongly supported *HhMAN1* as grouping with the *Bacillus* clade (Fig. 1*C*); trees in which *HhMAN1* was constrained to group with animal sequences had significantly lower likelihoods. This indicated that *HhMAN1* is an example of HGT and has a bacterial origin before transfer into the *H. hampei* genome.

***HhMAN1* Has Some Hallmarks of a Eukaryotic Gene and Is Integrated in the *H. hampei* Genome.** Despite showing strong overall sequence similarity to bacterial mannanase genes, sequence analysis of the *HhMAN1* cDNA and genomic region revealed that *HhMAN1* has adopted several features indicative of a eukaryotic context. A predicted polyadenylation signal (AATAAA) is present 34 bp downstream of the *HhMAN1* ORF, and the *HhMAN1* cDNA

that was recovered was indeed polyadenylated, as is typical for eukaryotic genes. Furthermore, there is no prokaryotic Shine–Delgarno sequence at the start of the gene. This is particularly notable because the phylogenetic analysis of *HhMAN1* revealed the closest homologs to be from the taxonomic class Bacilli (Fig. 1*C*), a group that is particularly stringent in its requirement for this sequence as a ribosomal binding site (22). In addition to these adaptations associated with eukaryotic transcription and mRNA maturation, an additional suggestion of the adaptation of *HhMAN1* to a eukaryotic context is seen in its predicted ER-targeting signal peptide, which has features more typical of a eukaryote rather than a Gram-positive prokaryote. Specifically, it is relatively short (21 aa) and has a leucine-rich hydrophobic region (23).

To exclude the possibility that the *HhMAN1* sequence was identified from contaminating bacteria, rather than the *H. hampei* genome, the genomic DNA sequences flanking *HhMAN1* were determined by genome-walking. Assembly of the contiguous sequences indicated that the *HhMAN1* coding sequence lies between two sequences with substantial similarity to eukaryotic transposable elements of the Tc1/mariner and hAT superfamilies, located in the upstream and downstream regions, respectively, of *HhMAN1* (Fig. 2*A*). More specifically, the closest transposon homologs were identified in the genomes of two insect species, *Nasonia vitripennis* (jewel wasp) and *Acyrthosiphon pisum* (pea aphid), respectively. PCR amplification of DNA isolated from dissected *H. hampei* further excluded contamination from bacteria present in the midgut, because the diagnostic PCR amplification products were detected in cDNAs from elytra and legs of the adult, in addition to the midgut of the second-stage larvae and the whole adult (Fig. 2*B*).

To address whether *HhMAN1* or a close homolog is present in other species that feed on coffee berries, PCR was similarly performed using DNA isolated from *H. obscurus* and *Araecerus fasciculatus*. *H. obscurus*, the tropical nut borer, is a close relative of *H. hampei* and a common pest of macadamia crops in several coffee-growing areas (24). Under controlled conditions, *H. obscurus* can bore into coffee beans, but unlike *H. hampei*, it is unable to develop successfully on coffee endosperm.* On the other hand, *A. fasciculatus*, the coffee bean weevil, feeds indiscriminately on a variety of stored grains, including coffee (25). Attempts to amplify mannanase genes from these species using our primers were unsuccessful (Fig. 2*C*), suggesting that they do not contain a close homolog of *HhMAN1*.

***HhMAN1* Is Present in a Geographically Diverse Collection of *H. hampei* Accessions.** We also conducted a broad geographic survey of *H. hampei* samples collected from 16 countries collectively spanning Asia, Africa, and the Americas to determine whether *HhMAN1* is broadly distributed across the entire geographic range of the pest or represents a very recent occurrence during its radiation. *HhMAN1* was detected in 37 accessions (Fig. 3), and no specific geographic pattern was apparent. The apparent ubiquity of *HhMAN1* in *H. hampei* further indicates that the HGT event preceded the invasions of *H. hampei* from the center of origin in West Africa to Latin America and Asia (26).

***HhMAN1* Encodes a Protein Capable of Hydrolyzing Galactomannan Derived from Coffee Berries.** Galactomannan is the most abundant polysaccharide in coffee beans (21), where it acts as a carbohydrate-storage reserve. To confirm that *H. hampei* is capable of hydrolyzing the storage polysaccharide galactomannan, we prepared crude enzyme extracts from the midguts of adult *H. hampei* and assayed for activity toward galactomannan isolated from coffee berries. For comparison, extracts were similarly prepared from the midguts of *H. obscurus* and *A. fasciculatus* adults. Whereas *H. hampei* extracts showed galactomannan hydrolysis activity that was nearly linear with time, galactomannan hydrolysis activities were essentially absent from the *H. obscurus* and *A. fasciculatus* extracts (Fig. 4*A*). The lack of mannanase activity in these two species provides important support for the PCR results indicating that *HhMAN1* is only present in *H. hampei* (Fig. 2*C*), given that the PCR primers used may not have been appropriate for amplifying more divergent mannanase gene sequences, and suggests the absence of mannanases in *H. obscurus* and *A. fasciculatus*. These results indicate that the acquisition of *HhMAN1* by *H. hampei* may have been a prerequisite for specialization on its only host: coffee beans.

To confirm that *HhMAN1* encodes an enzyme with mannanase activity, the recombinant protein with a hexahistidine tag (HhMAN1-His_6) was expressed by baculovirus transfection of insect cells and purified by nickel affinity chromatography (Fig. 4*B*). The purified protein showed hydrolytic activity with coffee berry galactomannan as a substrate (Fig. 4*C*), confirming the bioinformatic annotation of *HhMAN1* as a mannanase with an activity relevant to the digestion of coffee bean polysaccharides.

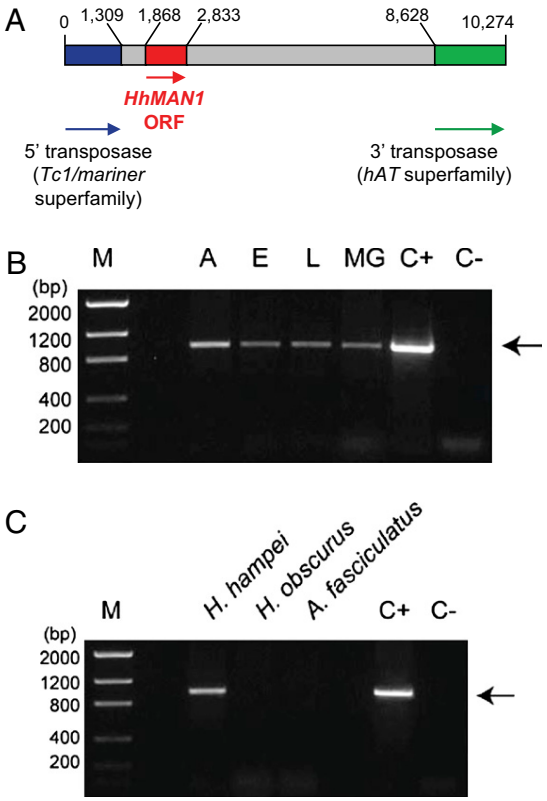

Fig. 2. *HhMAN1* is integrated into the *H. hampei* genome. (*A*) Structure of the *HhMAN1* gene highlighting the ORF, as well as flanking untranslated regions (gray) and predicted adjacent transposase-associated sequences. Positions (bases) within the genomic fragment (GenBank GQ375156) are indicated. (*B*) PCR amplification of *HhMAN1* from DNA isolated from various *H. hampei* body parts. A, whole adult; C+, positive control (HhMAN1 cDNA plasmid); C–, negative control (empty plasmid); E, elytra; L, legs; MG, midgut of second-stage larvae. The arrow indicates the diagnostic 1,007-bp *HhMAN1* PCR product. (*C*) PCR amplification of *HhMAN1* from DNA isolated from *H. hampei*, *H. obscurus*, and *A. fasciculatus*. Molecular mass markers (M) in base pairs are indicated.

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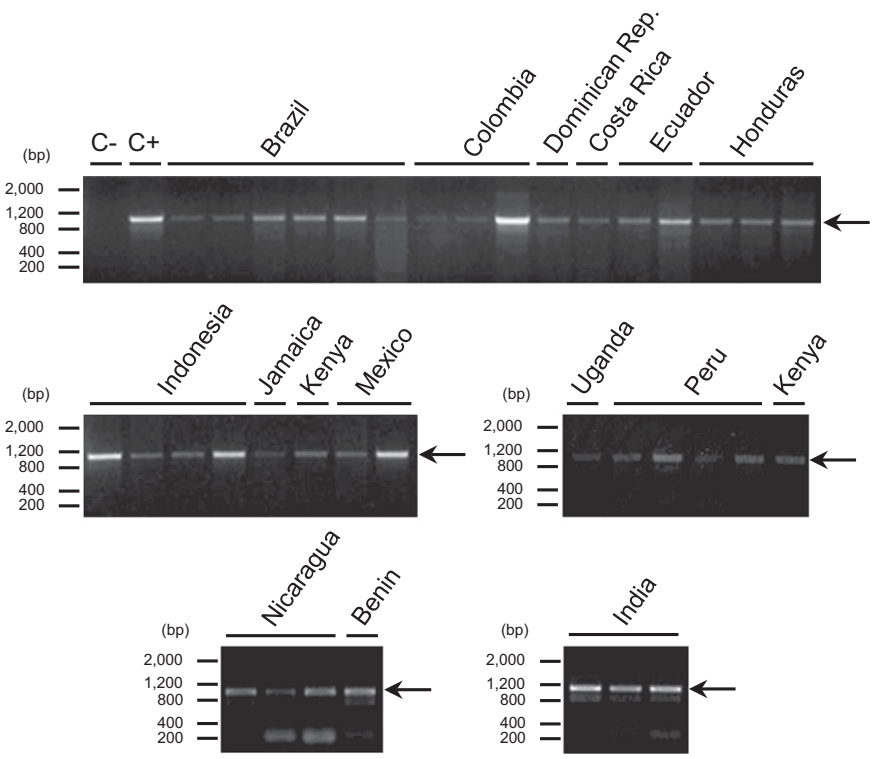

Fig. 3. Detection of *HhMAN1* in genomic DNA from a geographically broad range of *H. hampei* accessions. The arrows indicate the diagnostic amplified band. A positive control (C+, *HhMAN1* cDNA plasmid) and a negative control (C–, empty vector) are shown. Molecular mass markers in base pairs (bp) are indicated.

Discussion

Although HGT in prokaryotes has long been established as a central mechanism of evolutionary adaptation, its significance in animals is often debated, and there are relatively few examples. Furthermore, whereas the mechanisms of HGT in prokaryotes are well known (2, 27), the mechanisms of eukaryotic HGT remain elusive, and the functional significance of HGT is rarely evident. Here, we report that *HhMAN1*, which encodes a secreted mannanase that is active against the primary seed storage polysaccharide of coffee, is the result of HGT between bacteria and an insect. This transfer likely confers an adaptive advantage because *H. hampei* uses the coffee berry as a specific host.

Phylogenetic analysis of *HhMAN1* clearly indicates that the sequence is more closely related to eubacterial than eukaryotic genes, and, previously, there have been no mannanase sequences identified in insects. Because symbiotic microorganisms can be the source of polysaccharide hydrolytic activity required for plant biomass degradation (28), it was important to exclude the possibility of *HhMAN1* actually being encoded by the midgut microbiota of *H. hampei*. PCR amplification of genomic DNA derived from multiple organs and developmental stages of *H. hampei* established that the sequence was ubiquitously present and, thus, not a contaminant of midgut-derived material. The *HhMAN1* cDNA and genomic region revealed that *HhMAN1* has adopted several features indicative of a eukaryotic context, perhaps the most intriguing of which is that the *HhMAN1* gene is flanked by two eukaryotic transposons of the hAT and Tc1/mariner superfamilies. Not only does this provide further evidence that *HhMAN1* occurs in the genome of *H. hampei* and not that of a symbiotic microbe, but it also suggests a potential mechanism for HGT in animals. Genome recombination, mobilization, and reorganization are associated with transposable elements and HGT of the mariner transposon between several eukaryotic species has been reported previously (29), although, in this case, there was no observation of HGT of neighboring nontransposon genes. Such an association is suggested by our results, as well as a study of bdelloid rotifers that identified extensive examples of HGT in telomeric regions rich in retrotransposons and DNA transposons, including those of the hAT and Tc1/mariner superfamilies (11). We propose that transposon-mediated incorporation of foreign DNA could be one component of the poorly understood mechanism of HGT involving animals.

It has been suggested that HGT is particularly prevalent in the contexts of parasitic or pathogenic relationships or in the adaptation to specific environmental conditions (30). In support of this idea, recent examples involving nematodes (15), oomycetes (16), the spider mite (10), and the case we describe here of *HhMAN1* share a common feature in that they all involve the acquisition of secreted plant cell wall or other glycan-degrading enzymes by HGT that is associated with the adoption of a new plant host. It seems very likely that acquisition of a mannanase activity by *H. hampei* would confer a strong adaptive advantage to the insect because it adopted a lifestyle that is dependent on a host tissue that is rich in mannans.

Thus, *HhMAN1* appears to be a relatively recent case of HGT that would have been essential for adaptation to a new ecological niche by an important agricultural pest. Our results further suggest that intensive agriculture, and particularly monoculture, may provide a powerful selective pressure for HGT and that intestinal bacteria may provide a rich source of genes that can be acquired by eukaryotic hosts.

Materials and Methods

Insect Material. Tissue was dissected from larvae or adults, as indicated, and immediately placed in a chilled microcentrifuge tube with 100 μL of 5 mM phenylmethylsulfonyl fluoride or 0.1% RNA Later reagent (Qiagen) for protein or RNA isolation, respectively, and stored at −80 °C.

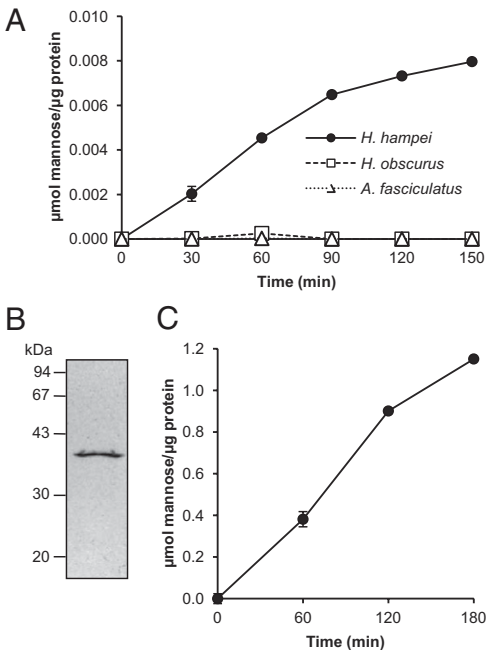

Fig. 4. Mannanase activity from extracts of the midgut of *H. hampei, H. obscurus*, and *A. fasciculatus* and recombinant HhMAN1. (*A*) Hydrolytic activity of midgut extracts from *H. hampei, H. obscurus*, and *A. fasciculatus* acting on coffee galactomannan substrate. (*B*) SDS/PAGE of recombinant HhMAN1-His_6 purified from transfected insect cells by nickel affinity chromatography. Molecular mas standards are indicated in kilodaltons. (*C*) Hydrolytic activity of purified HhMAN1-His_6 on coffee galactomannan substrate. Error bars are SD for $n = 3$.

Cloning and Phylogenetic Analysis of HhMAN1. RNA was extracted from 100 mg of midgut tissue and polyadenylated RNA isolated using an Oligotex mRNA Midi Kit (Qiagen) and used to generated a yeast secretion trap (YST) cDNA library (20). Following YST screening, oligonucleotide primers derived from the DNA sequence corresponding to the predicted signal peptide of HhMAN1 were used in 3′RACE PCR with the vector primer as an adapter primer. PCR products were subcloned into the pGEM-T Easy vector (Promega) and sequenced.

Phylogenetic Analysis. Protein sequences identified as likely homologs of HhMAN1 were identified by standard psi-BLAST and FlowerPower (31). Amino acid sequences were aligned in MUSCLE Version 3.7 (32) on the European Molecular Biology Laboratory European Bioinformatics Institute Web Server (www.ebi.ac.uk), producing a gapped alignment of 848 characters. The best model of protein evolution was determined in ProtTest (33) on the ProtTest Web Server according to the Akaike information criterion (34). The best-fit model was the LG+I+G+F model; this LG model uses a general amino acid replacement matrix (35) and incorporates a proportion of invariable sites (+I) (36), the gamma distribution for modeling rate heterogeneity (+G) (37), and empirical amino acid frequencies (+F) (38).

Phylogenetic relationships were estimated using maximum parsimony (MP) and maximum likelihood (ML). MP was conducted in PAUP* (39) with 1,000 heuristic searches using 10 random addition sequences per search and tree bisection and reconnection branching-swapping. Bootstrap support was estimated using 1,000 bootstrap replicates under the same conditions as above. Of 848 characters, 103 were constant and 576 were parsimony-informative. Six most-parsimonious trees were found with a score 5,253 and were summarized in a strict consensus tree. ML was conducted in RAxML Version 7.2.6 (40) using the best-fit model of protein evolution. Bootstrap support was obtained using 1,000 bootstrap replicates under the same model conditions. The best tree had a likelihood score of −25,568.28. In both analyses, *HhMAN1* was strongly supported as grouping with a clade of *Bacillus* sequences, separate from eukaryotic sequences.

To test the placement of *HhMAN1* within prokaryotes, we calculated the likelihood of a phylogeny in which *HhMAN1* was constrained to be monophyletic with other animal sequences. The best tree under constraint had a likelihood score of −25,604.48, a likelihood score significantly worse (below the 1% significance level) than the best ML tree under no constraints, as ascertained by the Shimodaira–Hasegawa test (41).

Genomic DNA Isolation and PCR Amplification of HhMAN1. Genomic DNA was extracted from dissected or intact insects as indicated using a DNeasy Tissue Kit (Qiagen). The sequence of DNA flanking the HhMAN1 ORF was determined following the protocol of the GenomeWalker Universal Kit (Clontech). To assay for the presence of *HhMAN1*, gene-specific primers flanking the ORF (5′-gctgatcgggtgtgtactca-3′ and 5′-ttaattgaatattgaacag-3′) were used for PCR. Products were subcloned and sequenced as above.

Protein Purification and Determination of Enzyme Activity. Crude extracts were prepared from adult midgut tissue homogenized in 200 mM sodium acetate (pH 5.0 buffer) at 4 °C. The extract was centrifuged at 18,000 × *g*, and protein content of the supernatant was quantified (42) before activity assays. For recombinant protein, the HhMAN1 coding sequence was subcloned into a baculovirus vector and transfected into *Spodoptera frugiperda* Sf9 cells for amplification and protein expression according to the BaculoDirect Baculovirus Expression System (Invitrogen) protocol. Recombinant HhMAN1-His_6 was purified from the culture medium using MagneHis Ni-Particles (Promega) according to the protocol of the manufacturer. Endo-β-mannanase activity of recombinant HhMAN1 protein or midgut tissues were determined (43) using galactomannan extracted from green coffee beans (21) as a substrate.

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